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Cover image:

Picture of all the speakers. (See P404 in this issue).

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Minimally invasive therapy for lung cancer: we are on the way for international consensus

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The Seventh China's Forum on Minimally Invasive Therapy for Lung Cancer & the Fourth Asia-Pacific Assembly on Video-Assisted Thoracoscopic Surgery (VATS) jointly sponsored by *Journal of Thoracic Disease*, *Annals of Cardiothoracic Surgery* and the Ming-Yi Medical Charity Foundation of Guangdong Province, and co-organized by the Professional Committee of Minimally Invasive Endoscopy in the Endoscopic Physician Branch of Chinese Medical Doctor Association (CMDA), State Key Laboratory of Respiratory Diseases/Guangzhou Institute of Respiratory Diseases, the First Affiliated Hospital of Guangzhou Medical University and the www.DXY.CN, was held at the First Affiliated Hospital of Guangzhou Medical University in Guangzhou, known as the flower city and "food palace" in China, during December 14th - 15th, 2013.

Professor Jianxing He, director of the First Affiliated Hospital of Guangzhou Medical University, and Professor Tristan D. Yan, Associate professor of University of Sydney, served as Co-Chairmans of the Forum (*Figures 1*,*2*). We welcomed national speakers include Professor Jie He (*Figure 3*), Professor Xiuyi Zhi (*Figure 4*), Drs. Xiaojing Zhao and Qun Wang etc. and for international Speakers, we have invited Professors Rene Peterson, Bernard Park, Dominique Gossot, Diego Gonzalez-Rivas, Michael Harden and Christopher Cao.

Each year, the Forum on Minimally Invasive Therapy for Lung Cancer held in Guangzhou provides an opportunity for the specialists to discuss ways to improve the results of the surgery. It is also an academic platform for the thoracic surgeons in China and even in Asia-Pacific Area to share and exchange the latest technologies and therapeutic experience on thoracic surgery.

The 2013's conference was dedicated to three aspects: the Chinese experts' consensus on VATS, video-assisted thoracoscopic segmentectomy *vs.* video-assisted thoracoscopic lobectomy for the early NSCLC, and the presentation of complex video-assisted thoracoscopic surgeries (*Figure 5*).

Before the opening ceremony, there is a video illustration of the enormous advancement we have achieved in the field of thoracic surgery. Just when the audience became inspired, Prof. He gave a passionate opening speech, pointing out the opportunities we have created and the goal for international experts' get-together. With high praise for the development of VATS technique in China, Prof. Yan expanded on the VATS Lobectomy 20th Consensus Statement. Prof. Xiuyi Zhi, who came to the conference hall just that morning, announced that the Chinese VATS group of the Chinese Medical Association is established, which laid a foundation of the future development of the VATS technique.

As a simple review in different sections, Prof. Harden introduced his experience regarding how to establish a VATS program, with a focus on addressing how to coordinate all the colleagues during an operation and how to train them to work as a close team (*Figure 6*). Known to all as a controversial issue, Prof Petersen gave a speech on Segmentectomy *vs.* Lobectomy demonstrated with excellent surgical video (*Figure 7*) and Dr. Christopher Cao then share the result of a Meta-analysis on Segmentectomy *vs.* Lobectomy to provide data proof (*Figure 8*). Prof. Gossot introduced French approach to segmentectomy (*Figure 9*) (1). There was little surprise that discussion on VATS to robotic surgery was ignited as Prof. Gossot mentioned his shift of research focus from VATS to robotic surgery, which was regarding a promising field by quite a few surgeons, including Dr. Shumin Wang, one of the national speakers.

Figure 1 Jianxing He is the Director and Professor of the Thoracic Surgery Department, The First Affiliated Hospital of Guangzhou Medical College, Guangzhou, China. He is also the Executive Editor-in-Chief of *Journal of Thoracic Disease*.

Figure 2 Tristan Yan is the Associate Professor of Department of Cardiothoracic Surgery, Royal Prince Alfred Hospital, University of Sydney, Sydney, Australia; The Collaborative Research (CORE) Group, Sydney, Australia. He is also the Editor-in-Chief of *Annals of Cardiothoracic Surgery*.

Figure 3 Jie He from Department of Thoracic Surgery, Cancer Hospital and Institute, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China.

Figure 4 Xiuyi Zhi, from Beijing Lung Cancer Center, Capital Medical University, Beijing, China.

Figure 5 The Scenario of conference hall during the conference.

Figure 6 Michael Harden from Department of Cardiothoracic Surgery, Royal North Shore Hospital, Australia

Figure 7 Rene Petersen was giving his speech.

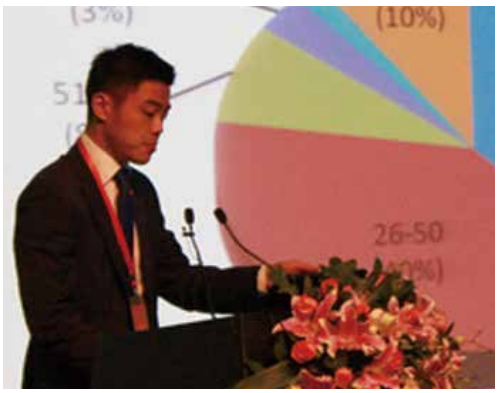

Figure 8 Christopher Cao was sharing the result of a Meta-analysis on Segmentectomy *vs*. Lobectomy.

Figure 9 Dominique Gossot was addressing the topic French approach to segmentectomy.

Following the "Segmentectomy Video demonstrations" section came Prof. Park's presentation on the Robotic-assisted Pulmonary Resection (*Figure 10*), as the key lecture in "the Robotic VATS technique" section. Then the application of da Vinci Surgical Robotics in Radical Lobectomy of Lung Carcinoma was introduced by Drs. Xiaojing Zhao (*Figure 11*) and Shumin Wang respectively. Prof. Gonzalez-Rivas further highlights the technique of Uniportal VATS in his speech (2). None-intubation VATS is one of the highlights for the conference, presented by Prof. He's surgical team (*Figure 12*).

Each speaker was awarded a commemorative certificate with his close-up after the speech (*Figures 13*,*14*). This is a witness of the significant event and a mark for the very moment: we are on the way for international consensus on

Figure 10 Bernard Park was talking on the Robotic-assisted Pulmonary Resection.

Figure 11 Xiaojing Zhao was giving a talk.

Figure 12 Wenlong Shao was presenting his talk.

Figure 13 Jianxing He presented the certificate to Tristan Yan as an acknowledgement for his excellent presentation.

Figure 14 Jianxing He and Diego Gonzalez-Rivas (left).

Figure 15 The attendees are taking a vote on the questions presented in the screen.

Figure 16 The Surgical video demonstration.

Minimally Invasive Therapy for Lung Cancer.

Simultaneous translation was available through the whole conference to make sure all the attendees better understand each speech. This conference also designated many sections for discussion between surgeons onsite (*Figures 15*,*16*). Some Guest Speakers like Profs. Petersen and Gonzalez-Rivas even spent one or two days around meeting agenda with a view to watching a live operation and having further communication with the surgeons in the hospital.

As Prof. He put it, this conference was close-knit (highly effective) and presents itself in a new form: video demonstration and the audience get a chance to communicate with the guest speakers in the section of Q&A. "*I was so satisfied with the conference as it is and I anticipate conference to be held in more innovative ways and format so that it can become both educational and interesting, and engage more international peers*", said Prof. He.

Successfully, the Seventh China's Forum on Minimally Invasive Therapy for Lung Cancer & the Fourth Asia-Pacific Assembly on VATS here came to a conclusion to the 2013 conference (*Figure 17*). Your participation in the upcoming next upcoming VATS conference will be most

Figure 17 Picture of all the speakers.

welcome. For your information, all of the speakers' latest work will be featured in the upcoming special issues in either *Journal of Thoracic Disease* (*JTD*) or *Annals of Cardiothoracic Surgery* (*ACS*) soon. They include two special issues on "Surgery for Esophageal Malignancy" (www.jthoracdis.com/announcement/view/63) and "Uniportal VATS and Live Surgery" (www.jthoracdis.com/announcement/view/62) published successively in *JTD* and the special issue on Sublobar Resection will be published in March in *ACS*. Please follow up and enjoy.

For more detailed information of the present conference (including Presentation PowerPoints and videos), please visit: http://meeting.dxy.cn/specials/jtd2013.

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Editorial

Chest tightness variant asthma (CTVA): reconfirmed and not generally appreciated

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In 2013, Shen *et al*. published a report in which they described 24 patients with asthma whose sole presenting clinical manifestation was chest tightness (1). In doing so, these investigators have reminded us that: (I) asthma can present with a variety of symptoms; (II) physiological testing for bronchial hyperresponsiveness is very important to assess for the possibility of symptomatic asthma; and (III) a favorable response to asthma therapy is necessary to confirm that the tests showing bronchial hyperresponsiveness are true and not false positive tests.

While the varied clinical presentations of asthma have been known for years, this information is not generally appreciated, perhaps because this work was published 30-40 years ago. With their recent study, Shen *et al*. have supported the validity of these earlier findings. In a review article on the clinical value of pharmacologic bronchoprovocation challenge that we published in 1990 (2), we summarized the literature on the clinical presentations of asthma in the following way: "Although the classic triad of symptoms associated with asthma is cough, shortness of breath, and wheeze occurring simultaneously, it is not unusual for one or more of these complaints to be absent. Moreover, asthma may present with other symptoms". For example, "It has been determined that asthma can present solely with cough, shortness of breath, chest discomfort (such as chest pain or tightness) (3,4), cough with expectoration, and the hyperventilation syndrome". Because asthma, a very common disease, can present with variant as well as classic symptoms, it behooves clinicians to be aware of the variety of presentations to avoid misdiagnoses as revealed by Shen *et al*. (1).

In the clinical realm, the diagnosis of asthma may not readily come to mind when patients present without wheezing. Moreover, the clinical evaluation alone even by asthma specialists uncovering a history of wheeze, a prior diagnosis of asthma and expiratory wheezing on physical examination has been shown to be an unreliable method of diagnosing asthma (5). Therefore, the diagnosis of asthma should be objectively confirmed by physiological testing. Such testing should assess for bronchial hyperresponsiveness that is a near universal finding in symptomatic asthma. As shown by Shen *et al*. (1), the presence of bronchial hyperresponsiveness can be revealed by bronchoprovocation challenge testing with an agent such as methacholine when baseline spirometry is normal or near normal, diurnal variation of peak expiratory flow rate, or spirometry before and after bronchodilator when there is baseline obstruction. When baseline spirometry is normal, one should not rely on improvement after bronchodilator to reveal bronchial hyperresponsiveness because it is unlikely that there is obstruction to reverse.

While bronchial hyperresponsiveness is consistent with the diagnosis of asthma, its presence is not diagnostic of asthma being the cause of the symptoms because bronchial hyperresponsiveness has been reported in a variety of other conditions (2). To determine that the test is diagnostic of asthma requires a favorable response with marked improvement or elimination of symptoms with asthma therapy. Such was done in the study of Shen *et al*. (1).

In an era when molecular biology and genetics research gets most of the headlines, the article by Shen *et al*. is noteworthy because it reminds us of the importance of clinical research and the relevance of lessons learned from excellent research from 30-40 years ago. These investigators

have not only reconfirmed a generally unappreciated variant presentation of asthma but also how to do so.

Acknowledgements

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Editorial

Chest tightness variant asthma: deja vu all over again

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Asthma is a serious problem that, according to WHO estimates, affects 235 million people (1). Apart from the typical symptoms of "recurrent episodes of wheezing, dyspnea, chest tightness, and coughing, particularly at night or in the early morning" (2), asthma may be manifested in various clinical phenotypes, and readily misdiagnosed as conditions such as chronic bronchitis, cardiovascular diseases, and mental disorders. Raising physicians' awareness of these clinical phenotypes can be of significant help in reducing misdiagnosis of asthma. Corrao *et al.* (3) first reported "cough variant asthma (CVA)", a phenotype of asthma presenting only with chronic cough. Of outpatients with chronic cough in China, one-third had CVA (4). The widely recognized definition of CVA greatly helped physicians reduce misdiagnosis and treat the patients correctly. In 1992, Zhong *et al.* reported "potential asthma," another clinical phenotype of asthma, and emphasized that people with asymptomatic bronchial hyperresponsiveness are at high risk of asthma (5). Potential asthma was further demonstrated by Laprise *et al.* in 1997 (6), and then by van den Nieuwenhof *et al.* (7) and Shaaban *et al.* (8) in 2008. Potential asthma may not always develop into symptomatic asthma if managed at an early stage. Recently, Shen *et al.* reported a notable clinical subtype of asthma, "chest tightness variant asthma (CTVA)" (9). Although chest tightness as a sole symptom in asthma was mentioned by Farr *et al.* 40 years ago (10), it is worthwhile reminding physicians that chest tightness may be related to asthma rather than cardiovascular diseases.

As common symptoms, either cough or chest tightness may be associated with different diseases. Only patients with both bronchial hyperresponsiveness (or diurnal peak expiratory flow variation of >20%) and good clinical response to beta-2 agonists (with or without inhaled corticosteroids), rather than improvement of bronchial hyperresponsiveness alone, can be confirmed with asthma.

Sufficient data are yet to be collected to clarify whether CTVA can be defined as an independent "clinical phenotype" regarding the prevalence, risk factors, course of disease, clinical features, management, and prognosis, as well as co-existing mental disorders. In Shen's cohort, 42% of CTVA patients showed concomitant anxiety. However, the causal relationship between asthma and anxiety remains unclear. Are patients with mental disorders prone to develop CTVA, or do patients develop mental disorders because they suffer from CTVA? There is no doubt that mental disorders should be treated properly in addition to asthma management, so as to break the vicious cycle of mental disorders and CTVA.

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Editorial

Asthma diagnosis: not always simple or straightforward…

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Asthma is a complex and multi-faceted condition, encompassing a variety of phenotypes and endotypes (1,2), and the diagnosis is often not easy in real-word clinical practice due to the lack of a simple 'gold standard' diagnostic test. Textbooks of medicine describe the 'classical' asthma presentation of a patient with intermittent and variable symptoms of wheeze, breathlessness, cough and chest tightness, typically with a diurnal pattern and often with symptoms following exposure to triggers such as exercise, viral infections or aero-allergens. With such a classical history, particularly in a patient with associated risk factors such as a personal or family history of atopy, asthma is very likely. The demonstration of variable or reversible airflow obstruction through basic spirometry and/or serial peak expiratory flow monitoring, and a clinical response to treatment is confirmatory. However, in some patients the history is less typical, and airflow obstruction may be difficult to demonstrate unless the patient is seen when symptomatic or after exposure to a trigger. In patients with a less classical history and non-confirmatory lung function measurements, it may be necessary to resort to more sophisticated diagnostic tests, such as bronchial provocation tests (for example with methacholine or histamine) or to tests of airways inflammation (3). Although guidelines advise clinicians on the criteria that should be met in order to apply a diagnosis of asthma, in the 'real world' of every-day clinical care, diagnostic algorithms may not always be applied rigorously. As a consequence, asthma may be 'missed' in patients with slightly unusual presentations, or indeed may be misdiagnosed in patients with an alternative explanation for their symptoms. The recognition of the pattern of symptoms is pivotal to raising the possibility of asthma in a clinician's mind, but the symptoms of asthma are variable between patients and in a single patient over time, and sometimes may be unusual. The classical symptoms of breathlessness, wheeze, chest tightness and cough are not specific to asthma but are shared with other diseases; there are a limited number of ways that pathology in the respiratory tract can manifest. Similar symptoms can be produced by other respiratory illnesses (for example infections, malignancies, fibrosis), but also by diseases of other systems, including cardiovascular disease and indeed by psychological illness, where subjective breathing and chest difficulties are common. This raises the possibility that asthma may be over-diagnosed in some and under-diagnosed in others resulting in a failure to prescribe the inhaled corticosteroids and bronchodilator treatments that are so effective in controlling asthma. There is current concern about both over-diagnosis in asthma, with a worry that some patents with non-specific symptoms are labeled as having asthma and commenced on (ineffective) asthma treatment without objective evidence (4), but also, as in the paper from Shen and colleagues on 'chest tightness variant asthma', of under-diagnosis or delayed diagnosis (5).

Shen *et al.* present a case series of 24 patients with chest tightness as their only presenting symptom, who undoubtedly did have asthma, but in whom it took a long time and a specialist assessment before the diagnosis was finally made. The authors propose that 'chest tightness variant asthma' should be recognised as a distinct phenotype of asthma. Although chest tightness is a recognised symptom of asthma, it is usually accompanied by other symptoms, and in isolation may fail to alert clinicians to asthma as the underlying cause. In this study, the patients underwent a rigorous objective assessment of airways dysfunction and inflammation, as well as assessment of atopic and psychological status and detailed testing to rule out alternative diagnoses that could explain the symptoms.

The asthma diagnosis was securely made, and the authors report that asthma treatment was successful when commenced.

This paper presents us with several important messages. Firstly, some patients who do indeed have asthma will not present with classical symptoms, and clinicians need to be aware of this possibility in those with unusual respiratory or chest symptoms, and test appropriately. Secondly, objective confirmation is necessary to make the diagnosis of asthma, particularly in those with unusual symptom patterns. This is likely to require referral to a specialist service, but is vital in a long-term (often life-long) condition like asthma, to allow effective treatment and information to be given and to prevent inappropriate or ineffective treatment (with associated side-effects and expense) and a failure to identify the true driver of symptoms. Thirdly, we need to be aware that all patients are different, and may present and behave in different ways. The same physiological or pathological impairment can result in very different symptoms and subjective experience in different patients. This is clear from the wide discrepancy between patient-reported outcomes, such as symptoms and quality of life on the one hand, and 'objective' measures of disease severity (such as lung function) on the other (6). Shen's paper reports a high level of psychological co-morbidity in these patients with atypical symptoms. There is increasing current interest in the overlap between the asthma and psychological dysfunction (7), with recognition of the effects of psychological and emotional state on the symptoms that a patient perceives. This is not to say that people are 'making up' or 'imagining' their symptoms or that 'it's all in their head', but to recognize the complexity of the interaction between body and brain. Neuro-imaging studies are increasingly revealing to us the 'neural substrate' of breathlessness and symptom perception in chronic lung disease (8), and reveal that the way a patient experiences a given impairment (e.g., bronchoconstriction or airways inflammation) will depend as much on their psychological as on their physical state. Patients with asthma and co-morbid psychological impairment need very careful assessment and appropriate treatments (which will often consist of both pharmacological and psychological-behavioral support) to achieve best results.

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Shen *et al.* are to be commended for reporting this interesting group of patients, but wider investigation in other patient groups, particularly in other countries and other linguistic and ethnic groups is needed to confirm that this group is generalizable enough to be described as a new phenotype. The importance of making a correct diagnosis and of recognizing the diversify of asthma is however a universal truth.

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Editorial

Endobronchial ultrasound-guided versus conventional transbronchial needle aspiration: time to re-evaluate the relationship?

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Conventional transbronchial needle aspiration (cTBNA) has a long history over three decades of utility for mediastinal sampling in lung cancer (1) especially in more bulky disease and can achieve high yields (nearly 80% or above) even in relatively new services (2). It has also been utilised in the diagnosis of benign mediastinal disease (3). The attractiveness of cTBNA is that it can be performed at the same sitting as conventional bronchoscopy by a respiratory physician/interventional pulmonologist under conscious sedation in an endoscopy suite without needing a thoracic surgeon, operating theatre, theatre team and associated expenses (4). A common application of cTBNA is to select out those patients with multi-station or bulky N2 disease who are not suitable for radical therapy but would be suitable for oncological therapies and thereby avoiding the invasiveness and cost of mediastinoscopy for diagnosis alone. More recently, in the last decade or so (5,6), endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) has come to the fore which has prompted a re-evaluation of all mediastinal sampling and staging techniques (4,7). There has also been further interest in the utility of both cTBNA and EBUS-TBNA beyond lung cancer in diagnosis of treatable benign mediastinal disease such as tuberculosis (3,6).

What is of interest however is how the relative roles of cTBNA and EBUS-TBNA have evolved and what they should be when the expertise and equipment is available in the same centre. Given the technical advantages of EBUS-TBNA [real-time sampling, imaging of surrounding vessels and nodal size and nature (8)], intuitively EBUS-TBNA should be superior for smaller nodes or those in more remote locations or juxtaposed to vessels. There are now several studies in the literature (albeit mainly retrospective or observational) that have compared the two techniques in both lung cancer and granulomatous disease (see *Tables 1* and *2*). The main findings consistently have been either superiority of diagnostic yield, sample adequacy or safety for EBUS-TBNA over cTBNA with variations depending on node size (smaller nodes favouring EBUS-TBNA) or location (station 7 often equivalent for both).

It is in this context that the paper in this issue of *Journal of Thoracic Disease* by Jiang *et al.* (16) attempts to compare the relative utilities of the two techniques in lung cancer. They report that cTBNA was non-inferior to EBUS-TBNA in lung cancer patients with mediastinal nodes. Unfortunately, the paper lacks other important data that would allow greater interpretation of the results and putting them in context. Firstly, the yields reported for EBUS-TBNA in the paper are low at ~78% not in keeping with published results of 88-93% (6,7) even in new services (17,18). This may partially relate to the particular cohort with lower cancer prevalence due to ethnicity and also reflect the utility of these techniques in benign disease as in the real world but further conclusions cannot be drawn as there are no data on node size, node location or staging especially given the limitations of positron emission tomography (PET) in radiological staging in sensitivity for mediastinal metastases. Potentially larger and more central nodes (not stations 2R, 2L) would favour cTBNA results as cTBNA would be expected to match EBUS-TBNA here. There are no data provided on surgical confirmation of results or clinical follow-up times which allow verification

Table 1 Comparative studies or EBUS-TBNA *vs.* cTBNA in lung cancer

Study	Type	Number	Inclusion criteria	Outcome	Prevalence (%)
Stoll *et al.* 2010 (9)	Retrospective	262	Possible lung cancer, referred for cTBNA or EBUS-TBNA	Favoured EBUS-TBNA (sensitivity 85.2% *vs.* 54.5% for cTBNA, 91.5% negative predictive value *vs.* 75% for cTBNA)	27.3
Wallace *et al.* 2008 (10)	Prospective blinded	150	Suspected lung cancer based on CT without distant metastases, surgical sampling of negative results and/or clinico-radiological followup	Favoured EBUS-TBNA (sensitivity 69% *vs.* 35.7% for cTBNA, 88.1% negative predictive value *vs.* 78% for cTBNA)	30.4
Rong *et al.* 2011 (11)	Unknown	95	Mediastinal lesions identified on CT amenable to cTBNA or EBUS-TBNA	EBUS-TBNA and cTBNA sensitivity not significantly different: 96% *vs.* 92% but sample adequacy rate higher with EBUS-TBNA: 41.1% *vs.* 23.2%, vascular puncture less with EBUS-TBNA: 4.2% *vs.* 9.9%	74.7
Arslan *et al.* 2011 (12)	Randomised trial	60	Mediastinal nodes >2 cm in short axis diameter on CT, 21 G EBUS-TBNA	Favoured EBUS-TBNA (sensitivity 66.7% *vs.* 33.3% for cTBNA), for station 7 no significant difference	>40 (data not available)
Bellinger *et al.* 2012 (13)	Retrospective	291	Clinical suspicion of lung cancer with mediastinal nodes	Sample adequacy not significantly different: 84% *vs.* 86% but for EBUS-TBNA nodes smaller (15 *vs.* 21 mm) and more often paratracheal (67% *vs.* 49%)	Data no provided

EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; cTBNA, conventional transbronchial needle aspiration.

Table 2 Comparative studies or EBUS-TBVNA *vs.* cTBNA in sarcoidosis

Study	Type	Number	Inclusion criteria	Outcome	Prevalence (%)
Tremblay *et al*. 2009 (14)	Randomised controlled trial	50	Suspected sarcoidosis with hilar/ mediastinal nodes, 19 G cTBNA	Favoured EBUS-TBNA (sensitivity 83.3% *vs.* 50.9% for cTBNA)	94
Gupta *et al.* 2014 (15)	Randomised controlled trial	130	Suspected sarcoidosis with right paratracheal and/or subcarinal nodes	Favoured EBUS-TBNA (sensitivity 74.5% *vs.* 48.4% for cTBNA)	90

EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; cTBNA, conventional transbronchial needle aspiration.

and clarification of "true negative" and "false negative" TBNA samples and identification of an alternative (benign) pathology. Learning curves for EBUS-TBNA may also be relevant here (discussed later on).

Secondly, they report 34 patients did not tolerate EBUS-TBNA (nearly 12% of the original cohort) which is far higher than would normally be expected in what is usually a well-tolerated procedure under conscious sedation (19,20). No information is provided on dosage of sedation, presence of an anaesthetist or procedure time but the fact that EBUS-TBNA was consistently performed after cTBNA may have been a factor. This also introduces bias of bronchoscopic landmarks for cTBNA puncture sites (for initial localisation prior to optimising position with the ultrasound) and also a small but theoretical risk of cross contamination of the EBUS-TBNA samples as the order was not randomised [a 7% rate of false positivity has been described with cTBNA (21)]. The non-inferior yield and poor patient tolerance of EBUS-TBNA compared to cTBNA may also reflect the ten times greater experience with cTBNA than for EBUS-TBNA in the study centre and it would be interesting to see a repeat study after longer experience with EBUS-TBNA to see if the results change. Existing studies have demonstrated the learning curve for EBUS-TBNA can be longer than thought and also individualised even amongst experienced conventional bronchoscopists (22,23). This is not surprising bearing in mind the endoscopic image is off-set with many systems and the scope is heavier and more rigid to manipulate (8).

Other limitations on the dataset include the fact that only one pathologist who not blinded to the study and reported all the results (it is common place now for double reporting of all lung pathology samples) so they were not analysed in blind fashion or independently which limited the internal validity. Only one to three samples were taken per station which may have lowered the sensitivity overall as standard practice suggest at least one visible core or three samples minimum should be taken per station (24). Moreover, the average number of samples taken was not provided and should be balanced to avoid bias as the change in sensitivity varies from 69% to 95% between one and three samples (24). There is no data provided on safety mid-procedure for both cTBNA and EBUS-TBNA which is of relevance as EBUS-TBNA would be expected to avoid vascular puncture due to real-time sampling.

Other factors that would have enhanced the study include data on ability to perform growth factor mutation analysis for epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase (ALK) as individualised treatment based on mutation status is now the aim of oncological therapy. It is known that EBUS-TBNA for example reports a high degree of success with providing samples for EGFR mutation testing (25,26). Additional information on needle gauge for both cTBNA and EBUS-TBNA would have been of interest as, depending on pathology set-up, there is more recent evidence that 21G EBUS-TBNA samples provide better subcharacterisation than 22G samples in a histopathological setup (18). Information on what benign diagnoses were made in the cancer negative samples would also been of interest to evaluate the performance of cTBNA and EBUS-TBNA in benign disease. In sarcoidosis for example, there is good randomised trial evidence to favour EBUS-TBNA over sarcoidosis (14). A cost analysis would also have been of interest given that the setup costs for EBUS-TBNA are far higher than for cTBNA but the importance of accurate clinical coding for these activities is even more important given the potential implications for loss of activity-based revenue in some healthcare systems which can be avoided by greater physician interaction with coders (2,27).

So what conclusions can be drawn from the Jiang *et al.* (14) study? In reality, this study has raised more questions than answers. It would be interesting to see a follow-up study to see if there is a learning effect with EBUS-TBNA as per cTBNA. Future randomised studies are needed but including data on follow-up, surgical confirmation, node size and location to definitively answer the question but one suspects the techniques should be complementary as suggested by national bodies (28). In the mean time, we know that cTBNA can perform well in selected patients in centres with relevant expertise but we do not have the evidence to conclude that cTBNA is non-inferior to EBUS-TBNA in all situations. However, we can infer cTBNA is non-inferior in the scenario of bulky or central mediastinal disease which is the probable context of the Jiang *et al.* study (16). In this respect, it is worth continuing to use cTBNA in centres with relevant expertise where the cost of setting up an EBUS-TBNA would be prohibitively high but EBUS-TBNA should be used for mediastinal staging of smaller nodes and at more distal nodal stations if available.

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Original Article

TBNA with and without EBUS: a comparative efficacy study for the diagnosis and staging of lung cancer

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Introduction: Conventional transbronchial needle aspiration (TBNA) has been around for over 30 years with sensitivities approaching 70-90%. Recent development of endobronchial ultrasound (EBUS) TBNA demonstrated even higher sensitivities among experts. However EBUS-TBNA is more costly and less available worldwide than conventional TBNA. A comparison study to determine the efficacy of TBNA with and without EBUS in the diagnosis and staging of lung cancer is described.

Methods: A total of 287 patients with mediastinal and hilar lymphadenopathy presenting for diagnosis and/or staging of lung cancer at enrolling institutions were included. Equal numbers of punctures were performed at the target lymph node stations using conventional TBNA techniques followed by EBUS-TBNA at the same sites. Patients and puncture sites that were biopsied by both methods and were positive for lung cancer were compared to establish efficacy of each technique on the same patients.

Results: In 253 patients at least one pair of specimens were obtained by conventional TBNA and EBUS-TBNA. In 83 of these patients malignancy was diagnosed. Among the 83 patients with a diagnosis of a malignancy there was no significant difference in the diagnostic yield of conventional TBNA versus EBUS-TBNA. When comparing diagnosis of malignancy for each lymph node sampled, there were a significantly greater number of positive (diagnostic for malignancy) lymph nodes sampled by EBUS-TBNA.

Conclusions: Recommendations for current practice depend on individual centers and bronchoscopist comfort level with TBNA (with or without EBUS). In our study, no significant difference was seen between the techniques for the diagnosis and staging of individual patients.

Keywords: Bronchoscopy; transbronchial needle aspiration (TBNA); endobronchial ultrasound (EBUS); lung cancer

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Introduction

Conventional transbronchial needle aspiration (TBNA) has been used for over 30 years to obtain histologic and cytological specimens from lymph nodes in the chest, with sensitivities approaching 80-90% (1-8). Recently endobronchial ultrasound (EBUS) TBNA has demonstrated even higher sensitivities among experts (8,9). However EBUS-TBNA is more complicated, requires additional training, less well tolerated by patients and more costly than conventional TBNA (c-TBNA) (10). A prospective comparison study was carried out to determine the efficacy of TBNA with and without EBUS in the diagnosis and staging of mediastinal and hilar malignant adenopathy.

Methods

A total of 287 patients with mediastinal and/or hilar lymphadenopathy presenting for diagnosis and/or staging were included in the study. All participating bronchoscopists

Table 1 Comparison of conventional TBNA (c-TBNA) and EBUS-TBNA cases (positive results represent cases in which malignancy was diagnosed from the biopsy)

	EBUS-TBNA positive	EBUS-TBNA negative	Total
c-TBNA positive	50	14	64
c-TBNA negative	12	177	189
Total	62	191	253

P=0.837. TBNA, transbronchial needle aspiration; EBUS, endobronchial ultrasound.

Table 2 Comparison of yield in malignant cases

TBNA Method	Diagnosed/total malignant cases	Percent yield (%)
c-TBNA	64/83	77.1
EBUS	62/83	74.7

P=0.717. TBNA, transbronchial needle aspiration; EBUS, endobronchial ultrasound.

Table 3 Comparison of positive (malignant) lymph node stations biopsied with c-TBNA and EBUS-TBNA

TBNA method	Diagnosed malignant/total lymph node stations	Percent yield (%)
c-TBNA	104/552	18.8
EBUS	122/441	27.7

P=0.001. TBNA, transbronchial needle aspiration; EBUS, endobronchial ultrasound.

were trained extensively by the senior investigator, KPW, and demonstrated competency in conventional TBNA and EBUS-TBNA prior to commencing study. Equal numbers of punctures (1 to 3 passes per lymph node) were performed at the target lymph node stations using conventional TBNA techniques followed by EBUS-TBNA at the same sites and by the same bronchoscopist on the same patient. In all cases, TBNA was performed first without EBUS so that there would be no puncture sites to guide the bronchoscopist. When nodal staging was the goal, the lymph node station that would give the highest stage was sampled first, followed by stations representing descending stages (i.e., N3-N2-N1 lymph node biopsy order). Rapid on-site cytology for adequacy of specimens was not used. Specimens were then smeared for cytology on slide and sent for cytospin and cellblock for any additional immunohistochemical or molecular marker studies. The adequacy of the specimen was graded by the quantity of lymphocytes and the quality of diagnostic tissue. A pathologist who was not part of the study team reviewed the slides and wrote a formal interpretation of each nodal station. The diagnostic results of each procedure and each lymph node biopsy station with and without EBUS were recorded and compared. Only the biopsies that were diagnostic for malignancy are presented in this report. Statistical analysis was done using STATA software and Pearson's chi squared test. The study was approved by the Institutional Review Boards of enrolling hospitals.

Results

From 27 NOV 2006 to 26 APR2013, 287 patients were studied. In 253 patients at least one pair of specimens were obtained by conventional TBNA and EBUS-TBNA. In 83 of these cases, malignancy was diagnosed. Of these 83 malignant cases, c-TBNA diagnosed 64 and EBUS-TBNA diagnosed 62. c-TBNA and EBUS-TBNA were both positive in 50 of these patients with exclusive positivity by c- TBNA in 14 patients and exclusive positivity by EBUS-TBNA in 12 patients (*Table 1*). Among the 83 patients with a diagnosis of a malignancy there was also no significant difference seen between the diagnostic yield of conventional TBNA and EBUS-TBNA (*Table 2*). There was a significant difference in the number of positive (diagnostic for malignancy) stations sampled by c-TBNA and EBUS-TBNA (*Table 3*).

Although 287 patients were studied only 253 patients had at least one pair of specimens obtained by c-TBNA and EBUS-TBNA. In 34 patients EBUS was not completed due to patient lack of tolerance of the procedure. All of these procedures were performed under local anesthesia with intravenous moderate sedation. Often, the patient would tolerate the normal bronchoscopy with c-TBNA well, but when the larger diameter EBUS scope with the rigid transducer tip was used the patients became too uncomfortable to continue. Patient intolerance accounted for the large number of stations that were not sampled by EBUS-TBNA compared with c-TBNA.

Discussion

The recent development of EBUS to guide TBNA (EBUS-

TBNA) has generated special attention and interest in the TBNA technique and has been shown to be more reliable and sensitive than conventional TBNA in some centers (11,12). EBUS-TBNA distinguishes itself from conventional TBNA in several significant ways. First is the ability to visualize and locate the target lymph node with ultrasound and then to perform the needle aspiration with real time ultrasound guidance. Second, the needle designed for the EBUS bronchoscope is longer and stiffer, making penetration through the tracheobronchial wall easier and allows for deeper penetration of the needle through the lymph node (13). Third, the needle apparatus is fixed to the scope at the working channel and the needle is moved independently, unlike conventional TBNA where the needle is moved with the sheath. By fixing the length of the sheath, the needle is prevented from being pushed too far out during the puncture attempt which is one of the most common problems when performing conventional TBNA. Another advantage for the EBUS-TBNA apparatus is that the needle sheath is locked to the scope and thus, the needle will not be pushed back into the scope channel when resistance is met. This is the second most common problem in conventional TBNA. Another major difference is the exit angle of the needle at the distal port of the working channel, where the EBUS bronchoscope automatically positions the needle at a good puncturing angle relative to the airway wall even with a straightened scope.

Despite these unique qualities of the EBUS scope and needle, the methodology for EBUS-TBNA is essentially the same as for conventional TBNA with only a few exceptions. First, location of the puncture site is identified visually using the endoscopic view of the airway and the natural landmarks that correlate to the CT image of the mediastinum as described in 1994 (14). This map included endobronchial, anatomic, and CT correlation for 11 of the most common locations for mediastinal adenopathy that can be reached from the airways. While this map was created for the bronchoscopist performing TBNA to enhance their ability to successfully locate and biopsy the target lymph nodes, it correlates very closely to the latest IASLC staging system (15). Unlike biopsy from the esophagus, the airways have distinctive landmarks to identify the areas where the lymph nodes consistently reside. These landmarks are very reliable and do not require ultrasound guidance to locate.

In 12 cases EBUS-TBNA was the only diagnostic specimen that was positive. It is reasonable to assume that the ultrasound guidance would be beneficial in lymph nodes located farther from endobronchial anatomic landmarks as in the case of a mid paratracheal lymph node (2R and 2L). Advantage in sampling smaller lymph nodes is also expected with EBUS real time visualization technology. Despite these 12 cases, it is interesting that in 14 different cases, conventional TBNA was the only diagnostic modality. No specific reason is obvious for this other than the patient tolerance of the procedure under moderate sedation or the larger and less flexible EBUS scope. In many of these cases only one puncture was made by each method, which should favor EBUS-TBNA punctures. It may also represent the learning curve of EBUS TBNA in which we are comparing a 30-year experience with conventional TBNA of the primary bronchoscopist versus a 3-year experience with EBUS-TBNA. With appropriate training and skill in both conventional TBNA and EBUS-TBNA, the difference between the two techniques may be minimized. In fact it is most interesting that in our first 100 patients, conventional TBNA was exclusively positive in 7 cases with zero cases exclusively positive by EBUS-TBNA. In the second set of 100 cases, there were 5 cases exclusively positive by conventional TBNA and 7 exclusively positive with EBUS-TBNA. This ratio shift between the two techniques as experience with EBUS-TBNA increased may support this speculation (16).

While there was no significant difference in the diagnosis of malignant cases, there were a significantly greater diagnostic percentage of overall positive (malignant) stations sampled by EBUS-TBNA. This is not surprising, as real-time visualization of the lymph node with EBUS should allow the bronchoscopist to puncture the target in fewer attempts than with c-TBNA, which is based off of anatomic landmarks and static CT scan correlation.

Using the EBUS bronchoscope to visualize the anatomy behind the tracheobronchial wall and the needle within the target lymph node is reassuring and often gives more detailed information about the lymph node that was not evident on CT scan. Despite this feature, the most important information needed for diagnosis and staging with any TBNA technique is still the cytology and histology. Any bronchoscopist with enough experience with EBUS-TBNA and rapid on site cytology (ROSE) knows very well that seeing the needle in the lymph node does not guarantee a diagnostic specimen. EBUS imaging

has demonstrated and confirmed the consistency of endobronchial and CT correlation for locating lymph nodes and has also demonstrated clearly the limitations that we have yet to overcome in TBNA. Understanding the variables in TBNA that prevent us from achieving closer to 100% yield on every pass that we visualize the needle in the lymph node is required for future improvements. Some combination of the instrument (scope and needles), the technique, or target lesion consistency or characteristics must play a role.

Conclusions

The diagnostic yield of c-TBNA and EBUS-TBNA performed sequentially under moderate sedation in cases of malignancy were not significantly different in our study. Recommendations for current practice depend on individual centers and bronchoscopist comfort level with TBNA (with or without EBUS). For the majority of lung cancer cases where diagnosis and staging is required with mediastinal adenopathy seen on CT, it is likely that conventional TBNA will be able to achieve the diagnosis and stage if EBUS is not available or if the patient will not tolerate the EBUS procedure. For those who desire maximal assurance that successful biopsy was achieved at the procedure, the addition of ROSE offers the most assurance but even that is not a guarantee since half of the slides are processed later and cellblock may contain specimens not present on specimens from the initial slides. The costs of all of these additional reassurances are significant and many institutions may not be able to afford the EBUS systems or ROSE. For centers that are comfortable with conventional TBNA, performing conventional first and only escalating to EBUS when conventional is non-diagnostic is supported by our findings.

As a bronchoscopist becomes more comfortable with the anatomy and TBNA technique, advancement from exclusive EBUS-TBNA to supplementing their practice with conventional TBNA will offer the greatest spectrum of care to the patients and allow for a more comfortable procedure with less sedation, lower costs, and similar yields if patient selection is matched with the bronchoscopist comfort level and skill in TBNA.

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Original Article

The Six-Minute-Walk Test in assessing respiratory function after tumor surgery of the lung: a cohort study

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Introduction: The Six-Minute-Walk Test (6-MWT) is an established and well-validated diagnostic procedure in cardiovascular and pulmonary diseases. The significance of the 6-MWT in the assessment of the respiratory function in tumor patients after lung surgery is yet unclear.

Methods: The retrospective study included 227 patients following oncological rehabilitation after lobectomy, pneumonectomy or wedge- and segmental resection due to a malignant tumor disease. Spirometry and 6-MWT were performed at the beginning (T1) and at the end (T2) of oncological rehabilitation and correlated with each other. A subgroup analysis on clinically relevant parameters was conducted as well.

Results: A significant improvement of the walking distance measured in 6-MWT as well as of forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) were detected within the scope of spirometry (all three $P<0.01$). This effect was demonstrable in all subgroups, except for patients who underwent pneumonectomy. However, a low correlation of the parameters walking distance and FEV1 was observed at both measurement points T1 (rho value =0.21) and T2 (rho value =0.25).

Conclusions: Measuring the walking distance in the 6-MWT could be a suitable parameter to assess respiratory function.

Keywords: Six-Minute-Walk Test (6-MWT); respiratory function; lung surgery; oncological; rehabilitation

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Introduction

Various functional test procedures are available for the assessment of cardio-pulmonary function and physical capacity. Many of these diagnostic procedures include complex evaluations of both cardiac and pulmonary function, require expensive equipment and are time-consuming. Frequently used functional diagnostic tests include spirometry, spiroergometry, whole-body plethysmography, Shuttle-Walk-Test, Cardiac-Stress-Test and Cardiopulmonary Exercise Test (1,2). Due to limited personal and technical resources in outpatient care as well as in community hospitals and rehabilitation facilities, simple patient surveys of lung function are more likely to be conducted; whereas complex diagnostic tests of cardio-pulmonary are often not feasible.

In 1963 Balke developed an easy to perform test of cardio-pulmonary function: measuring the walking distance in a defined time period (3). The 12-Minute Walking Test has been developed subsequently, which was used to measure the physical capacity in healthy individuals (4). This test has proven unsuitable in subjects with respiratory diseases due to its duration and physical strain. Subsequently the Six-Minute-Walk Test (6-MWT) was developed (5). The 6-MWT is similar to physical activity in daily life, is easier to perform, better tolerated by subjects and represents lung function similar as other more complex cardio-pulmonary tests (6). Moreover, the 6-MWT is potent a predictor of morbidity and mortality in subjects with chronic-obstructive pulmonary disease (COPD) (7). The 6-MWT is an established instrument to assess cardio-pulmonary status and has been used to assess subjects following surgical resections of benign tumors (8-11).

The significance of 6-MWT to determine respiratory function in subjects after surgical resections of malignant tumors is unclear. Moreover, it has no cohort been defined, if in this patient group a correlation between 6-MWT walking distance and lung function parameters such as the forced expiratory volume in one second (FEV1) exists. The aim of the present study is to analyze in a large cohort of subjects, who underwent surgical pulmonary resection of malignant tumors, the 6-MWT walking distance at the beginning and at the end of inpatient rehabilitation and to assess its correlation with concomitantly measured FEV1.

Materials and methods

Subjects

This is a retrospective study of subjects, who received oncological rehabilitation after lung surgery at a single center from April 2010 to July 2010. Inclusion criteria were malignant diseases in the form of non-small cell lung cancer (NSCLC), other histology of primary lung cancer or lung metastases. Exclusion criteria were: subjects' malignant mesothelioma, subjects' inability to perform spirometry or the 6-MWT subjects.

Inpatient rehabilitation

The study was designed as a retrospective analysis. All examinations and therapy measures were standard components of oncological rehabilitation. The rehabilitation included aerobics training, breath-therapeutic and physiotherapeutic exercises for the lungs and the muscles of the upper and the lower limb as well as of the auxiliary respiratory muscles. Rehabilitation therapies occurred daily from Monday to Saturday. The participation in the therapies was monitored by using attendance lists. Moreover, subjects were trained with regard to breathing technique and encouraged to perform breathing exercises independently. There was no additional intervention outside of the clinical routine. Both spirometry and 6-MWT were performed at the beginning (T1) as well as at the end (T2) of oncological rehabilitation.

Spirometry

Lung function was examined by measurement of the forced vital capacity FVC (FVC as a percentage of the predicted value) and FEV1 (FEV1 as a percentage of the predicted value), with a commercial spirometer (SpiroPerfect®, Welch Allyn, 4341 State Street Road, PO Box 220, Skaneateles, NY, USA). Spirometry was performed according to the American Thoracic Society recommendations (12). In order to obtain optimal measured values, measurements were done by well-trained technicians and were repeated on each examination. The subjects were coached to optimal performance in a standardized fashion. To assess for and to improve the reproducibility several measurements were carried out by each subject. All spirometric measurements were obtained as pre-bronchodilator tests and no additional broncholytic or other medications with effects on the respiratory system were administered and the examination was performed in conditions of rest.

Six-Minute-Walk Test (6-MWT)

6-MWT was performed according to the guidelines of the American Thoracic Society (12). The test was conducted along a 30-meter flat, straight surface at the clinic and subjects were requested to walk the maximum possible distance within six minutes, which was documented in meters. At T1 and T2 the test was carried out only once for each patient. Subjects were encouraged to walk as fast as possible and repeatedly asked to keep their walking speed. Heart rate, arterial blood pressure and peripheral oxygen saturation (pO_2) were measured with commercial available devices (LCD Pulsoxymeter, Quirumed, 46113 Valencia, Spain) both at the beginning and at the end of the test. When respiratory or muscular fatigue occurred during 6-MWT, walking speed was reduced to a tolerable maximum and in case of severe exhaustion or cardio-pulmonary symptoms, the test was discontinued.

Statistical analysis

Statistical analysis was performed with the help of the statistics software Statistika Basis® (StatSoft GmbH, Hoheluftchaussee 112, D-20253 Hamburg, Germany). In order to demonstrate "Before (T1) and After (T2)" differences, the Wilcoxon signed-rank test was chosen as a non-parametric test for dependent and not normally distributed data with constant measurement level. A P-value <0.05 was considered statistically significant. To determine a correlation between the results of 6-MWT and FEV1 at points T1 and T2, the Spearman's rho rang correlation coefficient was calculated and represented in relation to the severity of the respiratory functional impairment. A rho value of 0.2 to 0.29 was interpreted as a low correlation. Pre-specified subgroup analysis were performed by stratifying subjects according

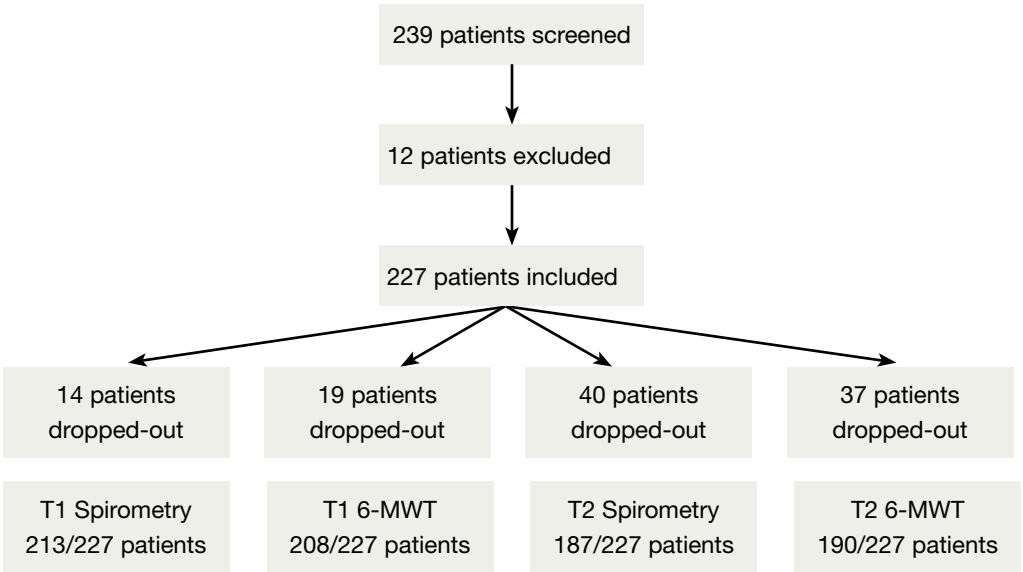

Figure 1 Consort diagramm. T1, at the beginning of the rehabilitation; T2, at the end of the rehabilitation; 6-MWT, Six-Minute-Walk Test.

surgical procedure (Lobectomy, Pneumonectomy and Wedge-/segment resection) and severity FEV1 reduction [normal function FEV1 >80% predicted, slight dysfunction (70-80%), moderate dysfunction (50-69%), severe dysfunction <50%].

Results

Patient characteristics

There were 239 subjects, who underwent rehabilitation after lung surgery from April 2010 to July 2010. Twelve subjects had exclusion criteria and the study cohort consisted of 227 subjects (*Figure 1*). Median age was 67 years (range, 33-87 years). With 120/227 (53%) subjects, male gender was slightly prevalent. On average, subjects were admitted to inpatient rehabilitation 25 days (range, 16-35 days) after lung operation and remained hospitalized for a median of 21 days (range, 8-42 days). The most frequently performed surgical technique was lobectomy in 108/227 subjects (47%) followed by wedge- and segment resection in 97/227 subjects (43%) and pneumonectomy 22/227 subjects (10%). The most common tumor diagnosis was NSCLC with 166/227 subjects (73%), followed by lung cancer of other histology in 26/227 subjects (12%) as well as pulmonary metastasis due to a solid extrapulmonary primary tumor (35/227 subjects, 15%). In the latter group there were 11 subjects with urothelial-/renal cell carcinoma, 8 subjects with colorectal carcinoma, 5 subjects with malignant melanomas and another 10 subjects with lung metastases of various other solid tumors. Depending on the tumor diagnosis, the selected surgical technique was lobectomy or pneumonectomy in subjects with primary lung cancer, each with systematic lymphadenectomy. Whereas wedge- and segment resection was used in metastasis surgery.

As expected, a history of smoking or active smoking was present in a large percentage (76%) of all lung cancer subjects. Clinically relevant comorbidities were found in 62% of the subjects, in particular COPD (22%), arterial hypertension (42%), compensated chronic heart failure (7%) and coronary heart disease (CHD) as well as chronic atrial fibrillation (20%). Patient characteristics are shown in *Table 1*.

Lung function parameters in spirometry

At T1, 213/227 subjects (94%) and at T2, 187/227 subjects (82%) could be evaluated with regard to spirometry parameters or delivered plausible values, respectively. The median time interval between T1 and T2 spirometry was 20 days (range, 17 to 23 days). In the overall cohort, when comparing parameters, there was a statistically significant improvement of the respiratory function from T1 to T2. Both FEV1 and FVC improved from median 59% to 64% and from 59% to 65% (P<0.01), respectively. This effect was observed after both lobectomy and wedge- and segment resection. There was no statistically significant improvement

Table 1 Patients characteristics (n=227)

	No.	%
Gender		
Male	120	53
Female	107	47
Age (years)		
Median	67	
Range	[33-87]	
BMI (kg/m²)		
Median	25	
Range	[16-51]	
ECOG performance status		
Grade 0	137	60
Grade 1	69	30
Grade 2	21	10
> Grade 2	0	0
Duration of rehabilitation (days)		
Median	21	
Range	[8-42]	
Diagnosis		
NSCLC	166	73
Lung cancer of other histology	26	15
Lung metastasis	35	22
Preoperative therapy		
None	186	82
Chemotherapy	32	14
Radiotherapy	3	1
Radiochemotherapy	6	3
Operation technique		
Lobectomy	108	47
Pneumectomy	22	10
Wedge- or segment resection	97	43
NSCLC stage (AJCC)		
IA/IB	75	45
IIA/IIB	33	20
IIIA	38	23
IIIB	14	8
IV	6	4
Nicotine abuse		
Never	55	24
Previous	153	68
Current	19	8
Comorbidities		
None	87	38
COPD	51	22
Essential hypertension	96	42
Chronic heart failure (NYHA II-IV)	16	7
CHD/AF	47	20
FEV1*		
>80% (normal)	27	12
70-80% (low)	26	11
50-69% (moderate)	84	37
<50% (high)	76	34
n.a.	14	6

BMI, body mass index; NSCLC, non-small cell lung cancer; AJCC, American Joint Committee of Cancer; COPD, chronic obstructive pulmonary disease; NYHA, New York Heart Association; CHD, coronary heart disease; AF, atrial fibrillation; FEV1, forced expiratory volume in one second; n.a., not available; *, at the beginning of the rehabilitation (T1).

of the lung function parameters in the subgroup of subjects after pneumonectomy (*Table 2*). Improvements were not affected by co-morbidities such as COPD, arterial hypertension and CHD/chronic atrial fibrillation. In the subgroup of subjects with chronic compensated heart failure, there were no significant differences between FEV1 and FVC at T1 and T2 (data not shown).

Measurement parameters of 6-MWT

At T1, 208/227 subjects (92%) and at T2, 190/227 subjects (84%) could complete 6-MWT. For the overall cohort as well as for each of the three subgroups with regard to surgical technique (lobectomy, pneumonectomy, wedge- and segment resection) a significant improvement of the distance walked was observed in 6-MWT (P<0.01). Except for a borderline significant improvement of heart rate in subjects who underwent lobectomy (P=0.04), there was no relevant change. Measurement of pO_2 did not show any significant difference in the comparison between T1 and T2 either (*Table 3*).

Within the scope of further subgroup analyses, a statistically significant improvement of the distance walked

Table 2 Lung function at T1 (n=213) and T2 (n=187) in according to the surgical technique

	All	Lobectomy	Pneumectomy	Wedge- and segment resection
FEV1 (% of the predicted value)				
T1, median [range]	59 [17-99]	59 [17-99]	44 [22-77]	62 [24-99]
T2, median [range]	64 [25-99]	65 [25-99]	47 [26-99]	66 [28-99]
P	<0.01	<0.01	0.42	<0.01
FVC (% of the predicted value)				
T1, median [range]	59 [14-99]	61 [(14-99]	43 [19-68]	61 [26-99]
T2, median [range]	65 [22-99]	67 [29-99]	47 [25-95]	66 [22-99]
P	<0.01	<0.01	0.30	<0.01

n, number of patients; FEV1, forced expiratory volume in one second; FVC, forced vital capacity.

Table 3 6-MWT at T1 (n= 208) and T2 (n=190) according to the surgical technique

	All	Lobectomy	Pneumectomy	Wedge- and segment resection
6-MWT (meter)				
T1, median (range)	224 [20-680]	235 [30-470]	175 [50-300]	223 [20-680]
T2, median (range)	327 [50-720]	345 [110-600]	255 [70-540]	325 [50-720]
P	<0.01	<0.01	<0.01	0.01
pO_2 (%)				
T, median [range]	95 [81-99]	95 [81-99]	95 [90-98]	96 [82-99]
T2, median [range]	95 [83-99]	95 [83-99]	95 [91-97]	96 [85-99]
P	0.3	0.67	0.8	0.1
HR, (beats per minute)				
T1, median [range]	95 [50-134]	94 [50-134]	95 [67-133]	95 [51-130]
T2, median [range]	96 [54-168]	99 [59-168]	95 [72-123]	94 [54-133]
P	0.1	0.04	0.27	0.83

n, number of patients; 6-MWT, Six-Minute-Walk Test; pO_2, peripheral oxygen saturation; HR, heart rate.

was found in 6-MWT with regard to comorbidities (COPD, arterial hypertension, chronic compensated heart failure, CHD/chronic atrial fibrillation) and to smoking behavior (data not shown). Chemotherapy performed prior to lung surgery did not influence 6-MWT and a statistically significant improvement of the walking distance at T1 and at T2 was observed in this subgroup as well (data not shown). Most likely due to the low number of subjects in the subgroups of radio- or radiochemotherapy, respectively (cf. *Table 1*), there was no significant difference between the distances walked at T1 and at T2 (data not shown).

Correlation of the walking distance with FEV1

With regard to their lung function at T1, subjects were divided into four groups according to their FEV1-value. The group without clinically relevant restriction (FEV1 >80%) counted 27/227 subjects (12%). The same number of subjects (26/227, 11%) were in the group with slight respiratory dysfunction (FEV1, 70-80%). Moderate (FEV1, 50-69%) and severe respiratory dysfunction (FEV1 <50%) was found in the majority of subjects (160/227, 71%) (*Table 1*). In all four groups, a statistically significant improvement of the walking distance was observed in 6-MWT in comparison between T1 and T2 with P<0.001 (*Table 4*). However, the correlations between the values of the walking distance in

Table 4 6-MWT at T1 (n=208) and T2 (n=190) in accordance to FEV1 at T1

	Normal function	Slight dysfunction	Moderate dysfunction	Severe dysfunction
FEV1 at T1	>80%	70-80%	50-69%	<50%
6-MWT (meter)				
T1, median [range]	240 [99-460]	255 [60-430]	210 [40-680]	205 [20-470]
T2, median [range]	380 [260-650]	390 [180-600]	335 [80-720]	270 [50-590]
P	<0.001	<0.001	<0.001	<0.001

6-MWT, Six-Minute-Walk Test; FEV1, forced expiratory volume in one second.

Table 5 Spearmans-Rho correlation between 6-MWT and FEV1

Variable pair	n	Spearman-Rho	t (N-2)
6-MWT-T1 *vs.* FEV1-T1	206	0.21	3,066.00
6-MWT-T2 *vs.* FEV1-T2	167	0.25	46,813.00

n, number of patients; 6-MWT-T1, Six Minute Walking Test at baseline T1; 6-MWT-T2, Six Minute Walking Test at the end T2; FEV1-T1, forced expiratory volume in one second at baseline T1; FEV1-T2, forced expiratory volume in one second at the end T2.

6-MWT and the FEV1 within the scope of spirometry at T1 (rho value =0.21) and at T2 (rho value =0.25) are low (*Table 5*).

Discussion

The 6-MWT is an established diagnostic procedure and well-validated across various age groups, in healthy subjects and in subjects with various cardiac and pulmonary diseases (13). The 6-MWT is used in particular in pulmonary hypertension, COPD exacerbations and chronic heart failure. In healthy subjects and in subjects with COPD, the 6-MWT closely correlates with FEV1 and the partial pressure of oxygen measured on a blood gas analysis (14,15). To the best of our knowledge this is the first study investigating the 6-MWT before and after inpatient rehabilitation in subjects, who had undergone lung surgery related to a malignant disease. In this study we identified a statistically significant improvement of the walking distance measured during 6-MWTs as well as of FEV1 and FVC after inpatient rehabilitation.

However, no improvement with regard to FEV1 and FVC was detected in subjects, who underwent pneumonectomy. Despite the absence of improved FEV1 and FVC in pneumonectoy group the 6-MWT showed a significant improvement after rehabilitation cohort. This finding is likely explained by the multi-dimensional effect of inpatient rehabilitation, which, however, can affect the performance during a 6-MWT. Apart from subjects after pneumonectomy, improvement of the walking distance in 6-MWT as well as lung function parameters (FEV1 and FVC) was not affected by the surgical technique (*Tables 2* and *3*). Moreover, the improvement of both walking distance and lung function values was also not affected by pretreatment. This, however, needs to be interpreted with caution due to the low number of individuals in this subgroup. Irrespective of the severity of the pulmonary dysfunction, graduated by FEV1 at the beginning of rehabilitation (T1), significant improvement of the walking distance was observed. In particular the subgroups with moderate and severe pulmonary dysfunction counted a sufficient number of subjects, so that walking distance has proven to be a reliable parameter at least in these subgroups (*Table 4*).

Regarding the relationship of the walking distance on 6-MWT with FEV1 at T1 and T2 respectively, at both time points a low correlation of the two parameters (spearman's rho for T1 =0.21 and for T2 =0.25) was observed. Thus, it can be postulated that besides FEV1 and FVC also the 6-MWT walking distance can be considered a clinical relevant parameter representing aspects of respiratory function. However, the rho values must be interpreted with caution. The patient cohort examined here is a very heterogeneous one with various tumor diseases. The largest group was composed of subjects with NSCLC, even though there were also subjects with pulmonary metastases of other solid tumor diseases. Even 26 subjects with lung cancer of other histology were included, who did undergo also functionally effective lobectomy or pneumonectomy. In the parameters of both 6-MWT and FEV1 and FVC, there are very large ranges, which suggest a highly inhomogeneous patient cohort with regard to pulmonary function. In particular the very low values in 6-MWT as

well as in spirometry were due to subjects who underwent pneumonectomy and to partly very old subjects with multiple co-morbidities. Twenty-two percent of the subjects had a previously known COPD already at baseline, which has possibly affected the lung function parameters in a negative way as well. In literature it is well described that a preoperative restriction of lung function with low FEV1 can only account for a slight improvement of the postoperative walking distance (16,17). Unfortunately in our analysis no data are available on preoperative pulmonary function and diffusing capacity.

An improvement of the pO_2 values as well as of heart rate, measured within the scope of 6-MWT, could not be shown. This negative result in our study was most likely due to the high median oxygen saturation values, which existed already at T1, and which have not improved any further in the remaining course of the study. Moreover, it is mentioned in literature that in particular pO_2 does not represent an exact measurement method for lung function or a patient's capacity (18).

Heart rate measurement in 6-MWT at T1 and T2 has not shown any significant change either, while the range was rather large. In particular this large range can be put down to heterogeneous training conditions, comorbidities, postoperative morbidity and subjects' age.

In conclusion, it can be stated based on the available data that walking distance in 6-MWT has a low correlation with the values measured by spirometry, such as FEV1. Measuring the walking distance could be a suitable parameter to assess the respiratory function in patients after lung surgery related to pulmonary tumor manifestations. Measuring the pO_2 or heart rate in 6-MWT is not suitable for this purpose. As a simple and cost-effective test, 6-MWT should therefore be firmly established in functional diagnostics in tumor patients after lung surgery.

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Original Article

Myocardial performance index for detection of subclinical abnormalities in patients with sarcoidosis

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Aim: The aim of this study was to evaluate ventricular functions in patients with sarcoidosis without an obvious heart disease by using tissue Doppler-derived left and right ventricular myocardial performance index (MPI).

Methods: The study population included 45 patient with sarcoidosis (29 men, 16 women; mean age, 44±10 years, mean disease duration, 4.2±2.7 years) and 45 healthy control subjects (31 men, 14 women; mean age, 41±8 years). Cardiac functions were determined using echocardiography, consisting of standard two-dimensional and conventional Doppler and tissue Doppler imaging (TDI). Myocardial tissue Doppler velocities [peak systolic (Sa), early diastolic (Ea), and late diastolic velocities (Aa)] were recorded using spectral pulsed Doppler from the LV free wall, septum, and RV free wall from the apical four chamber view. MPI was also calculated by TDI.

Results: The conventional echocardiographic parameters and tissue Doppler measurements were similar between the patients and controls. Left ventricular MPI (0.490±0.092 *vs.* 0.396±0.088, P=0.010) and right ventricular MPI (0.482±0.132 *vs.* 0.368±0.090, P=0.006) were significantly higher in patients with sarcoidosis than the control subjects. There was a correlation between the disease duration and right and left ventricular MPI (r=0.418, P=0.005; r=0.366, P=0.013, respectively). There was also a correlation between the systolic pulmonary arterial pressure and right ventricular MPI but not left ventricular MPI (r=0.370, P=0.012; r=0.248, P=0.109, respectively). In receiver operating characteristics curve analysis, the cutoff value of left ventricular MPI >0.46 had 92% sensitivity and 64% specificity in predicting left ventricular diastolic dysfunction.

Conclusions: We have demonstrated that tissue Doppler-derived myocardial left and right ventricular MPI were impaired in sarcoidosis patients, although systolic function parameters were comparable in the patients and controls, showed a subclinic impaired ventricular functions in patients with sarcoidosis.

Keywords: Sarcoidosis; cardiac involvement; echocardiography; tissue Doppler imaging (TDI); myocardial performance index (MPI)

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Introduction

Sarcoidosis is a granulomatous disease of unknown cause that involves multiple organ systems, including the lungs, heart, skin, and eyes (1). Involvement of the heart in sarcoidosis may lead to diastolic dysfunction, left ventricular wall motion abnormalities, reduced systolic function, cardiac arrhythmias, and sudden death (1-4).

Although sarcoidosis affects many organs, the most prominent complications are ventricular diastolic and systolic dysfunction and accounts for most of the morbidity, mortality, and sudden death (5). The treatment approach of sarcoidosis should aim at relieving symptoms, controlling the inflammatory process, preventing further worsening

in myocardial function and preventing sudden death. Physicians should not wait until the onset of symptoms to start therapy; the treatment should be started as soon as the diagnosis is made (5). Therefore, early recognition of cardiac abnormalities in these patients is very important for modifying their treatment and improving morbidity and mortality.

'To date, there are some reports investigating the abnormalities of the cardiac functions in patients with sarcoidosis in the literature (6-8). Conventional echocardiographic methods have been used for the assessment of cardiac function in these trials. However, several controversial results were obtained. Hence, a new method for more objectively estimating the cardiac functions in sarcoidosis is needed.'

Current developments in cardiac ultrasound permitted more precise echocardiographic assessment of cardiac functions. Tissue Doppler imaging (TDI) is a recently developed technique for the quantization of myocardial velocities in the left and right ventricle using low-velocity pulsed wave Doppler interrogation of the myocardium. Recently, a new non-invasive Doppler-derived myocardial performance index (MPI) was proposed by Tei *et al.* (9,10). MPI, which combines both systolic and diastolic function, may give a better reflection of the global left ventricular function than an isolated evaluation of either ejection or relaxation. Increased MPI was shown to be a prognostic index and independent predictor for cardiac death in various heart diseases (11-13).

The aim of this study was to evaluate ventricular functions in patients with sarcoidosis without an obvious heart disease by using tissue Doppler-derived left and right ventricular MPI and to validate this index against conventional measures of systolic and diastolic left ventricular function and pulmonary artery pressure.

Materials and methods

Study population

The study population included 45 consecutive patients with sarcoidosis (29 men; mean age, 44±10 years, mean disease duration, 4.2±2.7 years) and 45 healthy subjects as controls (31 men; mean age, 41±8 years). The patients were referred from our Chest Disease Department. The diagnosis of sarcoidosis was established according to the American Thoracic Society (ATS) recommendations (14). Age, gender, and body mass index (BMI) were recorded. Fasting blood glucose, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglyceride levels, and white blood cell count were recorded. The demographic characteristics, clinical features, and MRI findings of the patients and the controls are given in *Table 1*.

The control subjects had no cardiovascular or any other organ system disease and had normal physical examination, chest roentgenogram, electrocardiogram, and two-dimensional and Doppler echocardiogram. None of the patients had hypertension, renal failure, diabetes mellitus, left ventricle (LV) ejection fraction (EF) lower than 50%, severe valvular regurgitation and moderate or severe valvular stenosis, coronary artery disease, chronic obstructive pulmonary disease, and atrial fibrillation. The patients with poor echocardiographic window were also excluded. This study complied with the Declaration of Helsinki, was approved by the Ethics Committee and the institutional review board of Erciyes University Medical School, and informed consent was obtained from each patient.

Echocardiography

All patients underwent complete transthoracic echocardiographic studies including two-dimensional, color flow, and spectral Doppler as well as TDI with a GE-Vingmed Vivid 7 system (GE-Vingmed Ultrasound AS, Horten, Norway) using a 2.5-MHz transducer. Echocardiographic measurements were taken with patients in the left lateral decubitus position using standard parasternal long- and short-axis and apical views. At least three consecutive beats in sinus rhythm were recorded, and the average values were taken. All measurements were taken according to the American Society of Echocardiographists' recommendations (15). The LV end-diastolic and end-systolic dimensions (LVEDD and LVESD), interventricular septal and posterior wall thickness (IVSd and LPWd) were measured from M-mode recordings of LV cavity with the cursor at the tip of the mitral valve leaflets in the parasternal long axis view. Left ventricular EF was calculated using a modification of Simpson's rule. RV end-diastolic dimension, right and left atrial dimensions were measured in the apical four chamber view. The LV mass was calculated with Devereux formula. Pulmonary arterial systolic pressure (PASP) was calculated by the following formulas (16): RVSP =4 × (peak tricuspid regurgitation velocity)^2 + RAP. PASP = RVSP - peak PV gradient where RVSP = right ventricular systolic pressure, and RAP = right atrial pressure

Table 1 Demographic and clinical futures of the patients and the controls (mean ± SD)

	Sarcoidosis patients (n=45)	Healthy controls (n=45)	P value
Age, years	44±10	41±8	0.408
Gender, F/M	16/29	14/31	0.655
Body mass index, kg/m^2	25±4	24±3	0.480
Heart rate, beats/min	89±10	87±7	0.225
Blood pressure, mmHg			
Systolic blood pressure	129±8	127±6	0.456
Diastolic blood pressure	76±10	74±7	0.298
Systolic pulmonary artery pressure, mmHg	32±9	23±5	0.010
Fasting glucose, mg/dL	103±14	99±15	0.217
Total cholesterol, mg/dL	177±34	170±36	0.708
HDL-cholesterol, mg/dL	35±6	32±8	0.141
LDL-cholesterol , mg/dL	118±27	110±29	0.329
Plasma triglyceride, mg/dL	117±54	127±88	0.630
White blood cell, 1.000/mm^3	6.6±1.9	5.9±1.5	0.187
Clinical manifestation			
Dyspnea, nonspecific chest pain, n [%]	30 [67]	–	
Palpitations, n [%]	12 [27]	–	
Extrapulmonary involvement, n [%]	21 [47]	–	
Pulmonary stage 0-I-II, n [%]	35 [78]	–	
Pulmonary stage III-IV, n [%]	10 [22]	–	
Duration of disease, years	4.2±2.7	–	

BP, blood pressure.

(assessed by inferior vena cava size and collapsibility). Pulmonary hypertension (PH) was diagnosed if systolic pulmonary artery pressure exceeded the upper limits of normal for age- and BMI-adjusted reference ranges (17).

A 2 mm sized sample volume of pulsed wave Doppler was placed at the tip of mitral and tricuspid leaflets to record trans-mitral and trans-tricuspid Doppler velocities. Peak early diastolic velocity (E), peak atrial filling velocity (A), E/A ratio, E wave deceleration time (DT) and isovolumic relaxation time (IVRT) were measured from the LV and RV filling recordings.

Myocardial tissue Doppler velocities [peak systolic (Sa), early diastolic (Ea), and late diastolic velocities (Aa)] were recorded using spectral pulsed Doppler from the LV free wall, septum, and RV free wall from the apical four chamber view (18). The left and right ventricular MPI was calculated as the sum of the isometric contraction time and isometric relaxation time divided by ventricular ejection time (9,10). All echocardiographic measurements were carried out by two experienced observers who were unaware of the clinical data.

Reproducibility of the measurements

Intraobserver and intraobserver variability were assessed in 12 randomly chosen patients. Variability was calculated as the mean percent error, derived as the difference between two sets of measurements, divided by the mean of the observations. Both investigators were blinded to the patients' diagnosis.

Statistical analysis

Continuous variables were given as mean ± SD; categorical variables were defined as percentage. Independent-sample t test was used to compare the study variables between sarcoidosis patients and control subjects. Correlation analyses were performed using the Pearson coefficient of correlation. Receiver-operating characteristic (ROC) curve was used to determine best cutoff value of left ventricular MPI for prediction of left ventricular diastolic dysfunction. A probability value of $P<0.05$ was considered significant, and

Table 2 Comparison of M-mode and pulse wave derived variables of patients and controls (mean ± SD)

	Sarcoidosis patients (n=45)	Healthy controls (n=45)	P value
Ventricular M-mode derived variables			
Right ventricular end-diastolic diameter, mm	31.8±6.3	33.5±7.6	0.228
Left ventricular end-diastolic diameter, mm	45.9±5.2	46.7±6.0	0.793
Left ventricular end-systolic diameter, mm	28.6±4.1	29.7±3.4	0.302
Inter ventricular septum diameter, mm	10.5±2.1	10.0±1.4	0.131
Left ventricular post. wall diameter, mm	9.8±1.4	9.4±1.4	0.330
Left ventricular ejection fraction, %	66.5±6.6	68.7±9.0	0.345
Left atrial size, mm	31.0±4.9	31.3±3.8	0.869
Right atrial size, mm	30.7±4.2	32.7±5.6	0.135
Left ventricular mass, gr	164.1±35.2	155.6±40.5	0.409
Pulse wave Doppler derived mitral inflow variables			
E, cm/s	69.8±16.0	71.9±14.3	0.531
A, cm/s	65.1±14.2	60.3±11.8	0.242
E/A	1.1±0.3	1.2±0.2	0.150
Deceleration time, ms	231.0±59.1	218.7±22.8	0.322
Isovolumetric relaxation time, ms	91.3±15.8	84.6±21.5	0.099
Pulse wave Doppler derived tricuspid inflow variables			
E, cm/s	46.0±11.0	47.3±10.5	0.322
A, cm/s	46.2±11.7	42.7±12.4	0.487
E/A	1.1±0.3	1.2±0.2	0.132
Deceleration time, ms	195.1±32.4	209.6±38.8	0.157
Isovolumetric relaxation time, ms	89.8±16.9	83.6±26.8	0.372

Plus and minus values are means ± SD; RV, right ventricular; LV, left ventricular.

two-tailed P values were used for all statistics. All statistical analyses were carried out using statistical software (SPSS, version 13.0 for Windows; SPSS, Chicago, IL, USA).

Results

Clinical features

According to the basic clinical and demographic characteristics, both groups of the study were similar with regard to age, BMI, fasting glucose, white blood cell count, cholesterol level, and smoking status. All subjects were normotensive and no significant differences in systolic or diastolic blood pressures and heart rate between these two groups were observed. However, systolic pulmonary arterial pressure was significantly higher in sarcoidosis patients than in healthy controls (32±9 *vs.* 23±5 mmHg, P=0.010) (*Table 1*). Patients had received diagnoses of sarcoidosis a mean time of 4.2±2.7 years prior to study enrollment, and 94% of patients had pulmonary involvement.

Echocardiographic data

Comparisons of the baseline echocardiographic values among sarcoidosis patients and healthy controls are shown in *Tables 2* and *3*.

2D echocardiography and standard doppler flow measurements

The results of conventional echocardiographic examinations are summarized in *Table 2*. The LV diameters, EF, and LV diastolic filling parameters such as A wave, DT, and isovolumetric relaxation time except E wave and E/A ratio were comparable in sarcoidosis patients and healthy controls. RV diameters and tricuspid diastolic velocities (E and A), DT, and isovolumetric relaxation time except E wave and E/A ratio were also similar in both groups.

Table 3 Comparisons of myocardial performance index (MPI) data and tissue Doppler velocities among patients and controls (mean ± SD)

	Sarcoidosis patients (n=45)	Healthy controls (n=45)	P value
Tissue Doppler data			
Mitral			
Sa, cm/s	9.1±2.1	9.8±1.9	0.231
Ea, cm/s	11.2±2.7	12.5±2.2	0.074
Aa, cm/s	7.9±2.4	8.1±2.1	0.922
E/Ea	6.6±2.8	5.6±1.6	0.110
Septal			
Sa, cm/s	7.6±1.6	7.8±1.4	0.822
Ea, cm/s	9.0±1.9	8.3±2.3	0.203
Aa, cm/s	11.3±1.7	11.2±2.0	0.224
E/Ea	7.6±2.4	6.8±1.5	0.187
Tricuspid			
Sa, cm/s	11.3±1.6	11.2±2.0	0.952
Ea, cm/s	11.6±2.8	10.3±3.2	0.109
Aa, cm/s	12.3±2.1	10.3±1.8	0.126
E/Ea	4.4±1.2	4.7±1.7	0.329
Myocardial performance index			
LV MPI	0.490±0.092	0.396±0.088	0.010
RV MPI	0.482±0.132	0.368±0.090	0.006

Plus and minus values are means ± SD; MPI, myocardial performance index; RV, right ventricular; LV, left ventricular.

Tissue doppler and MPI data

Comparisons of the tissue Doppler parameters and MPI values among sarcoidosis patients and healthy controls are also summarized in *Table 3*. Systolic velocities of the both ventricles (Sa) were not different between the groups. Similarly, Ea, Aa, and E/Ea values were also comparable between the groups. "Left ventricular MPI (0.490±0.092 *vs.* 0.396±0.088, P=0.010) and right ventricular MPI (0.482±0.132 *vs.* 0.368±0.090, P=0.006) were significantly higher in patients with sarcoidosis than the control subjects". There was a correlation between the disease duration and left and right ventricular MPI (r=0.418, P=0.005; r=0.366, P=0.013, respectively) (*Figure 1*). There was also a correlation between the systolic pulmonary arterial pressure and right ventricular MPI but not left ventricular MPI (r=0.370, P=0.012; r=0.248, P=0.109, respectively) (*Figure 2*). Left ventricular diastolic dysfunction was detected in 18 (40%) of 45 patients; 13 patients presented with diastolic dysfunction I and 5 patients presented with diastolic dysfunction II.

On the basis of the ROC analysis, the cutoff value of left ventricular MPI >0.46 had 92% sensitivity and 64% specificity in predicting left ventricular diastolic dysfunction (*Figure 3*).

Intraobserver and interobserver variability

Intraobserver and interobserver variability for conventional Doppler and TDI-derived variables (LVEDD, LPWD, Sm, Em, Am, and MPI) ranged from 3% to 6%.

Discussion

In this study, we investigated the ventricular functions in patients with sarcoidosis by using high-usefulness TDI. We have demonstrated that tissue Doppler-derived left and right ventricular MPI were impaired in sarcoidosis patients, although systolic and diastolic function parameters were comparable in the patients and controls. We also showed a correlation between the systolic pulmonary arterial pressure and right ventricular MPI in patients with sarcoidosis. According to this study, left ventricular MPI >0.46 measured predicts left ventricular diastolic dysfunction with

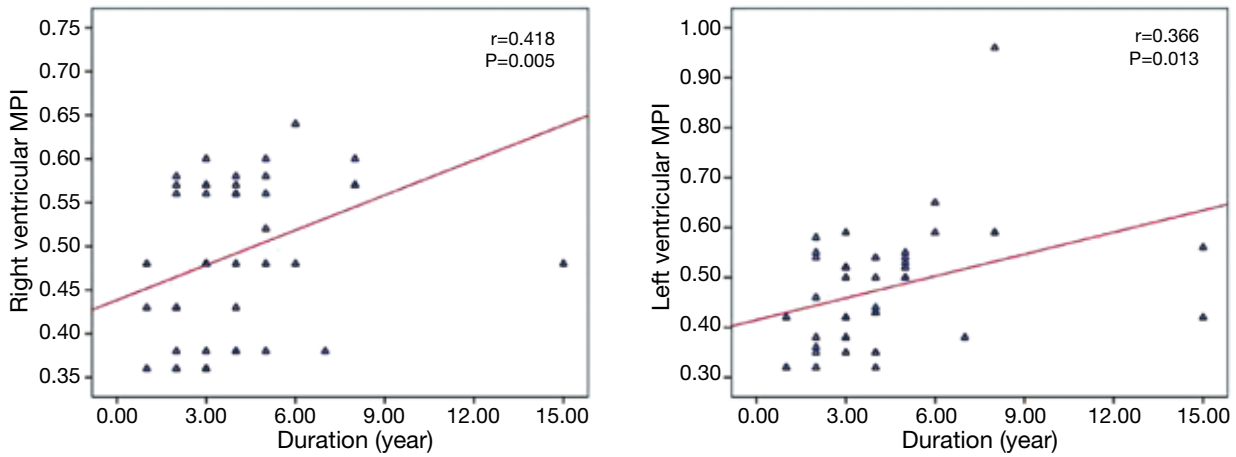

Figure 1 Correlation between the disease duration and right and left ventricular MPI. MPI, myocardial performance index.

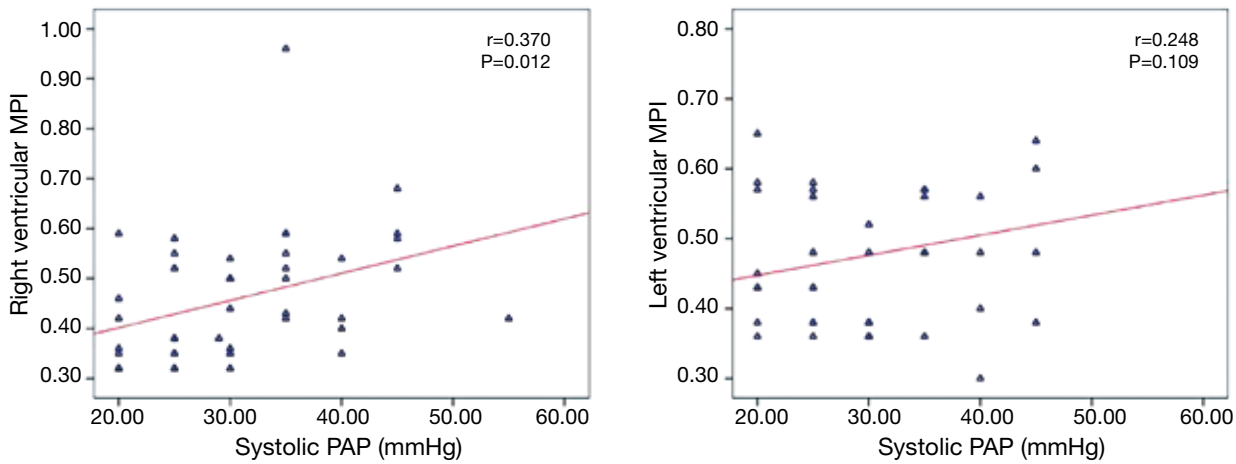

Figure 2 Correlation between the systolic pulmonary arterial pressure and right and left ventricular MPI. MPI, myocardial performance index.

92% sensitivity and 64% specificity.

There are only a few reports in the literature related to LV diastolic function in patients with sarcoidosis (6-8). The first related reports determined diastolic dysfunction according to the criteria based on the amount of patients who had E/A <1 in conventional Doppler echocardiography. Fahy *et al.* (6) studied 50 patients with sarcoidosis. Seven patients (14%) had diastolic dysfunction by mitral inflow pattern. In another study, Ucar *et al.* (7) examined 30 patients with pulmonary sarcoidosis in their prospective study. They found left ventricular diastolic dysfunction in 16 patients (63.3%) by pulse wave Doppler echocardiography. Sköld *et al.* (8) showed the presence of both systolic and diastolic dysfunction in patients with cardiac sarcoidosis documented by Doppler echocardiography and magnetic resonance imaging. Diastolic dysfunction was found in 56-59% of the patients. In our study, left ventricular diastolic dysfunction was detected in 18 (40%) of 45 patients by mitral inflow pattern.

Several mechanisms may be responsible for ventricular dysfunction in patients with sarcoidosis. One of the main possible mechanisms is cardiac infiltration by sarcoid granulomas which may cause left ventricular diastolic dysfunction due to ventricular stiffness or reduced systolic contractile function, or both (5).

MPI, as an echocardiographic parameter summarizing right and left ventricular systolic and diastolic function, was measured as the sum of the isometric contraction time and isometric relaxation time divided by ventricular ejection time (9). In this study, to find out more accurate and objective evaluation of cardiac changes in patients with sarcoidosis, we have used tissue Doppler-derived MPI in

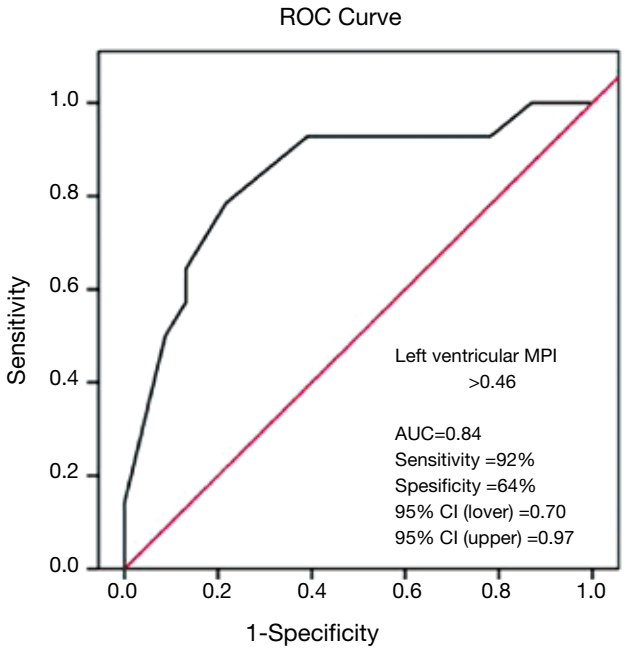

Figure 3 ROC curve of left ventricular MPI for predicting left ventricular diastolic dysfunction. RDC, receiver-operating characteristic; MPI, myocardial performance index.

addition to conventional echocardiographic measurements and other tissue Doppler modalities. We used MPI for the reason that MPI is easily measurable, reproducible, and have been clinically helpful in assessing global ventricular function in both left and right ventricles. The MPI is independent of heart rate and ventricular geometry and also superior to conventional methods, because it is relatively unaffected by significant changes in preload and afterload (9,10). In addition, MPI might be more accurate and reflective of overall cardiac dysfunction than systolic and diastolic measures alone. As a result, this new modality might be more sensitive for detecting subclinical abnormalities (19,20).

To our knowledge, there is only one report in the literature of ventricular function evaluated by MPI in patients with sarcoidosis (21). However, several studies have evaluated ventricular function using MPI in patients with PH, Behcet's disease, mitral stenosis, hypertension, and diabetes mellitus (22-26). Dyer *et al.* (22) reported that patients with idiopathic pulmonary artery hypertension had worse RV and LV MPI than healthy volunteers. Tavil *et al.* (23) also showed that RV MPI patients with Behcet' disease had higher RV and LV MPI values. Ozdemir *et al.* (24) demonstrated that TDI-derived RV MPI correlates well with pulmonary arterial pressure in patients with mitral stenosis. Karvounis and Yilmaz *et al.* showed that patients with hypertension and diabetes mellitus had higher ventricular MPI compared with healthy volunteers (25,26). Our study demonstrates that ventricular MPI of both ventricles were higher in patients with sarcoidosis than in healthy controls.

In our study, we also showed preserved RV systolic function in all sarcoidosis patients, as indicated by normal tricuspid annular systolic myocardial velocities. However, RV diastolic function was impaired in sarcoidosis patients, as indicated by increased late tricuspid annular velocity and a reduced ratio of early to late tricuspid annular velocities. We confirmed this RV diastolic dysfunction with assessment of RV MPI. While RV systolic function was found to be preserved in patients with sarcoidosis, the increased RV MPI is most likely explained by decreased diastolic function. The diastolic dysfunction of the right ventricle in patients with sarcoidosis seems to be an early subclinical manifestation of myocardial diastolic derangement, similar to that previously reported in other cardiac disease, including systemic hypertension and hypertrophic cardiomyopathy (27,28).

In the current study, we evaluated the left ventricular function using TDI and we calculated TDI-derived MPI in sarcoidosis patients. We demonstrated that LV systolic and diastolic functions are preserved in sarcoidosis patients, as indicated by normal mitral annular systolic and diastolic myocardial velocities and normal ratio of early to late mitral annular diastolic velocities.

PH is a well-known complication of sarcoidosis. The prevalence of PH has been reported to range from 4% to 44% in patients with sarcoidosis (29,30). Handa *et al.* (30) found that the frequency of PH was 5.7% evaluated with Doppler echocardiography in sarcoidosis patients. In our study, ten patients (22%) have PH and our results were comparable with previous studies. We found also a correlation between the systolic pulmonary arterial pressure and right ventricular MPI. Consequently, RV MPI can be considered noninvasive parameter in monitoring the progression of the disease.

The diastolic dysfunction of right ventricle may be a result of increased RV afterload due to PH. Pulmonary parenchymal involvement in sarcoidosis causes fibrosis and devastation of the pulmonary vessels, resulting in an irreversibly obliterated pulmonary vascular bed. Meanwhile, vascular involvement of sarcoidosis and extrinsic compression of pulmonary arteries by enlarged mediastinal lymph nodes can cause PH in the absence of significant pulmonary fibrosis can also cause PH in Sarcoidosis.

In addition, we also found a weak but significant association between disease duration and MPI in patients with sarcoidosis. If the patients had more disease duration, this correlation would be more prominent. For that reason, further studies that examine the long-term outcomes of cardiac involvements are needed to elucidate this association.

Conclusions

MPI is useful index to evaluate right and left ventricular functions in patients with sarcoidosis. In the present study, increased MPI in both ventricles, whereas LV EF was normal, showed a subclinic impaired ventricular functions in patients with sarcoidosis.

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Original Article

Effectiveness and safety of diagnostic flexi-rigid thoracoscopy in differentiating exudative pleural effusion of unknown etiology: a retrospective study of 215 patients

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Background: The aim of this study was to evaluate the effectiveness and safety of diagnostic flexi-rigid thoracoscopy in differentiating exudative pleural effusion of unknown etiology.

Methods: A total of 215 patients with undiagnosed exudative pleural effusion were consecutively recruited between January 2011 and February 2013. Thoracoscopy was carried out under local anesthesia, and multisite pleural biopsies were performed using a flexi-rigid thoracoscope. The tolerance of the patients, surgical complications and postoperative pathological diagnosis rate were used to evaluate the effectiveness and safety of the thoracoscopy procedures.

Results: All patients, Karnofsky performance status (KPS) >70, could tolerate both the thoracoscopic surgery and pleural biopsy; there were no severe complications. Thoracoscopic findings included pleural hyperaemia, fibrinous adhesion, nodular bulge and fester. The pathological biopsy confirmed diagnoses of malignant tumor (97 cases), tuberculous pleuritis (91 cases), tuberculous empyema (one case), pulmonary schistosomiasis (one case) and unknown etiology (25 cases). The total diagnosis rate was 88.4%. Subcutaneous emphysema occurred in ten cases and fever in six cases, all of which recovered completely with conservative treatment.

Conclusions: Flexi-rigid thoracoscopy had a high diagnosis rate, differentiating exudative pleural effusion of unknown etiology with satisfactory effectiveness and safety. There was high degree of relationship between thoracoscopic appearance and primary disease or tumor classification.

Keywords: Pleural effusion; flexi-rigid thoracoscopy; diagnosis

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Introduction

Pleural effusion, a condition commonly encountered in respiratory medicine, is classified as transudative or exudative based on criteria established by Light (1,2). Among the most common causes of exudative pleural effusion are tuberculosis, malignant tumors, parapneumonic effusion, and connective tissue disease. The accurate diagnosis of pleural effusion is challenging because even after thoracocentesis and/or closed pleural biopsy, 25-40% of pleural effusions remain undiagnosed (3). Although the accuracy of Light's criteria to identify pleural exudates is very high, a recent review has described a relatively lower specificity when applying these criteria (4). Recent reports have shown that after a series of tests including chest CT, bronchoscopy, experimental examination of hydrothorax and exfoliative cytology, about 1/4 of exudative pleural effusions were not diagnosed (5,6). Previously, such patients were given antituberculosis treatment, and malignant

pleural effusion was considered if there was no response to the initial therapy. However, accurate diagnosis required thoracotomy and video-assisted thoracoscopic surgery. General anesthesia, significant trauma, high cost and high risk are major disadvantages of these procedures. Medical thoracoscopy is now increasingly used in the diagnosis and treatment of pleural effusions following the availability of video-assisted thoracoscopic equipment and improvements in the clinical skills of respiratory physicians. Both rigid and flexi-rigid thoracoscopy have been shown effective in the diagnosis of pleural diseases.

Attempts to obtain a diagnosis for pleural effusion usually involve fluid aspiration and analysis and closed-needle biopsy specimens of the pleura. However, the results of such procedures are often not satisfactory (7). Flexi-rigid thoracoscopy has been available for clinical use in China since 2004. This relatively new technique has the advantages of requiring only local anaesthesia, ease of operation, low cost, wide vision, reduced complication rates and high patient tolerability. It has been used mainly for the diagnosis and treatment of pleural effusions that could be confirmed by the conventional methods (8,9). In particular, it has a high diagnostic value for malignant pleural effusions (10,11), and pleurodesis can be performed directly for malignant pleural effusions with increasing pleural fluid.

The aim of this retrospective study of 215 patients was to evaluate the effectiveness and safety of diagnostic flexi-rigid thoracoscopy in differentiating exudative pleural effusions of unknown etiology.

Materials and methods

Patients

A total of 215 patients with undiagnosed exudative pleural effusion were consecutively recruited in the Department of Respiratory Medicine, the First College of Clinical Medical Science of China Three Gorges University and Yichang Central People's Hospital, China, between January 2011 and February 2013. The cohort included 118 males and 97 females, with a mean age of 46.5 years (range, 16-80 years). The etiology had not been determined in any of the patients after a series of evaluations including chest CT scan, fiber bronchoscopy, laboratory examination of pleural effusions and exfoliative cytology. Blind percutaneous pleural biopsy was excluded because of its low diagnostic rate. Light's criteria and cholesterol determination in pleural fluid were used to exclude transudative pleural effusions. Histopathology of caseating granulomas or microbiology of acid-fast bacillus was used to make a diagnosis of tuberculous pleurisy. Postoperative patients with unknown etiology were registered and were entered into a strict follow-up system.

Flexi-rigid thoracoscope and ancillary equipment

The flexi-rigid thoracoscope that was used (LTF-240, Olympus Corporation, Tokyo, Japan) has a rigid shaft and a flexible tip. Its accessories and ancillary equipment include a light source (EVIS-240) and TV system, a flexible trocar, biopsy forceps, closed thoracic drainage tubes and bottles.

Preoperative preparation

Preoperative examinations included routine complete blood count, blood type, coagulation function, hepatic and renal function and an electrocardiogram, which, combined with the patient's general condition, assessed the feasibility of the procedure. All patients had a Karnofsky performance status (KPS) >70, and patients with surgical contraindications such as severe cardiopulmonary diseases were excluded. Emergency measures such as endotracheal intubation and cardiopulmonary resuscitation were prepared. Large amounts of free pleural effusion could be directly located with ultrasound; however, patients with small amounts of pleural effusion (78 cases) required a preoperative artificial pneumothorax. The edge of the capsule was chosen for tube insertion in patients with an encapsulated pleural effusion.

Thoracoscopy and postoperative evaluation

The patients were positioned on their uninjured side and were supplied with oxygen through a nasal catheter. Preoperative treatment included intramuscular injection of diazepam (10 mg) and tramadol (0.1). Insertion of the thoracoscope through the chest wall was performed under local anesthesia using 10 mL of 2% lidocaine. A 1-1.5 cm transverse skin incision for tube insertion was made with a scalpel parallel to the rib along the 5-7th intercostal space, between the anterior axillary line and midaxillary line. Blunt dissection proceeded from superficial fasciae to parietal pleura to expose the pleural cavity. Then, a flexible trocar was placed, and the flexi-rigid thoracoscope was inserted into the pleural cavity. The visceral, parietal and diaphragmatic pleura around the incision were inspected in a medial, anterior, superior, posterior, lateral and inferior

order. As much pleural fluid as possible was aspirated, and a pleural biopsy was performed. Any fibrous adhesions or pus mass, if present, was removed using biopsy forceps and a freezing probe. If necessary, normal saline and iodophor were used to wash the pleural cavity. At the end of the procedure, a closed thoracic drainage tube was inserted and attached to a closed drainage bottle. The optimal time of extubation was determined according to the amount of fluid in the drainage bottle. Intraoperative blood pressure, respiration, heart rate, cardiac rhythm and blood oxygen saturation was monitored. Postoperative treatments included ECG monitoring, oxygen therapy and wound care. The tolerance to operation, surgical complications and postoperative pathological diagnosis rate were recorded precisely.

Results

Preoperative diagnoses included 105 cases of right pleural effusion, 62 cases of left pleural effusion and 48 cases of bilateral pleural effusions. Chest radiography and ultrasound demonstrated large amounts of pleural effusion in 74 patients and encapsulated pleural effusion in 45 patients. Bloody pleural fluid was found in 117 of these patients, yellow or grass-green pleural fluid in 97, and chocolate-colored, turbid pleural fluid in one patient. Thoracoscopy and biopsy was successfully performed in all of the patients, including 38 patients who had pleural adhesions. There were no severe complications and the operative time averaged 35 min.

Thoracoscopic findings

Early tuberculous pleuritis was characterized by hyperemic, edematous pleura with uniformly distributed white nodules that were frequently seen in the costophrenic sinus. The pleura become adhesive, encapsulated and fibrinous at later stages. The appearance of metastatic pleural tumors of the parietal pleura or diaphragm varied from a diffuse infiltration to off-white nodules. Angiogenesis was observed around the lesions, some of which were shaped like a cauliflower. A single lesion was quite rare. Malignant pleural mesothelioma was semitransparent, yellowish white or claret-colored, smooth, sessile, with a 'cluster of grapes' appearance like multiple granulomas. The tumors were occasionally widespread along the pleural surface, with varied thickness, unclear boundaries and diffuse thickening. One patient with tuberculosis complicated by pyothorax presented with extensive, irregular, festering, pale nodules. A single off-white nodule was observed in a patient with pulmonary schistosomiasis. Thoracoscopy revealed glandular tissue hyperplasia on the costophrenic angle in a patient with lymphoma (*Figure 1*).

Pathological results of pleural biopsy

All of the patients were successfully taken a biopsy. There were 97 patients with malignant tumors. Adenocarcinoma occurred in 43 of the patients, squamous carcinoma in 15, small cell carcinoma in 19, malignant pleural mesothelioma in 16, malignant lymphoma in one. Atypical malignant cells unable to been accurately classified were seen in three patients. A total of 91 patients were diagnosed with tuberculous pleuritis by histopathology or microbiology. Chronic inflammation and fibrinous pleuritis of histopathology were considered as negative findings of semirigid thoracoscopy, which was found in 25 patients and they had regular follow-ups. Eleven of them were highly suspected and treated by antituberculosis therapy. One patient was pathologically confirmed to have tuberculosis complicated by empyema by a pleural decortication. Pulmonary schistosomiasis occurred in one patient, who had a positive reaction on a Paragonimus antigen intradermal test. A definitive diagnosis could not be made in 25 patients who had regular follow-ups. The total diagnosis rate was 88.4% (190/215).

Complications

All the patients had stable vital signs intraoperatively. Minimal to moderate hemorrhage observed at the biopsy site did not require special treatment. Subcutaneous emphysema occurred in ten cases postoperatively. The patients had complete remission following conservative treatments such as administration of oxygen. Six patients without preoperative fever had an elevated temperature of around 37.5-38.5 ℃ postoperatively. The body temperature of these patients returned to normal within five days after symptomatic treatments. There were no complications such as aeroembolism, pleural reaction or wound infection.

Discussion

Our retrospective analysis showed that the diagnostic rate of exudative pleural effusion with unknown etiology using flexi-rigid thoracoscopy was approximate to 90%. All the patients including the oldest one at 80 years of age were able to undergo thoracoscopic surgery and pleural biopsy. This confirmed the safety of the operation and its tolerance by

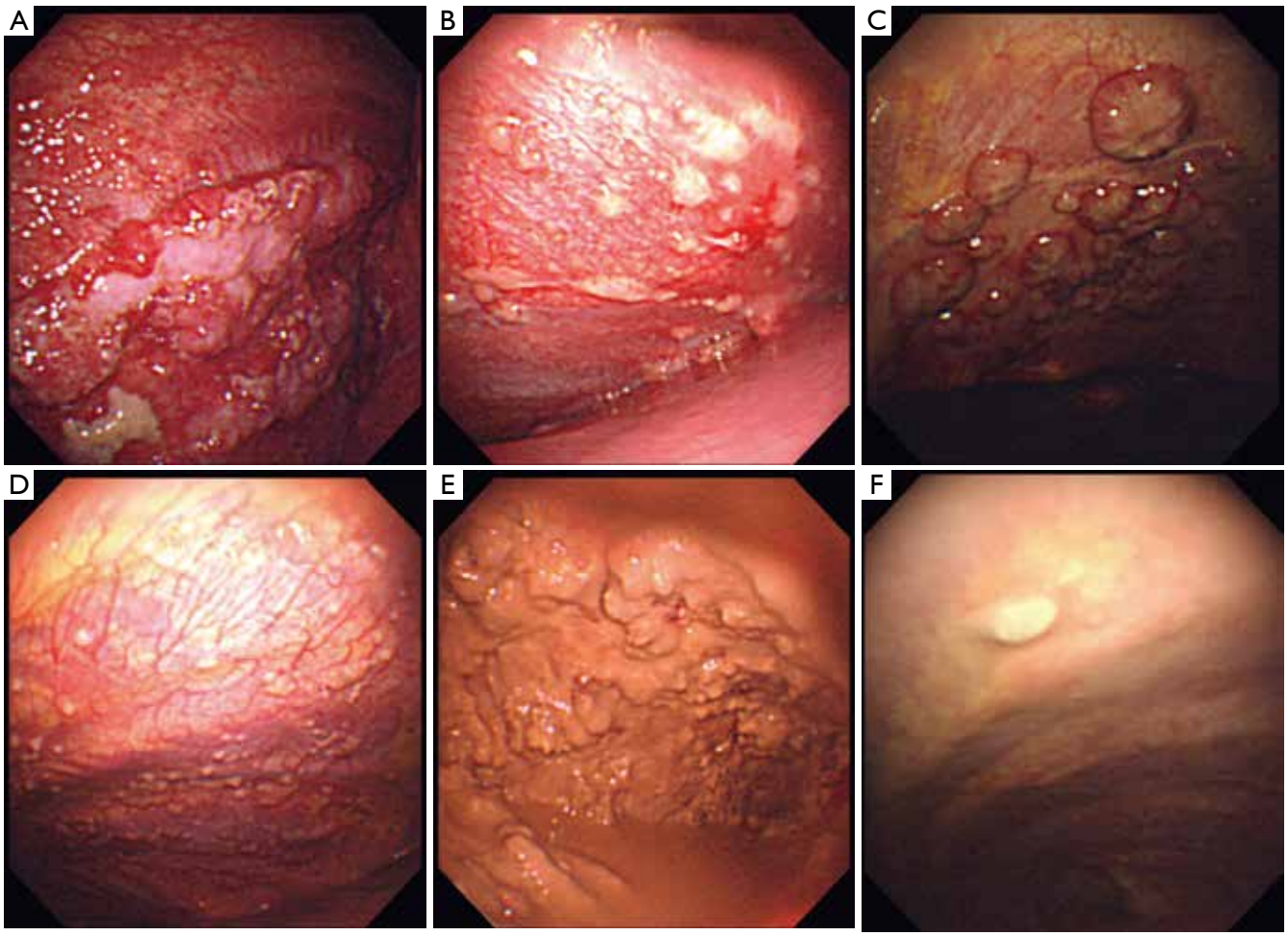

Figure 1 Gross findings under thoracoscopy. (A) squamous carcinoma; (B) adenocarcinoma; (C) malignant mesothelioma; (D) tuberculosis; (E) tuberculosis complicated by empyema; (F) pulmonary schistosomiasis.

patients. There were few surgical complications; ten cases of subcutaneous emphysema and six cases of postoperative fever among 215 patients. All were alleviated through symptomatic treatments. There was strong relationship between thoracoscopic appearance and primary disease or tumor classification. In this study, 91 patients (42.3%) were diagnosed with tuberculous pleuritis, and another 11 patients were considered to have tuberculosis because of their clinical manifestations although chronic inflammation was observed pathologically. The ADA values in tuberculous pleural effusions were 34-86 U/L (median: 52 U/L). Tuberculous pleuritis occurred in patients of all ages, most of which lacked the typical clinical symptoms of tuberculosis. The thoracoscopic characteristics of tuberculous pleuritis appeared as small nodules or wheat grain-like nodules with extensively fibrotic adhesions. Some patients at an early stage of progression had the appearance of inflammation such as hyperaemia and edema or a diffuse, single nodule could be observed. Thoracoscopic decortication can be applied directly to fibrotic tissue to avoid pleural adhesion and hypertrophy. Pulmonary schistosomiasis occurred in one patient, in whom a single white nodule was found on the pleura microscopically. Thus, careful observation is required for surgeons to find suspicious lesions and perform multi-site and multi-point biopsies.

About half of these patients (97/215, 45.1%) with pleural effusions of unknown etiology were finally diagnosed with malignant pleural effusions, in which lung and breast cancers were the most frequent. The lower part of pleural cavity (e.g., the dome of the diaphragm and Harrison's groove) was often involved in malignant pleural metastasis. Macroscopically, tumors had multiple nodules, and were

often shaped like a cauliflower or a bunch of grapes. Close attention should be paid when performing biopsies because of the possibility of angiogenesis surrounding lesions. Clinically, it was difficult to distinguish malignant pleural mesothelioma and metastatic adenocarcinoma. Thus, microscopic characteristics and immunohistochemistry were used to identify them. Thoracoscopy had a high diagnostic rate for malignant pleural effusions, and the small number of false negative results usually occurred in cases of serious pleural adhesions, early malignant mesothelioma and with inexperienced operators. The latest fluorescence-assisted thoracoscopy, when used in diagnosis and clinical staging of malignant pleural disease, was shown to be superior traditional thoracoscopy for detecting micrometastasis lesions that were invisible to the naked eye (10). A recent study indicated that the degree of pleural adhesion as seen by thoracoscopy was an important predictor of survival time of patients with malignant pleural effusions (12). There was also a positive correlation between the degree of pleural adhesion and prognosis (13). For repeatedly occurring malignant pleural effusions, pleurodesis was a better choice, with talc as an effective sclerosing agent (14,15). There was no significant difference of effectiveness between spray or perfusion application of talc. However, talc spray had a higher probability of success for malignant pleural effusion induced by primary lung or breast cancer (16,17). Patient tolerance and safety were both acceptable for talc pleurodesis.

The flexi-rigid thoracoscopy has been designed to combine the best features of rigid thoracoscope and flexible bronchoscope with a proximal rigid shaft and a distal flexible tip, and can be maneuvered akin to the flexible bronchoscope. It has good sensitivity, excellent specificity in diagnosis of exudative pleural effusion of undetermined etiology and the capability to treat pleural diseases directly. Compared with rigid thoracoscopy, flexi-rigid thoracoscopy has several benefits like less need for analgesia and sedation, minimal compression over the rib and the underlying neural structures, easier maneuvering and less complications. The disadvantage would be a smaller size of biopsy tissue and the difficulty to obtain tissue from fibrous pleura. Moreover, rigid thoracoscopy scores over flexi-rigid thoracoscopy in adhesiolysis and management of empyemas (18,19). Improved clinical techniques and understanding of the surgical indications and tolerance of patients allow for strict selection criteria and comprehensive preoperative assessment to avoid, or reduce occurrence of serious complications. Combining the guidelines with experience, thoracoscopy can be performed to directly identify the etiology of pleural effusions, of which the most likely are malignant pleural effusions (20). Once diagnosed with malignant pleural effusions, the survival time of patients is limited, with a poor prognosis. Early, standardized treatment could help to improve the quality of life and extend the survival time of such patients (21,22). For patients with tuberculous pleural effusion diagnosed by thoracoscopy, decisive application of corticosteroids, would promote the regression of pleural effusion when antituberculosis treatment was ineffective. Also, changing empiric antituberculosis treatment to antituberculosis treatment after diagnosis was beneficial to the decisive application of corticosteroids, which would promote the regression of pleural effusion when anti-tuberculosis treatment was ineffective. On the other hand, complete aspiration of pleural fluid using flexi-rigid thoracoscopy could reduce the production of fibrotic adhesions and toxic symptoms. Elimination of fibrotic adhesions in the pleural cavity could reduce the occurrence of pleural hypertrophy and chest deformity.

Although the accuracy of Light's criteria is very high, a decrease in specificity has been noted when applying these criteria. In our study, the concentration of pleural fluid cholesterol >1.55 mmol/L was chosen as an exclusion criterion to improve specificity, and still few transudate pleural effusion cases were probably included in this study (23). Also, for the patients definitively diagnosed with malignant pleural effusions, systemic chemotherapy or targeted therapy was the main choice considering the patient's tolerance. A small number of patients were given postoperative intrapleural chemotherapy but not talc pleurodesis under thoracoscope, which was considered in our subsequent studies.

Conclusions

Diagnostic flexi-rigid thoracoscopy, with the guidance role for primary disease, has the higher diagnosis rate in differentiating exudative pleural effusion of unknown etiology, and it is worthy to wider clinical use because of its satisfactory effectiveness and safety.

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Original Article

Characterization of primary symptoms leading to Chinese patients presenting at hospital with suspected obstructive sleep apnea

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Objectives: We identified the primary symptoms leading to Chinese patients presenting at hospital with suspected obstructive sleep apnea (OSA) and studied the prevalence and characteristics of OSA in confirmed cases.

Methods: We collected data on 350 consecutive patients (302 males and 43±11 years old) with suspected OSA who underwent overnight polysomnography (PSG).

Results: Among all patients, rankings of primary symptoms that led to the patients presenting at hospital for PSG were observed apnea (33%), snoring alone (29%), choking/gasping (13%), daytime sleepiness (5%) and other (20%). For severe OSA, prevalence rate was 61%, apnea hypopnea index (AHI) was 64±18, age was 44±10 years old, body mass index (BMI) was 28±3.5 kg/m^2, and hypertension rate was 28%.

Conclusions: Self-awareness of symptoms led a majority of the patients to present at hospital in China. Compared to currently available case series studies, our results suggest that OSA patients in East Asian countries are characterized by higher prevalence and more severe apnea, younger age, poorer sleep quality, but less obesity and less comorbidity with hypertension, relative to countries in North America, South America and Europe.

Keywords: Apnea; symptoms; prevalence; consecutive patients

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Introduction

Obstructive sleep apnea (OSA) is highly common in the general population and may affect 9% to 17% of adults with higher rates in men (1). Recurring episodes of apnea during sleep is the central pathological component of OSA, but symptoms and associated illnesses vary among individual patients. Symptoms typically include loud snoring, witnessed episodes of apnea during sleep, gasping and daytime sleepiness. OSA also has significant comorbidity with hypertension, cardiovascular diseases, stroke and diabetes as well as other illnesses (2). Effective treatment, even for mild OSA patients, may ameliorate comorbid conditions.

Though symptoms vary among OSA patients, there are two primary reasons for which potential patients present to a sleep laboratory for polysomnography (PSG). Patients may be self-aware of symptoms and seek medical assistance or they may be referred by a physician who notes OSA related symptoms in patients who have presented with hypertension, heart diseases, stroke or other comorbidities. Patient's self-awareness may largely arise from public awareness programs whereas referral likely reflects the cognizance of OSA by physicians. Clarifying these issues would be valuable for strategizing for the development and improvement of respiratory sleep medicine. For example, a low number of referred OSA patients would suggest that we may need to increase education related to respiratory sleep medicine knowledge to physicians. Further, these two patient pools could influence the evaluation and

Table 1 Number and percentage of PSG prescriptions ranked according to the outpatient departments where the patients initially presented

Department	Number	%
Sleep center	189	54.0
E.N.T.	113	32.3
Respiratory	23	6.6
Others	25	7.1
Total	350	100.0

PSG, polysomnography; E.N.T., ear, nose and throat.

characterization of OSA as well as differentially skew data regarding OSA prevalence in hospital based studies. For instance, if the data include fewer referred OSA patients, the average age of the pool may be younger. In the present investigation, we used a case series approach to identify the primary symptoms that lead to patients presenting at our sleep medicine center in China.

However, many complicated factors may impact the characteristics of OSA prevalence across races and countries, including biological and socioeconomic factors (3). In the current study, we found a particularly young average age and high prevalence of severe OSA in patients with suspected OSA. To further evaluate potential differences specific to Chinese OSA patients, we therefore compared our results to those in studies of populations of other countries in order to assess potential demographic differences in age and prevalence of severe OSA across nationalities.

Methods

Subjects

The data were collected from October, 2011 to December, 2011 at the Sleep Medicine Center of West China Hospital, Sichuan University (Chengdu, China). During this three months period, 350 (302 males and 48 females, average age 42.9±11.0 years old) consecutive patients with suspected OSA were referred to take an overnight PSG. Data from a subset of these patients, as identified below, were used in the study. This study was approved by the Research Ethics Board of the West China Hospital of Sichuan University.

Clinical data collection

Prior to PSG recording, clinic data collection mainly included three steps. First, we recorded the information concerning PSG prescribed by the physicians across departments. The results were summarized in *Table 1*. Second, routine information was collected for all patients who underwent PSG in our sleep medicine center. These included checklists for general information (e.g., age and gender, totally seven items), symptoms (e.g., snoring, observed apnea and leg movement, choking and morning headache, totally 13 items), comorbidities (e.g., hypertension and heart disease, totally ten items), lifestyle (smoking and alcohol, totally five items) and a checklist for physical examination (height, body weight, blood pressure and pharyngeal tonsils, totally ten items). The Epworth daytime sleepiness scale (ESS) was also evaluated. However, we only present some of most important data in *Table 2*.

Third, we specifically identified the primary symptoms that led to the patients presenting at the sleep medicine center for PSG for this study. In this step, we attempted to classify the primary symptoms as a distinct category. For example, patients presenting with a complaint of loud snoring alone with no other symptoms were classified as "snoring". However, many patients might have multiple symptoms. We carefully investigated what the single primary issue that led to present the hospital. For example, if a patient had symptoms of snoring, observed episodes of apnea, daytime sleepiness and hypertension, he might answered that "my wife observed the apnea and pushed me to come to the hospital", he then would be classified as "observed apnea"; if his answer was daytime sleepiness, he would be recognized into that category; if he stated that due to "I have suffered from hypertension and PSG was prescribed by my doctor of internal medicine", he would be categorized as "hypertension". In fact, most patients could clearly identify as one of them. However, if the patients presented with insomnia, regardless of whether the patients were suspected of OSA, they would be placed into an insomnia category in our registration system and excluded from the investigation.

Overnight PSG

The diagnosis of OSA was established by a standard overnight PSG. Overnight PSG consisted of continuous recordings from six electroencephalographic leads (F3-A2, F4-C1, C3-A2, C4-A1, O1-A2, O2-A1, international 10-20 system), two electrooculographic leads (ROCA1, LOC-A2), four electromyography leads (two submental and bilateral tibialis anterior), thermistors for nasal and oral airflow, strain gauges for thoracic and abdominal excursion,

Table 2 Clinical and polysomnographic characteristics in each group of AHI <5, 5~<15, 15~<30, ≥30 (different letters indicate significant differences across the groups of AHI <5, 5~<15, 15~<30, ≥30, as assessed by post hoc Tukey tests)

	AHI			
	<5 (n=35) (10%)	5~<15 (n=44) (12.6%)	15~<30 (n=57) (16.3%)	≥30 (n=214) (61.1%)
Age	37.0±12.8[b]	43.0±12.1[a]	43.1±11.0[a]	43.7±10.3[a]
BMI	26.0±4.0[b]	27.0±3.3[a,b]	26.1±3.2[b]	28.0±3.5[a]
ESS	7.7±5.9	9.1±6.4	8.3±6.3	10.7±6.6
Hypertension, n (%)	3 (8.6)[a,b]	4 (9.1)[b]	9 (15.8)[a,b]	60 (28.0)[a]
Diabetes, n (%)	3 (8.8)	4 (9.1)	2 (3.5)	9 (4.2)
TST (min)	391.9±78.5[c]	432.3±72.8[a,b]	417.4±79.2[c,b]	468.0±74.3[a]
N1 (min)	61.8±34.9[b]	78.0±31.4[b]	93.0±69.3[b]	172.5±115.4[a]
N2 (min)	216.1±66.0	238.1±50.0	221.9±76.2	203.1±96.6
N3 (min)	49.3±28.5[a]	43.3±25.7[a]	40.4±25.4[a]	25.0±26.4[b]
REM (min)	64.7±30.8	73.0±34.8	62.2±35.0	67.7±34.4
AHI	2.3±1.4[d]	11.0±2.9[c]	21.7±4.3[b]	63.6±18.0[a]
Arousal index	21.0±9.7[b]	26.1±9.7[b]	27.2±13.7[b]	51.8±19.8[a]

BMI, body mass index; ESS, Epworth daytime sleepiness scale; TST, total sleep time; REM, rapid eye movement sleep; N1-3, Non-REM sleep stage 1-3; AHI, apnea hypopnea index.

finger pulse oximetry, and electrocardiography. Thirty-second epochs were analyzed and sleep stages were scored according to the international criteria of American Academy of Sleep Medicine. An apnea was defined as more than 90% reduction in airflow for at least 10 s; hypopnea as 50% or more reduction of airflow for at least 10 s associated with 3% or more reduction in oxygen saturation.

Statistical analysis

Comparisons between both groups of patients were performed using independent *t*-tests and the post hoc Tukey test for normally and abnormally distributed data, respectively. Chi-square analyses were used for categorical data (hypertension and diabetes). Statistical significance was defined using a P value of 0.05. Statistical analyses were performed with Statistic Package for Social Science (SPSS) for Windows software (version 17.0). Results are shown as mean ± standard deviation (SD).

Results

As shown in *Table 1*, of the 350 patients, 54% and 32% (total 86%) patients directly came to the outpatient clinics of our sleep medicine center and ear, nose and throat (E.N.T.) department, respectively. Most had clear complaints of apnea and snoring. Another 7% presented at the outpatient clinic of respiratory medicine and the remaining 7% presented at the outpatient clinics in other departments, such as internal medicine, neurology, Chinese sleep medicine, etc.

Of the consecutive 350 patients with clinic suspicion of OSA, we identified 24 different primary symptoms that led to patients presenting at the clinic. As shown in *Figure 1*, the most prevalent primary symptoms were witnessed apnea (32.9%), snoring alone (28.9%), and choking/gasping (13.7%). *Figure 1* also presents eight primary symptoms with less percentages of occurrence [e.g., daytime sleepiness (4.9%), etc.], and rest of 13 primary symptoms were classified as "other" (6.3%) presented in *Figure 1*. The 13 symptoms included pharynx choking (n=3), bruxism (n=2), nose bleeds (n=3), numbness in hands and feet (n=2), tonsillitis (n=3), irritability (n=1), polycythemia (n=1), chronic bronchitis (n=1), excessive dreaming (n=1), regurgitation of food (n=1), rhinitis (n=2), turbinate hypertrophy (n=1), and pleurisy (n=1).

Table 3 presents the percentage and apnea hypopnea index (AHI) mean values for AHI 5~<15 (mild), 15~<30 (moderate), ≥30 (severe) for each grouping of primary symptoms. Considering the distribution of patients' number

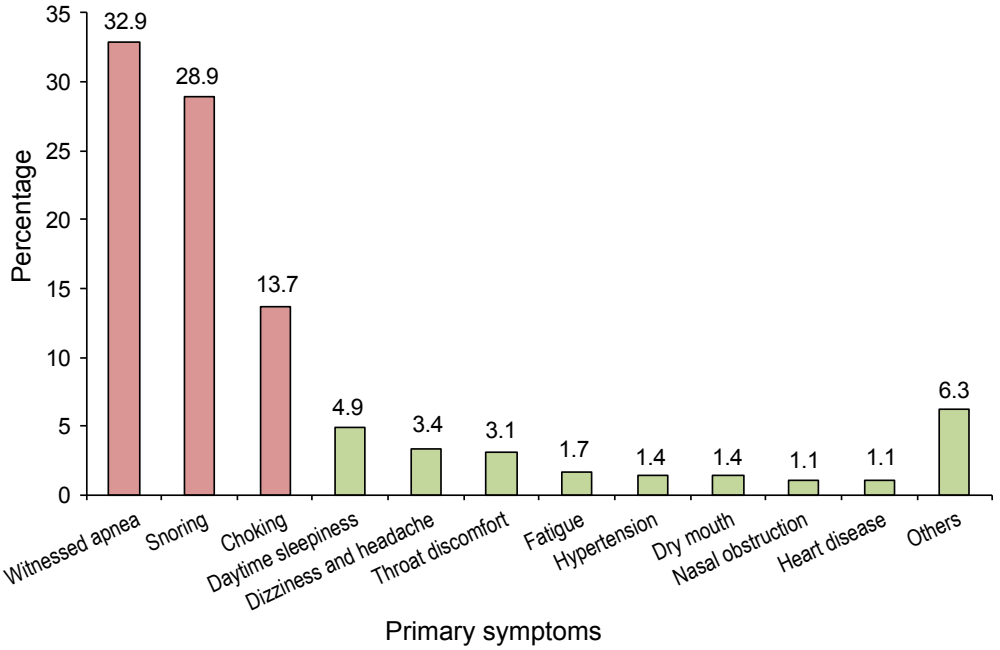

Figure 1 Percentage of primary symptoms for 350 patients with clinic suspicion of OSA who presented at sleep laboratory for overnight PSG. OSA, obstructive sleep apnea; PSG, polysomnography.

Table 3 Percentage and AHI mean values (± SD) for AHI 5~<15, 15~<30, ≥30 in each group of primary symptom patients (different letters indicate significant differences across the groups with different primary symptoms, assessed by post hoc Tukey tests)

	Witnessed apnea (n=115)	Snoring (n=101)	Choking (n=48)	Daytime sleepiness (n=17)	Others (n=69)
AHI 5~<15					
n (%)	12 (10.4)	18 (17.8)	6 (12.5)	1 (5.9)	7 (10.1)
Mean	9.3±2.9	11.9±2.9	12.3±2.3	12.2	10.0±2.4
AHI 15~<30					
n (%)	20 (17.4)	16 (15.8)	5 (10.4)	0 (0)	16 (23.2)
Mean	22.5±3.4	21.4±4.6	19.4±3.8	0.0	21.6±5.1
AHI ≥30					
n (%)	79 (68.7)	55 (54.5)	28 (58.3)	15 (88.2)	37 (53.6)
Mean	63.8±17.7[b]	60.0±14.5[b]	65.4±15.5[b]	82.5±20.1[a]	59.3±19.6[b]

AHI, apnea hypopnea index; SD, standard deviation.

in the different primary symptoms, we divided the patients into five different groups according to primary symptoms as shown in *Figure 1*. Using severe OSA criteria (AHI ≥30), the rankings of OSA percentage across symptom groups were daytime sleepiness (88%) > witnessed apnea (69%) > choking (58%) = snoring (55%) = rest (54%). The AHI in the daytime sleepiness group was significantly greater than that found in the other four symptom groups.

As shown in *Table 2*, 12.6%, 16.3% and 61.1% of the patients had AHI between 5~<15, 15~<30, and greater than 30, respectively. Compared to patients with AHI <5, those with mild, moderate and severe OSA, had significantly greater ages. Patients with severe OSA had greater BMI than those with AHI <5 and moderate OSA, higher likelihood of hypertension (28%) than those with mild OSA (9%). With regard to sleep parameters, patients with severe OSA had consistently greater arousal index and time spent in N1, and fewer amount of N3 time, compared to those with AHI <5,

mild and moderate OSA.

Discussion

Immediate data interpretation

Of the 350 patients who presented at our sleep medicine center to take an overnight study for OSA diagnosis, nearly 80% had self-aware symptoms of snoring alone, witnessed apnea, choking/gasping, daytime sleepiness and others (*Figure 1*). Additionally, 86% of patients presented at outpatient the clinics of the sleep medicine center (54%) and ENT (32%) had clear concerns for OSA; some patients seen at the ENT clinic had throat discomfort, nasal obstruction and other symptoms, and were referred due to their ENT physician's concerns regarding potential OSA. However, only 2.6% of patients with primary symptoms of hypertension and heart diseases were referred for OSA screening. Together, these results indicate that the majority of the patients were aware of their symptoms and presented at the hospital for the specific purpose of solving their sleep breathing problems. China has no established system for family physicians; thus, patients directly go to hospitals and departments based on their understanding of their own medical needs. Additionally, only medium and large sized hospitals have sleep disorder centers capable of conducting overnight PSG studies. The fact that witnessed apnea was the number one primary symptom (33%) that led to patients presenting in the hospital suggests that public education efforts for OSA aimed at middle aged spouses, particularly to women (the results showed that patients were 86% male, age was 43±11 years old, and most were married) is likely an important approach to enhance respiratory sleep medicine in China. In addition, for witnessed apnea and snoring alone, the percentage for severe OSA (AHI greater than 30) was higher among individuals with the complaint of witnessed apnea (69%) compared to those with the complaint of loud snoring alone (54%), but the differences were not as great as we expected. This suggests, for more severe OSA patients, that the apnea might not be noted by bed partners; thus, routine PSG screen for OSA is still necessary for loud snoring individuals.

Characterization of OSA in East Asians compared to other regions in the literature

In this case series of 350 patients, mean age was 43±11 years and the prevalent rate of severe OSA (AHI ≥30) was 61%. Among diagnosed severe OSA patients (n=214), age was 43.7±10.3 years old, BMI was 28±3.5 kg/m^2, AHI was 54±18, hypertension was 28%, and sleep architecture was characterized by a nearly threefold increase in N1 time and more than a twofold decrease in N3 time. Those are likely the most important parameters to evaluate OSA prevalence and severity.

By comparing our data to that of published studies, we noted that the age of patients appears to be younger and the prevalence of severe OSA appears to be higher in the countries of East Asian compared to other regions. In order assess these potential differences in a more detailed manner, through an extensive literature review, we obtained, through PubMed, 470 papers based on the search terms "sleep apnea and consecutive and age" that were published during the recent ten years. We eliminated non-case series and papers that focused on children, a specific male or female sample, a specific illness, a specific age, a community based study and so on. We found 28 cases serial studies conducted in 17 different countries, which we believe are comparable to our study. In *Figure 2*, including the results of current study (labeled as "Current"), we present average age in patients with suspected OSA from 29 reports (left panel) and prevalence of severe OSA (AHI >30) in 11 reports (right panel). As clearly reflect in *Figure 2*, eight studies with the youngest age (from left to right) were conducted in four different countries in Eastern Asia [South Korea, 42 years (4); Singapore, 43 and 45 years (5,6); China, 43 (current study) and 45 years (7); Japan, 46, 47 and 48 years (8-10)]. By comparison, in the remaining 21 studies with ages above 48 years, only three were reported from Japan (49, 50 and 50 years old) (12,15,16) and rest were reported from 13 different countries in North America (13,18,20,25), South America (23), Europe (17,21,22,24,26-31) and the Middle East (11,14,19). The average age in all 11 reports from the countries of Eastern Asia was 46 (range from 42 to 50) years, whereas it was 52 (range from 48 to 57) years in the 18 reports from the other countries. We found only ten papers that clearly indicated the prevalence of severe OSA prevalence among those we examined. As shown in the right panel of *Figure 2*, the three highest percentages were reported in the current study conducted in China (61%) and in two studies conducted in Japan (58%, 45%) (15,32), no studies reported the percentage above 45% (range, 45-16%) in the remaining 8 papers conduced in other regions (11,13,17,21,26,28,33,34). Even using the criteria of AHI >40, the prevalence still reached 44% in one study in China (7). Review of these reports plus our current study

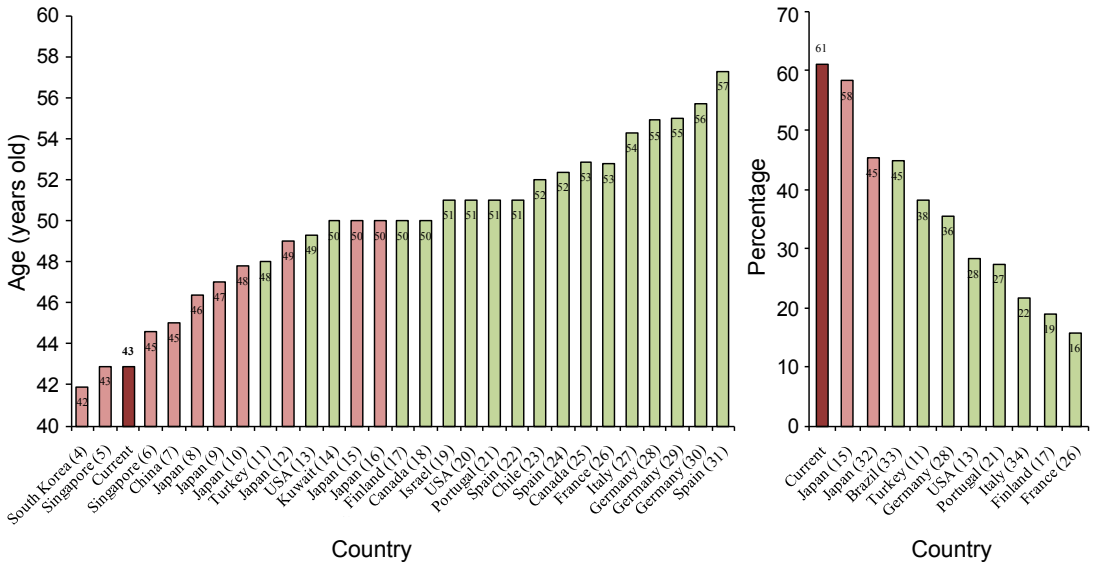

Figure 2 Summary of average ages (left panel) (4-14) in total observed cases (15-31) and prevalence rate (%) for severe OSA (AHI >30) (right panel) (11,13,15,17,21,26,28,32-34) across different countries determined through case series studies. Our results are indicated as Current. Pink bar indicate East Asia countries. OSA, obstructive sleep apnea; AHI, apnea hypopnea index.

suggest that the age onset of illness is considerably younger and that prevalence is remarkably higher for OSA in East Asia, compared to North America, South America and Europe.

OSA patients in East Asia may also have greater AHI, lower BMI, more severe objective sleep changes, and less hypertension comorbidity compared to those in North America, South America and Europe. For example, for severity of OSA, the current work shows that the means of AHI was 64/h for severe OSA patients, whereas mean AHI was 43/h for severe OSA patients in a similar study in Europe (34). Regarding BMI, the current investigation shows a mean of BMI of 28 kg/m^2, which is close to that in similar studies in the mainland of China (27 kg/m^2) (7), Singapore (28 kg/m^2) (5) and Japan (25-28 kg/m^2) (9,10,12,15). In contrast, BMI appeared to be markedly higher in the USA (34-37 kg/m^2) (13,20), Italy (36 kg/m^2) (27), Spain (30-32 kg/m^2) (22,24,31), Germany (32 kg/m^2) (29,30), Portugal (31 kg/m^2) (21), and in Canada (32 kg/m^2) (25). For hypertension in severe OSA patients, we found comorbidity was 28%, but studies report comorbidity of 67% in Italy (34), 44% in USA (13) and 41% in Turkey (11).

For mild, moderate and severe OSA, we found significant increases in the number of brief arousals and N1 time, and a decrease in N3 time, compared to patients with AHI less than five. Similar disruptions in objective sleep measures have been consistently reported in OSA studies, but the severity appears to be less than the level we found. For example, we found a threefold increase in N1 time and a nearly twofold decrease in N3 time in the severe OSA group relative to the group with AHI less than five. Previous studies have not reported similar levels of changes in sleep.

Biological or socioeconomic issues?

Through extensive literature review (described in the Results section) of currently available case series studies, our results suggest that OSA patients in countries of East Asia are characterized by higher prevalence, more severe apnea, younger age, poorer sleep quality, but less obesity and less comorbidity with hypertension, relative to countries in North America, South America and Europe. As extensively reviewed by Villaneuva *et al.* (3), we believe there are at least two major factors which may impact the prevalence of OSA in East Asian populations.

First, narrower upper airways, which are related to congenital ethnic characteristics of the skeletal craniofacial structure of East Asians compared to Caucasians (3), possibly lead to OSA patients in this population having higher prevalence, more severe symptoms and earlier age of onset, compared to other races. For the consistently large differences in prevalence and average age in the countries of East Asia compared to the countries of North America, South America and Europe, we believe that this

biological issue may play the major role in differences across races. In particular, the current investigation and previous studies showed that mean patient BMI was 25-29 kg/m^2 in East Asians (5,7,9,10,12,15), whereas mean BMI was 30-37 kg/m^2 in Caucasians (13,20-22,24,25,27,29-31). The characteristic of relatively low BMI may implicate that local anatomic factors may play more important role than systematic factors in the high prevalence of OSA in East Asians.

Secondly, complicated differences in socioeconomic levels and both general and medical education levels across institutes, societies, regions, and countries would also significantly affect the evaluation of prevalence in case series study. For example, cardiovascular physicians knowledgeable of sleep breathing disorders who regularly prescribe PSG examinations may significantly change the composition of data collected at a single sleep medicine center, driving an increased average age for hospital based studies. It is possible that more developed countries may have better training in sleep medicine, and thus physicians may pay more attention to OSA comorbidities which could lead to differences in the constitution of the patient pool. In addition, the fact of underdevelopment of sleep medicine in China may drive greater percentage of severe OSA patients to seek medical assistance. As we can see in the left panel of *Figure 2*, among the five countries of East Asia, Japan may be the most developed country and has the oldest reported ages in three studies collected in that country.

We believe that identifying the factors underlying this difference is an important issue that needs to be addressed in future research. However, we are aware that the current work does not distinguish many complicated factors underlying the characteristics of prevalence of OSA in hospital based studies. For example, the study was carried out in a single institute and did not reflect the characteristics of the prevalence of OSA in the entire nation of China. Secondly, for insomnia patients, snoring-associated issues are not normally the primary factors driving patients to seek medical assistance. The current study is focused on the characterization of primary symptoms in possible OSA patients. Therefore we did not include insomnia complaints into this study, though the exclusion of insomnia may have resulted in exclusion of some OSA patients, particularly elderly patients.

Conclusions

In summary, the current results and existing literature suggest that OSA in East Asian countries may have higher prevalence, younger onset age, greater severity and poorer sleep quality, but with less obesity and less comorbidity with hypertension, relative to OSA in Caucasians and other races.

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Original Article

Symptom prevalence and risk factors for asthma at the rural regions of Denizli, Turkey

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Objective: This study was conducted using a standardised method (ECRHS) to identify the prevalence of asthma symptoms and risk factors in adults who lives in the rural regions of Denizli province.

Methods: This study was designed as a cross-sectional study to investigate the prevalence of asthma and asthma symptoms in adults older than 18 years old, around the rural regions of Denizli. Data were collected by personal interview and filling in questionnaires between July 2009 and September 2009.

Results: A total of 1,343 individuals were enrolled. Prevalence of current asthma was 5.9%, having an asthma-like symptom was 34.0% and allergic rhinitis was 2.5%. Most common asthma symptoms were woken by an attack of breathlessness (20.5%, n=275), woken by attack of cough (19.9%, n=267) and wheezing (12.7%, n=171).

Conclusions: Asthma is an important disease that may occur not only in cities but also at country sides. In rural areas risk factors for asthma and asthma-like symptoms compared to urban areas may show some differences.

Keywords: Asthma; wheezing; dyspnea

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Introduction

Asthma is a heterogenous disorder that is characterized by variable airflow obstruction, airway inflammation and hyperresponsiveness, and reversibility either spontaneously or as a result of treatment (1). Chronic inflammation is related with airway hyperresponsiveness leading to wheezing, shortness of breath, chest tightness and coughing, particularly during night or early in the morning (2,3). Asthma is a global problem estimated to affect 300 million people around the world (4,5). Prevalence of asthma is variable around the world. According to some relatively standardised and comparable studies, prevalence rates of asthma varied between 1% and 18% for children and adults (4,5). Studies from Turkey conducted relying on European Community Respiratory Health Survey (ECRHS) methodology reported the prevalence of asthma between 0.3% and 7.6% and which are similar with most of the European countries (6). Prevalence of asthma varies among the regions of Turkey. It is more prevalent in seaside regions, city centers, big cities and among lower socioeconomic communities (2). Asthma prevalence studies from Turkey relying on standardized methods like ECHRS are usually conducted in city centers. Whereas prevalence and risk factors of asthma vary between city centers and rural regions due to variations in socio-demographic, geographic and economic conditions. Risk factors of asthma can be outlined under personal and environmental factors. Heredity, atopy, gender and obesity are the main subtitles of personal factors. Environmental factors comprise of allergens, infections (especially viruses), occupational sensitisers, smoking (active and also passive), air pollution (inner and outer) and lifestyle (rural life, diet, consumption of antibiotics etc.) (1,7). Symptoms of asthma are described as wheezing, shortness of breath and coughing. This study was conducted using a standardised method (ECRHS) to identify the prevalence of asthma

symptoms and risk factors in adults who lives in the rural regions of Denizli province.

Methods

Characteristics of the study region

Denizli is located in the southwest of Anadolu peninsula to the northeast of Ege Region, standing on a corridor among Ege-Central Anadolu and Akdeniz regions. Climate of Denizli is not uniformly resemble seaside climate of Ege and terrestrial climate of the central Anadolu contributes to climate around Denizli. Province of Denizli has 19 dependent towns with 81 big villages with municipality and 369 small villages. According to 2008 Residential Population Registry Database Denizli has an overall population of 917,836. City center and dependent towns and villages has an overall population of 508,870 while the other provincial towns and villages' population is 408,966. Gender distribution is 457,787 males to 459,049 females (8). Textile industry is the main industrial sector in Denizli. Semi-professional individual workplaces distributed around the town and villages which are sometimes settled at the backyard of houses, has significant contribution to this industrial sector.

Study space and sample size

This study was designed as a cross-sectional study to investigate the prevalence of asthma and asthma symptoms in adults older than 18 years old, around the rural regions of Denizli. Sample size was calculated as 1,063 cases with a 95% confidential interval and 0.03 error margin, and 1,343 individuals were enrolled in the study.

Data collection

Data were collected by personal interview and filling in questionnaires between July 2009 and September 2009. The questionnaire had two parts. In the first part of the questionnaire age, gender, marital status, occupation, education level, socioeconomic status, residence location (must be residing more than three years), population of the residence, conditions of the residence (in terms of humidity, exposure to sunlight), heating system of the residence (central heating or independent with solid fuel), presence and species of pets in the house, presence of cockroach, presence of carpets in the bedroom, proximity of the residence to motorways, status and frequency (packs/year) of smoking in the residence, smoking status of the parents, familial history of atopy and history of mumps were sought as predisposing factors of asthma. In the second part ECRHS questionnaire was utilized to investigate asthma and asthma symptoms. In ECHRS, asthma symptoms of wheezing, wheezing with shortness of breath, wheezing in the absence of a cold, waking with tightness in the chest, woken by an attack of breathlessness and by attack of cough, attack of asthma, treatment for asthma and history of allergic rhinitis were sought for the last year.

After completion of questionnaire pulmonary function test (PFT) was applied according to American Thoracic Society definitions with Spirobank No.A23643 device of MIR Srl Company with the patient in sitting position and every patient had undergone three repeated cycles of PFT (9). Best values for each patient enrolled and data of non-cooperative patients were excluded from the study.

According to ERCHS questionnaires, 'current asthma' was established if one had an attack of asthma and/or treatment for asthma in the last year. The ones who had at least one of the following symptoms were defined as 'the ones who has asthma-like symptoms'; wheezing, waking with tightness in the chest, woken by an attack of breathlessness and by attack of cough.

Statistical analysis

Statistical analyses were carried out using Statistical Program For Social Sciences (SPSS) for Windows (10.0 version) software. Besides definitive statistical methods, Pearson χ^2 test was applied for comparing the categorical variables and a parametric Pearson *t* test was applied for comparing the quantitative data. The effect of independent variables were evaluated with Binary logistic regression analysis. $P<0.05$ was accepted to be statistically significant.

Ethics committee approval

Ethics committee approval was obtained from the local ethics committee of Pamukkale University in accordance with the Declaration of Helsinki. Participants were informed about the study, and those who gave written consent were included.

Results

Gender distribution was 764 females (56.9%) to 579 males (43.1%) among the study group. Mean age of the group

Table 1 Risk factors for asthma

	n	%
Smoking		
Smoker	264	19.7
Ex-smoker	190	14.1
Never	888	66.2
Smoking inside the house		
Yes	364	27.2
No	974	72.8
Family history of atopy		
Yes	129	9.6
No	1,214	90.4
History of mumps		
Yes	200	14.9
No	1,143	85.1
Conditions of the residence		
Good	607	45.3
Moderate	694	51.7
Bed	40	3.0
Heating system of the residence		
Coal-fired stove	1,304	97.1
Gas stove	1	0.1
Central heating	38	2.8
Presence of pets in the house		
Yes	91	6.8
No	1,252	93.2
Presence of cockroach		
Yes	137	10.2
No	1,206	89.8
Presence of carpet in the bedroom		
Yes	1,334	99.3
No	9	0.7
Proximity of the residence to motorways		
Near	698	52.1
Less near	537	40.0
Far	106	7.9

Table 2 Prevalence of asthma and asthma-like symptoms

	n	%
Current asthma	79	5.9
Attack of asthma	31	2.3
Treatment for asthma	76	5.7
Asthma-like symptoms	457	34.0
Wheezing	171	12.7
Wheezing with shortness of breath	138	10.3
Wheezing in the absence of a cold	130	9.7
Waking with tightness in the chest	252	18.8
Woken by an attack of breathlessness	275	20.5
Woken by attack of cough	267	19.9
History of allergic rhinitis	33	2.5

was 52.45±16.13 years (18-89 years) with 51.51±16.15 years (18-88 years) for females and 53.70±16.04 years (18-89 years) for males. Sociodemographics were as follows: 4.1% were single (n=55), 83.3% married (n=1,117), 12.6% were widow or widower (n=169), 17.9% had no education (n=240), 6.0% is only capable of reading or writing (n=80), 58.8% had a primary school degree (n=790), 6.5% had a junior high school degree (n=87), 8.0% had a high school degree (n=107), and 2.8 % had a postgraduate degree (n=38). Socioeconomic level distribution in the study group was high in 21.7% (n=292), fair in 71.8% (n=963) and poor in 6.5% (n=87). Some individual and environmental risk factors for asthma were evaluated and shown in *Table 1*. In the study group the prevalence of current asthma was 5.9%, having an asthma-like symptom was 34.0% and allergic rhinitis was 2.5%. Most common asthma symptoms were woken by an attack of breathlessness (20.5%, n=275), woken by attack of cough (19.9%, n=267) and wheezing (12.7%, n=171) (*Table 2*). Waking with tightness in the chest, woken by an attack of breathlessness and by attack of cough and asthma-like symptoms were more prevalent in women than in men (P=0.013, P=0.003, P=0.005 and P=0.002 respectively) (*Table 3*). According to PFT results, mean FEV1/FVC values were significantly lower in patients with wheezing, wheezing with shortness of breath, wheezing in the absence of a cold findings, treatment for asthma and with current asthma (*Table 4*). When risk factors attributable to asthma development were evaluated against multiple variables with logistic regression analysis, family history of atopy was found to increase the risk for all symptoms in varying degrees and age was found to increase the risk one fold for all parameters except asthma-like symptoms. Presence of cockroach in the house increased the risk two to three times for many parameters. Female gender and pets inside the house also increased the risk one to tow times for some parameters. Presence of carpet in the bedroom was related with a slightly increased risk for 'wheezing in the absence of a cold (*Table 5*).

Table 3 Comparison of asthma and asthma-like symptoms for gender

	Female		Male		P*
	n	%	n	%	
Current asthma	53	6.9	26	4.5	0.059
Attack of asthma	21	2.7	10	1.7	0.217
Treatment for asthma	51	6.7	25	4.3	0.064
Asthma-like symptoms	287	37.6	170	29.4	0.002
Wheezing	97	12.7	74	12.8	0.963
Wheezing with shortness of breath	82	10.7	56	9.7	0.526
Wheezing in the absence of a cold	73	9.6	57	9.8	0.859
Waking with tightness in the chest	161	21.1	91	15.7	0.013
Woken by an attack of breathlessness	178	23.3	97	16.8	0.003
Woken by attack of cough	172	22.5	95	16.4	0.005
History of allergic rhinitis	26	3.4	7	1.2	0.010

*, Pearson χ^2.

Table 4 Comparison of mean values of FEV1/FVC for asthma and asthma-like symptoms

	n	Mean	SD	t	P*
Wheezing					
Yes	167	82.69	12.89	–3.791	<0.0001
No	1,159	86.26	11.16		
Wheezing with shortness of breath					
Yes	135	82.37	13.04	–3.701	<0.0001
No	1,191	86.20	11.20		
Wheezing in the absence of a cold					
Yes	127	82.34	12.46	–3.613	<0.0001
No	1,199	86.18	11.28		
Waking with tightness in the chest					
Yes	245	85.65	12.38	–0.238	0.812
No	1,081	85.85	11.23		
Woken by an attack of breathlessness					
Yes	268	85.46	12.80	–0.511	0.610
No	1,058	85.90	11.09		
Woken by attack of cough					
Yes	262	85.07	12.20	–1.168	0.243
No	1,064	85.99	11.25		
Attack of asthma					
Yes	30	83.34	9.63	–1.196	0.232
No	1,296	85.87	11.49		
Treatment for asthma					
Yes	74	82.12	11.17	–2.864	0.004
No	1,252	86.03	11.43		
Current asthma					
Yes	77	82.33	11.12	–2.753	0.006
No	1,249	86.03	11.44		
Asthma-like symptoms					
Yes	448	85.31	12.29	–1.091	0.276
No	878	86.07	10.99		

SD, standard deviation; *, Pearson *t* test.

Table 5 Logistic regression analysis of risk factors for asthma and asthma-like symptoms

	OR, 95% CI							
	Age	Gender	Smoking	Smoking inside the house	Cockroach	Famliy history of atopy	Carpet in the bedroom	Pets in the house
Wheezing	1.032, 1.020-1.045*	1.105, 0.682-1.789	1.321, 0.803-2.172	1.140, 0.762-1.706	2.932, 1.861-4.620*	4.993, 3.223-7.736*	0.264, 0.060-1.157	1.574, 0.852-2.907
Wheezing with shortness of breath	1.038, 1.024-1.052*	1.310, 0.766-2.242	1.398, 0.802-2.435	1.050, 0.670-1.647	2.969, 1.809-4.873*	5.922, 3.725-9.416*	0.399, 0.075-2.141	1.969, 1.036-3.741#
Wheezing in the absence of a cold	1.035, 1.022-1.049*	0.938, 0.551-1.597	1.093, 0.628-1.902	0.978, 0.613-1.562	2.898, 1.745-4.811*	5.736, 3.568-9.223*	0.191, 0.043-0.851#	2.174, 1.145-4.130#
Waking with tightness in the chest	1.019, 1.009-1.028*	1.800, 1.185-2.734ǂ	1.418, 0.920-2.186	1.138, 0.819-1.583	1.277, 0.822-1.985	2.277, 1.511-3.431*	0.917, 0.185-4.545	1.592, 0.958-2.643
Woken by an attack of breathlessness	1.031, 1.021-1.041*	2.148, 1.417-3.256*	1.608, 1.046-2.473#	1.127, 0.812-1.565	0.864, 0.537-1.392	2.454, 1.630-3.696*	0.634, 0.150-2.675	1.731, 1.048-2.858#
Woken by attack of cough	1.025, 1.016-1.035*	1.864, 1.234-2.816ǂ	1.394, 0.909-2.140	1.270, 0.918-1.755	0.731, 0.446-1.201	2.162, 1.432-3.263*	0.592, 0.143-2.461	1.520, 0.914-2.528
Attack of asthma	1.026, 1.001-1.052#	1.042, 0.373-2.910	0.685, 0.221-2.125	0.523, 0.189-1.451	3.107, 1.248-7.737#	8.871, 4.049-19.437*	0.186, 0.019-1.803	2.532, 0.816-7.859
Treatment for asthma	1.062, 1.040-1.083*	0.954, 0.471-1.932	0.515, 0.231-1.149	0.785, 0.396-1.554	2.131, 1.003-4.530#	24.856, 13.685-45.144*	0.654, 0.058-7.384	0.950, 0.298-3.032
Current asthma	1.054, 1.034-1.074*	1.046, 0.523-2.094	0.621, 0.287-1.343	0.781, 0.406-1.502	2.181, 1.062-4.479#	20.543, 11.629-36.292*	0.655, 0.060-7.118	1.173, 0.414-3.325
Asthma-like symptoms	1.018, 1.011-1.026	1.717, 1.226-2.405ǂ	1.294, 0.911-1.840	1.245, 0.947-1.636	1.282, 0.883-1.862	2.246, 1.544-3.265*	1.159, 0.281-4.775	1.153, 0.731-1.818

*, P<0.0001; #, P<0.05; ǂ, P<0.01; CI, confidential interval.

Discussion

Results of our study which was designed to evaluate prevalence of asthma symptoms and determine the risk factors of asthma among adults in rural regions of Denizli province were as follows; prevalence of asthma was 5.9%, asthma-like symptom was 34.0%. Prevalence of attack of asthma in the last year was 2.5% and treatment for asthma (spray, inhaler, pills) was 5.7%. In a study of Daloğlu *et al.* conducted among adults between 20 and 49 years in Denizli, the prevalence of attack of asthma in the last year and treatment for asthma were 1.5% and 2.3%, respectively (10). In another study of Bozkurt *et al.* conducted among patients 15 years old and older in Denizli, the prevalence of attack of asthma during the last year and treatment for asthma were 2.1% and 3.8%, respectively (11). Higher prevalence found in our study can be attributed to older population and higher allergen exposure in the rural regions. The finding that age is related with one fold increase in the risk of asthma and asthma like symptoms also supports this proposal. In a study conducted in the city center of Antalya province, prevalence of asthma was as 9.4% (12). The discrepancy between our results and this study can be attributed to the risk factors like higher humidity, crowded population and air pollution in Antalya province. Prevalence of current asthma in individuals 18 years and older in Manisa was reported as 1.2% by Şakar *et al.* (13). It is interesting that current asthma prevalence in rural regions of Denizli is higher than the city center of Manisa which has similar characteristics with Denizli. The same study reported the prevalence of asthma-like symptoms as 25.0%. This is lower than the prevalence found in our study. Also prevalence of attack of asthma during the last year and treatment for asthma (spray,

inhaler or pills) were 0.9% and 0.7%, respectively. These prevalences are also lower than our study. The fact that Şakar *et al.* evaluated current asthma, cumulative asthma and asthma-like symptoms on individuals between 20-44 years old, lower humidity and location of industrial structures outside the town in Manisa and difference of interview technique might be responsible for the discrepancies from our results. Older study population and higher exposure to allergens in our study might be potentially responsible for differences between results. In other reports outside Turkey which are conducted using ECRHS questionnaire method reported the prevalences of treatment for asthma and/or attack of asthma in the last year (diagnosed current asthma) as: 5.8-6.8% in Sweden, 2.1-4.4% in Germany, 3.5-5.5% in France, 7.5-8.4% in England, 2.9% in Greece, 3.3-4.5% in Italy, 2.1-6.3% in Spain (14). Similar prevalences with Spain may be attributed to geographic proximity and sociocultural resemblance. National Health Interview Survey (NHIS) study in United States of America between 2001 and 2010 years found the prevalence of current asthma 7.0% among the individuals 65 years and older (15). Again this higher prevalence might be attributed to the older study population.

In terms of asthma-like symptoms; the prevalences of waking with tightness in the chest, woken by an attack of breathlessness and by attack of cough in the last year were 18.8%, 20.5% and 19.9%, respectively. In a study conducted in central province of Denizli, prevalences of the most common symptoms of woken by attack of cough, wheezing and wheezing with shortness of breath were reported 17.4%, 16.8% and 11.1%, respectively (10). Again in another study conducted in central province of Denizli reported the prevalences of tightness in chest, shortness of breath and woken by attack of cough as 13.0%, 11.0% and 22.7%, respectively (11).

Our results indicated that mean FEV1/FVC values were lower in patients with wheezing, wheezing with shortness of breath, wheezing in the absence of a cold findings, current asthma and in patients who were on treatment for asthma. Decreased mean FEV1/FVC values are expected where air flow restriction is encountered.

Family history of atopy was related with increased risk for all symptoms in varying degrees. Cockroach presence in the house also was related with increased risk two or three fold for most of the symptoms. In a study conducted in rural and provincial regions of Nigeria, people with asthma had shown to have positive skin tests for many allergens including cockroach and total IgE levels were higher when compared with people without asthma (16). Our study was not designed to utilize blood and skin tests but self-report of individuals were investigated and similar results were found. Among the atopic disorders, allergic rhinitis was most commonly associated with asthma (17,18). In our study, prevalence of allergic rhinitis was found 2.5% and 3.4% of women and 1.2% of men had allergic rhinitis. Daloğlu *et al.* found 16.1% prevalence of allergic rhinitis with a predominance of women in central province of Denizli (10). Our results are lower but this discrepancy might be a reflection of the socioeconomic differences among the two study groups as allergic rhinitis is thought to be more common in urban areas then rural areas. Female gender and presence of pets in the house was related with one or two fold risk increase for some of the symptoms. Presence of carpet in the bedroom was related with only mild decrease in risk for 'wheezing in the absence of a cold findings'. The fact that having carpet in the bedroom to protect against cold is more prevalent in rural regions where central heating is rare climate is colder, might have a contribution to this finding. Smoking inside the house is less prevalent in rural regions than urban areas due to sociocultural factors. Prevalence of smoking in the house was found 27.2% which means a passive exposure to smoke. This might be a reason why smoking inside the house was not found as a risk factor in the multivariate analysis.

In conclusion, asthma is an important disease that may occur not only in cities but also at country sides. In rural areas risk factors for asthma and asthma-like symptoms compared to urban areas may show some differences. İn rural areas, more studies should be conducted to determine urban/rural differences.

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Original Article

Efficacy and safety of low-dose clopidogrel after 12-month dual antiplatelet therapy for patients having drug-eluting stent implantation

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Background: To prevent stent thrombosis (ST) after implantation of drug-eluting stents (DESs) in patients with coronary heart disease, 12-month dual antiplatelet therapy (DAPT) is recommended. However, the optimal long-term antiplatelet regimen is not clear for the patients who have completed the 12-month DAPT.

Methods: We reviewed the data of 755 consecutive patients who had undergone percutaneous coronary intervention (PCI) three years ago and completed 12-month DAPT. They were divided into three groups according to the antiplatelet medication they had used for two years after 12-month DAPT [low-dose clopidogrel (Talcom®, 25mg/d), clopidogrel (Plavix®, 75mg/d) and aspirin (100 mg/d)]. The efficacy (a composite incidence of cardiac death, myocardial infarction and target vessel revascularization) and safety (incidences of bleeding, gastrointestinal trouble and drug discontinuation) were compared among the three groups.

Results: The rates of multi-vessel lesions, prior MI, hemoglobin A1C (HbA1c) and low-density lipoprotein cholesterol were significantly higher in the clopidogrel (75 mg/day) group than in the other two groups (P>0.05 for both comparisons). There was no significant difference in the overall composite incidence of cardiac death, myocardial infarction and target vessel revascularization in the three groups at three years after PCI. The rates of bleeding (especially minor bleeding), gastrointestinal trouble, drug discontinuation and any blood transfusion were markedly lower in the low-dose clopidogrel (25 mg/d) group than in the other two treatment groups (P<0.05).

Conclusions: The 25-mg maintenance dose of clopidogrel after 12-month DAPT may be more preferable to Chinese patients who have undergone DES implantation, because of its lower cost but no less efficacy and safety.

Keywords: Clopidogrel; drug-eluting stent (DES); thrombosis; antiplatelet

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Introduction

The widespread use of drug-eluting stents (DESs) for coronary heart disease has significantly reduced the risk of in-stent restenosis (ISR) but along with a tendency in increasing the risk of stent thrombosis (ST), which is associated with a mortality rate of 20% to 45% (1,2). To prevent ST after percutaneous coronary intervention (PCI) with DES, dual antiplatelet therapy (DAPT) with aspirin (100 mg/d) and clopidogrel (75 mg/d) for at least 12 months has become a class I recommendation in the treatment guidelines. Premature discontinuation of DAPT has also been regarded as a risk factor for ST (3-5). Following DAPT, monotherapy with either aspirin or clopidpgrel (when aspirin is contraindicated or not

tolerated) is recommended for long-term use. However, there is serious concern about complications like bleeding and gastrointestinal intolerance during long-term, usually life-long, antiplatelet administration.

Clopidogrel, the key component of DAPT, is a prodrug modified through oxidation in liver and its active metabolite is selectively and irreversibly combined to platelet adenosine diphosphate receptors, thus inhibiting platelet aggregation. The efficacy and safety of clopidogrel in the secondary prevention of cardiovascular and cerebrovascular diseases have been confirmed by several large scale trials, such as CAPRIE, CURE, and COMMIT (6-8), most of which were carried out with the original clopidogrel (Plavix®, Sanofi-Synthelabo, France). However, the high cost of clopidogrel has been known as a factor in the premature discontinuation of therapy, resulting in an increase of major adverse cardiac events (MACEs) (9). In China, one alternative generic clopidogrel (Talcom®, Shenzen Salubris) has been released. It is much cheaper and has a lower content of 25 mg per tablet. As a result, the price advantage encouraged both doctors and patients to switch to the alternative clopidogrel product with a lower dose (Talcom®, 25-50 mg/d) from the innovator clopidogrel (Plavix®, 75 mg/d) that had been formerly received. Consequently, in the current clinical practice in China, DES-PCI patients after 12-month DAPT may have a choice to continue their antiplatelet therapy with any of the three drugs: aspirin (100 mg/d), clopidogrel (Plavix®, 75 mg/d) and clopidogrel (Talcom®, 25 mg/d).

This retrospective study aimed to compare the efficacy and safety among the three antiplatelet medications: low-dose clopidogrel (Talcom®, 25 mg/d), clopidogrel (Plavix®, 75 mg/d) and aspirin (100 mg/d) which were used in patients who had undergone DES implantation and completed 12-month DAPT.

Materials and methods

Study population and data collection

The clinical data were retrospectively collected between September 2008 and May 2013 from the 796 consecutive patients who had undergone PCI three years ago and completed 12-month DAPT at the Cardiac Center of The First Affiliated Hospital of Sun Yat-Sen University. The choice of DES was at operators' discretion and PCI was performed using standard techniques. Of these 796 patients we retrieved the records of long-term antiplatelet regimens. They had aspirin 100 mg/d, clopidogrel (Plavix®) 75 mg/d, or clopidogrel (Talcom®) 25 mg/d after 12-month DAPT. We also reviewed their demographic, clinical, angiographic and procedural characteristics at baseline, including age, sex, body mass index (BMI), systolic and diastolic blood pressure (SBP and DBP), history of hypertension and stroke, lipid profile and kidney function. We excluded the patients who had hypoxic encephalopathy, malignancies, or chronic hemodialysis (HD), were taking warfarin or single antiplatelet therapy, or had been transferred to other hospitals after a successful PCI at the first affiliated hospital of Sun Yat-sen University. The protocol was approved by the hospital ethics committee.

Definitions and follow-up

The primary endpoint for outcome efficacy was MACE defined as a combined incidence of cardiac death, myocardial infarction, urgent target vessel revascularization (coronary bypass surgery or PCI) due to myocardial infarction. Mortality data of cardiac death were collected from the medical records of the patients. The definition of myocardial infarction was development of pathologic Q waves (≥30 ms in duration and ≥0.1 mV in depth) in ≥2 contiguous precordial leads or ≥2 adjacent limb leads, or elevation of creatine kinase isoenzyme MB (CK-MB) ≥2 times the upper limit of normal. On follow-up coronary angiography (CAG), target lesion revascularization was considered clinically driven if prompted by symptoms or signs consistent with myocardial ischemia or if lesion diameter stenosis was more than 70% at follow-up.

The safety endpoints of the study were incidences of minor/major bleeding, gastrointestinal trouble, drug discontinuation at 36 months after DES placement. Major bleeding was defined as intracranial, intraocular, or retroperitoneal hemorrhage, clinically overt blood loss resulting in a decrease in hemoglobin of more than 3 g per deciliter, any decrease in hemoglobin of more than 4 g per deciliter, or transfusion of 2 or more units of packed red blood cells or whole blood (10). The gastrointestinal troubles referred to epigastric discomfort or pain, nausea, vomiting and diarrhea.

Statistical analysis

The data are presented as mean ± SD or frequencies (%). Categorical data were compared with the chi-squared test or Fisher's exact test when cell values were less than 5. Multiple testing of continuous data were analyzed using one-way ANOVA and post hoc Bonferroni's test since

Table 1 Baseline clinical characteristics of the study population

	Aspirin (100 mg/d) (n=283)	Clopidogrel (25 mg/d) (n=224)	Clopidogrel (75 mg/d) (n=248)
Age (years)	68.3±7.8	69.2±5.7	70.1±6.2
Hypertension, n (%)	192 (67.8)	149 (66.5)	168 (67.7)
Stroke, n (%)	73 (25.8)	51 (22.8)	59 (23.8)
Multivessel, n (%)[#†]	188 (66.4)	153 (68.3)	201 (81.4)
Prior CABG, n (%)	0	0	0
Prior MI, n (%)[#†]	47 (16.6)	35 (15.6)	72 (29.0)
BMI, kg/m^2	24.9±4.1	25.1±3.2	26.6±4.7
SBP, mmHg	152.2±22.3	148.3±19.2	150.8±18.9
DBP, mmHg	62.3±10.1	63.9±8.2	64.5±9.5
TC, mg/dL	192.4±30.2	188.5±28.5	187.3±27.9
LDL-C, mg/dL[#†]	121.6±30.4	124.9±24.4	142.7±27.5
HDL-C, mg/dL	42.4±11.6	41.2±9.5	38.9±10.4
TG, mg/dL	142.6±47.8	138.6±44.4	142.3±45.8
FBG, mg/dL	130.1±33.2	129.1±30.9	134.5±35.4
HbA1c, %[#†]	7.81±1.31	7.62±1.18	8.44±2.67
eGFR, mL/minute/1.73 m^2	64.3±18.5	63.6±15.9	61.6±16.2
CKD, n (%)	42 (14.8)	33 (14.7)	59 (23.8)
HD, n (%)	0	0	0

CABG, coronary artery bypass graft surgery; CKD, chronic kidney disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FBG, fasting blood glucose; HbA1c, Hemoglobin A1c; HD, hemodialysis; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; MI, myocardial infarction; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides; *, P<0.05 for aspirin group versus clopidogrel (25 mg/d) group; #, P<0.05 for aspirin group versus clopidogrel (75 mg/d) group; †, P<0.05 for clopidogrel (25 mg/d) group versus clopidogrel (75 mg/d) group.

the assumption of linearity and normal distribution of residuals were achieved. For categorical data, multiple testing was conducted with the chi-squared test followed by Bonferroni's posttest. Survival analysis was performed by the Kaplan-Meier method. Differences in the cumulative adverse cardiac events were assessed with the log-rank test, which allowed the calculation of odds ratio (OR) [95% confidence intervals (CI)] associated with clopidogrel (25 mg/d) group. A P value <0.05 was considered to indicate statistical significance. All data were analyzed using SPSS version 16.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Baseline characteristics of patients are shown in *Table 1*. Of the total 796 patients, 41 patients were further excluded for lack of data (n=11), and cannot follow-up in our hospital (n=30). Among the 755 patients included in the analysis, 283 were in aspirin (100 mg/day) group, 224 clopidogrel (25 mg/day) group and 248 clopidogrel (75 mg/day) group. The mean follow-up duration was 36±2.4 months after DES-PCI, with 12-month DAPT completed. The age, history of hypertension and stroke, BMI, SBP, DBP, high-density lipoprotein-cholesterol (HDL-C), triglycerides (TG), fasting blood glucose (FBG), estimated glomerular filtration rate (eGFR), chronic kidney disease (CKD) at baseline did not differ significantly among the three groups. However, there were significantly more patients with multivessel lesions, prior MI, hemoglobin A1c (HbA1c) in clopidogrel (75 mg/day) group than in clopidogrel (25 mg/day) and aspirin (100 mg/day) groups (P>0.05 for both comparisons). Moreover, clopidogrel (75 mg/day) group had a significantly higher level of low-density lipoprotein cholesterol (LDL-C) than the other two groups (P<0.05). No significant difference existed among the three groups regarding total and high-density lipoprotein cholesterol (HDL-C).

Table 2 shows medication profiles at discharge and

Table 2 Medications and angiographic profiles

	Aspirin (100 mg/d) (n=283)	Clopidogrel (25 mg/d) (n=224)	Clopidogrel (75 mg/d) (n=248)
ACEIs, n (%)	75 (26.5)	59 (26.3)	73 (29.4)
ARBs, n (%)	139 (49.1)	108 (48.2)	132 (53.2)
β-Blockers, n (%)	231 (81.6)	185 (82.5)	202 (81.5)
Statins, n (%)	224 (79.2)	185 (82.6)	204 (82.5)
LVEF, %	64±12	65±10	61±10
Number of treated vessels*#	1.4±1.2	3.1±0.9	3.2±1.1
Type of stents, (%)			
Sirolimus-eluting stent	89.6	89.2	90.1
Paclitaxel-eluting stent	10.4	10.8	9.9
Number of stents, n*#	2.3±0.4	2.9±0.5	3.5±0.9
Mean stent size, mm	2.71±0.39	2.75±0.32	2.84±0.29

ACEIs, angiotensin-converting enzyme inhibitors; ARBs, angiotensin receptor blockers; LVEF, left ventricular ejection fraction; *, P<0.05 for aspirin group versus clopidogrel (25 mg/d) group; #, P<0.05 for aspirin group versus clopidogrel (75 mg/d) group.

Table 3 Results for the efficacy endpoint at one year follow up after DAPT

	Aspirin (100 mg/d) (n=283)	Clopidogrel (25 mg/d) (n=224)	Clopidogrel (75 mg/d) (n=248)
Combined MACEs, n (%)	70 (24.7)	53 (23.7)	56 (22.6)
Cardiac death	25 (9.9)	18 (8)	18 (7.3)
Myocardial infarction	38 (13.4)	31 (13.8)	33 (13.3)
TVR	7 (2.5)	4 (1.8)	5 (2.0)

DAPT, dual antiplatelet therapy; MACEs, major adverse cardiac events; TVR, target vessel revascularization.

the angiographic features of the patients included in the present study. The medications of Angiotensin-Converting Enzyme Inhibitors (ACEIs), Angiotensin Receptor Blockers (ARBs) and statins use, as well as left ventricular ejection fraction, showed no difference in frequency among the three groups. The mean stent size and DES type used did not differ among the three groups, either. However, the numbers of treated vessel and stent were significantly lower in the aspirin treatment group than in the two clopidogrel treatment groups (P<0.05), but not significantly different between the latter two groups (P=0.16). As shown in *Table 3*, there were no significant difference in the overall composite incidence of cardiac death, myocardial infarction and target vessel revascularization in the three groups at three years after PCI. Specifically, 25 deaths occurred in aspirin (100 mg/day) group, 18 in clopidogrel (25 mg/day) group and 18 in clopidogrel (75 mg/day) group, giving a 3-year mortality rate of 9.9%, 8% and 7.3% respectively (P>0.05) (*Figure 1*). The cumulative incidence of overall mortality presented in *Figure 1* shows the Kaplan-Meier survival curve. There was no significant diverge in the overall mortality rate among the three groups at three years. *Table 4* shows the safety endpoints among the three groups. The bleeding (especially minor bleeding), gastrointestinal trouble, drug discontinuation and any blood transfusion in aspirin group were markedly higher than in the other two clopidogrel treatment groups (P<0.05). Moreover, compared with clopidogrel (75 mg/d) group, clopidogrel (25 mg/d) group had a lower event rate in terms of minor bleeding and gastrointestinal trouble.

Discussion

This retrospective study assessed the efficacy and safety of long term (approximately two years) administration of standard and low-dose clopidogrel (25 mg/d) and aspirin (100 mg/d) after 12-month DAPT in real world patients who had undergone DES implantation. The main findings

of this study are as follows: (I) in daily practice, there were no significant differences regarding the composite endpoint of cardiac death, myocardial infarction and urgent target vessel revascularization among patients receiving single treatment of different doses of clopidogrel and aspirin three years after DES implantation; (II) low-dose clopidogrel regime (Talcom®, 25 mg/d) resulted in fewer bleeding events, better gastrointestinal tolerance and medicine compliance than standard clopidogrel (Plavix®, 75 mg/d) and aspirin (100 mg/d) ones.

The delayed healing following DES placement (and therefore the optimal duration of anti-platelet therapy) has been the subject of much recent debate and an American advisory board has strongly recommended thienopyridine treatment for at least 12 months in all patients after DES implantation (9). After a standard duration of DAPT (12 months), the patients may have the option of aspirin or cliopidogrel (75 mg/d) for long-term single antiplatelet treatment, as there is no doubt that monotherapy of these agents are effective following DAPT in patients after DES-PCI (4). However, increasingly widespread use of the generic clopidogrel (Talcom®) in China has raised doubts about the pharmacokinetic and pharmacodynamic action as well as the tolerability of the copy form drug. Investigations were performed to clarify these doubts (11-13). One study failed to demonstrate significant differences either in the measure or in the tolerability of platelet aggregation between the two forms at the same dosage in healthy volunteers (11). On the contrary, our data showed that, during 3-year follow-ups, there were no significant differences in MACE and bleeding events between standard clopidogrel (75 mg/d) and low-dose clopidogrel (25 mg/d) after 12-month DAPT, indicating low-dose generic clopidogrel may serve as an effective alternative of the standard-dose original agent while it incurs a significantly lower cost. Moreover, our study revealed a significant lower drug discontinuation rate in the patients taking low-dose clopidogrel than those taking the other two agents, suggesting that much less cost of low-dose clopidogrel may be associated with better treatment compliance. A multicenter, prospective, randomized trial showed that, in patients undergoing selected PCI, there were no significant differences in MACE between domestic clopidogrel (Talcom®) and Plavix® (12). Moreover, another randomized trail evaluated the efficacy and safety of 50 mg clopidogrel in Japanese patients who underwent DES implantation (13).

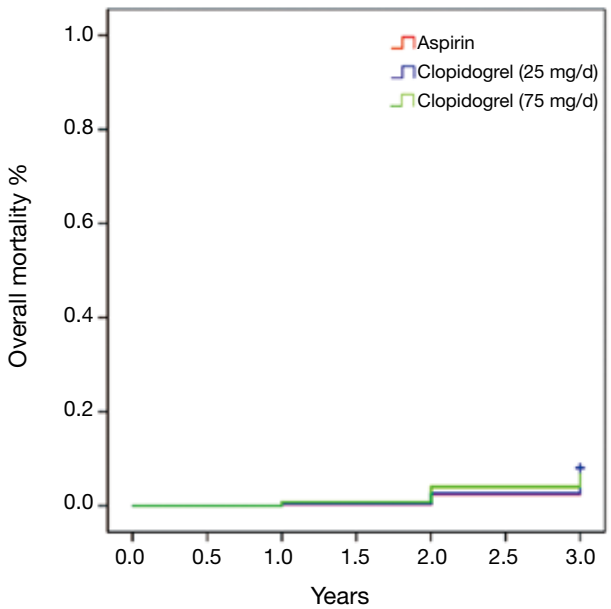

Figure 1 The 3-year composite incident of cardiac death, myocardial infarction, urgent target vessel revascularization due to myocardial infarction among patients taking low-dose clopidogrel (25 mg/d), clopidogrel (75 mg/d) and aspirin (100 mg/d).

Table 4 Results for the safety endpoint at one year follow-up after DAPT

	Aspirin (100 mg/d) (n=283)	Clopidogrel (25 mg/d) (n=224)	Clopidogrel (75 mg/d) (n=248)
Bleeding, n (%)*#†	54 (19.1)	6 (2.8)	20 (8.1)
Major bleeding*#	6 (2.1)	0	0
Minor bleeding*#†	48 (17.0)	6 (2.8)	20 (8.1)
Gastrointestinal trouble, n (%)*#†	72 (25.4)	14 (6.3)	43 (17.3)
Drug discontinuation, n (%)*#	64 (22.6)	11 (4.9)	15 (6.0)
Any blood transfusion, n (%)*#	18 (6.4)	0	0

DAPT, dual antiplatelet therapy. *, P<0.05 for aspirin group versus clopidogrel (25 mg/d) group; #, P<0.05 for aspirin group versus clopidogrel (75 mg/d) group; †, P<0.05 for clopidogrel (25 mg/d) group versus clopidogrel (75 mg/d) group.

During follow-up, no significant difference in cardiac death, myocardial infarction or ST was observed in low dose clopidogrel group compared with standard clopidogrel group, as well as side effects. This result can attribute to ethnic difference. A previous study showed that maintenance doses of some drugs differed between Asian patients and Caucasian patients (14,15). Similarly, Fukushima *et al.* reported a similar antiplatelet effect between 200 mg ticlopidine and 50 mg clopidogrel in Japanese patients, and 50 mg clopidogrel is much lower than the "standard" dosage (75 mg) for Caucasian patients (16). Taken together, given the potential benefit of lower incidence of side effects that low dose clopidogrel maintenance may bring about, as well as the ethnic difference, a lower maintenance dose of clopidogrel may be considered appropriate in Chinese patients after 12-month DAPT.

Studies have identified, besides the above antiplatelet agents, stent underexpansion, dissection, long stent length, and residual stenosis at stent edges as procedure-related risk factors of ST (17-19). Thus, besides optimal medication, it is important to achieve optimal stent deployment to prevent ST. Roy *et al.* have demonstrated a lower ST rate after DES implantation under intravascular ultrasound guidance when compared with angiographic guidance (20). Premature discontinuation or ineffectiveness of antiplatelet drugs predisposes the patients to ST (5). Analysis of the Dutch ST registry (21,009 patients and a total of 31,065 stents) showed that discontinuation of antiplatelet therapy with clopidogrel was a strong independent predictor of ST (21). Cessation of clopidogrel in the first 30 days after PCI enhanced the hazard ratio for ST to 36.5 (95% CI: 8.0 to 167.8), and the lack of clopidogrel therapy between 30 days and six months was also linked to a significantly increased risk of ST (hazard ratio 4.6, 95% CI: 1.4 to 15.3) (21). Similarly, the absence of aspirin therapy was also independently related to ST (21). In other words, the low-dose clopidogrel therapy may be applicable to those patients that procedure related risk factors of ST were under well-controlled, for example, by the use of intravascular ultrasound-guided DES implantation.

There were some limitations to our analysis. Firstly, our study was an observational one. It was possible that confounding factors could have accounted for the observed differences. Secondly, the relatively short follow-up duration did not allow us to monitor the overall effect of low-dose clopidogrel on the whole progression of cardiovascular disease. Further clinical trials in large cohorts of patients are necessary to compare antiplatelet agents. Thirdly, we did not assess plasma levels of the active metabolites of clopidogrel either, because the authorization of this retrospective study did not allow us to take extra blood samples from the patients for assessment of the clopidogrel plasma levels or platelet function.

Conclusions

This retrospective study shows safety and efficacy of 25 mg maintenance dose of clopidogrel after 12-month DAPT in Chinese patients undergoing DES implantation, justifying its advantage of low-cost over the original product. However, this conclusion should be interpreted with caution before large-scale randomized trials come to a definite one comparing doses of 25 and 75 mg clopidogrel in Chinese patients undergoing coronary stent implantation after DAPT.

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Original Article

Female breast cancer statistics of 2010 in China: estimates based on data from 145 population-based cancer registries

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Background: The aim of the study is to provide incidence and mortality data of female breast cancer at national level of China in 2010.

Methods: A total of 145 population-based cancer registries submitted qualified cancer incidence and mortality data to National Cancer Registration Center of China. Based on the qualified cancer registries' data, we estimated the overall breast cancer incidence and mortality data of China in 2010 and reported breast cancer statistics by age and geographical area.

Results: The estimated number of female breast cancer cases was about 208 thousand. The crude incidence rate, age-standardized rate by China and World population were 32.43 per 100,000, 25.89 per 100,000 and 24.20 per 100,000, respectively. The incidence rates were higher in urban area than in rural area. And the incidence rates in Eastern area and Middle area were similar and higher that those in Western areas. The estimated number of female breast cancer death in 2010 of China was about 55.5 thousand. The crude, age-standardized mortalities by China population and World population were 8.65 per 100,000, 6.56 per 100,000 and 6.36 per 100,000, respectively. The mortality rates by geographical area had similar pattern to the incidence rates.

Conclusions: Breast cancer is still a major health burden for Chinese women especially in urban area. Prevention strategies such as weight control, high-quality screening and diagnosis may help control the disease.

Keywords: Breast cancer; cancer registry; incidence; mortality; China; 2010

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Introduction

Breast cancer is the most common cancer among women in China for several years. It is also the principal cause of cancer death for females (1). As a result of "westernized lifestyles" and exogenous estrogen exposure, there is an increasing trend of breast cancer incidence in China in the latest decades (2).

Cancer registry is an information system designed for the collection, storage, management, and analysis of data on persons with cancer, usually covering a specific area. Cancer registration may provide accurate, up-to-date population-based cancer data of incidence, mortality and survival, which are vital for decision making regarding cancer prevention and control. The data may also provide basic information for cancer research and cancer surveillance. In China, population-based cancer registries collect cancer statistics from clinics and hospitals, health insurance database, death surveillance database, and cooperative health insurance database over many years. National Cancer Registration Center of China published annual cancer report based on the local cancer registries' data since 2008 (3).

The precise number of breast cancer cases diagnosed each year in the nation is unknown because the cancer registration is incomplete in China. Therefore, we estimated the status of the female breast cancer of 2010,

Table 1 The Quality control indicators of female breast cancer in Chinese cancer registries, 2010

Areas	New cases	New deaths	M/I	MV%	DCO%
All	30,819	7,615	0.25	89.88	0.70
Urban area	21,867	5,156	0.24	91.16	0.51
Rural area	8,952	2,459	0.27	86.75	1.15
Eastern area	22,846	5,359	0.23	91.01	0.49
Middle area	6,040	1,587	0.26	88.00	1.11
Western area	1,933	669	0.35	82.36	1.81

M/I, mortality to incidence ratio; MV%, percent of proportion of morphological verification; DCO%, percent of proportion of death certification only.

based on existing population-based cancer registries' data. To characterize the geographical distribution of breast cancer incidence and mortality, we examined the rates by different areas of China. Comparisons of disease rates by area and age can provide important clues to the underlying causes of diseases and the scope for preventive strategies.

Material and methods

We obtained the incidence and mortality information of invasive breast cancer for women from the National database of cancer registration of China. In 2013, 219 population-based cancer registries submitted cancer incidence and mortality data to National Cancer Registration Center. The cancer data quality was evaluated for quality control. The detailed criteria of the data inclusion were based on "Technical Protocols of Cancer Registration and Follow up" by Ministry of Health 2009, "Guideline of Chinese Cancer Registration" and "Cancer Incidence in Five Continents Volume IX" by The International Agency for Research on Cancer (IARC)/The International Agency for Cancer Registry (IACR) (4,5). And 145 cancer registries' data were accepted for further analysis.

The female population covered by the qualified cancer registration areas in 2010 was 78,048,060, accounting for 12.96% of overall female population of China. By geographical area, the female population covered by cancer registration in Eastern areas, Middle areas, and Western areas of China were 52,618,341, 17,735,155 and 7,694,564, respectively.

Among the 145 cancer registries, the mortality to incidence ratio (M/I), percent of proportion of morphological verification (MV%) and percent of proportion of death certification only (DCO%) were 0.25%, 89.88% and 0.70 respectively. In urban cancer registries, the MV%, DCO%, and M/I were 0.24, 91.16 and 0.51 respectively. In rural cancer registries, the MV%, DCO% and M/I were 0.27, 86.75 and 1.15 respectively (*Table 1*).

Based on the standard of National Bureau of Statistics in China, we classified qualified cancer registries into Eastern urban area, Eastern rural area, Middle urban area, Middle rural area, Western urban area and Western rural area (6). We firstly calculated the age-specific and sex-specific cancer registration incidence and mortality by these areas. According to the age-specific cancer incidence (mortality) by each strata, we further multiply the corresponding 2010 national census population data of each strata by the age-specific cancer incidence (mortality) data. And the overall estimated new cancer cases (deaths) were summed up. Overall female breast cancer incidence and mortality of China by age and area were further estimated.

Results

Breast cancer incidence

As shown in *Table 2*, the estimated number of female breast cancer cases was about 208 thousand. And the overall crude incidence rate was 32.43 per 100,000, accounting for 16.20% of all cancer cases in women, ranking first among all cancer incidences. After age standardization by China population and World population, the standardized rates were 25.89 per 100,000 and 24.20 per 100,000, respectively.

Stratified by area, the incidence rate of urban area were higher in urban area than in rural area, both for crude rate and age-standardized rate. The incidence rates in Eastern area and Middle area were similar, but in Western areas, the incidence rate of the disease was 23.47 per 100,000, lowest among all areas.

Age-specific incidence rates were presented in *Figure 1*. The age-specific incidence rate of female breast cancer was

Table 2 Estimated breast cancer incidence of China in 2010

Areas	Number of cases	Crude rate ($1/10^5$)	Ratio (%)	ASRcn ($1/10^5$)	ASRwld ($1/10^5$)	Cumulative rate 0-74 (%)	TASR 35-64 ($1/10^5$)	Rank
All	208,192	32.43	16.20	25.89	24.20	2.59	61.16	1
Urban area	127,707	39.47	17.64	30.50	28.56	3.09	71.02	1
Rural area	80,485	25.28	14.34	20.78	19.28	2.02	50.55	1
Eastern area	95,484	35.57	17.19	28.05	26.21	2.82	65.73	1
Middle area	73,624	35.58	17.31	28.05	26.36	2.86	66.88	1
Western area	39,084	23.47	12.85	19.48	18.03	1.88	45.74	2

ASRcn, Age-standardized rate (using China standard population, 2000); ASRwld, Age-standardized rate (using World standard population, 1985); TASR, Truncated age-standardized rate (using World standard population, 1985).

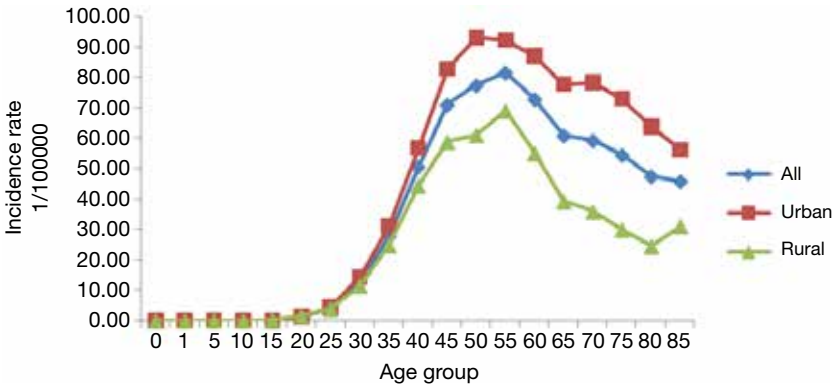

Figure 1 Age-specific incidence rates of female breast cancer of 2010 in China.

relatively low before 25 years old, but dramatically increased after then. The incidence rate reached peak at the age group of 55-59 years, and then gradually decreased. The trend of age-specific incidence in urban and in rural were similar as the overall incidence. And the age-specific incidence rates in urban was significantly higher than those in rural in all age groups after 24 years.

Breast cancer mortality

The estimated number of female breast cancer death in 2010 of China was about 55.5 thousand. And the overall crude mortality rate was 8.65 per 100,000, accounting for 7.90% of all cancer deaths in women, ranking fifth among all cancer deaths. After age standardization by China population and World population, the standardized rates were 6.56 per 100,000 and 6.36 per 100,000, respectively (*Table 3*).

Stratified by area, the mortality rate of female breast cancer in urban area were higher than that in rural area, both for crude rate and age-standardized rates. And the mortality rate in Middle area was highest among all three areas of China, with 9.18 per 100,000 for crude rate (*Table 3*).

The age-specific mortality rates of female breast cancer were shown in *Figure 2*. The mortality rate increased with age until 55-59 years old, and then slightly dropped after then. Since 65-69 years old, the mortality rate increased very quickly, reaching highest in age group of 85+ years. Stratified by urban area and rural area, both rates were very low in young age groups. And the age-specific mortality rates were higher in urban than in rural since 20 years old.

Discussion

In this paper, we described the epidemiology of female breast cancer in 2010, focusing on geographical variations in incidence and mortality of China. For female breast cancer the age standardized rate by World population as a whole was 24.20 per 100,000 for incidence and 6.36 per 100,000 for mortality. For incidence, the lowest rates were found in rural areas and Western areas. For mortality, similar spatial pattern

Table 3 Estimated breast cancer mortality of China in 2010

Areas	Number of cases	Crude rate ($1/10^5$)	Ratio (%)	ASRcn ($1/10^5$)	ASRwld ($1/10^5$)	Cumulative rate 0-74 (%)	TASR 35-64 ($1/10^5$)	Rank
All	55,500	8.65	7.90	6.56	6.36	0.69	14.10	5
Urban area	32,765	10.13	8.59	7.19	7.00	0.75	14.99	5
Rural area	22,735	7.14	7.09	5.78	5.57	0.62	13.11	5
Eastern area	22,309	8.31	7.37	6.07	5.93	0.65	12.32	6
Middle area	18,992	9.18	8.61	7.04	6.82	0.76	15.13	5
Western area	14,199	8.53	7.92	6.79	6.51	0.68	15.79	5

ASRcn, Age-standardized rate (using China standard population, 2000); ASRwld, Age-standardized rate (using World standard population, 1985); TASR, Truncated age-standardized rate (using World standard population, 1985).

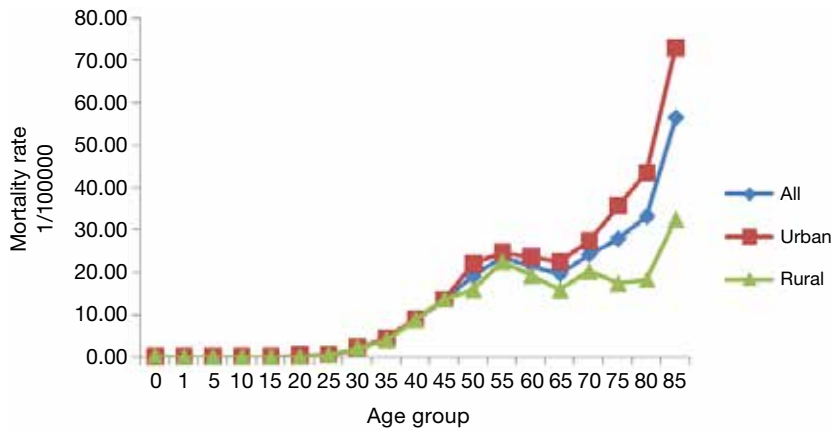

Figure 2 Age-specific mortality rates of female breast cancer of 2010 in China.

was shown as for incidence.

The interpretation of the breast cancer incidence and mortality patterns are complex due to the multiple risk factors such as reproductive, hormonal and nutritional factors, the implementation of breast cancer screening, and the improvement in cancer therapy (7,8). In general, the high rates of breast cancer are the consequence of a higher prevalence of known risk factors for the disease. The changing patterns of childbearing and breastfeed, exogenous hormonal intake, obesity and physical inactivity contributed to trends in incidence and mortality (9). For example, the prevalence of obesity was significantly higher in urban than in rural for several years in China (10-12).

Age-specific incidence curve of breast cancer was distinctive. The rates increased very fast before menopause and decreased after that, probably due to diminishing levels of circulating estrogens. And the age-specific incidence pattern was similar to that of Japan (13). The variation in mortality rates reflect in part variations in incidence, but mortality is also affected by case fatality. We found the range in mortality rates between regions is less than that for incidence because of the more favorable survival of breast cancer in developed regions of urban area and Eastern area. Advances in recent years for breast cancer therapy have made the subsequent stabilization of breast cancer death rates in some big cities of China such as Beijing and Shanghai (14).

The main strength of the study is we provided the updated data of overall female breast cancer statistics of China. Although population-based cancer registries in China have provided with cancer incidence and mortality data for many years, there are still many regions with low or no accurate registry data. We estimated the overall breast cancer incidence and mortality of China and by area with techniques. And the projected numbers of new cancer cases and deaths should be interpreted with caution because these estimates are model-based. Expansion of the existing registry network so that it may cover a more representative sample of the national population would increase the accuracy of the estimation.

Conclusions

Despite much research in understanding and controlling breast cancer, it is still a major health burden for Chinese women especially in urban area. Primary prevention strategies aimed at weight control and breastfeeding promotion may be beneficial though the prolonged endogenous hormonal exposure is not easily modifiable. Increased efforts to provide high-quality screening, diagnosis and treatment may also reduce the breast cancer mortality.

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Original Article

The association between airflow obstruction and radiologic change by tuberculosis

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Introduction: Cigarette smoking is the most commonly encountered risk factor for chronic obstructive pulmonary disease (COPD). However, it is not the only one and there is consistent evidence from epidemiologic studies that nonsmokers may develop chronic airflow limitation. A history of tuberculosis has recently been found to be associated with airflow obstruction in adults older than 40 years. The aim of this study was to evaluate the association between the radiologic changes by tuberculosis and airflow obstruction in a population based sample.

Methods: A nationwide COPD prevalence survey was conducted. We compared the prevalence of airflow obstruction according to the presence of the radiologic change by the tuberculosis.

Results: We analyzed 1,384 subjects who participated in the nationwide Korean COPD survey. All subjects were older than 40 years and took the spirometry and simple chest radiography. We defined the airflow obstruction as FEV_1/FVC <0.7. A total of 149 (10.8%) subjects showed airflow obstruction. A total of 167 (12.1%) subjects showed radiologic change by tuberculosis. Among these 167 subjects, 44 (26.3%) had airflow obstruction. For the subjects without radiologic change by tuberculosis, the prevalence of airflow obstruction was only 8.6%. The unadjusted odds ratio for airflow obstruction according to the radiologic change was 3.788 (95% CI: 2.544-5.642).

Conclusions: The radiologic change by tuberculosis was associated with airflow obstruction.

Keywords: Chronic obstructive pulmonary disease (COPD); tuberculosis; risk factor; airflow obstruction

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation which is not fully reversible. *Cigarette smoking is the most commonly encountered risk factor for* COPD. It is not the only factor, however, and there is consistent evidence from epidemiologic studies that nonsmokers may develop chronic airflow limitation. Thus, the current understanding of risk factors for COPD is in many respects incomplete (1).

A history of tuberculosis has been found to be associated with airflow obstruction in adults older than 40 years (2,3). Tuberculosis can cause chronic lung function impairment and is associated with a mean excess loss in FEV_1 and FVC. Lung function loss was greater among those with more severe or later clinical presentation of tuberculosis (4,5). In Korea, the new active pulmonary tuberculosis rate per 100,000 was 59.3 in 2009. This rate increased with the age of the patients (6).

A nationwide COPD prevalence survey in Korea in conjunction with the second South Korean National Health and Nutrition Examination Survey (Korean NHANES II) was conducted from 2001 to 2002. Using Global Initiative for Chronic Obstructive Lung Disease (GOLD) criterion

Table 1 Radiologic findings used in this study

Normal
Emphysema
Pulmonary tuberculosis, mild, inactive
Pulmonary tuberculosis, moderate, inactive
Pulmonary tuberculosis, severe, inactive
Pulmonary tuberculosis, mild, activity undetermined
Pulmonary tuberculosis, moderate, activity undetermined
Pulmonary tuberculosis, severe, activity undetermined
Pulmonary tuberculosis, mild, active
Pulmonary tuberculosis, moderate, active
Pulmonary tuberculosis, severe, active
Pleural effusion
Pleural thickening
Others*

*, including lung mass, solitary pulmonary nodule, bronchiectasis, mediastinal abnormality, diffuse interstitial infiltration, atelectasis, etc.

for defining airflow obstruction, 17.2% of Korean adults over the age of 45 years had COPD (7). In Korean NHANES II, chest X-ray films were taken in specially equipped mobile examination cars at the time of spirometry.

The aim of this study was to evaluate the association between the radiologic changes by tuberculosis and airflow obstruction in a population based sample.

Materials and methods

This study was performed as part of a nationwide COPD prevalence survey. It was conducted in conjunction with the second South Korean National Health and Nutrition Examination Survey (Korean NHANES II) from October 15, 2001, to January 20, 2002. Spirometry was performed by specially trained technicians who conformed to the 1994 American Thoracic Society (ATS) recommendations (8). A more detailed description of the nationwide COPD survey methodology and spirometric procedures has been previously published (7). The study protocol was approved by the ethics committee of Hallym University Sacred Heart Hospital, Anyang, Korea.

Study subjects

In 2005, ATS and the European Respiratory Society (ERS) published a new statement on spirometry (9). The acceptability and repeatability criteria of the spirometry in this study were adopted from the 2005 ATS/ERS recommendations. We analyzed the data of subjects older than 40 years with at least three acceptable spirometry performances and available chest X-ray films.

Airway obstruction is defined by the GOLD criteria as FEV_1/FVC of less than 70%. The severity of obstruction was also classified according to GOLD criteria.

The radiologic findings were evaluated by two qualified radiologists and categorized as *Table 1*. The 'TB scar positive' case was defined if their chest radiography showed any parenchymal changes by tuberculosis listed in *Table 1*, irrespective of tuberculosis activity.

Analysis

All data are expressed as means and standard deviations or frequencies. We compared the mean spirometry values and the frequency of airflow obstruction according to the status of "TB scar". We used student t-test and chi-square test for statistical analysis. The logistic regression models for the association between the presence of "TB scar" and airflow obstruction were also used. P value less than 0.05 was considered statistically significant.

Results

Study populations

A total of 1,384 subjects (male 629, female 755) were included for the analysis. The median age of the study population was 51 years. *Table 2* describes the demographic characteristics of the study population. Radiologic change by tuberculosis was found in 167 (12.1%) subjects. Most of them showed mild changes. None of them showed active lesion of tuberculosis (*Table 3*).

Spirometry results

The mean value of FEV_1, FVC and FEV_1/FVC of total subjects were 2.87±0.71 (L), 3.68±0.89 (L) and 0.78±0.73, respectively. The percentages of predicted value in FEV_1 and FVC were (95.7±14.2)% and (97.3±12.5)%. *Table 4* shows the spirometry results of total subjects according to the radiologic changes. Subjects with radiologic changes by tuberculosis showed significantly lower levels of percentage of predicted value for FEV_1 and FVC (*Table 5*).

Table 2 Demographic characteristics of study population

	Male (N=629)	Female (N=755)	Total (N=1,384)
Age group (years), N (%)			
40-49	294 (46.7)	330 (43.7)	624 (45.1)
50-59	185 (29.4)	229 (30.3)	414 (29.9)
60-69	117 (18.6)	156 (20.7)	273 (19.7)
≥70	33 (5.2)	40 (5.3)	73 (5.3)
Smoking amount (pack-year), N (%)			
0-10	93 (14.8)	18 (2.4)	111 (8.0)
10-20	120 (19.1)	7 (0.9)	127 (9.2)
≥20	269 (42.8)	8 (1.1)	277 (20.0)
Airflow obstruction, N (%)			
Present*	107 (17.0)	42 (5.6)	149 (10.8)
Absent	522 (83.0)	713 (94.4)	1,235 (89.2)
TB scar, N (%)			
Present†	107 (17.0)	60 (7.9)	167 (12.1)
Absent	522 (83.0)	695 (92.1)	1,217 (87.9)

*, defined as observed FEV_1/FVC <0.7; †, presence of any parenchymal change by tuberculosis listed in *Table 1*.

Table 3 Frequency of radiologic changes by tuberculosis

Type of radiology change	N (%)
Pulmonary tuberculosis, mild, inactive	154 (92.2)
Pulmonary tuberculosis, moderate, inactive	1 (0.6)
Pulmonary tuberculosis, severe, inactive	1 (0.6)
Pulmonary tuberculosis, mild, activity undetermined	9 (5.4)
Pulmonary tuberculosis, moderate, activity undetermined	0 (0)
Pulmonary tuberculosis, severe, activity undetermined	2 (1.2)
Pulmonary tuberculosis, mild, active	0 (0)
Pulmonary tuberculosis, moderate, active	0 (0)
Pulmonary tuberculosis, severe, active	0 (0)
Total	167 [100]

Prevalence of airflow obstruction

Among the 1,384 subjects, 149 (10.8%) showed airflow obstruction on spirometry. The presence of airflow obstruction was more frequent in subjects with radiologic change of tuberculosis. Among 167 subjects with radiologic changes by tuberculosis, 44 (26.3%) subjects showed airflow obstruction. Only 8.6 % of subjects without radiologic changes showed airflow obstruction. The difference of prevalence of airflow obstruction was statistically significant (P<0.001, chi-square test) (*Table 6*). The unadjusted odds ratio (OR) for airflow obstruction according to the presence of radiologic changes by tuberculosis was 3.8 (95% CI: 2.54-5.64). After adjustment for smoking status, the OR was 3.12 (95% CI: 2.01-4.67).

The distribution of airflow obstruction severity, according to the GOLD spirometry classification, was also evaluated. More subjects were classified into a more severe stage when they showed radiologic changes by tuberculosis. 11.3% of the subjects with radiologic changes were classified into GOLD 3 or 4, but only 4.8% of subjects without radiologic changes were in GOLD 3 or 4 (*Figure 1*).

Discussion

Pulmonary tuberculosis is associated with chronic airflow obstruction at diagnosis, during treatment, and several years after treatment ended (10). Tuberculosis can cause chronic lung function impairment in gold miners which increases incrementally with the number of episodes of tuberculosis, affecting approximately 18% of subjects with one episode, 27% of subjects with two episodes, and 35% of subjects with three episodes of tuberculosis (4). Prevalence of airflow obstruction varied from 28% to 68% (11,12) of patient with pulmonary tuberculosis. A history of tuberculosis was also revealed to be associated with airflow obstruction in a large population-based study (2,3). However, little is known about the effect of radiologic changes by tuberculosis on

Table 4 Spirometry results of total subjects by the radiologic change

	TB scar (+) (N=167)	TB scar (–) (N=1,217)	P
FEV_1 (L)	2.77±0.78	2.88±0.70	0.064
FEV_1 (% of predicted value)	90.02±17.05	96.51±13.53	<0.001
FVC (L)	3.75±0.95	3.66±0.88	0.236
FVC (% of predicted value)	95.01±14.10	97.65±12.24	0.022
FEV_1/FVC	73.81±10.21	78.90±6.70	<0.001

TB, tuberculosis; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity.

Table 5 Spirometry results of the subjects with airflow obstruction by the radiologic change

	TB scar (+) (N=44)	TB scar (–) (N=105)	P
FEV_1 (L)	2.25±0.73	2.48±0.75	0.083
FEV_1 (% of predicted value)	74.36±18.03	82.60±16.59	0.008
FVC (L)	3.69±0.97	3.84±1.06	0.401
FVC (% of predicted value)	91.71±14.51	97.60±15.86	0.036
FEV_1/FVC	60.57±9.04	64.27±6.10	0.015

TB, tuberculosis; FEV_1, forced expiratory volume in one second; FVC, forced vital capacity.

Table 6 Presence of airflow obstruction according to radiologic change

	TB scar (+) [%]	TB scar (–) [%]	Total [%]
Airflow obstruction (+)	44 [26.3]	105 [8.6]	149 [10.8]
Airflow obstruction (–)	123 [73.7]	1,112 [91.4]	1,235 [89.2]
Total	167 [100]	1,217 [100]	1,384 [100]

P<0.001, chi-square test. TB, tuberculosis.

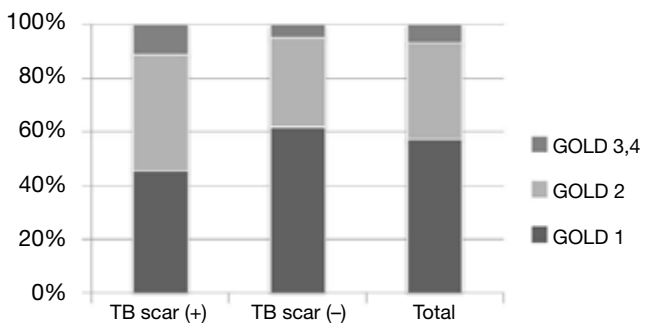

Figure 1 Distribution of GOLD spirometry classification according to radiologic change. GOLD, Global Initiative for Chronic Obstructive Lung Disease.

airflow obstruction. We think that this is the first study that evaluated the association between the radiologic changes by tuberculosis and airflow obstruction in the general population.

In this population-based study, 12.1% of the study subjects showed radiologic changes by tuberculosis. Most of them showed mild change. Among the subjects with radiologic changes by tuberculosis, 26.3% of them had airflow obstruction. This figure was much higher than that of subjects without radiologic changes and statistically significant. The smoking-adjusted odds ratio for airflow obstruction according to the presence of radiologic changes by tuberculosis was over 3.12, which was statistically significant. Also, subjects with radiologic changes were categorized into more severe stages in GOLD spirometry classification. These findings suggest that tuberculosis can be not only a risk factor but also a prognostic factor for COPD.

Extensive lesions by tuberculosis may produce restrictive changes but obstructive alterations were also identified (13,14). Patients with more extensive disease at presentation had lower FEV_1 at follow-up (11). The degree of airflow obstruction was correlated with the extent of disease assessed by radiography (11), despite of the radiologic improvement after six months of antimicrobial chemotherapy (12). However, as shown in *Table 3*, almost all (97.6%) of radiologic changes by tuberculosis detected in this study were minimal, so the possibility of airflow obstruction caused by extensive lesion can be ruled out. There might be another mechanism for airflow obstruction such as chronic airway inflammation rather than airway fibrosis especially in the case of minimal radiologic changes by tuberculosis.

We also found that subjects with radiologic changes by tuberculosis also showed lower percentages of predicted values in FEV_1 and FVC than subjects without radiologic changes irrespective of the presence of airflow obstruction (*Tables 4,5*). This confirms the previous finding by Ross *et al.* that pulmonary tuberculosis is associated with excess loss of lung function (5).

The present findings are relevant for public health. COPD is projected to be the third leading cause of death by 2020. Unfortunately, current pharmacological treatment

does not reduce the mortality rate or modify the natural disease course, so, prevention is an important strategy for managing COPD. COPD can be prevented in three levels. Primary preventions could be achieved by modification or reduction of the disease's main causal factor (e.g., cigarette smoking). Secondary prevention could focus on screening or early detection of COPD. Tertiary prevention might include management of identified COPD patients to augment health status, reduce or slow disease progression or diminish exacerbations and other adverse outcomes (15). Several issues relate to early diagnosis in terms of cost and benefits of identification of groups with increasing risk and severity. The current findings suggest that subjects with radiologic change by tuberculosis could be candidate for screening COPD. Therefore, early diagnosis of COPD might be possible cost-effectively.

This study has some limitations. The spirometric definitions of COPD generally required a post-bronchodilator measurement (1,16), the spirometry examination in this nationwide survey did not include a test of reversibility of obstruction. While some subset of the study population had a probability of having pure asthma, we believe the presence of airflow obstruction using pre-bronchodilator FEV_1/FVC is reasonable as a criterion of airflow obstruction after the age of 40 years in this large epidemiologic survey. As the current views of reversibility misclassify large segments of the population (17,18), the degree of reversibility of airflow limitation is no longer recommended for differential diagnosis with asthma (1). Safety issue was another consideration in this large epidemiologic survey. Fatal or near-fatal paradoxical bronchospasms after administration of a bronchodilator are described elsewhere (19). FEV_1 and other respiratory indices obtained without bronchodilation are good markers of overall health (20).

In conclusion, a presence of radiologic change even mild, by tuberculosis was associated with airflow obstruction. The present findings would reduce the burden of airflow obstruction by the contribution of early detection of airflow obstruction.

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Original Article

Hemoglobin in normal range, the lower the better?—Evidence from a study from Chinese community-dwelling participants

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Objective: To examine the association between hemoglobin (Hb) levels and cardiovascular risk factors in a large community-dwelling cohort.

Methods: A total of 4,186 women and 4,851 men were enrolled in the study. Data on personal history, physical examination and biochemical parameters were collected. Subjects were categorized by gender and divided into different group according to the level of Hb or blood pressure, and the association between Hb levels and cardiovascular risk factors was examined using Pearson's correlation analysis.

Results: In both men and women even with normal Hb level, tertiles of Hb levels were positively associated with body mass index (BMI), total-cholesterol (TC), triglyceride (TG), uric acid (UA), diastolic blood pressures (DBP) and fasting plasma glucose (FPG) (all P=0.000 in men and women). Furthermore, significantly increased incidence of hyperuricemia (P=0.000 both in men and women) and obesity (P=0.000 both in men and women) were observed with the gradually increased Hb level. In addition, Pearson's correlation analysis revealed obvious correlation between Hb level and various cardiovascular risk factors including blood pressure and UA. Binary logistic regression analysis further demonstrated that the level of Hb was an important risk factor for elevated blood pressure (OR =1.216; 95% CI: 1.138-1.293, P=0.000 in men; OR =1.287; 95% CI: 1.229-1.363, P=0.000 in women).

Conclusions: Increasing Hb levels, even in subjects with normal level were associated with increasing prevalence of cardiovascular risk factors, suggesting that a slightly low Hb level might be beneficial to Chinese community-dwelling individuals.

Keywords: Hemoglobin; cardiovascular risk; blood pressure

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Introduction

Cardiovascular disease is the leading cause of mortality in the world, which accounts for about 30% of all causes of deaths. Of these deaths, high blood pressure is regarded as the most important risk factor and underlying cause of cardiovascular disease (1-3). Furthermore, such risk factors as high level of serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), uric acid (UA) and diabetic mellitus contributed greatly to the

development of the cardiovascular disease. Therefore, to maintain the blood pressure values at an appropriate level and decrease the risk factors to the minimal level are especially important. And it is imperative for us to obtain enough knowledge about factors that are associated with blood pressure or those cardiovascular risk factors.

Previous reports indicate that systolic blood pressure (SBP) and diastolic blood pressure (DBP) may increase with increasing hemoglobin (Hb) levels not only in hypertensive patients, but also in healthy patients (4,5). In addition, increased Hb values often accompany insulin resistance and compensatory hyperinsulinemia in humans (6-8). Conversely, anemia has also been suggested to be responsible for insulin resistance (9), thus suggesting a reasonable postulation that J- or U-shaped relationship might exist between the level of Hb and other cardiovascular risk factors. However, to our knowledge, studies that demonstrate a relationship between hematological parameters such as Hb and blood pressure and other cardiovascular risk factors are very few. And drawbacks of these earlier studies were too small individuals included or the lack of adjustment for potential confounding factors, such as age, gender and heart rate (4,10). In that, this study aimed to investigate the relationship between Hb and the traditional cardiovascular risk factors such as hypertension, hyperglycemia, and dyslipidemia by using cross-sectional data in large community-dwelling subjects.

Materials and methods

Subjects

Participants were recruited at the time of their health examination in a single community. All of the visits in the period from Jun. 1, 2012, to Nov. 30, 2012, were eligible for inclusion in the study. Information on medical history, present conditions, and drugs was obtained by interview. All of enrolled participants have no knowledge of their blood pressure previously and none of them had received any anti-hypertension medication before enrollment. During the physical examination, TC, TG, LDL-C, high-density lipoprotein cholesterol (HDL-C), fasting plasma glucose (FPG) and UA were measured. Eligibility criteria include a body weight ≥40 kg, regular pulse, an SBP of ≥90 and ≤200 mmHg and a DBP of ≥50 and ≤110 mmHg, and an Hb level of ≥84 mmol/L for men and ≥78 g/L for women.

Blood pressure measurements

Blood pressure measurements were obtained by manual auscultation with a mercury-gravity manometer via a standardized protocol by trained physicians. Three blood pressure readings were obtained after the participant had been seated, with feet on the ground and back supported, and resting quietly for ≥5 minutes. Each reading was obtained 30 seconds apart, and a fourth reading was obtained if ≥1 of the previous readings had been interrupted (11,12). Mean SBP and DBP for each participant were calculated from the recorded readings. Because we only obtained blood pressure measurements at a single study visit, and thus, a formal diagnosis of hypertension is not possible, participants were characterized as having "elevated blood pressure" if the mean SBP ≥140 mmHg and/or DBP ≥90 mmHg, and "normal blood pressure" if the mean SBP/DBP were <140/90 mmHg. Furthermore, we recorded such information as date of birth, sex, height and weight. We calculated the body mass index (BMI) by dividing weight (in kilograms) by height squared (in meters squared). All of the participants gave written informed consent before they were included in the study.

Biochemical analysis

Hyperuricemia was defined as serum UA ≥420 μmol/L in men or ≥360 μmol/L in women (13). Diabetes was defined as FPG ≥7.0 mmol/L, and/or diabetes history with an antidiabetic drug treatment. Impaired fasting glucose (IFG) was defined as subjects without previously confirmed or treated diabetes but with FPG from 5.6 to below 7.0 mmol/L. General obesity was defined as BMI ≥25 kg/m^2. Dyslipidemia was defined as subjects with TG level ≥1.7 mmol/L and/or high TC ≥5.2 mmol/L (14).

Statistical analysis

All data analyses were conducted using SPSS 13.0. Pearson χ^2 test was used for the comparison of categorical variables, and continuous data was assessed using analysis of variance (ANOVA). All values are expressed as mean ± standard deviation (SD), unless otherwise specified. Differences based on quartiles of Hb status within gender were analyzed by one-way ANOVA or Kruskal-Wallis H test. Correlation analysis was used for continuous variables between Hb level and various confounding cardiovascular risk factors

including blood pressure with adjustment of age. Binary logistic regression analysis was used to determine the factors associated with blood pressure in the entire population, and the results are presented with p for trend. Statistical significance was set at a two-tailed P<0.05.

Results

Baseline characteristics

The subjects comprised 4,851 men and 4,186 women, and all the patients were divided into six groups based on their Hb levels and categorized by gender. The individuals were divided into the following groups according to the quartiles of Hb status and gender. Men: Group 1, ≤119 g/L; Group 2, 120-129 g/L; Group 3, 130-139 g/L; Group 4, 140-149 g/L; Group 5, 150-159 g/L; and Group 6, ≥160 g/L. Women: Group 1, ≤109 g/L; Group 2, 110-119 g/dL; Group 3, 120-129 g/L; Group 4, 130-139g/L; Group 5, 140-149 g/L; and Group 6, ≥150 g/L. Both in men and woman, the Hb level in 6th group were above the upper limit of normal range and the level of Hb in 1th group was under the normal range. The whole patients were evaluated at baseline and found to be eligible for the present analysis. The demographic characteristics of these people are summarized in *Table 1*.

Associations of blood pressure and other cardiovascular risk factors with the titre of Hb

Table 2 shows that whether in men or women, elevated Hb levels were positively associated with DBP, MBP, UA, TC, TG, fasting GLU, BMI and age (P=0.00 from 1st tertile to the highest Hb level). Meanwhile, no significant difference in SBP level was observed among the groups of Hb in men (P=0.14). Furthermore, the incidence of hyperuricemia and obesity gradually increased with the increase level of Hb, and Chi-square test demonstrated that significant difference can be seen in different Hb groups (both P=0.00 in women and men for hyperuricemia and obesity). Those benefits, however, seem to be disappeared in patients with anemia.

The relationship of blood pressure and other risk factors including Hb

To evaluate the association between Hb and the blood pressure, the enrolled individuals were divided into two group of normal and elevated blood pressure group (described in subjects and data collection section). The binary logistic regression analysis demonstrated that whether in men or women, the level of Hb was an important independent risk factor for elevated blood pressure (OR =1.216; 95% CI: 1.138-1.293, P=0.000 in men; OR =1.287; 95% CI: 1.229-1.363, P=0.000 in women), as shown in *Table 3*.

Discussion

The current study for the first time showed that increasing Hb were associated with consistent increased levels of a range of cardiovascular risk factors, including general obesity, adverse lipid profile, higher blood pressure, IFG and high UA and increased blood pressure, and this positive association still can be seen in patients within normal Hb levels.

Some earlier cross-sectional studies found significant Pearson correlations between Hb level and arterial blood pressure, TC and UA in healthy or hypertensive persons. A recent study has demonstrated that Hb levels were positively related to the level of blood pressure in a large cohort of healthy blood donors (5). In accordance with their results, our results also demonstrated that the blood pressure is increased with the increasing of Hb level both in SBP and DBP in normal individuals. In addition, our results demonstrated that the level of Hb was an important risk factor for hypertension both in men and women participants, and positive correlation remained between Hb and blood pressure when adjusted with age whether in SBP or DBP in women. Although significant difference between Hb and blood pressure can be observed in our study and reported elsewhere, the exact mechanisms for Hb lead to an elevated blood pressure are not entirely known. Present viewpoints indicate that Hb can have direct or indirect effects on vascular system. Studies have demonstrated that several biological mechanisms for the Hb-blood pressure association might partial explain their relationship. Firstly, Hb may be a scavenger of NO, which is produced in endothelial cells. As the NO can relax the muscle cells surroundings the vessel, the changed levels of Hb may then control the blood pressure (15). Furthermore, Hb has been reported to be strongly related to arterial stiffness which, in turn, can increase SBP and DBP (10). Another explanation for blood pressure increase with increased Hb levels would be increased blood viscosity. It has been reported that elevation of hematocrit and Hb levels increases blood viscosity and that increased viscosity partly through an effect on blood pressure may worsen cardiovascular function (16).

Besides with relationship to blood pressure, our study

Table 1 Characteristics of subjects categorized by gender and hemoglobin status

	Hemoglobin level (g/L)						
	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	P
Men							
Number	45	153	554	1,490	1,733	876	–
Characteristic	≤119	120-129	130-139	140-149	150-159	≥160	–
Age (y)	60.9±13.1	60.9±14.2	56.2±14.3	52.2±13.7	48.8±13.7	47.2±14.2	0.000
Current smoking (%)	29.8	28.7	27.4	29.3	30.4	29.8	0.860
DM (%)	6.7	6.5	7.1	7.8	8.8	10.1	0.269
Hyperuricemia (%)	20.9	18.2	20.6	25.0	27.2	31.3	0.000
Obesity (%)	37.8	26.1	30.1	36.8	41.3	45.7	0.000
SBP (mmHg)	128.5±19.4	127.7±19.2	126.5±17.0	125.5±17.0	125.2±17.0	126.6±17.1	0.140
DBP (mmHg)	77.2±9.5	78.1±11.7	78.1±11.3	79.2±10.4	80.2±10.6	82.2±10.6	0.000
MBP (mmHg)	94.3±11.0	94.6±12.4	94.2±12.5	94.7±11.3	95.2±11.4	97.0±11.7	0.000
HR (beat/min)	68±9	67±9	70±11	67±10	71±9	68±9	0.270
TG (mmol/L)	1.1 (0.8-1.6)	1.4 (1.0-1.8)	1.3 (1.0-1.9)	1.5 (1.0-2.2)	1.6 (1.2-2.3)	1.8 (1.3-2.6)	0.000
TC (mmol/L)	4.4±1.0	4.5±0.9	4.6±1.0	4.8±1.0	4.9±1.0	5.0±1.0	0.000
UA (μmol/L)	356.2±91.9	337.5±92.3	353.1±89.6	369.6±85.5	378.4±83.2	383.8±84.8	0.000
Glu (mmol/L)	5.1±0.9	5.4±1.6	5.2±1.1	5.2±1.1	5.2±1.3	5.4±1.5	0.010
H (cm)	165.3±6.1	165.9±6.1	167.2±6.4	169.1±5.9	169.6±5.9	169.8±5.8	0.000
W (kg)	65.5±9.8	64.4±10.7	66.2±9.5	68.8±9.1	70.3±9.3	71.7±9.1	0.000
BMI (kg/m^2)	23.9±3.2	23.4±4.1	23.7±3.0	24.0±2.8	24.4±2.9	24.9±3.0	0.000
Women							
Number	112	296	1,222	1,650	781	125	–
Characteristic	≤109	110-119	120-129	130-139	140-149	≥150	–
Age (y)	44.9±13.6	45.5±14.2	46.5±14.1	48.5±13.7	52.5±13.0	53.8±11.6	0.000
Current smoking (%)	6.8	8.5	7.4	6.6	5.8	6.1	0.645
DM (%)	5.9	5.8	6.3	6.9	7.7	9.2	0.617
Hyperuricemia (%)	8.0	7.4	10.0	11.9	17.9	24.0	0.000
Obesity (%)	19.6	14.5	18.9	28.4	35.3	48.0	0.000
SBP (mmHg)	118.5±16.7	116.5±16.6	117.1±17.9	121.0±18.6	124.8±18.4	131.8±18.8	0.000
DBP (mmHg)	71.7±11.6	72.5±10.2	73.6±10.7	76.1±11.1	78.4±10.9	97.9±12.9	0.000
MBP (mmHg)	87.3±12.9	87.0±11.4	88.1±11.9	91.1±12.3	93.9±12.1	97.0±11.7	0.000
HR (beat/min)	71±11	68±9	73±10	72±9	68±10	65±10	0.330
TG (mmol/L)	1.0 (0.7-1.5)	1.1 (0.8-1.5)	0.8 (1.1-1.6)	1.3 (0.9-1.9)	1.4 (1.0-2.0)	1.6 (1.3-2.4)	0.000
TC (mmol/L)	4.5±0.9	4.5±0.9	4.7±1.0	5.0±1.0	5.1±1.0	5.2±0.9	0.000
UA (μmol/L)	252.7±70.3	246.8±68.6	266.6±69.6	279.5±70.0	298.0±73.0	317.2±71.2	0.000
Glu (mmol/L)	4.5±0.9	4.5±1.6	4.7±1.0	5.0±1.0	5.1±1.0	5.2±0.9	0.000
H (cm)	158.7±6.4	158.7±6.0	158.2±5.9	157.9±5.4	157.5±5.5	160.0±5.7	0.080
BW (kg)	54.7±6.9	55.5±7.1	56.2±8.0	58.2±8.2	60.4±9.4	63.4±7.9	0.000
BMI (kg/m^2)	21.7±2.6	22.3±3.2	22.5±3.1	23.4±3.3	24.4±3.5	25.2±3.1	0.000

Data are presented as mean ± SD unless otherwise indicated. Abbreviations: DM, diabetes mellitus; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; HR, heart rate; TG, triglyceride; TC, total cholesterol; Glu, glucose; UA, uric acid; H, height; BW, body weight; BMI, body mass index.

further demonstrated that even modestly elevated levels of Hb were associated with well recognized cardiovascular risk factors such as diabetes mellitus, hypertension and obesity, and the association is a continuum. Interestingly, Kawamoto *et al.* (10) also found that Hb per se was associated with other confounding factors such as increased BMI, blood pressure, and TG. Furthermore, our results demonstrated that high levels of Hb were associated with high incidence of obesity and hyperuricemia, even the incidence of diabetes mellitus also has the trend to increase with Hb level. Hyperuricemia has been well proved to link to endothelial cells dysfunction, inflammatory cytokines release and thus increase blood pressure, which is an independent predictor for cardiovascular death (17-19). Although the exact mechanisms between such cardiovascular risk factors and the level of Hb remain unknown, we still may tentatively say that slightly decreased level of Hb within normal range may be beneficial to Chinese community-dwellings.

Table 2 Relationship between hemoglobin and various confounding factors including blood pressure of subjects categorized by gender

Characteristic	Age-adjusted Pearson's correlation			
	Men (N=4,851)		Women (N=4,186)	
	r	P	r	P
SBP (mmHg)	0.062	0.070	0.107	0.000
DBP (mmHg)	0.146	0.000	0.158	0.000
MBP (mmHg)	0.122	0.000	0.151	0.000
UA (μmol/L)	0.106	0.000	0.159	0.000
TC (mmol/L)	0.156	0.000	0.130	0.000
TG (mmol/L)	0.131	0.000	0.130	0.000
Glu (mmol/L)	0.101	0.000	0.155	0.000
BMI (kg/m^2)	0.163	0.000	0.179	0.000
BW (kg)	0.180	0.000	0.181	0.000

SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure; UA, uric acid; TG, triglyceride; TC, total cholesterol; Glu, glucose; BW, body weight; BMI, body mass index.

The limitations in our study still should be noted. The first limitation was the cross-sectional nature of the data. We therefore could not assess the temporal relationship between Hb level and the cardiovascular risk factors. Furthermore, Hb levels and blood pressure values were measured within the same visits. Consequently, drawing conclusions about causality is difficult. However compared to most previous studies, our study has a large sample size, allowing the data to be analyzed separately for men and women. We also controlled for a wide range of potential confounders. In conclusion, there was a significant dose-response association between a wide range of cardiovascular risk factors, especially the blood pressure with Hb level even in subjects with both normal blood pressure and normal Hb levels. Therefore, further studies are thus warranted to quantify the clinical significance in those subjects with high Hb state.

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Table 3 Binary logistic regression analysis of independent risk factors for elevated blood pressure (methods: forward:conditional)

Characteristic	Binary logistic regression			
	Men (N=4,851)		Women (N=4,186)	
	OR (95% CI)	P	OR (95% CI)	P
Age (y)	1.034 (1.029-1.039)	0.000	1.056 (1.048-1.063)	0.004
Smoking (Yes =1, No =0)	1.164 (1.108-1.215)	0.003	1.123 (1.104-1.145)	0.009
Hgb (Group 2 =1, Group 3, 4, 5, 6 =0)	1.216 (1.138-1.293)	0.000	1.287 (1.229-1.363)	0.000
Hyperuricemia (Yes =1; No =0)	1.004 (1.001-1.006)	0.039	–	0.238
Dyslipidemia (Yes =1, No =0)	1.115 (1.089-1.146)	0.009	1.139 (1.072-1.194)	0.004
DM (Yes =1, No =0)	1.265 (1.115-1.347)	0.000	1.154 (1.097-1.208)	0.012
Obesity (Yes =1, No =0)	1.109 (1.083-1.131)	0.013	1.088 (1.064-1.115)	0.034

DM, diabetes mellitus.

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Original Article

Diagnostic and prognostic significance of lysophosphatidic acid in malignant pleural effusions

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Background: Lysophosphatidic acid (LPA) is an important extracellular signal transmitter and intracellular second messenger in body fluids. It can be detected in the ascitic fluid of patients with ovarian cancer. Increasing evidence shows that LPA can stimulate cancer cell proliferation and promote tumor invasion and metastasis. Our study aimed to evaluate the diagnostic value of LPA in differentiating between malignant pleural effusions (MPEs) and benign pleural effusions (BPEs) and to evaluate the association between the level of LPA in MPE and the prognosis of lung cancer patients.

Patients and methods: The level of LPA in the pleural effusions (PEs) of 123 patients (94 MPE, 29 BPE) with lung cancer was evaluated using an enzyme-linked immunosorbent assay. The performance of LPA was analyzed by standard Receiver operator characteristic curve (ROC) analysis methods, using the area under the curve (AUC) as a measure of accuracy. Overall survival (OS) curves and progression-free survival (PFS) curves were based on the Kaplan-Meier method, and the survival differences between subgroups were analyzed using the log-rank or Breslow test (SPSS software). A multivariate Cox proportional hazards model was used to assess whether LPA independently predicted lung cancer survival.

Results: The levels of LPA differed significantly between MPE (22.08±8.72 μg/L) and BPE (14.61±5.12 μg/L) ($P<0.05$). Using a cutoff point of 18.93 μg/L, LPA had a sensitivity of 60% and a specificity of 83% to distinguish MPEs from BPEs with an AUC of 0.769±0.045 (*SE*) (P=0.000) (95% CI, 0.68-0.857). In the three pathological types of lung cancer patients with MPE, there were no significant associations between LPA levels and the length of PFS and OS (P=0.58 and 0.186, respectively). Interestingly, in the patients with MPE caused by lung adenocarcinoma there were significant associations between the LPA levels and the PFS and OS (P=0.018 and 0.026, respectively). Multivariate analysis showed that the LPA level was an independent prognostic factor for PFS in lung adenocarcinoma.

Conclusions: Our results indicate that LPA can be used as a new biomarker for the diagnosis of MPE caused by lung cancer and that higher levels of LPA are related to shorter PFS in adenocarcinoma of the lung.

Keywords: Lysopohsphatidic acid (LPA); malignant pleural effusions (MPEs); lung cancer; diagnosis; prognosis

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Introduction

Malignant pleural effusion (MPE), the accumulation of pleural fluid due to metastasis of cancer to the pleural space, is a common clinical manifestations with an annual incidence rate of approximately 500 patients/million in the United States (1,2). Malignancies account for approximately 40% of all pleural effusions; lung cancer is the most common metastatic tumor associated with MPE (3,4). In fact, at the time of diagnosis, 14% of patients with non-small-cell lung cancer have a pleural effusion, because this cancer disseminates to the pleura, approximately 50% will eventually develop a pleural effusion (5). Whereas, the most common causes of benign pleural effusion (BPE) are tuberculosis and pneumonia. Therapeutic management differs for these two types of effusions; therefore, it is clinically important to differentiate between them rapidly and accurately. Finding malignant cells, in either the pleural fluid by cytological examination or in a pleural biopsy, has traditionally been the primary diagnostic method for MPE (6). However, it is often difficult to find malignant cells in MPE, and because pleural biopsy is an invasive procedure it is not readily accepted by some patients. Although several tumor markers, such as carcinoembryonic antigen (CEA), cancer antigen 125 (CA-125), and cytokeratin fragment 19 (CYFRA 21-1), can assist the diagnosis of MPE, their specificity and sensitivity are limited (7). Therefore, new indices that are more accurate are needed. Several factors that participate in the formation of pleural effusion been identified; however, our current understanding of the basic mechanisms by which effusion accumulates within the pleural space is poor. Metastasis of tumor cells into the pleural space may lead to production of large amounts of PE. In addition, angiogenic factors released by the infiltrated tumor cells or stromal cells play an important role in the development of PE (8).

Lysophosphatidic acid (LPA) is a bioactive phospholipid signaling mediator that is present in almost all mammalian cells and tissues (9). Recent research indicates that LPA promotes the proliferation and invasion of cancer cells (10) and regulates tumor angiogenesis (9). *In vitro* experiments found that A549 lung carcinoma cells express endogenous LPA receptors and that LPA can activate this receptor, enhance the degradation of the p53 tumor suppressor and thereby allow cancer cell proliferation and motility (11) {Murph, 2007 #6516}. Therefore, LPA also plays an important role in lung cancer.

Previous studies reported that malignant effusions contained LPA-like activity, but they primarily evaluated ascites of ovarian cancer patients (12), and the method for detecting LPA, by the neurite retraction assay, was limited. Most importantly, whether LPA is actually present in MPE and whether there is a significant difference in LPA levels between MPE and BPE remained unknown.

The aims of our study were to explore the diagnostic value of LPA in differentiating between MPE and BPE and to evaluate the prognostic value of LPA in MPE caused by lung cancer.

Patients and methods

Patients

This retrospective cohort study included 123 patients with PE who were hospitalized in the Respiratory Department, Nanjing General Hospital of Nanjing Military Command in China, from September 2009 through September 2013. Written consent was obtained from all the patients who participated in this study. Twenty-nine patients (20 males and nine females; mean age, 61.21 years) had BPEs, and 94 had MPEs caused by lung cancer (55 males and 39 females; mean age, 63.29 years). Only patients diagnosed with primary malignancies were included; otherwise they were excluded.

Diagnostic criteria for pleural effusions

In our study, BPE mainly included tuberculosis PE (TPE) and parapneumonic PE. The diagnostic criteria for TPE were as follows: identification of M. tuberculosis, pleural biopsy revealing granulomatous tissue and positive response to anti-tuberculosis treatment. Pleural effusions were considered pneumonic if they had the following these characteristics: an acute febrile condition along with cough, yellow sputum, chest CT with pulmonary infiltration, leukocytosis with neutrophilic predominance in the PE and response to antibiotic treatment. The diagnostic criteria for MPE were malignant cells present either in the cytology of the pleural fluid or seen on histopathologic examination of a biopsy specimen of the pleura.

Sample collection and biochemical analyses

Pleural effusions were collected by routine thoracentesis performed after patients gave written the consent. Samples were centrifuged at 1,500 ×g for 10 min at –4 ℃. The supernatants were dispensed into 1.5 mL Eppendorf tubes and were stored at –80 ℃. LPA was determined using a

Table 1 Descriptive statistics of patient characteristics

Variables	MPE (n=94)	BPE (n=29)	P value
Age	63.29±12.31	61.21±18.18	0.568
Male/female	55/39	20/9	0.313
Smoke/non-smoke	47/47	18/11	0.225
MPE			
Adenocarcinoma	74 (78.7%)		
Squamous cell carcinoma	12 (12.8%)		
Small cell lung carcinoma	8 (8.5%)		
Cytology			
Positive	28 (29.8%)		
Negative	66 (70.2%)		

MPE, malignant pleural effusions; BPE, benign pleural effusions.

commercial human LPA ELISA Kit (CUSABIO, China; Catalog Number CSB-EQ 028005HU). The assay was performed following the manufacturer instructions. The technicians were blinded to the clinical data.

Statistical methods

Statistical analyses were performed with SPSS Software 17.0. Patient demographics and disease characteristics were summarized using descriptive statistics. The student's *t*-test was used to assess the difference of the LPA level between MPE and BPE. One-way ANOVA was used to compare the difference of LPA level in subgroups of MPE (adenocarcinoma, squamous cell carcinoma and small cell lung carcinoma) patients. Receiver operator characteristic curve (ROC) analysis was used to select the threshold value of LPA that best differentiated MPE from BPE, specifically the value that maximized the sum of specificity and sensitivity. OS was defined as the time interval from the date of diagnosis to the date of death from any cause or to the date on which the patient was last known to be alive. PFS was defined as the interval from the date of diagnosis to the date that progression was detected or to the date when the patient was last known to be disease free. Kaplan-Meier survival curves were used to examine the associations between the LPA concentration and the OS and PFS; significance of these associations was analyzed using the log-rank or Breslow test. A multivariate Cox proportional hazards model was used to assess whether LPA was an independent predictor of survival. For the above comparisons, P<0.05 was considered statistically significant.

Results

Our study was carried out with 123 patients (75 males and 48 females; age range, 17-83 years) with MPE (94 MPE) caused by lung cancer or BPE (29 BPE) caused by tuberculous pleuritis or pneumonia. The patient characteristics are shown in *Table 1*. There were no significant differences in gender, age, and smoking history between the MPE and BPE groups (P>0.05). Of the MPE cases, 74 (78.7%) had adenocarcinoma, 12 (12.8%) squamous carcinoma and eight (8.5%) small cell lung cancer. The MPE cytology results were 28 (29.8%) positive and 66 (70.2%) negative. There were 19 patients with TBE and ten with pneumonic PE in BPE group.

LPA levels in PE

As shown in *Figure 1A*, the level of LPA was significantly higher in patients with MPE (22.08±8.72 μg/L) than in those with BPE (14.61±5.12 μg/L; P=0.000). Subgroup analysis (*Figure 1B*) found that the levels of LPA in MPEs caused by adenocarcinoma, squamous cell carcinoma and small cell lung carcinoma, 22.01±9.28, 20.76±4.65, and 24.75±8.72 μg/L, respectively, were not significantly different (P=0.603).

Relationship between LPA concentration and clinicopathologic factors in lung cancer patients with MPE

Because the mean LPA concentration was significantly higher in MPEs than in BPEs, we evaluated the relationship between the levels of LPA and gender, age, smoking history, histologic type of tumor, and a positive cytology result. However, as shown in *Table 2*, no significant associations between LPA concentration and these clinicopathologic factors were found.

Discriminative power of LPA in MPE

ROC curve was created to find sensitivity and specificity of LPA in MPE versus BPE group. The area under the ROC curve was 0.769±0.045 (*SE*) (P=0.000, 95% CI, 0.68-0.857). Using a threshold value of 18.93 μg/L, LPA had a sensitivity of 60%, a specificity of 83%. The ROC curve is shown in *Figure 2*.

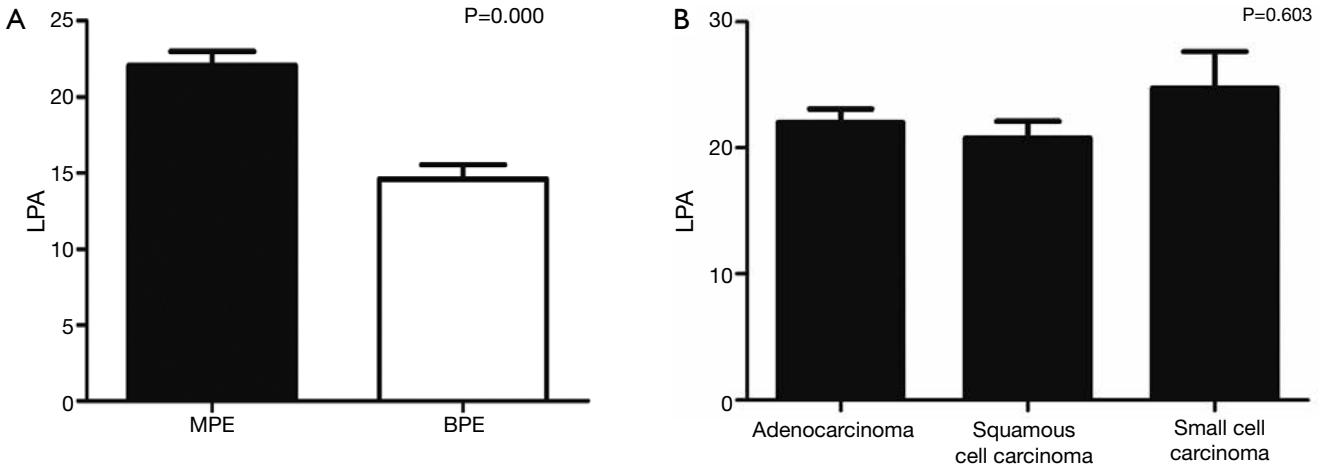

Figure 1 (A) Comparisons of LPA levels between MPE and BPE. The value between the MPE and BPE groups (22.08±8.72 versus 14.61±5.12) has significant difference. P=0.000. (B) Subgroups comparisons of LPA levels in MPE caused by adenocarcinoma, squamous cell carcinoma and small cell lung carcinoma. No significant differences were found among the three groups (P=0.603). LPA, lysophosphatidic acid; MPE, malignant pleural effusions; BPE, benign pleural effusions.

Table 2 LPA concentration in pleural effusion of lung cancer patients

Clinical variables	No. of patients	LPA (μg/L), mean ± SD	P value
Age (years)			0.41
≥60	56	20.68±9.30	
<60	38	21.20±7.84	
Gender			0.789
Male	55	21.87±8.10	
Female	39	22.48±9.63	
Smoke condition			0.838
Smoke	47	22.27±8.03	
Non-smoke	47	21.90±9.44	
Histologic type			0.603
Adenocarcinoma	74	22.01±9.28	
Squamous cell carcinoma	12	20.76±4.65	
Small cell lung cancer	8	24.75±8.19	
Cytologic examination			0.234
Positive	28	24.10±11.68	
Negative	66	21.23±11.68	

LPA, lysophosphatidic acid.

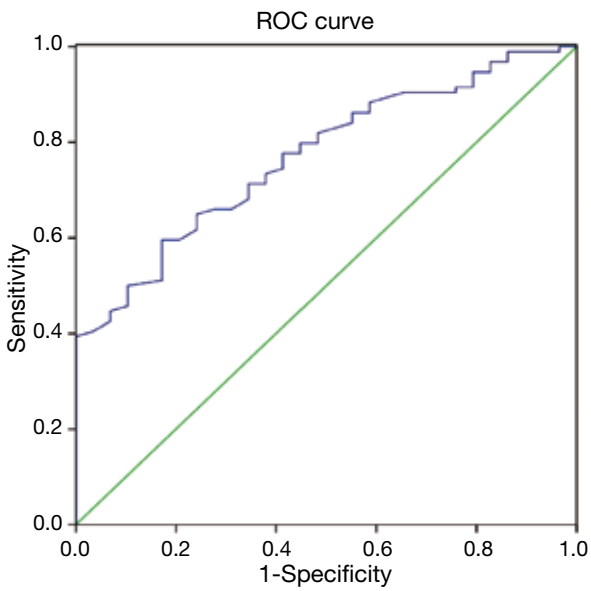

Figure 2 ROC of LPA for the diagnosis of MPE *vs.* BPE. The plot was constructed by computing the sensitivity *vs.* (1-specificity) for the different possible cutoff points of the LPA ELISA assay. ROC, receiver operator characteristic curve; LPA, lysophosphatidic acid; MPE, malignant pleural effusions; BPE, benign pleural effusions.

Prognostic value of LPA among MPE patients

For this analysis, the patients with MPE were divided into two groups based on the LPA level in their PE, either above or below the median LPA concentration, 20.57 μg/L. *Figure 3A,B* showed no significant differences in the OS rate [P=0.580, Log-rank (Mantel-Cox)] and the PFS rate [P=0.186, Log-rank (Mantel-Cox)] between the high LPA and low LPA concentration groups.

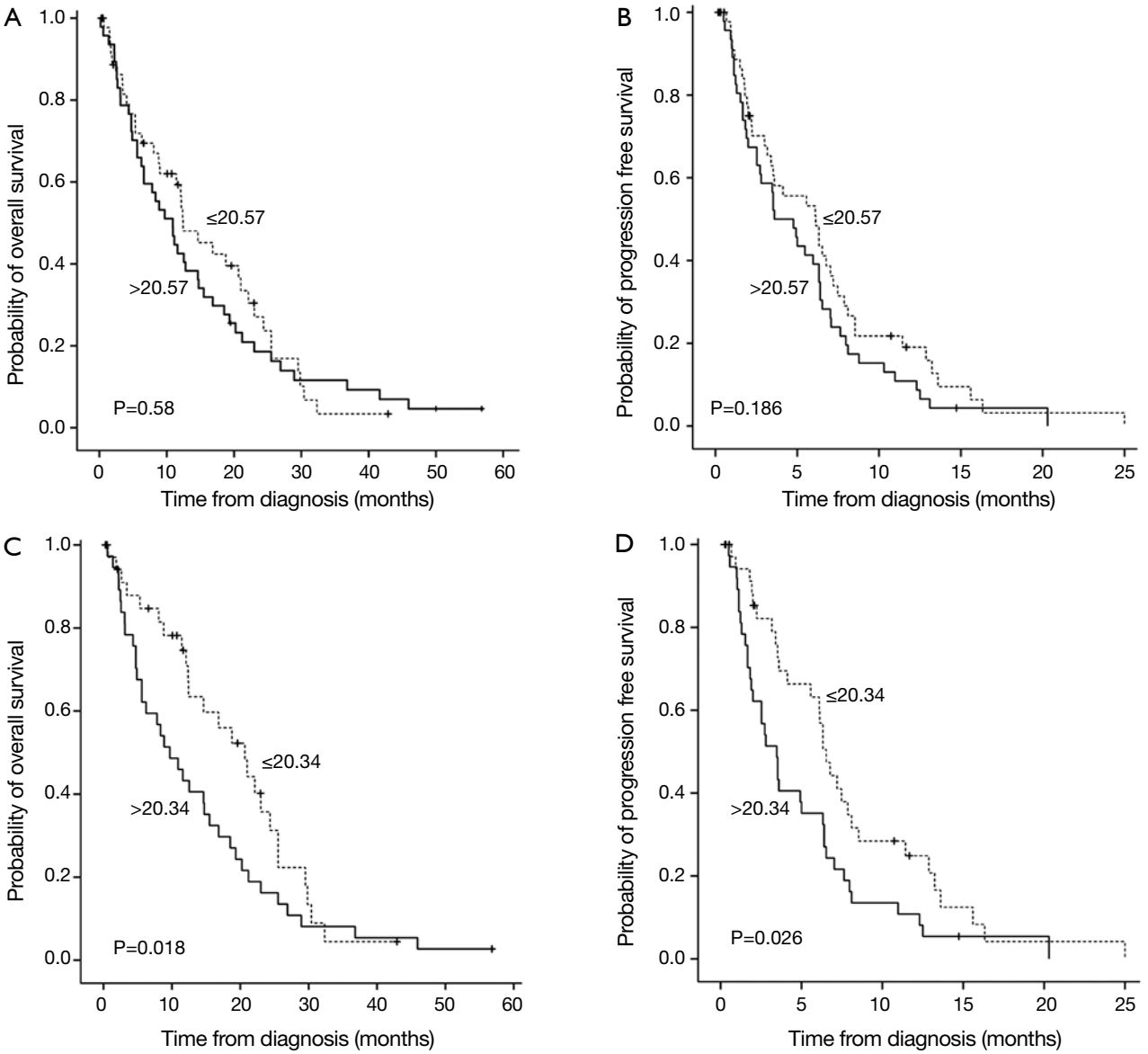

Figure 3 Kaplan-Meier analysis for OS and PFS according to the level of LPA in MPE. Data were dichotomized at the median value for each parameter. (A,B) LPA in MPE caused by the three types of lung cancer; (C,D) LPA in MPE caused by lung adenocarcinoma. For *Figure 3C*, when using Log-rank test, it didn't reach a statistical significance (P=0.076), while using Breslow (Generalized Wilcoxon) test reach a statistical significance (P=0.018). The *Figure 3* showed a P value using the latter test method. OS, overall survival; PFS, progression-free survival; LPA, lysophosphatidic acid; MPE, malignant pleural effusions.

We also carried out a subgroup analysis based on the three types of lung cancer. The number of patients with MPE caused by squamous cell carcinoma and small cell lung carcinoma was too small to provide meaningful statistical results, so we only further analyzed the relationship between the LPA concentration and patient's OS and PFS in patients with adenocarcinoma of the lung in the MPE group. The median LPA level, 20.34 μg/L, was used to separate these patients into high and low LPA concentration groups. The median OS intervals were 20.67 and 9.7 months, respectively, for the patients in the low and high LPA concentration groups [P=0.018, Breslow (generalized Wilcoxon); P=0.076, Log-rank (Mantel-Cox)] as depicted in *Figure 3C*. The median PFS intervals were 6.53 and 3.47 months, respectively, for the patients with low and high LPA concentrations [P=0.026, Log Rank (Mantel-Cox); P=0.009,

Table 3 Cox regression analysis of PFS in patients with lung adenocarcinoma

	e^{β}	SE	P value	β
LPA	0.028	0.013	0.034	1.029

Model χ^2:4.527 (P=0.041). PFS, progression-free survival; LPA, lysophosphatidic acid; β, regression coefficient; SE, standard error; e^{β}, HR (hazard ratio).

Breslow (generalized Wilcoxon)] as depicted in *Figure 3D*.

Multivariate cox proportional hazards analysis

LPA concentration and other factors, including gender, age, smoking history, p-T status, lymph node metastasis and extrapulmonary distant metastasis, were analyzed by Cox's proportional hazards regression models in the 74 patients with MPE caused by adenocarcinoma of the lung. We found that LPA concentration was an independent prognostic factor for PFS (P=0.034) but not for OS (P=0.112). Only LPA level was retained as a significant variable in the forward regression model, when controlling for other variables, based on the likelihood ratio test (*Table 3*).

Discussion

This is the first study to investigate the potential of LPA, measured by ELISA, as a possible marker to differentiate MPE due to lung cancer from BPE. We not only showed that the level of LPA was higher in MPE than in BPE, but also that LPA concentration was an independent prognostic factor for PFS in patients with MPE who had adenocarcinoma of the lung.

LPA is a naturally occurring phospholipid produced by activated platelets, mesothelial cells, fibroblasts, adipocytes and some cancer cells (10,13) and now is recognized as an extracellular lipid mediator that evokes growth-factor-like responses in almost every cell type (10). LPA was first detected in serum and ascites from ovarian cancer patients. In 1998, Westermann *et al.* (12) found that malignant effusions contained LPA-like activity using a bioassay of neurite retraction in differentiated NIE-115 neuroblastoma cells described by Jalink *et al.* (14). Their study included malignant effusions caused by various types of cancers, but not benign effusion as a control group. Furthermore, malignant effusions caused by each type of cancer had a limited number of samples and only seven samples were from non-small lung cancer. That study's primary finding was that the LPA-like activity in the effusions of ovarian cancer patients was significantly higher than in the effusions caused by other cancers.

In our study, we focused on MPE caused by lung cancer and we compared the levels of LPA between MPE and BPE using an ELISA to measure LPA, a more accurate detection method in a larger study population than that reported by Westermann *et al.* ROC curves for MPE versus BPE were analyzed and the cutoff value of 18.93 μg/L had a sensitivity of 60% and a specificity of 83%. Other tumor markers, such as CEA, CA-125, and CYFRA 21-1 have been widely used to distinguish between MPE and BPE. Huan-Zhong Shi *et al.* performed a meta-analysis of forty-five studies and found that the sensitivity and specificity of CEA for the diagnosis of MPE were 54% and 94%, respectively (15). Another meta-analysis reported that the sensitivity/specificity of these tumor markers for the diagnosis of MPE were as follows: CA 125, 48%/85%; and CYFRA 21-1, 55%/91%, respectively (16). In contrast, the specificity of LPA for the diagnoses of MPE was slightly lower than that of these tumor markers. However, much less research has been conducted on the diagnostic value of LPA in MPE than on these tumor markers, and the 123 PE samples in our study were too few to support definitive recommendations. Therefore, our results only suggest that LPA could be a new informative tumor marker for the diagnosis of MPE caused by lung cancer. More high quality studies with larger samples need to be performed.

LPA, an inducer of cell proliferation, migration and survival, has actions that are concordant with many of the 'hallmarks of cancer' (17) and suggestive of a role for LPA in the initiation or progression of malignant disease (10). In our study, we evaluated the prognostic value of LPA in MPE caused by three pathological types of lung cancer and in MPE caused only by adenocarcinoma of the lung. We found that LPA concentration was not significantly associated with the OS or PFS of the three types of lung cancer. These results agreed with those reported by Westermann *et al.* (12). However, when we only considered the patients with MPE caused by lung adenocarcinoma we found that those patients with a lower LPA concentration in their MPE survived markedly longer (20.67 months, median OS) than patients with a higher LPA concentration in their MPE (9.7 months, median OS) based on univariate analysis of OS and PFS. Multivariate Cox proportional hazards analysis found that LPA concentration was significantly and independently associated with PFS but not with OS. These findings mean that patients with lower LPA concentrations

in their MPE have a longer PFS than those with higher LPA concentrations. Westermann *et al.* (12) reported that disease free and overall survival were not in any way linked to LPA-equivalent levels in malignant effusions. The reason for these different results may be that they evaluated MPE due to various cancers; whereas, we specifically analyzed MPE caused by lung adenocarcinoma. Accordingly, we propose that LPA may contribute to the progression of MPE caused by lung adenocarcinoma.

Several potential biologic mechanisms might explain our findings and are worthy of further study. One possible explanation is that LPA stimulates lung adenocarcinoma cells more than it stimulates squamous cell or small cell growth at concentrations just as LPA present in ascitic fluid markedly stimulates ovarian tumor growth (18). Therefore, those patients with both lung adenocarcinoma and a higher LPA level in their MPE had a shorter OS and PFS time. Furthermore, LPA has been found to stimulate hypoxia-inducible factor-1-independent VEGF expression that promotes angiogenesis and vascular permeability and leads to ascites formation (18-20). Xiaoyu Xu found that LPA antagonist BrP-LPA inhibited tumor growth and angiogenesis in an engineered three-dimensional lung cancer xenograft model (20). According to our clinical results and these previous studies, we propose that LPA facilitates MPE caused by lung adenocarcinoma by promoting angiogenesis and that LPA may be a potential target for MPE therapy. However, these are only our hypotheses. Additional studies with larger number of samples are necessary to determine whether or how LPA affects the pathogenesis of pleural effusions in lung cancer.

Conclusions

Our findings indicate that the level of LPA in PE can assist to distinguish MPE from BPE and the level of LPA in MPE is inversely related to the length of OS and PFS in lung adenocarcinoma. LPA may contribute to the progression of MPE caused by lung adenocarcinoma. We believe that LPA may be a therapeutic target to reduce or inhibit MPE (12). Additional studies that are more extensive are needed to understand the role and mechanism of LPA in the formation of MPE.

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Original Article

Analysis of mutations in 7 candidate genes for dextro-Transposition of the great arteries in Chinese population

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Background: Transposition of great arteries (TGA) represents the most frequent cyanotic heart defect diagnosed in the neonatal period. Several genes had been identified to be associated with the pathogenesis of dextro-transposition of the great arteries (d-TGA). These genes are located in different chromosomes and their mutations can only explain few clinical cases. Besides, no genetic scan for TGA has been implemented in China.

Methods: To evaluate whether aberrations in any of the 13 reported mutations in seven genes (*MED13L*, *ZIC3*, *CFC1*, *NODAL*, *FOXH1*, *GDF1* and *NKX2-5*) could completely or in part be the genetic component involved in TGA in Chinese population, we screened 102 Chinese patients with d-TGA by direct sequencing for mutations within the seven genes.

Results: We found none of the reported 13 mutations in those 102 Chinese d-TGA patients.

Conclusions: These reported 13 mutations may not be a common cause of d-TGA in Chinese population due to racial variation and genetic heterogeneity of TGA. New approaches including the whole exome sequencing technology are required to effectively identify genetic variants in TGA patients in China.

Keywords: dextro-transposition of the great arteries (d-TGA); mutation; Chinese

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Introduction

Transposition of the great arteries (TGA) is the most common cyanotic congenital heart lesion that presents in neonates. The hallmark of TGA is ventriculoarterial discordance, in which the aorta arises from the morphologic right ventricle and the pulmonary artery arises from the morphologic left ventricle. Despite its overall low prevalence, TGA is the most common etiology for cyanotic congenital heart disease (CHD) in the newborn (1). This lesion presents in 5-7% of all patients with CHD. The overall annual incidence is 20-30 per 100,000 live births, and inheritance is multifactorial. TGA is isolated in 90% of patients and is rarely associated with syndromes or extracardiac malformations. This congenital heart defect is more common in infants of diabetic mothers (2). The mortality rate in untreated patients is approximately 30% in the first week, 50% in the first month, and 90% by the end of the first year. With improved diagnostic, medical, and surgical techniques, the overall short-term and midterm survival rate exceeds 90% (3).

The exact etiology of TGA is still unknown. The controversy between environmental factors and genetic causes

has been discussed for years. Some studies have postulated associated risk factors, such as gestational diabetes mellitus (4,5) , maternal exposure to rodenticides and herbicides (6), and maternal use of antiepileptic drugs (7). However, significant progresses have been achieved in understanding the genetic causes of this disease. The genes being thought to be associated with the pathogenesis of TGA so far are the thyroid hormone receptor-associated protein-2 gene (*MED13L*) (8), nucleocytoplasmic shuttling protein gene (*ZIC3*) (9), the forkhead activin signal transducer 1 *(FOXH1)* (10), cryptic family 1 *(CFC1)* (11), growth differentiation factor 1 *(GDF1)* (11) and TGF-beta gene *(NODAL)*, the latter five genes of which affect the LR-axis development (12). NK2 homeobox 5 *(NKX2-5)* are rarely involved in TGA pathogenesis (13). These genes are located in different chromosomes and their mutations only explain few clinical cases. The chromosomal region 22q11 was also suggested to be in the involvement of the pathogenesis of TGA (14).

Since no genetic scan for TGA has been implemented in China, this study was initiated to evaluate whether aberrations in any of the reported 13 mutations (*Table 1*) in seven genes (*MED13L*, *ZIC3*, *CFC1*, *NODAL*, *FOXH1*, *GDF1* and *NKX2-5*) could completely or in part be the genetic component involved in TGA in Chinese population. The basis for selecting these 13 mutations was their possible involvement in malformations of TGA. The purpose of the study was to perform mutational screening of these 13 mutations in d-TGA patients in China.

Patients and methods

Patients

Charts for patients with d-TGA in Guangdong General Hospital between Jan 1, 2000 and August 30, 2012 were reviewed. A total of 102 patients diagnosed by echocardiography, with age ranged from 10 days to 12-year-old were selected. The male-to-female ratio was 2.4:1. Fifty-four patients were TGA with intact ventricular septum (TGA/IVS), 31 patients were TGA with ventricular septum defect (TGA/VSD), 17 patients were combined with other complex situations as coarctation of aorta (CoA), pulmonary valve stenosis (Ps), single ventrical (SV) *et al.*

Genomic DNA extraction and mutational screening

After informed consent was obtained with approval by the Medical Ethical Review Committee of Guangdong General Hospital, constitutional DNA was prepared from peripheral blood with the PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations. The sequences of 13 regions in *MED13L*, *ZIC3*, *CFC1*, *NODAL*, *FOXH1*, *GDF1* and *NKX2-5* were analyzed for mutations using direct sequencing analysis. Polymerase chain reaction (PCR) products were generated by exon flanking primers according to the reported 13 mutations in seven genes which might be associated with pathogenesis of TGA in human (*Table 1*).

Primers are given in *Table 1*. PCR reactions were performed in Eppendorf5332 PCR system in a total volume of 20 μL, using the mixture containing 10 μL GoTaq Green Master Mix (Promega), 10 μM forward primer, 10 μM reverse primer, 200 ng genomic DNA and 4 μL Nuclease-Free Water. The following amplification conditions was used: initial denaturation at 95 ℃ for 5 min, followed by 40 cycles including denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s, and elongation at 72 ℃ for 1 min. The procedure was completed by a final incubation at 72 ℃ for 5 min. The PCR products were then sent to Invitrogen Co. (Guangzhou, China) for sequencing on ABI 3730. Sequence analysis was carried out using Chromas.exe, and the homozygous and heterozygous alleles were scored manually.

Results

Patients' clinical characteristics are given in *Table 2*. The percentage of males is 70.6% (72/102). The mean age at first diagnosis was 3.28 months (Standard Deviation, SD 1.21), while the mean age at first operation was 3.42 months (SD 1.33). 1% (n=1) of patients had family history of CHD, with the mother diagnosed of atrial septal defect (ASD). 18.6% (n=19) of mothers had suffered from upper respiratory tract infection in the early stage of pregnancy and 2.9% (n=3) were diagnosed with gestational diabetes mellitus. No mothers had been exposed to rodenticides and herbicides or took any antiepileptic drugs. All the patients were isolated TGA with the absence of extracardiac anomalies.

We sequenced candidate genomic regions where the 13 mutations were exactly located in *MED13L*, *ZIC3*, *CFC1*, *NODAL*, *FOXH1*, *GDF1 and NKX2-5*, and found none of the reported 13 mutations in those 102 Chinese d-TGA patients.

Discussion

TGA is the second most common congenital heart defect

Table 1 Candidate mutations for d-TGA screening

Gene	Aliases	Location	Genomic region	Mutations (Coding DNA)	Primers
CFC1	CFC1B CRYPTIC DTGA2 HTX2	2q21.1	exon 4	c.623-627 + 17dup	GCCGCACCCCTGATAGTTTGT GGGCGCCGCATTCACTGAGAA
GDF1	DORV DTGA3 RAI	19p12	exon 3	c.681C > A	GGCCGGTGCTGCTCCGCCAGT CCCACCTCGCGGAAGCTCACG
FOXH1	FOH1 FAST1	8q24.3	exon 1	c.61C > T	CACCCAAAGGCAACTCAGGGGT GTGGGGAACGAGTCTGGGGAAA
NODAL	HTX5	10q22.1	exon 2	c.700indel	TGGGTCCACCTTGCTGTGGGAA TTACTGCCTCCCCTCCCCCTCAC
			intron 2	c.891+1G > A	GACAATGCGCGCGAAACGCT GCGTTTGTGGATTGCGCCCC
			intron 2	c.892-1G > C	CTGTTTAAAGGCTGTTTTCAC GCACTCTGCCATTATCCACAT
MED13L	PROSIT240 THRAP2 TRAP240L	12q24.21	exon 6	c.752A > G	TCTCCTCCCCTCCATAGTACTGGT GGACCAAGGGTGCTTCAGGCA
			exon 25	c.5615G > A	GGGGAATTATTAGAGACCTGC TTTCCTCTGTCTTCTGCAAAT
			exon 28	c.6068A > G	GTGTGTCCATAATAGCCATAT TTCCCACACTTATTTCTCTTG
ZIC3	RP1-137H15.3 HTX	Xq26.2	exon 2	c.1741A > T	GAGCTCAGTCTCCTGCTGCTTGC ATGAACCACCCACCCTCGGG
	HTX1 VACTERLX		exon 1	c.763T > G	CCCACAGGGCACGTGGACAAC CGGATGTGGTTGACCAGTTTG
	ZNF203		exon 1	c.49G > T	CACTTCGGCCGGATCGCCTG CCGAACCCTGCGGCGTGAAA
NKX2-5	CHNG5 CSX CSX1 HLHS2 NKX2.5 NKX2E NKX4-1 VSD3	5q34	exon 2	c.448G > A	CTGCCAGCCGGGAGAGAATTC AGCGGCTTGACCTACGGAGC

CFC1, cryptic family 1 gene; *GDF1*, growth differentiation factor 1 gene; *FOXH1*, forkhead activin signal transducer 1 gene; *NODAL*, TGF-beta gene; *MED13L*, thyroid hormone receptor-associated protein-2 gene; *ZIC3*, nucleocytoplasmic shuttling protein gene; *NKX2-5*, NK2 homeobox 5 gene; dup, duplication; c.700INDEL, c.700_723delinsTTGACTTCC.

Table 2 Clinical data on d-TGA patients participating in mutation screening

Number of patients		102
Percentage [N] of males	70.6%	[72]
Mean (SD) age at first diagnosis (months)	3.28	(1.21)
Mean (SD) age at first operation (months)	3.42	(1.33)
Family history of CHD—percentage [N]	1%	[1]
Percentage [N] of mothers suffered from URTI in the early stage of pregnancy	18.6%	[19]
Percentage [N] of mothers with gestational diabetes mellitus	2.9%	[3]
Percentage [N] of maternal exposure to rodenticides and herbicides	0	
Percentage [N] of maternal use of antiepileptic drugs	0	
Percentage [N] of patients with extracardiac anomalies	0	
Categories—percentage [N]		
TGA/IVS	52.9	[54]
TGA/VSD	30.4	[31]
TGA/IVS with other cardiac malformations (CoA, Ps, SV *et al*.)	10.8	[11]
TGA/IVS with other cardiac malformations (CoA, Ps, SV *et al*.)	5.9	[6]

CHD, congenital heart disease; URTI, upper respiratory tract infection; IVS, intact ventricular septum; VSD, ventricular septum defect; CoA, coarctation of aorta; Ps, pulmonary valve stenosis; SV, single ventrical; SD, standard deviation; N, number; d-TGA, dextro-transposition of the great arteries.

that causes problems in early infancy. Previous studies had focus on asymmetric cardiogenesis and several mutations have been implicated as the cause of this disease. However the total number of mutations detected so far is not sufficient to explain the high incidence of TGA. Besides, genetic screenings of TGA patients are rare in Chinese population. In this study we present the results of our efforts to screen a Chinese group of 102 d-TGA patients for the reported 13 mutations in *MED13L*, *ZIC3*, *CFC1*, *NODAL*, *FOXH1*, *GDF1 and NKX2-5*. Mutation analysis showed the absence of identical mutations in the 13 regions.

Two major hypotheses for TGA development have been suggested from the embryological point of view: an anomalous infundibular rotation and an aorticopulmonary septum anomaly (11). Nodal signaling pathway is responsible for early embryonic development, mesoderm and endoderm formation and left-right axis patterning in vertebrate embryos (15). The 6 genes of *ZIC3*, *CFC1*, *NODAL*, *FOXH1*, *GDF1* and *NKX2-5* are essential components of the NODAL signaling pathway, which are responsible for a subset of laterality defects. *MED13L* had been showed to affect nuclear receptor and cause severe defects during embryonic development (9).

For the pathogenesis of d-TGA, the involvement of 7 genes has already been discussed. In Muncke's study (8), 97 patients with d-TGA were screened in *MED13L* for mutations and 6 intronic polymorphisms, 6 silent mutations, and 4 missense mutations were found, the mutation analyze strongly suggested 3 missense mutations (Glu251Gly, Arg1872His, and Asp2023Gly) were involved in the pathogenesis of d-TGA. De Luca and colleges (11) screened probands of seven families with isolated TGA and a family history of concordant or discordant CHD for mutations within the *ZIC3*, *ACVR2B*, *LEFTYA*, *CFC1*, *NODAL*, *FOXH1*, *GDF1*, *CRELD1*, *GATA4* and *NKX2-5* genes. Two missense mutations in *FOXH1* (Pro21Ser) and *ZIC3* (Gly17Cys), a splice site variant in *NODAL* (IVS2-1G/C) were detected. Also the role of *EGF-CFC* gene was investigated in gene-targeting studies in mice, knockout of *CFC1* gene in mouse results in laterality defects and complex cardiac malformations including TGA (16,17). Two distinct mutations of *CFC1* were identified in three independent patients with laterality defects and d-TGA in Bamford's research (18). *GDF1* mutated in a variety of CHD including TGA (19), and *NKX2-5*mutations have also previously been identified in patients with CHD including septal defects, but they are very rarely detected in TGA (20). These results confirm that genetic heterogeneity of this congenital heart defect is related to the heterogeneity of the mechanisms that finally produce the same phenotype.

In our study, none of the 13 mutations was found in 102 Chinese patients. There could be several possible

explanations to the lack of mutations in this study. First of all, we did not examined the whole sequence of the seven genes and only checked thirteen mutations in them, thus we cannot exclude the possibility that certain mutations were missed by our direct sequencing approach. Secondly, we may not be able to predict whether causal mutations could be found in TGA, and whether the mutations are loss- or gain-of-function mutations. If mutations of the former category were involved, they can account for the missing mutations according to the fact that loss-of-function mutations are located in the promoter region, or in other regulatory sequences affecting gene expression levels, it might not be detected by our mutation analysis approach. The same is true for deletions or insertions that interfere with any of the primer binding sites. Thirdly, some genes of L-R signaling pathways, which perturbing the development of human laterality and resulting in discordant orientation of great artery, like *Lefty1* and *Lefty2*, were not tested. Moreover, the sample size might be not large enough. However, the results presented here strongly indicate that the 13 missense or deletion/insertion mutations in these seven genes may not be a common cause of TGA in Chinese population.

The low mutation frequency of *MED13L*, *ZIC3*, *CFC1*, *NODAL*, *FOXH1*, *GDF1 and NKX2-5* in d-TGA patients underlines the heterogeneity of this disease, in which the candidate gene screening had limited success. Therefore, new approaches are required to identify genetic variants in TGA patients. Whole exome sequencing technology, with its high effectiveness to detect common and rare variations, identify the genes responsible for complex diseases, will be applied for sequencing the exome (1% of genome) to discover most of the diseases-related variations in exons in TGA.

This study highlights the fact that the underlying genetic etiology of d-TGA can be complex. Thirteen known mutations in *MED13L*, *ZIC3*, *CFC1*, *NODAL*, *FOXH1*, *GDF1* and *NKX2-5* may not be a common cause of d-TGA in Chinese population due to racial variation. Next-generation sequencing approaches including the whole exome sequencing technology could effectively identify disease-related genetic variants of d-TGA patients in China, which will be very helpful in understanding the pathogenesis of the disease and clinical diagnosis.

Conclusions

These reported 13 mutations may not be a common cause of d- TGA in Chinese population due to racial variation and genetic heterogeneity of TGA. New approaches including the whole exome sequencing technology are required to effectively identify genetic variants in TGA patients in China.

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Original Article

Anti-endothelial cell antibodies in connective tissue diseases associated with pulmonary arterial hypertension

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Objective: To investigate the prevalence of anti-endothelial cell antibodies (AECA) in connective tissue diseases (CTD) associated with pulmonary arterial hypertension (PAH) and to corroborate the pathologic function of AECA in PAH-associated CTDs.

Methods: AECA were detected by cellular enzyme-linked immunosorbent assay (ELISA) in sera of 19 PAH-associated CTD patients, 22 CTD patients without PAH involvement, and 20 age- and sex-matched healthy individuals as controls. Using IgG purified from the sera of AECA-positive, AECA-negative, and healthy subjects, the effects of AECA on the expression of ICAM-1 and the chemokine regulated upon activation normal T-cell expressed and secreted (RANTES) in cultured endothelial cells were also evaluated.

Results: A total of 12 of the 19 (63.2%) CTD patients with PAH, 9 of the 22 (40.9%) CTD patients without PAH, and 1 of the 20 (5%) healthy controls were positive for AECA, which were calculated as ELISA ratio (ER) values. ER values in PAH-associated CTD patients were significantly higher than those with CTD without PAH (3.68±2.05 versus 1.67±1.07, P<0.001). IgG purified from AECA-positive sera induced a significantly increased level of ICAM-1 expression after 48 h incubation (795.2±32.5 pg/mL) compared with AECA-negative or healthy control IgG (231.5±27.1 and 192.8±33.4 pg/mL, respectively; P<0.001). In addition, RANTES production by cultured human pulmonary arterial endothelial cells (HPAECs) increased in both a time- and concentration-dependent manner in response to incubation with purified AECA-positive IgG.

Conclusions: AECA could be involved in CTD and might participate in the pathogenesis of PAH-associated CTD.

Keywords: Connective tissue diseases (CTD); pulmonary arterial hypertension (PAH); anti-endothelial cell antibodies (AECA)

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Introduction

Pulmonary arterial hypertension (PAH) is a rare disease characterized by endothelial dysfunction and cellular proliferation, resulting in progressively elevated pulmonary arterial resistance and, ultimately, right-sided heart failure (1). PAH complicated by connective tissue diseases (CTD) is associated with a poorer prognosis than idiopathic PAH. Nevertheless, the pathophysiologic mechanisms of PAH are currently poorly defined, despite understanding of the underlying inflammatory processes involved (2).

Anti-endothelial cell antibodies (AECA) are a heterogeneous family of antibodies that can specifically recognize endothelial cell proteins and molecules present on the surface of endothelial cells (3). AECA have been detected in patients with various collagen vascular diseases, and their levels fluctuate with disease activities (4,5), indicating that AECA may interfere with pathological processes rather than being an independent existence. Studies using human umbilical vein endothelial cells have suggested that AECA can up-regulate the expression

of adhesion molecules and the secretion of cytokines, which may play roles in vascular lesions (6,7). In addition, increased expression of chemokines, such as regulated upon activation normal T-cell expressed and secreted (RANTES), have also been noted in severe cases of PAH (8), highlighting the possible role of AECA-related inflammatory mechanisms in this condition.

Since pulmonary endothelial cell dysfunction appears to play an integral role in the initiation and progression of PAH in humans (9), we hypothesized that production of anti-human pulmonary arterial endothelial cell antibodies (anti-HPAEC) and their association with endothelial dysfunction may be a key step in the PAH pathological process, especially when associated with CTD. We therefore performed the current study to evaluate the prevalence of anti-HPAEC in CTD, in cases associated and not associated with PAH, and examined the relationship between anti-HPAEC and clinical features. The results of this study should help to corroborate the pathologic function of anti-HPAEC in PAH, especially when combined with CTD.

Materials and methods

Patients and controls

Patients were screened for PAH using Doppler echocardiography based on systolic pulmonary artery pressure >40 mmHg, and confirmed by right-side heart catheterization with mean pulmonary artery pressure at rest >25 mmHg. A total of 41 CTD patients admitted to the Rheumatology Department of China Medical University between 2008 and 2011 were enrolled in the study, including the study group (PAH-associated CTD group, n=19) and disease control group (no-PAH CTD group, n=22). In the 19 PAH-associated CTD patients, the underlying diseases included systemic sclerosis (SSc, n=8), systemic lupus erythematosis (SLE, n=3), mixed connective tissue disease (MCTD, n=4), primary Sjögren syndrome (pSS, n=2), and undifferentiated connective tissue disease (uCTD, n=2), with no significant distribution difference compared to the diseases of the no-PAH CTD group. Sera samples were collected at the time of diagnosis when none of the patients were under systemic steroid or immunosuppressive therapy. Twenty age- and sex-matched healthy volunteers served as the control group. The study protocol was approved by our institutional review board, and each patient gave informed consent.

Cellular enzyme-linked immunosorbent assay (ELISA) for AECA

HPAECs (ScienCell Corp.) at passage 4 were seeded onto 96-microculture plates and allowed to reach confluence. AECA were detected using a cyto-ELISA method as described previously (10). In brief, after being fixed with 0.1% glutaraldehyde, sera samples diluted 1:400 were added to triplicate wells for 1 h at 37 ℃. After washing, horseradish peroxidase-conjugated goat anti-human IgG-F(ab')$_2$ diluted 1:1,000 was added for 1 h at 37 ℃. Then 3,3', 5,5'-tetramethylbenzidine was added as a substrate and the color reaction was terminated by the addition of 1 mol/L H_2SO_4. Optical density was measured at 450 nm and the results are expressed as the ELISA ratio (ER) = (S – A)/(B – A), where S is the absorbance of the sample, and A and B are the absorbances of negative and positive reference serum, respectively. Positivity was defined as ER values > mean +3 standard deviations (SD) of the healthy control values. Intra- and inter-assay coefficients of variation were 5% and 12%, respectively. Sera from six AECA-positive patients, six AECA-negative patients, and five AECA-negative healthy subjects were pooled and purified using Protein G Sepharose (GE Hitrap) chromatography.

ICAM-1 and RANTES production by HPAECs

To identify the effect of AECA on cultured HPAECs, we incubated arterial endothelial cells forming a monolayer with IgG purified from AECA-positive, AECA-negative, or healthy control sera. ICAM-1 and RANTES levels were measured using a commercial ELISA kit (USCN Life Science Inc., Wuhan, China), following the manufacturer's instructions.

Statistical analysis

All data are presented as means ± SD or as percentages. The *t*-test and χ^2 test were used for statistical comparisons. Statistical analysis was performed with the SPSS software version 17.0, and $P<0.05$ was considered statistically significant.

Results

Characteristics of patients

The clinical characteristics of 41 CTD patients are summarized in *Table 1*. Analysis of serological factors

Table 1 Clinical characteristic of CTD patients

Clinical characteristic	PAH-associated CTD	No-PAH CTD
No. of patients	19	22
Males/females	3/16	5/17
Age, years	49±12	46±18
Mean disease duration, years	5.64±6.21	6.44±5.16
Systolic blood pressure, mmHg	126.03±14.9	126.32±18.04
Diastolic blood pressure, mmHg	79.16±4.91	74.39±5.18
White blood cells, $\times10^9$/L	5.72±3.37	5.35±2.46
Macrophages, %	7.86±5.79	6.54±3.25
Lymphocytes, %	24.96±9.87	27.53±10.58
Platelet, $\times10^9$/L	155±67.47**	201.57±75.2
Total cholesterol, mmol/L	4.3±1.28	4.22±0.9
Triglycerides, mmol/L	1.68±0.98*	0.92±0.44
Fasting plasma glucose, mmol/L	5.18±1.52	5.38±0.78
IgG concentration (mg/mL)	21.26±8.26	16.22±5.92
IgM concentration (mg/mL)	1.93±1.35	1.39±1.24
hsCRP, mg/dL	16.38±27.06*	13.02±21.92
ESR, mm/h	37.33±18.63	33±19.33
Raynaud's syndrome	13*	8

Data are presented as N or mean ± SD. ESR, erythrocyte sedimentation rate; CTD, connective tissue diseases; PAH, pulmonary arterial hypertension; SD, standard deviations. *, P<0.05, PAH-associated CTD *vs.* no-PAH CTD; **, P<0.01, PAH-associated CTD *vs.* no-PAH CTD.

revealed that PAH-associated CTD patients had significantly higher levels of triglycerides and high-sensitivity C-reactive protein (hsCRP) (P<0.05), but lower platelet numbers (P<0.001) than the no-PAH CTD patients. PAH-associated CTD patients showed an increased tendency to have Raynaud's syndrome (P<0.05).

Elevated levels of AECA in patients with PAH-associated CTD

We selected the mean +3 SD of healthy controls (ER =0.93±0.49) as the cut-off AECA value (2.4), and with this criterion, 12 of the 19 (63.2%) PAH-associated CTD patients, 9 of the 22 (40.9%) no-PAH CTD patients, and 1 of the 20 (5%) healthy controls were positive for AECA. AECA levels in PAH-associated CTD patients were significantly higher than those of the no-PAH CTD patients (3.68±2.05 versus 1.67±1.07, P<0.001) or healthy controls (*Figure 1A*).

Correlation between AECA and clinical features

A positive correlation was observed between ER values of AECA and levels of hsCRP in all CTD patients (P<0.05, r=0.417). The sera levels of AECA were not correlated with other clinical parameters including pulmonary function, blood cell counts, D-dimer, triglycerides, total cholesterol, or erythrocyte sedimentation rate (*Figure 1B*).

Purified AECA IgG increased the secretion of ICAM-1 and RANTES by HPAECs

The levels of RANTES secreted by cultured HPAECs increased in response to stimulation from purified AECA-positive IgG in both a time- and concentration-dependent manner, but this effect was not observed in response to purified AECA-negative IgG or healthy control IgG (*Figure 2*). After 48 h of incubation with IgG purified from AECA-positive sera, the expression level of RANTES was 35.6±1.79 pg/mL, which was significantly higher than that obtained following incubation with AECA-negative or healthy control IgG (17.6±1.04 and 13.8±0.28 pg/mL, respectively; P<0.05; *Figure 2*). IgG purified from AECA-positive sera (100 μg/mL) also induced a significantly increased level of ICAM-1 expression by HPAECs after 48 h incubation (795.2±32.5 pg/mL), which was significantly higher than that obtained with AECA-negative or healthy control IgG (231.5±27.1 and 192.8±33.4 pg/mL, respectively; P<0.001).

Discussion

AECA, a heterogeneous group of antibodies that is distinct from the autoantibodies family, have been detected in a variety of diseases, especially in different types of CTD such as SLE, SSc, and MCTD. In CTD, AECA titers are always found to fluctuate with disease activities, indicating their possible pathologic role rather than a simple phenomenon. Regarding the possible association between AECA and organ manifestations in CTD, Negi (11) reported that AECA were found in 40% of patients with diffuse SSc disease, with a tendency to be associated with digital ischemia and PAH, but no such correlation was reported in other CTD diseases. In this study, we mainly focused on AECA in CTD associated with PAH, and aimed to evaluate the pathogenic value of AECA in pulmonary vascular lesions, as well as to

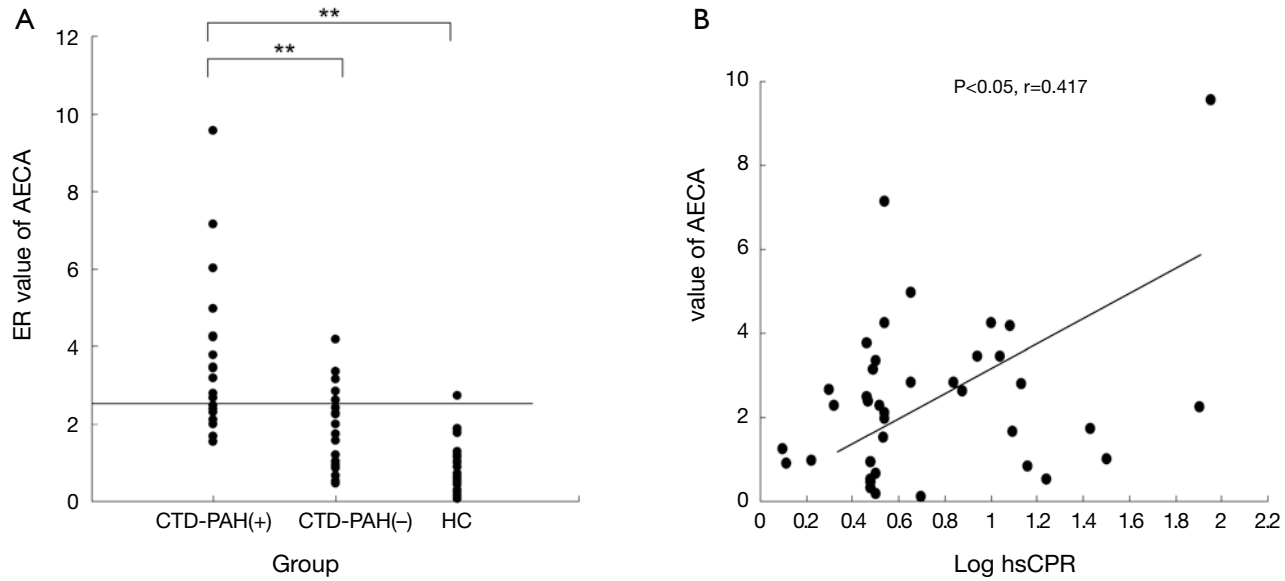

Figure 1 Detection of sera anti-endothelial cell antibodies (AECA). (A) AECA levels in patients with connective tissue disease (CTD) with or without pulmonary arterial hypertension (PAH) and healthy controls (HC). Levels are expressed as the enzyme-linked immunosorbent assay (ELISA) ratio (ER). The horizontal line indicates the cutoff value of the normal serum. **, P<0.001; (B) correlation between sera AECA levels and high-sensitivity C-reactive protein (hsCRP).

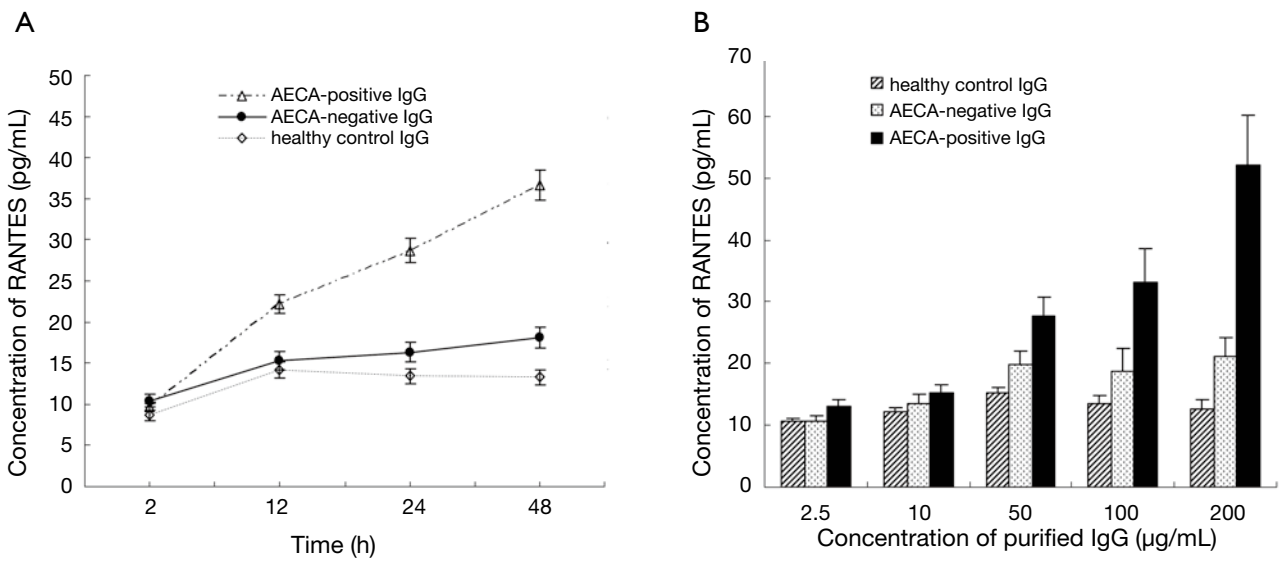

Figure 2 Time- and concentration-dependent regulated upon activation, normal T-cell expressed and secreted (RANTES) induction by human pulmonary arterial endothelial cells in response to IgG purified from anti-endothelial cell antibodies (AECA)-positive, AECA-negative, and healthy control sera. (A) Endothelial cells were incubated for up to 48 h with IgG (100 μg/mL) purified from AECA-positive, AECA-negative, and healthy control sera; (B) endothelial cells were incubated for 24 h with 2.5, 10, 50, 100, and 200 μg/mL IgG purified from AECA-positive, AECA-negative, and healthy control sera.

further corroborate previous findings describing the role of AECA in PAH-associated CTD (11). We observed a high prevalence of AECA in CTD patients, and their levels were closely related to levels of the inflammatory marker hsCRP. Moreover, our data showed that AECA levels were significantly higher in PAH-associated CTD patients than in CTD patients without PAH or healthy controls. Therefore, these results were in accordance with previous findings and further support the role of AECA in PAH-associated disease.

Currently, the precise mechanism of the involvement of PAH to CTD remains largely unknown, but there is some convincing evidence showing that endothelial dysfunction is one of the earliest pathogenic events of the process (12). Experimental *in vitro* and *in vivo* models showed that AECA might be pathogenic, especially by inducing autoimmune vascular disease (13,14). AECA may bind to endothelial cells' membrane antigens, and activate endothelial cells by up-regulating the expression of adhesion molecules (e.g., E-selectin, ICAM-1, and VCAM-1), which will in turn cause leukocyte recruitment and adhesion (7). Production of cytokines has also been demonstrated in PAH, especially in PAH combined with CTD, indicating the possible influence of inflammatory mechanisms in this condition.

The chemokine RANTES is known to be an important chemoattractant for T cells and monocytes, facilitating the tight adhesion of circulating leukocytes to the vascular endothelium. RANTES plays a key role in a number of arterial inflammatory processes. Using *in situ* hybridization and immunohistochemistry methods, Dorfmuller *et al.* (15) confirmed that RANTES expression was predominant in vascular lesions, and that endothelial cells were the major source of RANTES within the pulmonary artery wall; RANTES expression was associated with CD45+ inflammatory cell infiltrates. Furthermore, RANTES can exert an indirect role in pulmonary hypertension by induction of endothelin-converting enzyme-1 and endothelin-1 system, which serve as potent factors for vasoconstrictive and mitogenic action.

Considering that the endothelial cells of the pulmonary artery wall are the major source of RANTES, and the possibility of differences in phenotype presentation on different endothelial cell surfaces, we selected normal HPAECs as cell substrates in this study to evaluate the effect of AECA on endothelial dysfunction in order to investigate the underlying pathogenic role of AECA in CTD pulmonary vascular lesions. We hypothesized that production of the chemokine RANTES may be associated with AECA, together with up-regulation of the adhesion molecule ICAM-1, a marker of endothelial cell activation, which might be the perpetuating mechanism to amplifying inflammatory responses in PAH-associated CTD. Indeed, we observed increasing levels of RANTES secretion in a time- and dose-dependent manner in response to incubation with purified AECA-positive IgG. These results support a pathologic role of AECA on endothelial cell interactions, and indicate that RANTES is one of the factors involved in the autoimmune and inflammatory multiple step process of PAH in CTD.

In conclusion, we reported the presence of AECA in CTD patients associated with PAH, further corroborating the pathogenic role of AECA in this group of conditions, which appears to occur via the induction of RANTES secretion.

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Author contributions: Sheng-Yu Guo, Li-Li Yang and Xiao-Li Zhang designed the study. Wen-Yi Fu collected and analyzed the data. Xiao-Dan Liu performed all the experiments and prepared the manuscript. Xiao-Fei Wang supervised and controlled the quality of this study.
Disclosure: The authors declare no conflict of interest.

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Brief Report

Infrequent *ERBB2* mutations in Chinese patients with non-small cell lung cancer

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Abstract: *ERBB2* mutations have been reported to occur in a subset of patients with lung adenocarcinomas or lung squamous cell carcinomas for some ethnicities, but it is unclear for Chinese patients with lung squamous cell carcinomas up to now. We retrospectively evaluated the status of *ERBB2* mutations in a large cross-sectional cohort of 212 Chinese patients with non-small cell lung cancer (NSCLC) diagnosed in several hospitals from southern China during a time period of 1.5 years by polymerase chain reaction (PCR)-based direct sequencing and PCR-single strand conformation polymorphism (PCR-SSCP) analysis. *ERBB2* mutation was found in 1 of 49 lung adenocarcinomas (2.0%) and none in lung squamous cell carcinomas and lung adenosquamous carcinomas. It implies the occurrence of *ERBB2* mutations is infrequent in Chinese patients with NSCLC, especially in lung squamous cell carcinomas.

Keywords: *ERBB2/HER2*; mutation; non-small cell lung cancer (NSCLC); molecular

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Introduction

Members of c-erb B family of oncogenes, which include epidermal growth factor receptor (*EGFR*, *ERBB1*), *ERBB2* (*HER2*), *ERBB3* (*HER3*), and *ERBB4* (*HER4*), play an important role in the development and progression of non-small cell lung cancer (NSCLC) by promoting cell growth and preventing apoptosis by regulating downstream effectors such as mitogen-activated protein kinase, protein kinase B, and signal transducer and activator of transcription 3 (1,2). Mutations in the kinase domain of *ERBB2* gene have been reported to occur in lung adenocarcinomas (3-5) and lung squamous cell carcinomas (6) for some ethnicities, which could potentially result in the activation of the tyrosine kinase activity of ERBB2 protein and play a critical role in oncogenesis in a manner similar to *EGFR* mutations (7,8). However, to date, it is unclear that the status of *ERBB2* mutations in Chinese patients with lung squamous cell carcinomas that are the majority of NSCLC.

To find out the overall status of *ERBB2* mutations in Chinese patients with NSCLC, in this study we conducted a detailed search for the *ERBB2* mutations in tumor tissues derived from a large cross-sectional cohort of Chinese patients with NSCLC who underwent tumor resection in several hospitals from southern China during a time period of 1.5 years.

Methods

Patients and tissue specimens

From June 2006 to November 2007, tumor tissues and

paired normal lung tissues were obtained from 212 Chinese patients with NSCLC who underwent curative resection in the Tumor Hospital of Hunan Province and the Xiangya Hospital and the Xiangya 2nd Hospital of Central South University which are top three hospitals in Hunan Province, China. None of these patients had received chemotherapy or radiotherapy before surgery. Written informed consent was obtained from each patient before the surgery. This study was approved by the institutional review boards respectively from the Tumor Hospital of Hunan Province and the Xiangya Hospital and the Xiangya 2nd Hospital of Central South University. Pathological histology type was determined according to WHO criteria (9). All of the tumor and macroscopically normal lung tissue samples were obtained at the time of surgery, and then they were rapidly frozen in liquid nitrogen and stored at –80 ℃. Only tumors with 80% or more tumor component were sent for DNA extraction and mutational analysis. The normal lung tissue specimens were obtained from either the opposite end of resected surgical samples or as distant as possible from the tumor. All of the macroscopically normal samples were confirmed as normal under hematoxylin and eosin staining.

Mutational analysis of *ERBB2* gene

Genomic DNA was obtained from frozen lung tissues by overnight digestion with sodium dodecyl sulfate and proteinase K [Tiangen Biotech (Beijing) Co., Ltd, China] at 37 ℃ followed by standard phenole chloroform (1:1) extraction and ethanol precipitation. PCR-based direct sequencing and polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) were used to detect mutations of exons 19-20 of the TK domain of *ERBB2* gene. The PCR was performed in a total volume of 25 μL containing 100 ng genomic DNA, 0.2 mmol/L of each primer, and 0.2 mmol/L dNTPs, 1 unit of Taq polymerase [Tiangen Biotech (Beijing) Co., Ltd, China], and 1 reaction buffer (10 mmol/L Tris-HCl, pH 8.3; 50 mmol/L KCl; and 1.5 mmol/L $MgCl_2$). The PCR cycle conditions consisted of an initial denaturation step at 95 ℃ for 5 minutes followed by 30 cycles of 30 seconds at 95 ℃ and 60 seconds at 72 ℃, and a final elongation at 72 ℃ for 7 minutes. The primers used for exon 19 were: forward, 5'-TGG AGG ACA AGT AAT GAT CTC CTG G-3' and reverse, 5'-AAG AGA GAC CAG AGC CCA GAC CTG-3', amplifying a 160-bp fragment; and for exon 20 were: forward, 5'-GCC ATG GCT GTG GTT TGT GAT GG-3' and reverse, 5'-ATC CTA GCC CCT TGT GGA CAT AGG-3', amplifying a 249-bp fragment. The PCR products were purified using a PCR Products Purification kit (Bio Basic Inc., CA), then sequencing was done using an ABI Prism 3100 Genetic Analyzer [Sangon Biotech (Shanghai) Co., Ltd, China]. Sequence chromatograms were analyzed by Mutation Surveyor 2.60, followed by manual review. At the same time, PCR-SSCP analysis was also performed for each sample. The purified PCR products were diluted 1:5 in loading buffer (95% formamide, 2 mM EDTA, pH 8.3), and then the diluted samples (12 μL) were denatured (5 min at 90 ℃), immediately cooled on ice and loaded into a 12% non-denaturing polyacrylamide gel. Electrophoresis was carried out for 4 hr at 15 ℃ at 5 Watt. Upon completing migration the gels were subject to staining using ethidium bromide for 20 minutes.

Results

Patient characteristics

Between June 2006 and November 2007, tumor specimens suitable for genetic analysis were available from 212 patients with NSCLC. The cohort included patients with age at diagnosis ranging from 41 to 77 (median age, 63) in 172 males and from 33 to 72 (median age, 57) in 40 females. There were 153 Squamous cell carcinomas (72.2%), 49 adenocarcinomas (23.1%) and 10 adenosquamous carcinomas (4.7%) in the cohort including 148 smokers (69.8%) and 64 never-smokers (30.2%).

The status of ERBB2 mutation

The *ERBB2* mutation was found in 1 of 49 lung adenocarcinomas (2.0%), and none in 153 lung squamous cell carcinomas and 10 lung adenosquamous carcinomas. The single adenocarcinoma case with *ERBB2* mutation was a never-smoker, female and 44 years of age. The category of *ERBB2* mutation was a heterozygous in-frame insertion occurring within exon 20: 2327-2329insTTT (G776V, insC) (*Figure 1*). Likewise, PCR-SSCP analysis revealed no more than one *ERBB2* mutation within exon 20 in the study cohort. The mutated *ERBB2* presented two different electrophoresis bands distinguished from normal one (*Figure 2*).

Discussion

In the present study, we found only 1 *ERBB2* mutation in 49 lung adenocarcinomas (2.0%) and no *ERBB2* mutation

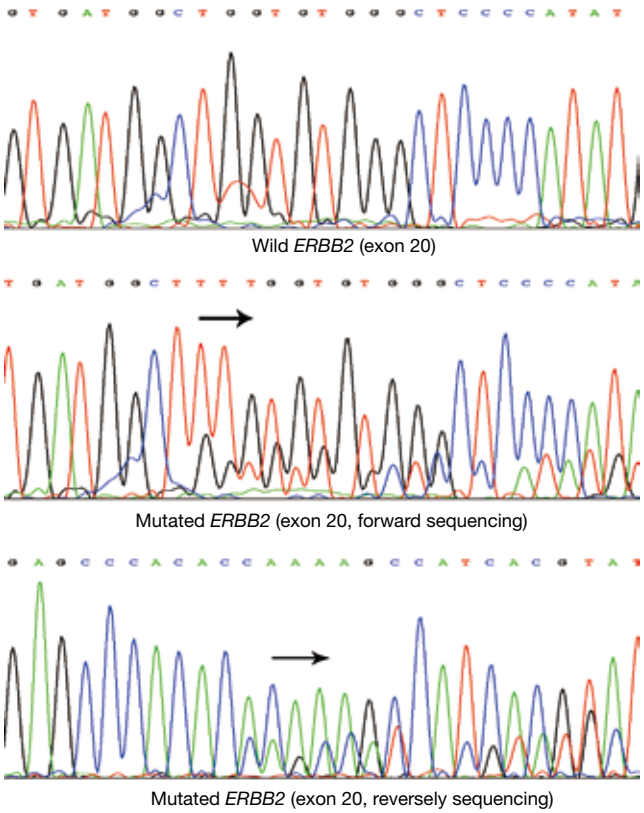

Figure 1 Sequencing map of wild and mutated *ERBB2* (exon 20).

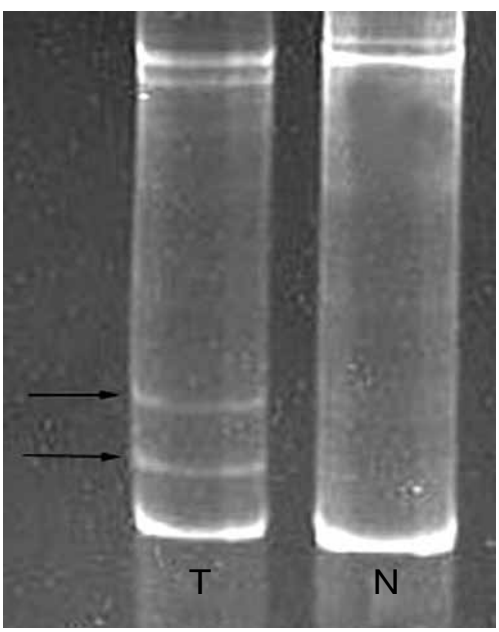

Figure 2 Electrophoretogram of mutated *ERBB2* by PCR-SSCP analysis (T, tumor tissue; N, normal tissue; Arrows, abnormal bands). PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism.

in lung squamous cell carcinomas and lung adenosquamous carcinomas. The rate of *ERBB2* mutation in this study is lower than those of previous researches (10,11), which didn't involve lung squamous cell carcinomas and lung adenosquamous carcinomas. This difference may be due to the different distributions of sex and smoking status of study sample. In addition, the differences of environment and histological types should be considered too. Similar to our findings, Yokoyama *et al.* didn't find *ERBB2* mutations in Japanese lung squamous cell carcinomas (12).

As with *EGFR* mutations, *ERBB2* mutations, which may be a therapeutic target in lung cancer patients (13), are more common in Asians, adenocarcinomas, females, and never-smokers (4,5,14). The patient with *ERBB2* mutation in this study was never-smoker, female and adenocarcinomas. Thus, *ERBB2* mutations may play a key role in the development of adenocarcinomas in nonsmokers. A 3-bp in-frame insertion mutation within exon 20 in our patients is not the major type of mutation found by other investigators (3-5,13), which may be resulted from a small sized study sample, but it is consistent with those of Shigematsu and colleagues (4). Further investigation will be needed to clarify the relationship between *ERBB2* mutations and clinicopathologic features in Chinese patients with NSCLC.

In conclusion, our results suggest that *ERBB2* mutations occur infrequently in Chinese patients with NSCLC, especially in lung squamous cell carcinomas. So it is possible of limited value for molecular target therapy based on the mutated *ERBB2* in Chinese patients with NSCLC.

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Rapid laboratory diagnosis for respiratory infectious diseases by using MALDI-TOF mass spectrometry

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Abstract: It is still challenging to prevent and treat respiratory infectious diseases. One critical step in the successful treatment of respiratory infections is rapid diagnosis by identifying the causative microorganisms in a timely fashion. However, traditional methods for identification of causative agents could not satisfy the need for rapid and accurate testing due to the limitations of technology-used. In recent years, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) has been validated and used for rapid identification of microorganism and for potential discovery of diseases associated biomarkers. We reviewed recent advances of MALDI-TOF-MS as the laboratory diagnostic tool for the rapid laboratory diagnosis of microorganisms associated with respiratory infectious diseases, with the focus on rapid identification of pathogenic bacteria and molecular markers discovery using MALDI-TOF-MS. With the advanced technologies such as MALDI-TOF, early and targeted therapies based on rapid identification of pathogens and could lead to quick and effective treatment of respiratory infections and better patient management.

Keywords: Respiratory infectious diseases; rapid laboratory diagnosis; matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS); pathogenic bacteria

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Introduction

According to a recent report offered by World Health Organization (WHO), respiratory infections are the third leading cause of death worldwide and result in over three million deaths per year (1). Community acquired and hospital acquired respiratory infections are common and can be serious which can lead to complications such as pneumonia, bronchitis, sinus infections, and a general worsening of chronic conditions (2). As is the case with other infectious diseases, management of respiratory infections often requires a definitive laboratory identification of the causative agents in addition to clinical diagnosis for quick and direct optimal treatment.

Laboratory diagnosis of pathogens causing respiratory infections depends on many factors, which include but not limit to pathogen types and pathogen loads, specimen types, laboratory tools and methods used for staining, culture and detection.

The conventional microbial diagnosis of pathogens primarily relies on the culture of respiratory specimens such as sputum. Other respiratory specimen types include induced sputum, tracheal aspiration, bronchial wash, bronchial brush, and bronchoalveolar lavage (BAL). The quantification of colony forming unit (CFU) of certain specimens can be performed. If microorganism were recovered from culture, antimicrobial susceptibility testing (AST) should be subsequently performed to determine the antibiotic resistance (3). Unfortunately, traditional methods for microorganism identification take a few days and could not satisfy the need for rapid testing. Thus, rapid laboratory diagnosis of respiratory infections has been challenging.

Recently, the technology called matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) has been demonstrated in identifying microorganisms at species level. MALDI-TOF MS is regarded as a novel rapid clinical diagnostic strategy for identification of pathogens infections (4). Here, we review recent advances of MALDI-TOF-MS as the laboratory diagnostic tools for the rapid diagnosis of microorganisms associated with infectious diseases, and impact on AST and study.

Common pathogenic microorganisms and conventional diagnostic methods

There are many types of pathogenic microorganisms that can cause respiratory infections. Among those pathogens, *Streptococcus pneumonia*, *Hemophilus influenza* and *Moraxella catarrhalis* are most common in causing bacterial respiratory tract infections including acute otitis media (AOM), sinusitis, laryngitis and pneumonia. In addition, *Legionella pneumophila* is mainly responsible for atypical pneumonia, and *Mycobacterium tuberculosis* leads to tuberculosis, etc. However, the etiologic agent remains unknown in nearly 30% of cases, suggesting that the existing methods lack accuracy and sensitivity for identification of unknown pathogens including viral pathogens in respiratory infection cases.

Gram stain and culture of respiratory samples have been traditionally used for the detection and distinguish of bacterial pathogens. In general, it takes 48 to 96 hours to have bacterial identification and resistance determination. In the meantime when identification and resistant results are not available, patients may be treated with broad-based antibiotics that are often unnecessary, inappropriate, even harmful, which eventually lead to resistance, even multiple resistances. Thus, new technologies are needed to shorten the time of detection, identification, and AST and to provide rapid laboratory results to clinicians for better patient care and control of antimicrobial resistance.

MALDI-TOF mass spectrometry (MALDI-TOF-MS), a new diagnostic technology for rapid diagnosis of pathogenic microorganisms in respiratory infection

Rapid identification of pathogenic bacteria using MALDI-TOF-MS

In general, earlier diagnosis of respiratory infections can lead to better outcomes. Generally, MALDI-TOF-MS provides effective bacterial identification and antibiotic resistance determination directly from positive blood culture specimens up to 24 hours faster than conventional method (*Figure 1*). Targeted therapies, based on the rapid identification of causative pathogens and antimicrobial resistance, shall become more effective and timely. It is essential for each clinical microbiology laboratory to develop techniques that identify these bacteria correctly and rapidly. Advanced microbiological methods are important for earlier bacteria detection. MALDI-TOF-MS has shown its fast and reliable feature for identifying microorganisms including pathogenic bacteria and yeasts from samples with positive culture, which may represent a rapid, inexpensive and alternative assay for identification of bacteria at the species level.

Here we only list a few studies to prove the concept. The *Streptococcus mitis* group is a set of closely related species in which conventional identification methods cannot reliably make differentiation analysis. The most important pathogen within the *S. mitis* group, *S. pneumonia*, is conventionally distinguished from the others on the basis of its susceptibility to optochin or its solubility in bile (5). In a recent study, the Vitek MS v2.0 System (MALDI-TOF-MS technology) accurately distinguished *Streptococcus pneumonia* from nonpneumococcal *S. mitis* group species. Only 1 of 116 nonpneumococcal isolates (<1%) was misidentified as *S. pneumoniae*. None of 95 pneumococcal isolates was misidentified. In this case, MALDI-TOF-MS provides a rapid, simple means of discriminating among these challenging organisms (6).

Infections caused by *Legionella* species other than *L. pneumophila* are also often lack of laboratory diagnosis, owing to limitations of the conventional diagnostic methods that are biased towards the detection of *L. pneumophila* (7). Recently, MALDI-TOF-MS was used to identify the specific species of the *Legionella* non-*pneumophila* isolates; and the result was consistent with the data of reference method and macrophage infectivity potentiator gene (mip) sequencing (8).

Corynebacteria have been recognized as opportunistic pathogens in causing various types of healthcare-associated infections in immunocompromised hosts (9). Identification of *Corynebacteria* by conventional methods is suboptimal and it is likely that their true prevalence in clinical specimens either as colonizers or as pathogens remains largely underestimated. Rapid identification of toxigenic *Corynebacterium* species and non-*diphtheria Corynebacterium*, including *C. triatum* can be achieved by using the MALDI-TOF MS (10).

Another application of MALDI TOF is the identification of yeasts. As the number of patients with profound immunosuppression continues to rise, the morbidity and

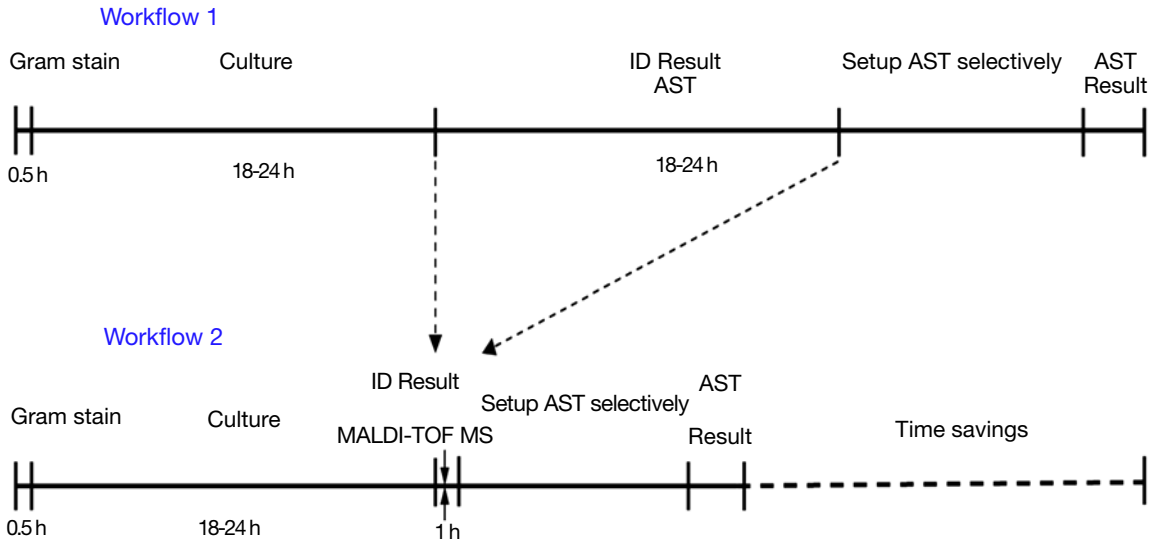

Figure 1 Comparison of new method workflow with traditional culture workflow. In the ideal situation when there is one predominant colony grow from the culture. If there are mixed cultures with different colonies, the time of saving could be two days as the subcultures are needed for pure colony and for identification by conventional method. Workflow 1, conventional culture; workflow 2, new method with MALDI-TOF MS; ID, identification; AST, antimicrobial susceptibility testing.

mortality burdens due to invasive fungal infections are significant. Traditional methods used to identify clinical yeast isolates are time-consuming and may result in low-discrimination identifications (11). In a recent study MALDI-TOF Vitek MS was applied on the identification of yeasts isolated from clinical specimens. A collection of 852 isolates was tested, in total, 823 isolates (96.6%) were identified to the genus level and 819 isolates (96.1%) were identified to species level, 24 isolates (2.8%) were not identified and 5 misidentified, which indicated that MALDI-TOF-MS offers a balance between speed and highly accurate yeast identification (12).

MALDI-TOF-MS: our experience

Here at Grady Memorial Hospital in metropolitan Atlanta area, we believe the need of culture based methods for laboratory diagnosis of infection causing microorganisms. We started to see the emergence of community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) USA300 genotype as a major cause of healthcare-associated blood stream infections as early as 2004 (13), and we noticed emergence of community-acquired MRSA USA300 Clone as the predominant cause of *S. aureus* skin and soft tissue Infections in Atlanta (14). The bacteria isolated from clinical cultures can be used for surveillance study of antimicrobial resistance (15) as well as the immune response study of antibiotic-resistant nosocomial bacteria (16). Study on current or new CLSI breakpoints of antimicrobial susceptibility (17) and even the population-based active surveillance method for multidrug resistant gram-negative bacilli (18) or the inoculum effect study among bloodstream isolates of methicillin-susceptible *Staphylococcus aureus* (19) rely on positive isolates from cultures.

For the above reasons, we recognized the importance of clinical utility of new culture based technologies such as MALDI-TOF MS as an important laboratory tool to battle antibiotic resistance (20) and we potential tool for improved clinical microbiology laboratory automation (21).

We reported a few clinical cases as evidence-based medicine for clinical utility of MALDI-TOF MS to help diagnosis of rare case of bacterial infection (22) and rare case of fungal infection (23). We tried to improve the method for rapid identification of bacteria and yeasts from positive blood culture bottles by using a lysis-filtration method and MALDI-TOF mass spectrum analysis (24), and recently we had success in making the same day identification and full panel AST of bacteria from positive blood culture bottles possible by a combined lysis-filtration method with MALDI-TOF VITEK mass spectrometry and the VITEK2 system (25). Those methods can certainly help rapid laboratory diagnosis of respiratory infections.

In addition to bacterial and yeast identification, we studied the method to improve the diagnosis of mycobacteria including *M. tuberculosis*, an important respiratory pathogen. We compared the heat inactivation method and cell disruption protocols for identification of mycobacteria from solid culture media using MALDI-TOF VITEK MS (26).

Translation research: molecular markers discovery in microorganisms by MALDI-TOF MS

MALDI-TOF MS can be used for translational study. Most proteins in bacteria are adhesins/virulence factors that not only can trigger the immune response, but also aid bacteria to evade the host defense. Identification these proteins shall be helpful to understand pathogenic mechanisms and resistance mechanisms so that clinicians can optimize the treatment plan to ensure the correct and timely therapy.

Outer membrane vesicles (OMVs) secreted by bacteria can be recognized as long-distance delivery vehicles which transport diverse virulence factors and allow pathogens to interact with the host. A total of 57 proteins in *Moraxella Catarrhalis* OMVs were identified by using MALDI-TOF-MS analysis and gained new insights in the biological function of these protein-carrying lipid structures (27), and a novel molecular basis could play a role of OMVs in *Moraxella Catarrhalis* pathogenesis (27).

Surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) can be considered as an extension of MALDI-TOF MS, which utilizes ProteinChip arrays with a MALDI-TOF-MS-based analytical platform, has been used for the discovery of diseases associated biomarkers (28). Tuberculosis is a major global concerned disease; its diagnosis is always problematic especially for latent tuberculosis and early disease. Recently, two biomarker-proteins, transthyretin and SAA des Arg were detected by SELDI-TOF-MS, which combined with neopterin and C-reactive protein, significantly improved the sensitivity and specificity for tuberculosis diagnosis (>80%) (29). Thus, MALDI-TOF-MS and its extension SELDI-TOF-MS shows promise in identifying novel biomarkers in respiratory infection that lead to advances in our understanding in pathophysiology and is useful for monitoring disease progression or treatment.

Summary

Microbiological diagnosis is often required to confirm clinical suspicions concerning infections. It is of great importance that microbiological diagnostic tools become more efficient and powerful, particularly for polymicrobial infections. MALDI-TOF MS as a new frontier technology is likely to have a major impact in clinical microbiology and provide platform for microbiology laboratory automation. The clinical utility of culture and proteomic based MALDI-TOF MS technologies by the microbial laboratories will enable laboratory diagnosis of common and rare pathogenic agents for respiratory infections quickly and accurately. More studies on identification of mycobacterial and fungal infection and clinical outcome analysis are needed.

Acknowledgements

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Application status of MALDI-TOF mass spectrometry in the identification and drug resistance of *Mycobacterium tuberculosis*

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Abstract: Characterizing *Mycobacterium tuberculosis* (MTB) and detecting its drug resistance are challenging for clinical laboratory diagnosis, largely due to its slow growth and higher rate of genetic mutation. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a revolutionary technique for the routine identification of microorganisms. In this review, we discuss the application status of mass spectrometry in the identification and drug resistance of *M. tuberculosis*.

Keywords: *Mycobacterium tuberculosis* (MTB); identification; drug resistance; matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)

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Introduction

Tuberculosis (TB) remains one of the most serious health threats facing several parts of the world (1). Methods for identifying *Mycobacterium tuberculosis* (MTB) are conventionally based on biochemical tests, typically require several weeks for adequate growth, and accurate results are often difficult to achieve. Besides the lengthy time required for identification, the emergence and transmission of multiple drug-resistant tuberculosis (MDR-TB) and extensive drug-resistant tuberculosis (XDR-TB) render control and prevention of tuberculosis even more difficult. The MDR phenotype is defined as resistant to at least isoniazid and rifampicin—the two most effective drugs recommended by the WHO for the first-line treatment of TB (2). Thus, the principal problems confront us exist in two respects. The first is to reduce the time and costs associated during the diagnosis of tuberculosis by conventional methods. Another is to speed up the efficiency and accuracy of drug susceptibility and resistance testing.

New strategies have been developed largely using molecular biology tools (3). Techniques based on DNA hybridization are sensitive, fast, and simple, but the available commercial assays (AccuProbe; Gen-Probe, San Diego, CA, USA) are able to identify only four species and two complexes of mycobacteria. Techniques requiring amplification followed by a hybridization step on a solid support are more complete than probes, but commercially available kits are limited to 5 (Geno-Type MTBC; Hain Lifescience GmbH, Nehren, Germany), 16 (Inno-LiPa Mycobacteria v2; Innogenetics, Gent, Belgium), or 30 (GenoType Mycobacterium; Hain Life science GmbH, Germany) species (4). Systems based on sequencing or enzymatic restriction targeting the *hsp65*, *16S rRNA*, *sod*, and *rpoB* genes allow good identification of all mycobacteria at the species level but remain limited to specialized laboratories (5). In addition, they are expensive and time consuming and require highly-qualified operators. More recently, alternatives based on the analysis of mycolic acid by high-performance liquid chromatography (HPLC) or electrospray ionization-tandem mass spectrometry analysis (6) have been proposed. In this review, the authors discuss the application status of mass spectrometry, to the identification and drug resistance of *M. tuberculosis*.

Identification of *M. tuberculosis* using MALDI-TOF MS

The identification of bacteria by mass spectrometry dates back to the 1970s, where researchers first described the use of mass spectrometry to identify bacteria (7). Despite the promise of this work and the development of significant advances in specimen preparation, adoption of this technology by clinical laboratories occurred slowly. Early studies targeted the analysis of polar fatty acids that comprised 5% to 8% of the dry cell weight of bacteria, whereas more recent studies have focused on analysis of basic proteins, primarily in the mass range of 2,000 to 20,000 Da (60% to 70% of the dry cell weight of bacteria).

An overview of MALDI-TOF MS

Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as a novel tool for rapid and reliable identification of microorganisms by analysis of protein profiles from either disrupted cells (8) or intact bacterial cells (9). Bacterial colonies can be removed from agar culture plates, mixed with an excess of UV-absorbing matrix, and dried on steel target plates. The dried preparations are then exposed to laser pulses, resulting in energy transfer from the matrix to the nonvolatile analyte molecules, with desorption of the analyte into a gas phase. The ionized molecules are accelerated by electric potentials through a flight tube to the mass spectrometer, with separation of the biomarkers determined by their mass/charge ratio (m/z). The profile of biomarkers acquired by the mass spectrometer system is then compared to profiles of a collection of well-characterized organisms contained in a knowledgebase (10).

Identification of M. tuberculosis by MALDI-TOF MS

Recently, several studies have provided the proof-of-concept that MALDI-TOF MS can also identify mycobacteria (11). Indeed, this technique has emerged over the last few years as a revolutionary technique for the routine identification of bacterial isolates. El *et al.* (12) proposed an original protocol for the MALDI-TOF MS identification of heat-inactivated mycobacteria after dissociation in Tween-20, mechanical breaking of the cell wall, and protein extraction with formic acid and acetonitrile. By applying this protocol to as few as 10^5 colony-forming units of reference isolates of *M.tuberculosis*, *Mycobacterium avium*, and 20 other *Mycobacterium* species, they obtained species-specific mass spectra for the creation of a local database. Using this database, their protocol enabled the identification by MALDI-TOF MS of 87 *M. tuberculosis*, 25 *M.avium* and 12 non-tuberculosis clinical isolates with identification scores ≥2 within 2.5 hours. Thus MALDI-TOF MS appears to be an alternative first-line approach to the routine identification of a large majority of bacteria commonly cultured in the clinical microbiology laboratory. Nevertheless, the experimental conditions must be carefully managed, as the MALDI mass spectra acquired are heavily dependent on both bacterial culture and MALDI sample preparation conditions (13).

In order to address these above problems, Lotz *et al.* (14) engineered a strategy to identify mycobacterial strains using MALDI-TOF MS without cell extraction, and instead based on the choice of a limited number of species-specific profiles. A total of 311 strains belonging to 31 distinct species and 4 species complexes grown in Lowenstein-Jensen (LJ) and liquid [mycobacterium growth indicator tube (MGIT)] media were analyzed to validate the mycobacterial database that was constructed for mycobacterial species isolated from human pathology. Correct identifications were obtained for 97% of strains from LJ and 77% from MGIT media. The result suggested that this system may represent a legitimate alternative for clinical laboratories to identify mycobacterial species.

Machen *et al.* (15) developed two novel protocols for inactivation and extraction to identify 107 Mycobacterium clinical isolates, including *M. tuberculosis* complex, from solid cultures using Vitek matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (bioMérieux, Marcy l'Etoile France). The protocol using heat inactivation with sonication and cell disruption with glass beads resulted in 82.2% and 88.8% species and genus level identification, respectively.

These previously published protocols are relatively complex, involve significant preparation time, and the required materials are not usually present in the typical clinical laboratory. In 2013, Mather *et al.* (16) tested two simplified protein extraction protocols developed at the University of Washington (UW) and by bioMerieux (BMX) using two different mass spectrometry platforms (MALDI Biotyper by Bruker Daltonics; and VITEK MS by bioMérieux). Both extraction protocols included vortexing with silica beads in the presence of ethanol. The commercial Bruker database was also augmented with an in-house database composed of 123 clinical Mycobacterium strains. A total of 198 clinical strains, representing 18 *Mycobacterium* species, were correctly identified to the species level 94.9%

of the time when extracted using the UW protocol and compared to the augmented database. The BMX protocol and VITEK MS system resulted in correct species level identifications for 94.4% of these strains. In contrast, only 79.3% of strains were identified to the species level by the non-augmented Bruker database, although use of a lower identification score threshold (≥1.7) increased the identification rate to 93.9%, with two misidentifications that were unlikely to be clinically relevant. The two simplified protein extraction protocols described in this study are easy to use and can be used to identify commonly encountered Mycobacterium species.

Other methods based on mass spectrometry

In addition, several other methods based on mass spectrometry are being explored. Dang *et al.* (17) developed a 20-compound model to distinguish between MTB and NTM using gas chromatography-mass spectrometry and chemometrics. They reported validation of this model with two independent sample sets, one consisting of 39 MTB and 17 non-tuberculous Mycobacteria (NTM) isolates from the Netherlands, the other comprising 103 isolates (91 MTB and 12 NTM) from South Africa. All of the MTB strains in the 56 Dutch samples were correctly identified and the model had a sensitivity of 100% and a specificity of 94%. For the South African samples the model had a sensitivity of 88% and specificity of 100%. Kataria *et al.* (18) conducted a proteomic analysis of tuberculous meningitis (TBM) cerebrospinal fluid (n=20) with 2-dimensional difference gel electrophoresis (2D-DIGE) and mass spectrometry. They found that arachidonate 5-lipoxygenase may be considered for validation as a potential marker for diagnosis of TBM.

Identification of mycobacteria and other acid-fast organisms by mass spectrometry still poses a considerable challenge. However, as a rapid, relatively inexpensive method for the identification of mycobacteria, this technology may quickly become a widespread application in routine clinical practice.

Detection of drug resistance in *M. tuberculosis* by MALDI-TOF MS

Genetic mutation associated with drug-resistant M.tuberculosis

Bacteria can resist antibiotic actions by the following mechanisms: the production of enzymes that inactivate antibiotic molecules (e.g., β-lactamases and aminoglycoside-modifying enzymes) (19), the hyperproduction or production of novel efflux pumps and other changes in the cell wall (e.g., porin alterations) (20), mutations in target genes [e.g., in ribosomal protein genes or in genes coding for penicillin-binding proteins (PBPs)] (21), the bypass of a metabolic pathway (e.g., the expression of acquired PBPs with a low affinity for antibiotic molecules), and the production of proteins that protect the target site (e.g., quinolone resistance mediated by Qnr) or of target site-modifying enzymes. For *M. tuberculosis*, data have been analyzed to outline a set of SNPs which are appropriate as markers of the RIF and INH resistance of *M. tuberculosis* strains (22).

Detection of first-line drug resistance in M. tuberculosis

Ikryannikova *et al.* (22) developed a novel MALDI-TOF MS-based minisequencing method for the analysis of rifampin and isoniazid resistance of *M.tuberculosis* strains. Jiang *et al.* (23) compared the proteomes of isoniazid-resistant *M. tuberculosis* strains and isoniazid-susceptible strains by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) and MALDI-TOF-MS. The data obtained from peptide mass fingerprinting were used to conduct a protein database search, and the genes of all strains were sequenced. They concluded in all protein spot differences, five protein spots were upregulated in isoniazid-resistant strains and identified as Rv1446c, Rv3028c, Rv0491, Rv2971, and Rv2145 by (MALDI-TOF-MS) and these results suggested that the differentially expressed proteins from isoniazid-resistant strains might be used as potential immunodiagnostic antigens and candidate novel drug targets against drug resistant tuberculosis.

Determination of second-line drug resistance in M. tuberculosis

Simner *et al.* (24) used PCR coupled with electrospray ionization mass spectrometry (PCR-ESI-MS) for detection and identification of Mycobacterium spp. and *M. tuberculosis* complex (MTBC) resistance determinants from solid and broth Middlebrook culture media. The performance of the MDR-TB assay was compared to identification using nucleic acid hybridization probes and *16S rRNA* gene sequencing for 68 MTBC and 97 NTM isolates grown on agar and 107 cultures grown in Bactec MGIT broth. MTBC resistance profiles from the MDR-TB assay were compared to results with the agar proportion method.

The PCR-ESI-MS system correctly identified all MTBC isolates and 97.9% and 95.8% of the NTM isolates from characterized agar cultures and MGIT broth cultures to the species level, respectively. In comparison to the agar proportion method, the sensitivity and specificity for the detection of drug resistance using the MDR-TB assay were 100% and 92.3% for rifampin, 100% and 93.8% for isoniazid, 91.6% and 94.4% for ethambutol, and 100% and 100% for fluoroquinolones, respectively. The MDR-TB assay appears to be a rapid and accurate method for the simultaneous detection and identification of mycobacterial species and resistance determinants of MTBC from culture.

Kumar *et al*. performed a proteomic analysis of *M. tuberculosis* isolates resistant to kanamycin and amikacin using two dimensional gel electrophoresis (2DGE), MALDI-TOF and bioinformatic tools such as BLASTP, InterProScan, KEGG motif scan and molecular docking. The major finding implicates that two hypothetical proteins (Rv3867 and Rv3224) might be playing some crucial role in contributing resistance to second line drugs. Further application in this direction may lead to the development of newer therapeutics against tuberculosis (25).

In brief, MALDI-TOF MS is used to detect the probable proteins or oligonucleotides related to the resistance, based on the specific peptide mass fingerprinting in protein database, and the drug susceptibility of *M. tuberculosis* can be resolved. These results also suggest that the differentially expressed proteins from resistant strains might be used as potential immuno-diagnostic antigens and novel drug candidates against drug resistant tuberculosis.

Future prospects

MALDI-TOF MS has been successfully adapted for the routine identification of mycobacteria with standard protocols for sample preparation and standard approaches to quantify reproducibility. This revolutionary technique allows for easier, cheaper and faster diagnosis of mycobacterial pathogens compared with conventional phenotypic identification methods. However, detection of drug resistance by mass spectrometry is still in the initial stages of exploration, and more efforts should be focused on this aspect in the future.

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MALDI-TOF MS versus VITEK 2 ANC card for identification of anaerobic bacteria

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Background: Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) is an accurate, rapid and inexpensive technique that has initiated a revolution in the clinical microbiology laboratory for identification of pathogens. The Vitek 2 anaerobe and *Corynebacterium* (ANC) identification card is a newly developed method for identification of corynebacteria and anaerobic species. The aim of this study was to evaluate the effectiveness of the ANC card and MALDI-TOF MS techniques for identification of clinical anaerobic isolates.

Methods: Five reference strains and a total of 50 anaerobic bacteria clinical isolates comprising ten different genera and 14 species were identified and analyzed by the ANC card together with Vitek 2 identification system and Vitek MS together with version 2.0 database respectively. 16S rRNA gene sequencing was used as reference method for accuracy in the identification.

Results: Vitek 2 ANC card and Vitek MS provided comparable results at species level for the five reference strains. Of 50 clinical strains, the Vitek MS provided identification for 46 strains (92%) to the species level, 47 (94%) to genus level, one (2%) low discrimination, two (4%) no identification and one (2%) misidentification. The Vitek 2 ANC card provided identification for 43 strains (86%) correct to the species level, 47 (94%) correct to the genus level, three (6%) low discrimination, three (6%) no identification and one (2%) misidentification.

Conclusions: Both Vitek MS and Vitek 2 ANC card can be used for accurate routine clinical anaerobe identification. Comparing to the Vitek 2 ANC card, Vitek MS is easier, faster and more economic for each test. The databases currently available for both systems should be updated and further developed to enhance performance.

Keywords: Anaerobe; matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS); Vitek 2 anaerobe and *Corynebacterium* card (Vitek 2 ANC card); identification

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Introduction

Anaerobic bacteria are a significant component of human mucous membranes bacterial flora, and often are the causative agent of respiratory, gastrointestinal and female genital tract infections and bacteremia. Anaerobes that combine with aerobic bacteria can cause serious mixed infections, and they are frequently overlooked. Rapid and accurate identification of anaerobes play an important role in timely and appropriate treatments.

Conventional identification of anaerobes has long been

mainly based on the detection of phenotypic characteristics, such as Gram staining, colony morphology, microscopic examination, differential growth on selective media and various manual biochemical tests. Most of these conventional methods are laborious and time-consuming processes. While the development and popularization of automated and semi-automated systems continue for the identification of isolates, clinical application studies using these systems to identify anaerobes are limited. This is especially the case for the newly developed Vitek 2 anaerobe and *Corynebacterium* (ANC) card (bioMérieux, France) and the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) system.

To expand the capabilities for identification of corynebacteria and anaerobic species on the Vitek 2 system, bioMérieux, Inc. has developed the new ANC card. Previous studies have confirmed that the Vitek 2 ANC card is a simple, rapid, and satisfactory method for the identification of anaerobes in a clinical microbiology laboratory (1-3). However, less information is provided in evaluating the Vitek 2 ANC card comparing to other identification systems (4). The ANC card has a database that includes 49 taxa of anaerobic bacteria belonging to the genera *Actinomyces*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Collinsella*, *Eggerthella*, *Eubacterium*, *Finegoldia*, *Fusobacterium*, *Lactobacillus*, *Parabacteroides*, *Parvimonas*, *Peptoniphilus*, *Peptostreptococcus*, *Prevotella*, *Propionibacterium*, and *Veillonella*. *Bifidobacterium spp.* and *Veillonella spp.* are identified only at the genus level in this system. Identification is accomplished within approximately 6 h incubation time using a 64 microwell card that contains dehydrated biochemical substrates.

MALDI-TOF MS technology for the identification of bacteria is now gaining increased attention due to its accurate, inexpensive and rapid performance efficiencies (5-9). This technique is a soft ionization method, which allows desorption of peptides/proteins from both whole different cultured bacteria and crude bacterial extracts (10). Identification is based on the comparison of the tested isolate mass spectrum to a reference database. Bruker MS and Shimadzu MS, two types of MALDI-TOF MS systems, were frequently studied for anaerobic bacteria identification, proving varied rates of identification for anaerobes (11-14). The majority of previous studies have compared MALDI-TOF identification with conventional identification or reference standard methods simply for the identification of anaerobic bacteria (15-21).

In this study, we evaluated the Vitek MS and the Vitek 2 ANC card for the identification of most common clinical anaerobic isolates. 16S rRNA gene sequencing was used as a reference method (22).

Materials and methods

Bacterial strains and culture condition

A total of 50 fresh and frozen anaerobic clinical isolates comprising ten different genera and 14 species were included in the study. All isolates were recovered from routine examination of clinical specimens submitted to the First Affiliated Hospital of Nanjing Medical University. The five reference strains, *Bacteroides fragilis* ATCC 25285, *Clostridium difficile* ATCC 43255, *Propionibacterium acnes* ATCC 11827, *Propionibacterium acnes* ATCC 6919 and *Lactobacillus acidophilus* ATCC 4356, were tested routinely. Prior to testing, all strains were subcultured twice onto Columbia blood agar (bioMérieux, Shanghai, China) and incubated in an anaerobic atmosphere produced by GENbag (bioMérieux, Shanghai, China) for 48 h at 35 ℃.

Vitek 2 ANC card

Bacterial colonies were suspended in 0.45% sodium chloride with a turbidity of 2.7-3.3 McFarland. Inoculums were then introduced into an ANC card in the Vitek 2 Compact automated identification system and incubated for approximately 6 h. Through the three additional tests of Gram staining, cell morphology, and aerotolerance testing, the Vitek 2 system deduced interpretations for final identifications. Isolates initially resulting in no identification were retested.

MALDI-TOF MS system

Vitek MS is an automated microbial identification system based on MALDI-TOF technology. Each isolate was directly smeared onto a disposable target slide and then covered by a small drop of matrix solution (Vitek MS-CHCA) and air dried. The loaded slide was then inserted into the Vitek MS system. The quality standard performed on each group was a spot of *E. coli* ATCC 8739. Microbial identification is achieved by obtaining a composite mass spectrum using MALDI-TOF technology and comparing the sample spectra to the reference spectra contained within the Vitek MS version 2.0 database.

Table 1 Reference strains identified by Vitek MS and Vitek 2 ANC card

Reference strains	Identification of isolates by	
	Vitek 2 ANC card	Vitek MS
ATCC 25285 *Bacteroides fragilis*	*B. fragilis*	*B. fragilis*
ATCC 43255 *Clostridium difficile*	*C. difficile*	*C. difficile*
ATCC 4356 *Lactobacillus acidophilus*	*L. acidophilus*	*L. acidophilus/L. gasseri*
ATCC 11827 *Propionibacterium acnes*	*P. acnes*	*P. acnes*
ATCC 6919 *Propionibacterium acnes*	*P. acnes*	*P. acnes*

Vitek MS, Vitek mass spectrometry; Vitek 2 ANC card, Vitek 2 anaerobe and *Corynebacterium* card.

16S rRNA gene sequencing

When discrepancies in identification were observed between the VITEK 2 ANC card and the VITEK MS system, or no identification achieved in both, 16S rRNA gene sequencing was used to confirm the result. Bacterial DNA was extracted using the TIANamp Bacteria DNA kit (TIANGEN, Beijing, China), and the sequencing reactions were performed with a 16S rDNA Bacterial Identification PCR Kit (Takara, Dalian, China) according to the manufacturer's instructions. PCR products were purified and sequenced by Genscript Corporation. The obtained 16S rRNA gene sequences were subjected to BLAST analysis against the NCBI nucleotide database.

Data analysis

Data obtained were classified into the following categories: (I) correct identification to the species level; (II) correct identification to the genus level, including a multiple choice within the same genus; (III) low discrimination, between two or more species, including the correct species requiring additional tests; (IV) no identification; and (V) misidentification, the final identification in which the genus or species were incorrect compared to that of the reference 16S rRNA gene sequence.

Results

In this study, five ATCC reference strains were selected for evaluating the accuracy of Vitek MS and Vitek 2 ANC card. All of these five reference strains were identified routinely and showed consistent results except for ATCC 4356 *Lactobacillus acidophilus* (*Table 1*). The Vitek MS cannot provide the accurate result of *Lactobacillus acidophilus* at the species level, but rather only provides a selection of low discrimination identification between *Lactobacillus acidophilus* and *Lactobacillus gasseri*, while ANC card can identify the *Lactobacillus acidophilus* accurately.

To further evaluate the capability of Vitek MS and Vitek 2 ANC card, we selected 50 clinical isolates from our clinical anaerobe bank. Each strain was identified with the two systems respectively. For the 50 isolates belonging to ten genera and 14 different species (*Table 2*), Vitek MS provided correct identification for 46 (92%) isolates to species level, 47 (94%) isolates to the genus level, but one (2%) with low discrimination, two (4%) with no identification, and one (2%) with misidentification. In comparison, the Vitek 2 ANC card achieved 43 (86%) correct identification to the species level, 47 (94%) correct identification to the genus level, three (6%) low discrimination, three (6%) no identification, and one (2%) misidentification.

As seen in *Table 3*, there are eight discrepant results among the 50 pairs of identifications produced by Vitek MS and Vitek 2 ANC card. All of these discrepant strains were confirmed by 16S rRNA gene sequencing. One minor error by Vitek ANC card was an identification of *Bacteroides stercoris* instead of *Bacteroides thetaiotaomicron*. *Bacteroides vulgatus* displayed mixed genera identification of *Bacteroides eggerthii* and *Bacteroides vulgatus* by the Vitek MS system. The Vitek MS produced superior accurate results for identification of *Clostridium difficile* while the Vitek 2 ANC card performed low discrimination consisting of *Clostridium spp.* of three isolates of *Clostridium difficile*. *C. difficile* requires further tests of indole and lipase for distinction from *C. bifermentans* and *C. sporogenes*. Our results also revealed that *Anaerococcus tetradius* was not included in the Vitek MS database and was misidentified as *Brevibacillus spp*. The Vitek 2 ANC card cannot identify this species too. Moreover, two strains of *Parabacteroides goldsteinii* were not identified by either system.

Table 2 Clinical isolates identified by Vitek MS and Vitek 2 ANC card

Organisms [No. of isolates]	No. [%] of isolates identified by									
	Vitek 2 ANC Card					Vitek MS				
	Correct identification to		Low discrimination	No identification	Mis-identification	Correct identification to		Low discrimination	No identification	Mis-identification
	Species level	Genus level				Species level	Genus level			
Gram-negative bacilli [35]	32	33	0	2	1	32	33	1	2	0
Bacteroides spp. [31]	30	31	0	0	1	30	31	1	0	0
B. Fragilis [26]	26	26	0	0	0	26	26	0	0	0
B. Thetaiotaomicron [3]	3	3	0	0	0	3	3	0	0	0
B. Stercoris [1]	0	1	0	0	1	1	1	0	0	0
B. Vulgatus [1]	1	1	0	0	0	0	1	1	0	0
Parabacteroides spp. [3]	1	1	0	2	0	1	1	0	2	0
P. Distasonis [1]	1	1	0	0	0	1	1	0	0	0
P. goldsteinii [2]	0	0	0	2	0	0	0	0	2	0
Fusobacterium spp. [1]										
F. Nucleatum [1]	1	1	0	0	0	1	1	0	0	0
Gram-positive cocci [6]	5	5	0	1	0	5	5	0	0	1
Peptostreptococcus anaerobius [2]	2	2	0	0	0	2	2	0	0	0
Parvimonas micra [2]	2	2	0	0	0	2	2	0	0	0
Finegoldia magna [1]	1	1	0	0	0	1	1	0	0	0
Anaerococcus tetradius [1]	0	0	0	1	0	0	0	0	0	1
Non-spore forming Gram-positive bacilli [2]	2	2	0	0	0	2	2	0	0	0
Collinsella aerofaciens [1]	1	1	0	0	0	1	1	0	0	0
Propionibacterium acnes [1]	1	1	0	0	0	1	1	0	0	0
Spore forming Gram-positive bacilli [7]										
Clostridium difficile [7]	4	7	3	0	0	7	7	0	0	0
Cumulative totals [50]	43 [86]	47 [94]	3 [6]	3 [6]	1 [2]	46 [92]	47 [94]	1 [2]	2 [4]	1 [2]

Vitek MS, Vitek mass spectrometry; Vitek 2 ANC card, Vitek 2 anaerobe and *Corynebacterium* card.

Table 3 Discrepant results between Vitek MS and Vitek 2 ANC card confirmed by 16s rRNA gene sequencing

Organism identified by DNA sequencing	GenBank accession number	Identification of isolates by	
		VITEK 2 ANC card	VITEK MS
Parabacteroides goldsteinii	KJ130487	No identification	No identification
	KJ130488	No identification	No identification
Anaerococcus tetradius	KJ130489	No identification	*Brevibacillus spp.*
Bacteroides stercoris	KJ130490	*B. thetaiotaomicron*	*B. stercoris*
Bacteroides vulgatus	KJ101610	*B. vulgatus*	*B. eggerthii, B.vulgatus*
Clostridium difficile	KJ130491	*C. difficile, C. bifermentans, or C. sporogenes*	*C. difficile*
	KJ130492	*C. difficile, C. bifermentans, or C. sporogenes*	*C. difficile*
	KJ130493	*C. difficile, C. bifermentans, or C. sporogenes*	*C. difficile*

Vitek MS, Vitek mass spectrometry; Vitek 2 ANC card, Vitek 2 anaerobe and *Corynebacterium* card.

Discussion

Up to now, clinical anaerobe identification is still a time consuming and skilled process. There are a few automated systems or rapid identification reagents presently available for clinical laboratory use. The Vitek 2 ANC card and the Vitek MS are two new developed methods for anaerobe identification. Whether if they can improve clinical anaerobe identification? Which system should be better for clinical use? No study has answered these questions till now. In this study, five reference strains and 50 clinical isolates were selected to evaluate the two commercial automated systems in identifying anaerobic bacteria. From the results contained in *Tables 1*,*2*, we can see that both the ANC card and the Vitek MS can identify most of the reference strains and clinical isolates accurately. For the 50 clinical anaerobe strains, the two identification systems achieved the same percentage (94%) of correct identification at the genus level, which meets the requirements of clinical routine anaerobe identification. However, at the species level, Vitek MS got a considerably higher rate of identification accuracy of 92% compared to the Vitek ANC card of 86%. This data revealed that the Vitek MS is superior to the ANC card for species identification. Our results using Vitek MS are similar to a recent multi-centre evaluation of anaerobic bacteria, which exhibited 92.5% of correct identification at the genus level and 91.2% at the species level (15). Comparable results of Vitek ANC card were presented with 98.5% accuracy rate at genus level and 86.5% at species level by Francine Mory *et al.* (2). Moreover, both systems in our study generated excellent results in achieving 100% correct identification to the species level for *Bacteroides fragilis*.

However, three of seven of *Clostridium difficile* gave low-discrimination identification results at the species level when using ANC card. These findings are consistent with the previous work stating that the identification of *Clostridia* at the species level using the ANC card should be enhanced (1,2). But by contrast, 100% of *Clostridium difficile* isolates were identified correctly to the species level using Vitek MS. Among the 8 sequenced strains, there are four sequence results supporting the Vitek MS identification results, and only one isolate matches Vitek 2 ANC card identification result at species level, as shown in *Table 3*. Unsuccessful identification of *Anaerococcus tetradius* and *Parabacteroides goldsteinii* with both of the identification systems is due to the species not being included in the database. The reliability of the identification depends on the quality and composition of the reference spectra present in the database (3,11,23,24). The databases currently available for both systems need to be optimized with more spectra for certain genera and species and the very rare species need to be included to increase the identification capability of both automated systems.

As we known, only one anaerobe colony is required for identification using Vitek MS while bacterial turbidity need to be 2.7-3.3 McFarland using Vitek 2 ANC card. For slow growing of most anaerobic bacteria, the Vitek ANC card needs at least 24 h longer incubation time than the Vitek MS. On the other hand, all the isolates we studied can be identified on the first attempt by Vitek MS while more than 20% of the isolates had to be identified on a second or third attempt for discrepancies or no identification results were obtained by Vitek 2 ANC card. All these may increase the costs and extend the turnaround time of each test on the Vitek 2 ANC card additionally. A recent cost assessment study also demonstrated that the MALDI protocol provided

identifications 1.45 days earlier on average and can reduce reagent and labor costs of identification by $102,424, or 56.9% less when compared with standard protocols (25). Besides the advantages mentioned before, Vitek MS is easy to perform even for a relatively inexperienced technician.

Overall, both the Vitek 2 ANC card and the Vitek MS can provide accurate identification of anaerobic bacteria and meet routine clinical requirements. However, the Vitek MS system is an easier, faster and cheaper method than the Vitek 2 ANC card for identification of most clinically important anaerobic bacteria, except for the initial cost of the Vitek MS instrument is significantly higher than the Vitek 2 instrument. Both our study and others found that there are still many bacteria not included in the databases currently available for both systems. Future developments of the databases to include an expanded number of new species and more robust mass spectra profiles for current species will greatly improve the performance and utility of these automated systems for bacterial identification.

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Performance of mass spectrometric identification of bacteria and yeasts routinely isolated in a clinical microbiology laboratory using MALDI-TOF MS

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Background: Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is an emerging technology newly applied to identifying bacterial and yeast strains. The aim of this study was to evaluate the clinical performance of the VITEK® MS system in the identification of bacteria and yeast strains routinely isolated from clinical samples.

Methods: We prospectively analyzed routine MALDI-TOF mass spectrometry identification in parallel with conventional phenotypic identification of bacteria and yeasts regardless of phylum or source of isolation. Discordant results were resolved with 16S rDNA or internal transcribed spacer (ITS) gene sequencing. Colonies (a single deposit on a MALDI disposable target without any prior extraction step) were analyzed using the VITEK® MS system. Peptide spectra acquired by the system were compared with the VITEK® MS IVD database Version 2.0, and the identification scores were recorded.

Results: Of the 1,181 isolates (1,061 bacterial isolates and 120 yeast isolates) analyzed, 99.5% were correctly identified by MALDI-TOF mass spectrometry; 95.7% identified to the species level, 3.6% identified to the genus level, and 0.3% identified within a range of species belonging to different genera. Conversely, 0.1% of isolates were misidentified and 0.4% were unidentified, partly because the species were not included in the database. Re-testing using a second deposit provided a successful identification for 0.5% of isolates unidentified with the first deposit. Our results show that the VITEK® MS system has exceptional performance in identifying bacteria and yeast by comparing acquired peptide spectra to those contained in its database.

Conclusions: MALDI-TOF mass spectrometry is a rapid, accurate, and relatively inexpensive method for bacterial and yeast identification. Our results demonstrate that the VITEK® MS system is a fast and reliable technique, and has the potential to replace conventional phenotypic identification for most bacterial and yeast strains routinely isolated in clinical microbiology laboratories.

Keywords: Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS); VITEK-MS; bacteria; yeasts; identification

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Introduction

Infectious disease is the most common clinical disease, and fast and accurate diagnosis is paramount to control infection. Traditionally, the identification of pathogenic bacteria and yeast has relied on conventional culture, isolation and biochemical identification methods (1), the latter of which can be complicated and requires prolonged turnaround times. In addition, the biochemical characteristics of atypical bacteria are often difficult to identify, which causes concern with regards to choosing effective antibiotic therapy in a timely manner. Empirical application of broad-spectrum antibiotics to treat unidentified pathogenic bacteria and yeast leads to the emergence of more resistant strains, which further increases the effectiveness and costliness of clinical

treatment. Therefore, identification of clinical pathogenic bacteria and yeast not only promotes rapid diagnosis and treatment disease, but it also helps reduce both the emergence of drug-resistant strains and the costs associated with drug-resistant strains present in the clinic.

While techniques in molecular biology (i.e., ribosomal gene sequence analysis, real-time quantitative PCR, gene chips) (2) provide rapid methods for identification of bacteria and yeast, their high cost and complexity often prohibit these molecular techniques from being applied to routine testing in the clinical microbiology laboratory. In order to meet clinical needs, it is urgent to establish a rapid diagnostic method for routinely identifying pathogenic bacteria and yeast. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) is a new platform that is being increasingly applied to the clinical microbiology laboratory for use of rapid and accurate identification of pathogenic bacteria and yeast (3-16). The present study was conducted to explore the accuracy and feasibility of MALDI-TOF-MS in identifying clinically isolated bacteria and yeast.

Materials and methods

Bacterial and yeast isolates

All isolates were prospectively recovered over a 14-week period from various clinical specimens (such as blood, cerebrospinal fluid, urine, pus, biopsy, swab from any site of the body, pleural effusion, hydroperitoneum, respiratory tract, and wound specimens) sourced from different medical departments. Isolate duplicates (i.e., from the same patient) were discarded. The isolates were recovered after aerobic and anaerobic incubation of clinical specimens on 5% sheep-blood and chocolate agar media (bioMérieux). After semi-automated Gram staining (bioMérieux) and determination of catalase and oxidase activities, isolates were identified by using either the Vitek 2-Compact system (bioMérieux) or an appropriate API identification strip (bioMérieux). In parallel, a single colony of a (sub) culture was directly deposited on a MALDI-TOF plate (VITEK® MS, bioMérieux). Technicians performing one method of identification were blind to the results obtained from the other method.

Mass spectrometry

Technical training

Three technicians were trained for sample and slide preparation by performing three slides of 48 deposits with duplicate deposits per isolate during three independent days (one slide per day). Mucoid and rough isolates were included only in the third slide performed by each operator. A proficiency test using 16 strains with single deposits was passed by each technician.

Plate preparation

The disposable plate preparation was performed with the Vitek® MS preparation station software to link sample information to the mass spectrometer using the single-use FlexiMass MALDI target plates, supplied in a 48-well microscope slide format, divided into three acquisition groups of 16 spots, and by smearing the bacteria or yeast directly onto the plate (mostly one colony/deposit). The preparations for bacteria were overlaid with 1 µL of ready-to-use α-cyano-4-hydroxycinnamic acid (CHCA) matrix (bioMérieux) and air dehydrated for 1 to 2 min at room temperature. For yeast preparations were lysed with 0.5 µL 25% formic acid. After drying completely at room temperature (1 to 2 min), 1 µL of CHCA matrix (bioMérieux) was applied to the spot, which was also allowed to dry completely (1 min). As recommended by the manufacturer's instructions, the Escherichia coli ATCC 8739 strain, used as a calibrator and internal ID control, was inoculated on the calibration spots of each acquisition group (small spot in the middle of each acquisition group). Each bacterial isolate had been tested with a unique deposit.

Generation of mass spectra

Mass spectra were generated with a Vitek® MS Axima Assurance mass spectrometer (bioMérieux) in positive linear mode at a laser frequency of 50 Hz with an acceleration voltage of 20 kV and an extraction delay time of 200 ns. For each spectrum, 500 shots in 5-shot steps from different positions of the target spot (automatic mode) were collected by the mass spectrometer operating in conjunction with the Acquisition Station software (Vitek® MS version 2.0). Measured mass spectra ranged from 2,000 to 20,000 Da.

MS identification

For each bacterial or yeast sample, mass fingerprints were processed by the compute engine and the advanced spectrum classifier (ASC) algorithm associated with the Vitek® MS system, which then automatically identifies the organism by comparing the characteristics of the spectrum obtained (presence and absence of specific peaks) with those of the typical spectrum of each claimed species contained in

the database.

The ASC algorithm compared the generated spectra to the expected spectrum of each organism or organism group of the database to provide identification. A percent probability, which represents the similarity in terms of presence/absence of specific peaks between the generated spectrum and the database spectra, was calculated by the algorithm. Isolates with scores from 60% to 99.9% with a single organism choice were considered a good identification. For isolates with probability scores >60% and a choice of 2-4 organisms, a genus level identification was recorded if all choices were within the same genus. However, no valid identification was recorded if the organism choices were of multiple genera. Scores of <60% were considered to have no valid identification.

When a human error or a poor-quality deposit occurred (including the warning messages "bad spectrum", "not enough peaks", "too many peaks", and "too much background noise" or in the case of calibration/control failure), the isolates were retested with a single deposit and the second result used for analysis. For informative purposes, samples with "no ID" or "mis-ID" first-spot results were secondarily retested with a single spot.

Criteria for identification of isolates

Accurate identification of isolates using the Vitek 2-Compact system or the API system was confirmed when the percentage of identification was >90%. As for MALDI-TOF analysis, when a probability score between 60% and 100% represents a high discrimination value and a reliable identification, MALDI-TOF MS identification was considered final. Discrepant results were regarded as follows: (I) a probability score that is >60% is found in a low discrimination identification that consists of a list of two to four choices for an identification match; (II) scores of <60% were considered to have no valid identification; and (III) a report of no identification is produced when either no match is found for the composite spectra, or not enough spectral peaks were obtained in the analysis. In the case of discrepant results or no identification with one or both methods, 16S rDNA or ITS sequencing resolved final identification.

Sequence data

Isolates that yielded discrepant results between routine phenotypic identification and MALDI-TOF MS identification were subjected to partial *16S rDNA* or *ITS* gene sequencing by an outside reference laboratory. DNA was extracted with the MagNAPure LC DNA isolation kit II (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. PCR amplification of gene was performed using the primers for 16S rDNA (F-AGAGTTTGATCCTGGCTCAG and R-TACGGCTACCTTGTTACGACTT) or the primers for ITS (F-TCCGTAGGTGAACCTGCGG and R-TCCTCCGCTTATTGATATGC). Amplicons were purified and double-strand sequenced using the primers for 16S rDNA or ITS. Fragments were analyzed using an automatic DNA sequencer (ABI Prism 3730 XL genetic analyzer; Applied Biosystems, Foster City, CA, USA) and queried against NCBI/GenBank databases. A per cent similarity of ≥99% between the unknown sequence and the closest matching sequence from the GenBank database was used as the criterion to classify an isolate to the species level.

Calculation of global assessment indices

For the MALDI-TOF based identification method, positive predictive values to the genus level and to the species level, considering isolates with correct identifications to the genus level true positives and isolates with correct identifications to the species level true positives, respectively, were calculated. Misidentified isolates were considered false positives. Negative predictive value, considering isolates with an absence of identification and belonging to species not included in the database true negatives and isolates with an absence of identifications and belonging to species included in the database false negatives, was calculated.

Results

Global identification performances

During the study period, 1,181 isolates were analyzed by the Vitek® MS system and the conventional Vitek2-compact system in parallel. Implementation of DNA-based identification methods to manage discrepancies or to obtain a more accurate fine identification (to the species or subspecies level) was performed for 9 (0.8%) isolates. Fine identification proposed by the Vitek® MS as a single choice, whatever the confidence value, or included in a multiple-choice result were considered overall correct identifications. Of 1,181 isolates encompassing 80 species and 39 genera, 1,175 (99.5%) isolates were correctly identified by MALDI-TOF MS as defined previously (*Table 1*). No IDs and discordant results (mis-IDs) were obtained for 0.4% and 0.1% of the isolates, respectively.

Table 1 Valid Vitek® MS results of 1,181 bacterial and yeast isolates

Fine identification	No. of isolates	No. [%] of isolates with the indicated result[a]				
		Correct identification to the level of:			No identification	Misidentification
		Species	Genus	Above genus		
Enterobacteriaceae	560	522 [93.2]	38 [6.8]			
Citrobacter amalonaticus	1	1 [100]				
Citrobacter freundii	3	3 [100]				
Citrobacter koseri	2	2 [100]				
Enterobacter aerogenes	5	5 [100]				
Enterobacter cloacae[e]	36		36 [100]			
Escherichia coli	224	224 [100]				
Klebsiella oxytoca	7	7 [100]				
Klebsiella pneumoniae	178	178 [100]				
Morganella morganii	22	22 [100]				
Pantoea agglomerans	1		1 [100]			
Proteus mirabilis	6	6 [100]				
Proteus vulgaris	1	1 [100]				
Salmonella typhi[g]	1		1 [100]			
Serratia marcescens	73	73 [100]				
Nonfermentative Gram-negative rods	311	307 [98.7]	1 [0.3]	2 [0.6]	1 [0.3]	
Acinetobacter baumannii complex[g]	247	247 [100]				
Acinetobacter bouvetii	1			1[b] [100]		
Acinetobacter johnsonii	1	1 [100)				
Acinetobacter junii	1		1 [100]			
Burkholderia cepacia	12	12 [100]				
Burkholderia pickettii	1	1 [100]				
Moraxella catarrhalis	1	1 [100]				
Moraxella osloensis	1			1[c] [100]		
Pseudomonas aeruginosa	35	35 [100]				
Pseudomonas putida	6	6 [100]				
Pseudomonas fluorescens	4	4 [100]				
Pseudoxanthomonas mexicana	1				1 [100]	
Gram-positive cocci	130	127 [97.7]		1 [0.8]	2 [1.5]	
Staphylococcus aureus	27	27 [100]				
Staphylococcus epidermidis	26	26 [100]				
Staphylococcus haemolyticus	9	9 [100]				
Staphylococcus hominis	7	7 [100]				
Staphylococcus saprophyticus	2	2 [100]				
Staphylococcus pasteuric	2				2 [100]	
Enterococcus avium	3	3 [100]				
Enterococcus casseliflavus	1	1 [100]				
Enterococcus faecalis	19	19 [100]				
Enterococcus faecium	18	18 [100]				
Enterococcus gallinarum	2	2 [100]				
Enterococcus raffinosus	1			1[d] [100]		
Streptococcus agalactiae	3	3 [100]				
Streptococcus dysgalactiae	2	2 [100]				
Streptococcus anginosus	4	4 [100]				
Streptococcus constellatus	3	3 [100]				
Streptococcus pneumoniae	1	1 [100]				

Table 1 (*continued*)

Table 1 (*continued*)

Fine identification	No. of isolates	No. [%] of isolates with the indicated result[a]				
		Correct identification to the level of:			No identification	Misidentification
		Species	Genus	Above genus		
Anaerobes	12	12 [100]				
Bacteroides fragilis	2	2 [100]				
Bacteroides thetaiotaomicron	1	1 [100]				
Bacteroides ovatus	1	1 [100]				
Clostridium difficile	1	1 [100]				
Eggerthella lenta	1	1 [100]				
Fusobacterium varium	1	1 [100]				
Parabacteroides distasonis	1	1 [100]				
Peptostreptococcus anaerobius	1	1 [100]				
Propionibacterium acnes	1	1 [100]				
Prevotella intermedia	1	1 [100]				
Prevotella bivia	1	1 [100]				
Miscellaneous bacteria	48	45 [93.8]	3 [6.3]			
Aeromonas hydrophila[f]	2		2 [100]			
Aeromonas caviae[f]	1		1 [100]			
Brucella melitensis	1	1 [100]				
Cardiobacterium hominis	2	2 [100]				
Gardneralla vaginalis	2	2 [100]				
Haemophilus influenzae	20	20 [100]				
Haemophilus parahaemolyticus	1	1 [100]				
Haemophilus parainfluenzae	9	9 [100]				
Listeria monocytogenes	2	2 [100]				
Neisseria gonorrhoeae	2	2 [100]				
Pasteurella multocida	1	1 [100]				
Vibrio parahaemolyticus	2	2 [100]				
Vibrio vulnificus	3	3 [100]				
Yeast	120	117 [97.5]			2 [1.7]	1 [0.8]
Candida albicans	6	6 [100]				
Candida butyri	2				2 [100]	
Candida glabrata	19	19 [100]				
Candida guilliermondii	7	7 [100]				
Candida haemulonii	10	10 [100]				
Candida lusitaniae	1	1 [100]				
Candida parapsilosis	20	20 [100]				
Candida pelliculosa	14	14 [100]				
Candida tropicalis	33	33 [100]				
Cryptococcus neoformans	3	3 [100]				
Rhodosporidium fluviale	1					1 [100]
Saccharomyces cerevisiae	1	1 [100]				
Trichosporon asahii	3	3 [100]				
Total	1,181	1,130 [95.7]	42 [3.6]	3 [0.3]	5 [0.4]	1 [0.1]

[a], Species, correct identification at the species level (single choice or low discrimination at the subspecies level); genus, correct identification at the genus level (low discrimination at the species level); above genus, correct identification proposed among a set of low-discrimination results including species of different genera; [b], The 1st identified as Acinetobacter johnsonii (confidence value 99.9%) by the Vitek® MS system, 2nd identified as Acinetobacter bouvetii (confidence value 99.9%), 3rd identified as Acinetobacter bouvetii (confidence value 99.9%); [c], The 1st identified as Moraxella osloensis (confidence value 99.9%) by the Vitek® MS system, 2nd identified as Alcaligenes faecalis (confidence value 99.9%), 3rd identified as Moraxella osloensis (confidence value 98.9%); [d], The 1st identified as Enterococcus raffinosus (confidence value 96.9%) by the Vitek® MS system, 2nd identified as Enterococcus avium (confidence value 99.9%), 3rd identified as Enterococcus raffinosus (confidence value 99.9%); [e], The Enterobacter cloacae/asburiae species group is displayed as a species group result by the Vitek® MS; [f], The Aeromonas hydrophila/caviae species group is displayed as a species group result by the Vitek® MS; [g], The species group is the final Vitek® MS identification. The subspecies or species included in each species group are as follows: for the Salmonella group, S. enterica subsp. enterica, S. enterica serovar Enteritidis, S. enterica serovar Paratyphi B, S. enterica serovar Paratyphi C, S. enterica serovar Typhimurium, and Salmonella spp.; for the Acinetobacter baumannii complex, A. baumannii, A. calcoaceticus, Acinetobacter genomospecies 3, and Acinetobacter genoh-3ospecies TU13.

Table 2 Discrepancies and errors in routine phenotypic tests and the Vitek® MS system identification

Gene sequencing identification (≥99% sequence matching)	No. of isolates			
	Vitek® MS identification		Current methods of identification	
	No identification	Misidentification	No identification	Misidentification
Pseudoxanthomonas Mexicana (n=1)	1	0	0	1[a]
Staphylococcus pasteuric (n=2)	2	0	2	0
Moraxella osloensis (n=1)	0	0	0	1[b]
Acinetobacter bouvetii (n=1)	0	0	0	1[c]
Candida butyri (n=2)	2	0	2	0
Rhodosporidium fluviale (n=1)	0	1[d]	0	1[e]
Total (n=8)	5	1	4	4

[a], Brevundimonas diminuta; [b], Alcaligenes faecalis; [c], Acinetobacter lwoffii; [d], Rhodotorula mucilaginosa; [e], Rhodotorula glutinis.

Correct MALDI-TOF mass spectrometry identifications

Among the 1,181 isolates, 1,130 (95.7%) isolates had correct species identification using Vitek® MS. Results of MALDI-TOF MS identifications for Enterobacteriaceae, nonfermentative Gram-negative rods, a group of miscellaneous bacteria, Gram-positive cocci, anaerobes and yeast are depicted separately in *Table 1*. A correct identification to the genus level only, that is, the correct species ID was included in a multiple-choice result of species from the same genus, was obtained for 3.6% (n=42) of the isolates including species complexes such as Enterobacter cloacae/asburiae (n=36), Pantoea agglomerans/dispersa (n=1), Salmonella spp (n=1), Acinetobacter junii/johnsonii (n=1), and Aeromonas hydrophila/caviae (n=3). Low discrimination results above the genus level, that is, with the correct ID proposed among species of different genera, were obtained for 0.3% (n=3) of the isolates (*Table 1*), some of which also seem to be recurrent , like the Moraxella osloensis/*Alcaligenes faecalis* low discrimination result for Moraxella osloensis isolate. An identical and high confidence value was mostly obtained for each proposed species in the case of a low discrimination result to the species level or above the genus level. In the few cases in which a confidence value difference occurred, it argued either for or against the correct species ID.

Lack of identification and erroneous MALDI-TOF mass spectrometry identification

The Vitek® MS system gave an absence of identification for 5 (0.4%) isolates that were tested again using one deposit for informative purposes (*Table 2*). These isolates included one Pseudoxanthomonas mexicana isolate, two Staphylococcus pasteuric isolates, and two Candida butyri isolates, for which the system gave the same "no identification" answer after reading a second deposit. These isolates among three tested in the study again gave a "no identification" result despite the additional retest. An additional one Rhodosporidium fluviale isolate (0.1%) was erroneously identified as Rhodotorula glutinis by MALDI-TOF mass spectrometry even though a confidence value of 99.9% was obtained (*Table 2*).

Phenotype erroneous identifications

The current methods of identification failed for four isolates (0.3%), which were two Staphylococcus strains and two strains of yeast (*Table 2*). Phenotypic identification was erroneous for four isolates (0.3%). One isolate phenotypically identified as Brevundimonas diminuta was not identified by MALDI-TOF mass spectrometry and was confirmed to be Pseudoxanthomonas mexicana by *16S rDNA* gene sequencing. One isolate phenotypically identified as Alcaligenes faecalis was identified as Moraxella osloensis by MALDI-TOF mass spectrometry and as Moraxella osloensis by *16S rDNA* gene sequencing. One isolate phenotypically identified as Acinetobacter lwoffii was identified as Acinetobacte bouvetii by MALDI-TOF mass spectrometry and as Acinetobacter bouvetii by *16S rDNA* gene sequencing. One isolate phenotypically identified as Rhodotorula glutinis isolate was identified as Rhodotorula mucilaginosa by MALDI-TOF mass spectrometry and was confirmed to be Rhodosporidium fluviale by ITS gene sequencing.

Global assessment indices

According to the criteria detailed in the Materials and methods section, all of the positive predictive values to the genus level and to the species level of the Vitek® MS system were 99.7, and the negative predictive value was 83.3.

Discussion

Culturing, isolating, and then identifying microorganisms remains the gold standard procedure in etiological diagnosis of infectious diseases. However, for many fastidious pathogens, including Mycobacterium tuberculosis and various fungi species, the growth cycle can be long and tenuous, and the costs associated with longer turn-around-times to phenotypic identification for these organisms are quite high. Matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) based proteomics has quickly developed in recent years in the area of microbiology. The basic principle of MALDI-TOF MS technology is the use of a matrix solution which co-crystallization of sample and matrix substrate following solvent evaporation. The matrix-sample formation will absorb the energy when fired upon by a laser, transferring the ionic charge from matrix to sample. When the charged samples enter the vacuum tube and accelerating electric field of the system, the charged sample fragments will be separated based on their mass-to-charge ratio, and the flight detector analyzes this separation based on mass and charge and generates what is known as mass spectra. Using software and algorithmic analysis, the mass spectra for the sample will be compared against the mass spectra for known species contained in the system database.

The VITEK®-MS v2.0 MALDI-TOF system has been implemented in our laboratory for efficient, cost-effective, rapid, and routine identification of bacterial and yeast isolates (14,17,18). The results of our prospective analysis of 1,181 clinical isolates revealed exceptional performance of the VITEK®-MS v2.0 MALDI-TOF system in comparison to conventional identification techniques. Overall, the performance of this system was highly accurate (95.7% correct to species-level identification), where only 0.5% of the total tested isolates were not identified or misidentified.

Until now, most studies have reported on the proof-of-concept of MALDI-TOF MS for specific microorganisms (6,9,12,13,19). The majority of these studies included strains from reference and culture collections. Recently, a study by Seng *et al.* concluded that MALDI-TOF MS can replace conventional systems for identification of bacteria in a conventional laboratory (20). In our study, the performance of MALDI-TOF MS was specified in detail for different groups of microorganisms. In accordance with the results of Seng *et al.*, >95% of our clinical isolates could be identified to species levels by MALDI-TOF MS. Enterobacteriaceae and Nonfermentative Gram-negative rods, both clinically relevant pathogens accounting for a large majority of aerobic Gram-negative rods in a conventional medical microbial laboratory, were accurately identified to the species level (>93%), even for very closely related species.

In the present study, the VITEK®-MS system was not able to distinguish Enterobacter cloacae isolates from Enterobacter asburiae, while the conventional systems correctly identified all isolates to the species level. Similarly, the VITEK®-MS system could not resolve identification for Aeromonas hydrophila/caviae. These findings are consistent with other reports in the literature (21). This may be because species show similar pathogenicities and antibiotics susceptibility patterns (for example, Enterobacter cloacae/asburiae), thus requiring some biochemical tests (for example, exercise test for Enterobacter cloacae/asburiae, VP test for Aeromonas hydrophila/caviae) to distinguish. These results clearly demonstrate that updating the database is essential for bacterial identification and that there are improvements to be made on the current database.

An important advantage of MALDI-TOF MS is rapid identification gram-positive cocci, including staphylococci, enterococci and streptococci. Coagulase negative staphylococcus (CoNS) are the most common blood isolates in culture, accounting for about 45% of the isolated bacteria in blood culture. However, about 60-80% CoNS isolated from blood culture are suspected to be contaminated bacteria. With the increase of invasive surgical operations, 27-38% catheter-related bloodstream infection is estimated to be caused by CoNS. So identification to the exact species level may be very useful as some CoNS can contaminate cultures from true infections by staphylococcus species. Most studies report a number of viridans streptococci and pneumococci were misidentified by MALDI-TOF MS. Seng *et al.* found that nearly 50% of S. pneumoniae isolates were misidentified as Streptococcus parasanguinis because the database included only three S. pneumoniae and two S. parasanguinis reference spectra (20). Therefore, the database also needs improvement, with more spectra of well-identified streptococcal species. However, in our study the performance of MALDI-TOF MS was specified in the identification of viridans streptococci and pneumococci.

This may be related to the number of identification bacteria associated with the existing database.

MALDI-TOF MS performed well for identification of yeasts in our study, with correct identification of 97.5% of 120 isolates encompassing 13 different species without laborious sample preparation procedures. Over the past decade the significance of infections by yeasts has increased, especially those caused by germ tube negative yeasts. Given the variable susceptibility of different species of yeasts to antifungal agents, the rapid and correct identification is of clinical importance. Furthermore, the identification of yeast isolates to the species level makes it possible to study the epidemiology of colonization and infection and the transmission of infections in hospitals. Conventional identification methods, however, are laborious and time-consuming. Additionally, high-resolution DNA-based molecular techniques, such as 16S or 18S rRNA or ITS DNA sequencing and real-time PCR assays, are expensive and also time-consuming. Lohmann *et al.* studied 312 clinical isolates and concluded that MALDI-TOF MS is a rapid and reliable tool for the identification of yeasts and yeast-like fungi, with low expenditure of consumables, easy interpretation of results, and a fast turnaround time (16). Misidentifications in our study and the Lohmann study were attributed to the use of an incomplete database.

In the study presented here, five isolates were insufficiently identified because of missing reference spectra in the VITEK®-MS database: Pseudoxanthomonas Mexicana, Staphylococcus pasteuric (n=2) and Candida butyri (n=2) were declared "unknown spectrum", and Rhodosporidium fluviale was misidentified as Rhodotorula mucilaginosa. This misidentification may be due to a technical error during sample preparation on the target slide.

The strengths of our study are the implementation of MALDI-TOF MS in a routine setting, the comparison of MALDI-TOF MS with conventional identification systems on clinical isolates, the use of 16S rDNA and ITS sequencing for analysis of discrepancies, and the inclusion of yeasts in addition to bacteria. The main limitation of this study is the lack of inclusion of sufficient Gram-positive aerobic rods and enteropathogens. During the study period, aerobic Gram-positive rods and enteropathogens were isolated sporadically (n=2 and n=8 isolates, respectively). Conventional identification methods for aerobic Gram-positive rods are cumbersome and time consuming. Using MALDI-TOF MS for identifying aerobic Gram-positive rods would certainly increase the number of species identifications since it can be applied directly from bacterial colonies on the primary culture plates (11,22). Moreover, MALDI-TOF MS technology is a powerful tool that can be used in routine laboratories for the diagnosis of enteric diseases. It is particularly useful for the rapid discrimination of Normal flora from potential pathogens that are isolated from stool samples. For pathogen identification itself, the limitations of MALDI-TOF MS must be considered. Initially, the identification of Shigella or E. coli will still require additional tests according to the nature of the sample (20,23). Secondly, biochemical and serological tests will still be required to accurately identify Salmonella species (23). Additional studies should be conducted in order to evaluate the ability of the Vitek MS database to differentiate S. typhi from other Salmonella serotypes. This indeed is of major interest from both the clinical management and public health perspectives.

Our results suggest that the major factors that may influence the quality of MALDI-TOF MS identifications are the purity of the strain, the amount of biological material smeared on the target plate and the experience of the technologist. Without an intensive training background of the technicians, the technical ownership of the Vitek® MS system is straightforward and fast, as previously mentioned (24). However, the technician must remain vigilant in routine practice during sample preparation because of reduced interspot distances (especially for spots near the E. coli calibrant spot) that can mix two bacterial deposits, particularly during the matrix application step, as happened during the training period. In addition, it is extremely important to cultivate conditions and colony solvent treatment. Need to strictly according to the manufacturer's operating standards, in order to ensure the consistency of the identification results.

Rapid and reliable identification of bacteria and fungi is paramount for effective therapy. The VITEK®-MS is an accurate system for identifying clinically relevant bacteria and yeasts with only one deposit of crude bacteria and yeasts, and without any extraction step required. Implementation of this technology in the clinical microbiology laboratory will lead to decreased turnaround times for identification, having a large impact on clinical outcome and dramatically reducing healthcare costs. MALDI-TOF MS can also be used in addition to traditional methods, such as colony morphology and Gram stain tests, for organisms that are difficult to identify. The introduction of MALDI-TOF MS into the clinical microbiology laboratory represents a significant shift in the diagnosis of bacterial and yeast infections, and ultimately enhances patient care.

Conclusions

In summary, MALDI-TOF MS-based identification provides cheaper and faster bacterial and yeast species identification than conventional phenotypic identification methods, with equal or better accuracy. Our results demonstrate that the VITEK® MS system is a rapid and reliable technique, and has the potential to replace conventional phenotypic identification for most bacterial and yeast strains routinely isolated in clinical microbiology laboratories. However, spectral databases should be regularly updated by suppliers to improve identification rates.

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Comparative study of MALDI-TOF MS and VITEK 2 in bacteria identification

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Background: Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has recently been introduced in diagnostic microbiology laboratories for the identification of bacterial and yeast strains isolated from clinical samples. This study aimed to evaluate the accuracy of MALDI-TOF MS in clinical microbiology diagnosis by comparing it with commonly-used VITEK 2 or gene sequencing.

Methods: The performances of MALDI-TOF MS and VITEK 2 were compared retrospectively for identifying routine isolates. Discrepancies were analyzed by gene sequencing analysis of the *16S* genes.

Results: For 1,025 isolates, classified as 55 species of 25 genera, 1,021 (99.60%) isolates were accurately identified at the genus level, and 957 (93.37%) isolates at the species level by using MALDI-TOF MS. A total of 949 (92.59%) isolates were completely matched by both methods. Both methods found 76 unmatched isolates among which one strain had no definite identification by MALDI-TOF MS and VITEK 2 respectively. However, MALDI-TOF MS made no errors at the genus level while VITEK 2 made 6 (0.58%) errors at the genus level. At the species level, the identification error rates for MALDI-TOF MS and VITEK 2 were 5.56% and 6.24%, respectively.

Conclusions: With a lower identification error rate, MALDI-TOF MS has better performance than VITEK 2 in identifying bacteria found routinely in the clinical laboratory. It is a quick and cost-effective technique, and has the potential to replace conventional phenotype methods in identifying common bacterial isolates in clinical microbiology laboratories.

Keywords: Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS); VITEK2; bacteria identification

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Introduction

Traditionally, bacterial and fungal identifications in clinical microbiology laboratories are mainly carried out according to phenotype characteristics, including identifications of culture media, colony morphology, gram stain and various biochemical reactions (1). Although all of these methods can achieve high accuracies, it usually takes minimum one day or longer to complete the whole identification process. Molecular methods, such as real-time PCR, gene sequencing and microarray analysis, are quick methods for bacterial and fungal identification, but they come at a very high cost and require highly-trained technicians. Therefore, molecular methods are not routinely used for bacterial identification. A faster and easier technique for microbial identification would greatly enhance the conventional laboratory in providing more timely feedback to clinic. This is especially true in cases when patients are critically ill suffering from infectious diseases and where therapeutic intervention is urgently needed.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) can be used to obtain protein fingerprinting from whole bacterial cells (2). Through comparing these fingerprints to a reference database by the use of various algorithms, bacteria can be rapidly identified. The earliest application of this technique

for bacterial identification dates back to 1975 (3), while the first related article was published in 1996 (Holland, *et al.*) (4). Studies in this field have been progressively advancing for the last decade (5). Until now, domestic comparative studies on mass spectrometry and other methods are relatively few. In order to compare the performance differences between MALDI-TOF MS and VITEK 2, the latter being the most-used automated identification technique in current microbiology laboratories, we employed the two systems in parallele to identify and analyze 1,025 isolates routinely isolated in 2012 at a microbiology laboratory of PLA General Hospital.

Methods

Bacteria isolates

A total of 1,025 isolates, composed of 1,020 bacterial strains and 5 fungal strains representing 25 different genera were selected for analysis. These bacteria were routinely isolated from clinical patients, such as *Pseudomonas spp*, *Acinetobacter spp* that cause lung infections, *Enterobacteriaceae* that cause blood and urinary tract infections. In addition, there are also some bacteria which grow slowly but have clinical significance such as *Eikenella corrodens*, *Listeria monotytogens*, *Haemophilus infuenzae*, etc.

VITEK 2 identification

GP, GN, YST, ANC and NH were selected to run identification analysis according to the different strains to be tested. The identification rate was expected to be above 93% by VITEK 2 (bioMérieux) (5), with quality control by stages via ATCC25922, ATCC27853 and ATCC25923.

MALDI-TOF MS identification

Strains were identified by MALDI-TOF MS using the VITEK MS system (bioMérieux). Loops were used to select and smear the subject isolates onto the sample spots on the target slide. Then 1 μL VITEK MS-CHCA matrix was applied over the sample and air dried until the matrix and sample co-crystallized. The target slide with all prepared samples then was loaded into the VITEK MS system to acquire the mass spectra of whole bacterial cell protein, mainly composed of ribosomal protein, for each sample. In the case of yeasts, 0.5 μL VITEK MS-FA was added to each sample on the target slide and allowed to air dry it before adding 1 μL VITEK MS-CHCA matrix. Finally, the mass spectra acquired for each sample were compared to the known mass spectra contained in the database for, and given a confidence score according to how close the acquired spectra matched those contained in the database. ATCC8739 was used as the quality control strain.

Gene sequencing

Discrepancies between VITEK 2 and MALDI-TOF MS were resolved by *16S rRNA* gene sequencing. The results would be considered valid if the homologous rate was above 99%.

Standards for identification

In cases where the isolate was correctly identified at the species level or at the generic level by both VITEK MS and VITEK 2, the results were considered to be in accordance with one another. In instances that the two methods produced different results, or if the results from one method were not definite, *16S rRNA* gene sequencing was used to resolve identification discrepancies. For isolates misidentified at the genus level, the results were considered a major mistake in the identification analysis. For isolates misidentified at species level but correctly identified at the genus level, the results were considered a minor mistake. Results not able to distinguish two different bacteria of the same genus also were considered as minor mistakes (6,7).

Results

Identification results by MALDI-TOF MS

Among the 1,025 total isolates, 1,021 (99.60%) isolates were accurately identified at the genus level and 957 (93.37%) isolates at the species level by MALDI-TOF MS.

Matched results by the two identification methods

Identification results for 949 (92.59%) isolates, belonging to 23 genera and 48 species, were in agreement (see *Table 1*) using both methods.

Unmatched results by the two identification methods

Among the 1,025 isolates, 76 (7.4%) isolates produced discordant results between the two identification methods. One strain had no definitive identification for each

Table 1 Matched results by both MALDI-TOF MS and VITEK 2

Bacterial genera	Number of strains
Acinetobacter banmannii complex	30
Corynebacterium jeikeium	1
Citrobacter braakii	2
Citrobacter freundii	6
Citrobacter koseri	1
Escherichia coli	297
Enterobacter aerogenes	13
Enterococcus casseliflavus	2
Enterococcus faecalis	10
Enterococcus faecium	24
Enterococcus gallinarum	1
Enterococcus hirae	3
Klebsiella oxytoca	10
Klebsiella pneumonia	66
Micrococcus ssp	3
Morganella morganii	7
Pseudomonas aeruginosa	126
Pseudomonas putida	1
Stenotrophomonas maltophilia	33
Proteus mirabilis	75
Proteus vul.gr/proteus pen.	9
Procidencia rettgeri	2
Staphylococcus aureus	76
Staphylococcus epidermidis	36
Staphylococcus hominisssphominis	37
Staphylococcus saprophyticus	1
Staphylococcus haemolyticus	14
Staphylococcus cohnii	1
Staphylococcus capitis	5
Serratia marcescens	23
Streptococcus parasanguinis	1
Streptococcus pneumonia	4
Streptococcus mitls/Steptococcusoralis	3
Streptococcus gallolyticus	1
Streptococcus gordonii	1
Streptococcus sailvarius	1
Streptococcus agalatiae	5
Streptococcus sanguinis	1
Shewanella algea	3
Vibrio parahaemolyticus	1
Candida parapsilosis	2
Aeromonasssp	3
Chryseobacterium indologenes	1
Eikenella corrodens	1
Salmonella group	2
Listeria monotytogens	1
Haemophilus infuenzae	1
Burkholderia cepacia	1
Total	949

MALDI-TOF MS and VITEK 2. However, MALDI-TOF MS made no errors at the genus level while VITEK 2 made 6 (0.58%) errors at the genus level. At the species level, the identification error rates of the two methods were 5.56% and 6.24% for MALDI-TOF MS and VITEK 2 respectively (*Table 2*).

Discussion and Conclusions

In our laboratory, MALDI-TOF MS is an efficient, quick and relatively inexpensive per isolate method for identifying pathogenic microorganisms including bacteria and fungi. MALDI-TOF MS identification also is largely compatible with a large range of culture media and culture conditions, and is the fastest means to detect microbes in positive blood culture (8). Furthermore, MALDI-TOF MS has broader application prospects in the field of testing for drug resistance (9,10).

Shortening the turnaround time to pathogen identification and offering quicker results to the clinic remain the most important issues needed to be solved in the microbiology laboratory. Based on our data, experienced clinicians can treat critically infected patients appropriate antibiotic treatment given effective and timely identification of pathogenic bacteria. Shortening the turnaround time permits the clinician to treat patients faster, ultimately reducing the fatality rates, the length of patient stay in the clinic, and healthcare costs associated with patient care. MALDI-TOF MS offers one promising solution to shorten the turnaround time and relieve these pressures in the clinic.

In the present study, we chose 1,025 pathogenic bacteria routinely found in our laboratory for identification, and compared the performance of MALDI-TOF MS to the VITEK 2 system, the latter which is based on conventional biochemical identification that is presently found in most microbiology laboratories. Our results showed that MALDI-TOF MS offered higher identification accuracy and lower error rates at the species level when compared to VITEK 2. Additionally, MALDI-TOF MS dramatically shortened identification time from 6-8 hours to just a few minutes.

MALDI-TOF MS identified 99.60% of isolates to the genus level and 93.37% of isolates to the species level, which are slightly higher rates than those previously reported (5,7,11-14). Van Veen *et al.*, for example, achieved accuracy rates of 97.1% and 92% to the genus and species levels, respectively. The difference in accuracy is most likely due to the different choices of strains. The strains in van Veen's study included 61 yeast strains, among which 96.7%

Table 2 Unmatched results by both MALDI-TOF MS and VITEK 2

Bacterial species	Number of strains	VITEK MS		VITEK 2	
		Generic errors	Species errors	Generic errors	Species errors
Acinetobacter banmannii	2			2	
Acinetobacter jonnosonii	2			2	
Citrobacter freundii	1		1		
Enterobacter cloacae	46		46		46
Enterococcus faecium	1		1		
Pseudomonas putida	1				1
Peudomonas aeruginosa	1		1		
Staphylococcus hominis	4				4
Staphylococcus aureus	4			1	3
Staphylococcus epidermidis	1				1
Streptococcus agalactiae	2				2
Streptococcus mitis	3		3		3
Candida guilliermondii	1				1
Rhodotorula mucilaginosa	1		1		1
Achromobacter xylosoxidans	3		3		
Chryse.indologenes	1		1		1
Listeria monocytogenes	1				1
Cryptococcus neoformans	1			1	
Total	76	0 (0)	57 (5.56%)	6 (0.58%)	6 (6.24%)

were identified to the genus level and 82.5% to the species level. Streptococcus viridans, streptococcus pneumoniae and anaerobic bacteria, all difficult pathogens to identify notably were included in van Veen's study and not in our study. Most of the strains analyzed in our study were largely more commonly found pathogens, and the construction of the MALDI-TOF MS database may offer higher identification accuracies for these pathogens.

In conclusion, MALDI-TOF MS is a rapid, easy, relatively inexpensive and high-throughput method for identifying clinically relevant bacteria and fungi. MALDI-TOF MS offers very high accuracy, often higher than conventional methods, in identifying common microorganisms. Due to these key advantages and with continued development of MALDI-TOF MS technology and its clinical knowledgebase, MALDI-TOF MS will become a rapid, routine method for identifying pathogenic bacteria found in the conventional clinical microbiology laboratory.

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Comparison of MALDI-TOF MS, gene sequencing and the Vitek 2 for identification of seventy-three clinical isolates of enteropathogens

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Objective: This study was performed to evaluate the analytical and practical performance of the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) compared to the sequencing method and the Vitek 2 system for identification of enteropathogens in the clinical microbiology laboratory.

Methods: Ten type strains and 73 clinical isolates of enteropathogens representing eight genera were analyzed by MALDI-TOF MS. All isolates were also characterized by gene sequencing allowing interpretation of the results from MALDI-TOF MS. In addition, MALDI-TOF MS was compared with the Vitek 2 system for the identification of ten isolates of *Aeromonas* and six of *Salmonella*.

Results: As previously known, identification between *Shigella* and *Escherichia coli* is not possible to distinguish. MALDI-TOF MS produced the correct identifications for all other type strains and clinical isolates to the genus level. Fifteen *Campylobacter jejuni*, six *Campylobacter coli*, three *Plesiomonas shigelloides*, three *Yersinia enterocolitica*, two *Clostridium difficile*, one *Vibrio parahaemolyticus*, one *Vibrio fluvialis*, and one *Vibrio cholera* were all correctly identified to the species level. Genus and species identifications of ten *Aeromonas* and six *Salmonella* isolates by MALDI-TOF MS were consistent with those by the Vitek 2, but with much less cost and about ten times faster.

Conclusions: This study demonstrates that MALDI-TOF MS is a powerful tool for fast, accurate and low-cost identification of enteropathogens in the clinical microbiology laboratory.

Keywords: Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS); enteropathogens; microbial identification; clinical microbiology

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Introduction

Bacterial diarrhea is induced by a number of different pathogens, including *Salmonella*, *Escherichia coli*, *Shigelle*, *Campylobater*, *Aeromonas*, *Plesiomonas shigelloides*, *Yersinia enterocolitica*, and pathogenic *Vibrio*, etc. The microbiologic etiology of diarrhea is often not clinically obvious, thus, the rapid and sensitive identification of the enteropathogens in the clinical laboratory is essential for early and accurate diagnosis and timely therapy. For decades, enteropathogens have been routinely identified in clinical microbiology laboratories with biochemical methods and serological tests which are usually time-consuming and require large amounts of biological materials. Moreover, species identification based on biochemical test results is sometime

unreliable and gives controversial results. Molecular methods have been demonstrated to have complementary value, however, the high cost and high technical expertise required have hindered their routine uses in clinical laboratories. More recently, however, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been developed for identification of microorganisms based on the comparison of sample mass spectral fingerprints that are unique protein signatures for each microorganism to reference mass spectral fingerprints. One or a set of characteristic peaks in the mass spectral fingerprints, which appear to be conserved for a certain type of microorganism, are used for the identification. Since being commercialized in 2008, MALDI-TOF MS has been evaluated in terms of its analytical accuracy to identify clinical isolates by many diagnostic laboratories.

A couple MALDI-TOF MS systems have entered the market of microbial identification tools. It was reported that MALDI-TOF MS provided rapid and reliable identifications of medical bacteria and fungi isolated from patients (1). However, the analytical accuracy varies according to isolate species and the different MS systems, and it was suggested that unsatisfactory identifications of some strains were related mainly to the databases or known limitations of the MALDI-TOF MS technique (2). The application of MALDI-TOF MS in identification of routine isolates in clinical laboratories has been reported (3,4), but its performance for enteropathogens is not as clear. We report here the clinical evaluation of MALDI-TOF MS for identification of enteropathogens.

Methods

Bacteria and culture conditions

Salmonella enterica (ATCC14028), *Shigelle sonnei* (GIM1.239), *S. flexneri* (GIM1.238), *S. dysenteriae* (GIM1.236), *Campylobacter jejuni* (ATCC33291), *Aeromonas hydrophila* (GIM1.172), *Yersinia enterocolitica* (GIM1.265), *Vibrio parahaemolyticus* (ATCC17802), *V. fluvialis* (GIM1.488) were purchased from the Guangdong Provincial Institute of Microbial Culture Collection (China), and the *V. cholera* attenuated strain was provided by Guangzhou CDC (China). Quality control strain of *Escherichia coli* (ATCC8739) for the Vitek MS system was provided by bioMérieux (France). Seventy-three clinical isolates of enteropathogens covering seven different genera were isolated from patients with diarrheal disease between October 2012 and September 2013 in our hospital. Bacteria were recovered from –80 ℃. *Clostridium difficile* was cultured anaerobically on anaerobic blood agar at 35 ℃ for 48 h. All others were cultured on Columbia blood agar but under different conditions: *Salmonella*, *Shigelle*, *Aeromonas*, *P. shigelloides*, *V. parahaemolyticus*, *V. fluvialis*, and the *V. cholera* attenuated strain were cultured at 35 ℃ in 5% CO_2 for 24 h; *Campylobacter* strains at 42 ℃ in microaerophilic condition for 48 h; and *Y. enterocolitica* at 28 ℃ for 48 h. All culture media were purchased from Guangzhou Detgerm Microbiology Technology (China).

MALDI-TOF MS analysis

A portion of a fresh colony of the bacterium was smeared onto a 48-well target plate and then immediately covered with 1 μL of α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution (bioMérieux, France). After drying, the target plate was loaded into the matrix-assisted laser desorption ionization-time of flight mass spectrometry-based Vitek MS system (bioMérieux, France). The mass spectral fingerprint was generated and compared automatically to the Vitek MS database version 2. Both the technician's working time (preanalytical procedure to prepare samples) and the turnaround time (automated analytical procedure to obtain results) needed for identification of ten *Aeromonas* and six *Salmonella* isolates were recorded and compared to those from the Vitek 2 system.

Gene sequence analysis

The identification results of 73 clinical isolates of enteropathogens from the Vitek MS were arbitrated by gene sequencing.

Nucleic acid extraction

Nucleic acid was extracted from bacteria using the QIAamp MinElute Virus Spin kit (Qiagen, Germany) according to the manufacturer's instructions.

Polymerase chain reaction

Polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-PCR) were performed to obtain specific gene sequences. Primers (see *Table 1*) specific for conserved regions of enteropathogens were synthesized by Sangon Biotech (China) according to the literature (5-7) and used for amplification and sequencing. Conditions for PCR and RT-PCR assays were optimized to yield gene sequences.

Table 1 Primers used in this study

Strain	Target	Sequence (5' to 3')
Salmonella	*invA*	F: GTGAAATTATCGCCACGTTCGGGCAA
		R: TCATCGCACCGTCAAAGGAACC
C. jejuni	*mapA*	F: CTATTTTATTTTTGAGTGCTTGTG
		R: GCTTTATTTGCCATTTGTTTTATTA
C. coli	*ceuE*	F: ATTTGAAAATTGCTCCAACTATG
		R: TGATTTTATTATTTGTAGCAGCG
Shigelle	*ipaH*	F: CTCGGCACGTTTTAATAGTCTGG
		R: GTGGAGAGCTGAAGTTTCTCTGC
Universal	16S rDNA	F: CCAGCAGCCGCGGTAATACG
		R: ATCGGYTACCTTGTTACGACTTC

Table 2 Identification results of the type strains

Type strain	Vitek MS		Gene sequencing	
	genus ID	species ID	genus ID	species ID
Salmonella enterica	+	–	+	+
C. jejuni	+	+	+	+
A. hydrophila	+	+/–	+	+/–
Y. enterocolitica	+	+	+	+
V. parahaemolyticus	+	+	+	+
V. fluvialis	+	+	+	+
V. cholera	+	+	+	+
Shigella sonnei	+/–	–	+	+
S. dysenteriae	+/–	–	+	+/–
S. flexneri	+/–	–	+	+

ID, identification; +, positive identification; +/–, uncertain identification result; –, unidentification.

Nucleic acid amplification with region-specific primers for *Salmonella*, *Shigelle*, *C. jejuni*, *C. coli* using 2× Tag PCR Master Mix kit (Aidlab Biotechnologies, China), and amplification with 16S rDNA universal primers for *Aeromonas*, *Plesiomonas shigelloides*, *Y. enterocolitica*, *C. difficile*, *V. parahaemolyticus*, *V. fluvialis*, and *V. cholerae* by the PrimeScript™ one step RT-PCR version 2.0 Kit (Takara, Japan) were performed on a C1000 thermocycler (Bio-Rad, USA) according to the manufacturers' instructions.

Sequencing

Sequencing was completed by a commercial service (BGI tech, China) and data were subsequently analyzed using the online BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Vitek 2 analysis

Ten *Aeromonas* and six *Salmonella* isolates were identified by the Vitek 2 system (bioMérieux, France) according to the manufacturer's instructions. Both the technician's working time (preanalytical procedure to prepare samples) and the turnaround time (automated analytical procedure to obtain results) needed for bacterial identification were recorded.

Results

All strains gave gene sequencing results with ≥99% similarity to published species. The identification results of strain types and 73 clinical isolates of enteropathogens from both the Vitek MS system and the sequencing method are listed in *Tables 2* and *3*, respectively. The genus

Table 3 Identification results of 73 clinical isolates of enteropathogens

Isolates	Number of genus identified		Number of species identified	
	Gene sequencing	Vitek MS	Gene sequencing	Vitek MS
Salmonella	33	33	+/–	0
C. jejuni	14	14	14	14
C. coli	6	6	6	6
Aeromonas	10	10	+/–	+/–
P. shigelloides	3	3	3	3
Y. enterocolitica	2	2	2	2
C. difficile	2	2	2	2
S. sonnei	3	+/–	3	0

+/–, uncertain identification result.

Table 4 Species identification of *Aeromonas* isolates by the Vitek MS and Vitek 2

Vitek 2		Vitek MS	
Isolate	Probability (%)	Isolates	Probability (%)
A. hydrophila/caviae	98.29	*A. hydrophila/caviae*	99.9
A. hydrophila/caviae	99.0	*A. hydrophila/caviae*	99.9
A. hydrophila/caviae	99.0	*A. hydrophila/caviae*	99.9
A. hydrophila/caviae	99.0	*A. hydrophila/caviae*	99.9
A. hydrophila/caviae	98.29	*A. hydrophila/caviae*	99.9
A. hydrophila/caviae	98.29	*A. hydrophila/caviae*	99.9
A. hydrophila/caviae	99.0	*A. hydrophila/caviae*	99.9
A. hydrophila/caviae	98.29	*A. hydrophila/caviae; A. sobria*	50; 50
A. sobria	98.03	*A. sobria; A. veronii*	51.2; 48.7
A. sobria	94.21	*A. sobria; A. veronii*	50; 50

Table 5 The cost and time required for identification

	Vitek MS	Vitek 2
Cost (Yuan) per strain	20	56
Time (min)/16 strains	48	510

Time, both technician working time and turnaround time.

identification results of *Aeromonas* isolates and *Salmonella* isolates by the Vitek MS system were consistent with those from gene sequence analysis. *C. jejuni*, *C. coli*, *P. shigelloides*, *Y. enterocolitica*, *C. difficile*, *V. parahaemolyticus*, *V. fluvialis*, and *V. cholera* were all correctly identified to the species level by the Vitek MS system. All *Shigella* isolates were identified as *E. coli* by the Vitek MS system, which was accompanied by a comment explaining the inability to discriminate between these two genera, and the identification result of the type strain of *A. hydrophila* was reported as *A. hydrophila/caviae*.

Identification of *Aeromonas* and *Salmonella* isolates by the Vitek MS system were further compared to those of the Vitek 2 system. *Table 4* shows the species identification of ten *Aeromonas* isolates by the Vitek MS and Vitek 2 system. The identification results obtained from the Vitek MS system were essentially consistent with those from the Vitek 2 system. Furthermore, identification results of six *Salmonella* isolates from the Vitek MS system were exactly the same as those from the Vitek 2 system (data not listed).

The cost of consumables and time required for identification of ten *Aeromonas* and six *Salmonella* isolates by the Vitek MS and Vitek 2 system are listed in *Table 5*. Consumables of the Vitek MS system include matrix solution, target plates, inoculation loops and tips. For the Vitek 2 system, the consumables include gram-negative identification cards, bacterial suspension pipes, cotton swabs and salt solution. The consumables for the Vitek MS system were in total far cheaper than those for the Vitek 2 system, and Vitek MS generated results ten times faster than the Vitek 2 system in identification of 16 isolates.

Discussion

The development of MALDI-TOF MS technology has potentially revolutionized the routine identification of microorganisms in clinical microbiology laboratories by introducing a simple, rapid, high throughput, and low-cost identification technique (3). In this study, MALDI-TOF MS is showed to be satisfactional in analytical and practical performance in identification of enteropathogens. With the exception of not being able to distinguish identification between *Shigella* and *E.coli*, MALDI-TOF MS produced correct identifications for all type strains and clinical isolates of enteropathogens representing 8 genera to the genus level. Most of the strains, including *C. jejuni*, *C. coli*, *P. shigelloides*, *Y. enterocolitica*, *C. difficile*, *V. parahaemolyticus*, *V. fluvialis*, *V. cholera* were correctly identified to the species level. Some fastidious enteropathogens such as *Campylobacter* used to be diagnosed by isolation of the organism on selective medium and identified manually by biochemical tests. MALDI-TOF MS especially makes identification of *Campylobacter* and *C. difficile* easier and quicker, and no longer needs large amounts of biological material and special culture environments. In addition, MALDI-TOF MS offers a clear advantage over conventional biochemical tools such as the

Vitek 2, as it achieves similar analytical performance, but at lower cost and much shorter time for identification.

However, a few issues appeared in using MALDI-TOF MS for identification of enteropathogens. First, MALDI-TOF MS is unable to discriminate between *E. coli* and *Shigella*. The system reports a *Shigella* isolate as *E. coli*. The misidentification is due to the limited resolution of the MALDI-TOF MS in distinguishing between *E. coli* and *Shigella*, which are very closely related to each other and have almost identical mass spectrum. The discrimination of *E. coli* and *Shigella* will require additional tests depending on the characteristics of the isolates. Genomic studies have in fact indicated that *Shigella* spp. belong to the species *E. coli*, rather than forming a separate genus (8). Our 16S rDNA sequencing of *Shigella* demonstrated such similarity to *E. coli* (data not show). However, as *Shigella* causes a distinct set of disease syndromes, *Shigella* is still classified and reported separately from *E. coli*. In clinical microbiology laboratories, *Shigella* is preliminary distinguished from *E. coli* on selective agar based on special colony morphology, and then the suspected colony is further confirmed by phenotypic methods and serological tests. In general, *Shigella* is correctly identified in our routine practice.

Second, using MALDI-TOF MS for *Salmonella* typing, which is of great importance for clinical and epidemiological purposes, poses a challenge for clinical laboratories. It was reported that a larger number of reproducible peaks were required for subspecies identification, therefore, rigorous control of the sample preparation and optimization of testing parameters was critical for strain typing with MALDI-TOF MS but not practical in clinical laboratories (9). Currently, serological tests are still needed for *Salmonella* spp. typing. Third, although a study from Donohue MJ indicated that the mass spectral data contained sufficient information to discriminate between genera, species, and strains (10), MALDI-TOF MS sometimes produced uncertain species identification results within the *Aeromonas* genus. Updates to the databases may solve the problem with this species. In addition, MALDI-TOF MS cannot presently discriminate between *A. hydrophila* and *A. caviae*. This problem has been temporarily settled by identifying strains as *A. hydrophila/caviae*. The solution is satisfactory in the routine practice since definitive identification does not normally impact clinical management. Further defining species identification of *Aeromonas* and other species are hoped to be achieved with continued database development.

Conclusions

Gene sequence analysis is the "gold standard" for bacterial identification and classification while the Vitek 2 system is a popular commercial method commonly used in clinical microbiology laboratories for bacterial identification. In this study, we evaluate the analytical and practical performance of MALDI-TOF MS for identification of enteropathogens in our clinical microbiology laboratory. MALDI-TOF MS is showed to be a simple, rapid, accurate, and low-cost tool for identification of enteropathogens.

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The value of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in identifying clinically relevant bacteria: a comparison with automated microbiology system

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Background: Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been developed as a new-type soft ionization mass spectrometry in the recent year. Increasing number of clinical microbiological laboratories consider it as an innovate approach for bacterial identification.

Methods: A total of 876 clinical strains, comprising 52 species in 27 genus, were obtained from Fudan University Affiliated Zhongshan Hospital. We compared the identification accuracy of the Vitek MS system (bioMerieux, Marcy l'Etoile) to other conventional methods for bacterial identification. *16S rRNA* gene sequencing was performed as a reference identification method in cases of discrepant results.

Results: The Vitek MS system consistently produced accurate results within minutes of loading, while conventional methods required several hours to produce identification results. Among the 876 isolates, the overall performance of Vitek MS was significantly better than the conventional method both for correct species identification (830, 94.7% *vs.* 746, 85.2%, respectively, P=0.000).

Conclusions: Compared to traditional identification methods, MALDI-TOF MS is a rapid, accurate and economical technique to enhance the clinical value of microorganism identification.

Keywords: Mass spectrometry; bacteria; identification

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Introduction

Rapid and accurate identification of pathogens is an important function of the clinical microbiology laboratory, particularly as it relates to choosing effective antibiotic therapies, reducing costs and shortening lengths of hospital stays. However, current bacterial identification methods are that are generally based on conventional phenotypic and biochemical methods usually require a significant time commitment ranging from four to twelve hours, in addition to higher consumable costs. Molecular methods are demonstrated to have complementary a value in bacterial identification, but not practical for routine use due to their associated complicated procedures and high cost levels.

As a result, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), a new-type soft ionization mass spectrometry method developed in the past several years, has been introduced for use by an increasing number of microbiology laboratories in multiple areas that include medical diagnostics, food quality control and environmental monitoring (1).

Materials and methods

Strains

We studied a collection of 876 non-repetitive isolates recovered from clinical sources including blood, urine, pus, sterile body fluid, and respiratory specimens received by our institution from July 2012 to March 2013. Among

those isolates, there were 270 enterobacteriaceae, 134 non-fermentative bacteria, 92 enterococci, 298 staphylococci, among which 217 isolates obtained from blood were coagulase negative staphylococci, 81 *staphylococcus* aureus, 70 *streptococcus* and 12 other bacteria.

Methods

Isolates were identified both by MALDI-TOF MS and conventional methods. In our routine workflow, an automated microbiology system was applied to identify the Gram-positive bacteria (BD PhoenixPID, panel448505), Gram-negative bacteria (BDPhoenixNID, panel448911) and streptococcus (BD PhoenixSID, panel448505). In cases where no identification result was achieved, the procedure was repeated to obtain a result. For *salmonella*, *Shigella spp*. and *legionella spp*., we chose serum plate agglutination, whereas for haemophilus influenza, the identification depended on the growing states in the plate added factor V and factor X. In cases where microorganisms could not initially be identified by MALDI-TOF MS due to reasons such as no presence of unique reference species, low mass spectra resolution, or simply no identification results, the samples were retested. When MALDI-TOF MS and the conventional method produced the same identification to the species level, the identification was considered final. In cases of discrepant results, partial *16S rRNA* gene sequencing was implemented to obtain final identification.

Devices

The following devices were applied in our research: (I) Vitek MS with IVD database V2.0 (BioMerieux, Marcy l'Etoile, France); (II) Phoenix System for bacterial identification and susceptibility (BD Company, Franklin Lakes, USA); and (III) Polymerase Chain Reaction Cycler (Applied Biosystems, Foster City, USA).

Reagents

The samples were cultured using a variety of medium types that are traditionally used in our practice, including blood plates (BioMerieux, Marcy l'Etoile, France), bacteria identification strips PMIC/ID, NMIC/ID and SMIC/ID (BD, Franklin Lakes, USA), Vitek MS-DS target plates and Vitek MS-CHCA matrix solution(BioMerieux, Marcy l'Etoile, France), 16s RNA primer (Sangon Biotech, China), Taq enzyme and PCR relevant reagents (Thermo Fisher Scientific, Waltham, USA).

Biochemical Identification

The Phoenix System for bacterial identification was used in accordance with the standard procedure recommended by the manufacturer.

MS identification

Pre-analytic preparation of samples was performed by using a sterile tip to pick bacterial colony isolates freshly grown on defined agar medium and then smearing a thin film onto a ground steel MALDI target plate. The dried microbial film was then overlaid with 1.0 μL α-cyano-4-hydroxycinnamic acid MALDI-TOF CHCA matrix. Then the sample-matrix mixture was dried at room temperature and subsequently inserted into the Vitek MS system for data acquisition. Quality controls were internally calibrated by using *Escherichia coli* ATCC8739 supplied by BioMerieux, which followed the same procedure aforementioned, with the exception of adding the clolnies to the calibration spot only. The data were processed automatically by the instrument software and the spectra were compared with reference libraries for bacterial identification matching.

16S rRNA identification

16S rRNA PCR and sequencing were performed after heat extraction of bacterial DNA. Polymerase chain reaction (PCR) was operated according to instructions by Hwang SM, *et al.*, 2011 (2), which included the amplification primer, reaction conditions and system. Then the PCR amplification products were delivered to the Liuhe Bejing Genomics Institute of Science and Technology Co., Ltd for sequencing, where gene sequences were compared using the BLAST website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistic methods

Categorical variables were compared using Pearson's chi-square test of Fisher's exact chi-square test. Statistical significance was defined as ≤ 0.05 in a two-tailed test. All statistical analyses were performed using the SPSS 17.0 package.

Results

The 876 isolates contained 27 genus and 52 species. Using the BD Phoenix automated microbiology system, 746 isolates (85.2%) were correctly identified to the species level and 853 isolates (97.3%) to the genus level. In comparison, the Vitek MS system correctly identified 830 isolates (94.7%) to the species level and 866 isolates (98.9%) to the genus level (*Table 1*).

Of the 270 *Enterobacteriaceae* isolates, there was a significant difference (P=0.019) between the BD Phoenix automated system and the Vitek MS system for bacterial identification to the species level. The Phoenix system identified 258 isolates to the species level, whereas the Vitek MS system identified 243 isolates to the species level. A total of 27 isolates, accounting for 10% of *Enterobacteriaceae* isolates, could not be identified to the species level. A total of 16 isolates could not be distinguished between *Enterobacter asburiae* and *Enterobacter cloacae*, four *Shigella* isolates were misjudged to be *Escherichia coli*, four *Salmonella* isolates could not be resolved to the species level, and the remaining two *Enterobacter aerogenes* isolates were misidentified as *Enterobacter cloacae*. In the contrary, there was no significant difference identifying non fermentative bacilli when comparing the two species-level identification methods (P=0.769). Vitek MS correctly identified 129 nonfermentative bacilli compared to 127 by BD Phoenix. The misidentified results produced by Vitek MS included one *Burkholderia cepacia*, whose reference identification also consists of *Burkholderia vietnamiensis*, two *Achromobacter xylosoxidans*, which could only be identified to genus rather than species level, and one *ochrobactrum anthropi* which generated no result.

The VitekMS system proved far superior at identifying *Staphylococcus* species, correctly identifying 212 isolates (97.7%) out of 217 *Coagulase negative staphylococcus* compared to only 165 isolates (76.0%) by the Phoenix automatic microbiology system (P=0.000). Five isolates, four *Staphylococcus epidermidis* and one *Staphylococcus hominis*, failed to be identified accurately by Vitek MS. The Phoenix automatic microbiology system didn't yield species-level identification for a total of 52 isolates, including 30 *Staphylococcus epidermidis*, 12 *Staphylococcus hominis*, three *Staphylococcus haemolyticus*, one *Staphylococcus capitis*, two *Staphylococcus warneri*, one *Staphylococcus lugdunensis* and three *Staphylococcus cohnii*. In addition, two *Staphylococcus aureus* isolates were misidentified as *Coagulase negative staphylococcus*.

For both *Enterococcus and Streptococcus* species, there were also significant differences comparing the Phoenix system to the Vitek MS system, (P value =0.000, P value =0.007, respectively). A total of 92 *Enterococcus* isolates were successfully identified to the species level using Vitek MS, whereas the Phoenix system yielded 26 (28.3%) misidentifications. In addition, 22 *Streptococcus* isolates failed species-level identification using the Phoenix system, compared to 8 isolates using Vitek MS. Further details are shown in *Table 2*.

In addition, we summarized the strains which failed to be identified by Vitek MS for detail in *Table 3*. A total of 46 isolates were not in accordance with reference identification methods, among which 39 isolates (84.8%) were identified incorrectly to the species level but correctly to the genus level which we defined as minor error, and 7 isolates (15.2%) were identified incorrectly to the genus level which was defined as major error.

Discussion

Until recently, conventional methods for bacterial identification based primarily on biochemical and phenotypic techniques have prevailed in the clinical microbiological laboratory. All manual and many automated methods are time-consuming, and often require complex procedures and large amounts of biological material, which is particularly difficult to achieve for fastidious microorganisms with atypical biochemical characteristics (3). Molecular methods, including *16S rRNA* gene sequencing, have been demonstrated to have complementary value, but they are not practical for routine use due to their high cost and high burden on laboratory technicians. MALDI-TOF MS, a technology which is based on generating characteristic mass spectral fingerprints unique for each microorganism, has been proven as a more accurate and direct method for bacterial identification when comparing with conventional methods.

In our study, we focused on comparing the identification efficiencies for clinically common bacteria using the Vitek MS system and one conventional biochemical method offered in the BD Phoenix system.

Four MS target plates each containing 48 sample spots can simultaneously be loaded into the Vitek MS system for bacterial identification, achieving an identification result for each sample within an average of one to two minutes per sample. In comparison, the Phoenix system generally takes several hours to achieve identification results. Additionally, the Vitek MS system operates at much lower cost per sample volume compared to conventional methods.

Since MALDI-TOF MS was introduced to the clinical

Table 1 The comparison of identification results of bacterial isolates using Vitek MS and automated microbiology system

	No. of isolates	NO. of isolates with the indicated results using automated microbiology system		NO. of isolates with the indicated results using MALDI-TOF MS	
		Identification to the level of species	Identification to the level of genus	Identification to the level of species	Identification to the level of genus
Enterobacteriaceae	270	258	264	243	265
Escherichia coli	154	153	153	154	154
Klebsiella Pneumoniae	52	51	51	52	52
Enterobacter cloacae	16	15	16	0	16
Proteus mirabilis	9	9	9	9	9
Enterobacter aerogenes	6	6	6	4	6
Morganella morganii	7	7	7	7	7
Klebsiella oxytoca	2	2	2	2	2
Serratia marcescens	11	9	9	11	11
Serratia rubidaea	1	1	1	1	1
Citrobacter koseri	1	0	0	1	1
Citrobacter freundii	1	0	1	1	1
Enterobacter gergoviae	1	1	1	1	1
Shigella	5	2	5	0	0
Salmonella	4	2	3	0	4
Nonfermentative Bacilli	134	127	130	129	132
Acinetobacter baumannii	58	58	58	58	58
Pseudomonas aeruginosa	51	49	50	51	51
Stenotrophomonas maltophilia	6	5	5	6	6
Burkholderia cepacia	7	6	6	5	6
Pseudomonas alcaligenes	2	2	2	2	2
Acinetobacter junii	2	0	2	2	2
Elizabethkingia meningoseptica	2	2	2	2	2
Achromobacter xylosoxidans	2	2	2	0	2
Sphingomonas paucimobilis	1	1	1	1	1
Burkholderia multivorans	1	0	0	1	1
Chromobacterium indologenes	1	1	1	1	1
Ochrobactrum anthropi	1	1	1	0	0
Staphylococcus	298	244	295	293	296
Staphylococcus aureus	81	79	81	81	81
Staphylococcus epidermidis	101	71	98	97	99
Staphylococcus hominis	27	15	27	26	27
Staphylococcus haemolyticus	38	35	38	38	38
Staphylococcus capitis	30	29	30	30	30
Staphylococcus warneri	9	7	9	9	9
Staphylococcus caprae	3	3	3	3	3
Staphylococcus sciuri	3	3	3	3	3
Staphylococcus cohnii	5	2	5	5	5
Staphylococcus lugdunensis	1	0	1	1	1

Table 1 (*continued*)

Table 1 (*continued*)

	No. of isolates	NO. of isolates with the indicated results using automated microbiology system		NO. of isolates with the indicated results using MALDI-TOF MS	
		Identification to the level of species	Identification to the level of genus	Identification to the level of species	Identification to the level of genus
Enterococcus	92	66	91	92	92
Enterococcus faecalis	60	52	60	60	60
Enterococcus faecium	31	14	31	31	31
Enterococcus casselifavus	1	0	0	1	1
Streptococcus	70	48	68	62	69
Streptococcus agalactiae	6	6	6	6	6
Streptococcus anginosus	15	10	15	14	15
Streptococcus intermadius	2	1	2	2	2
Streptococcus constellatus	5	4	5	4	5
Streptococcus dysgalactiae	21	10	20	17	21
Streptococcus pyogenes	10	8	10	9	10
Streptococcus pneumoniae	11	9	10	10	10
Other bacterium	12	3	5	11	12
Aerococcus viridans	1	0	0	1	1
Micrococcus luteus	1	0	0	1	1
Corynebacterium aurimucosum	1	0	0	1	1
Microbacterium aurum	1	0	0	0	1
Corynebacterium jeikeium	5	3	4	5	5
Listeria monocytogenes	3	0	1	3	3
Total (%)	876	746 (85.2)	853 (97.3)	830 (94.7)	866 (98.9)

Table 2 Comparison of Phoenix and Vitek MS in identifying 864 isolates

	No. of isolates	No. of isolates identified to species level by			No. of isolates identified to: genus level by		
		Automated microbiology system	MS	P	Automated microbiology system	MS	P
Enterobacteriaceae spp.	270	258	243	0.019	264	265	1.000
Nonfermentative Bacilli	134	127	129	0.769	130	132	0.684
Staphylococcus spp.	298	244	293	0.000	295	296	1.000
Staphylococcus aureus	81	79	81	0.497	81	81	–
Coagulase negative staphylococcus (CoNS)	217	165	212	0.000	214	213	1.000
Enterococcus spp.	92	66	92	0.000	91	92	1.000
Streptococcus spp.	70	48	62	0.007	68	69	1.000
Total	864	743	819	0.000	848	854	0.236

Table 3 Isolates misidentified or unidentified by Vitek MS

Reference ID*	No.	Vitek MS	No.	Identification parameter	
				Minor error (%)	Major error (%)
Enterobacter cloacae	16	*Enterobacter cloacae/Enterobacter asburiae*	16	16	0
Enterobacter aerogenes	2	*Enterobacter cloacae/Enterobacter asburiae*	2	2	0
Shigella ssp	5	*Escherichia coli*	5	0	5
Salmonella typhi	2	*Salmonella group*	4	4	0
Salmonella enteritidis	1				
Salmonella choleraesuis	1				
Burkholderia cepacia	2	*Burkholderia cepacia/Burkholderia vietnamiensis*	2	2	0
Achromobacter denitrificans	2	*Achromobacter denitrificans Achromobacter xylosoxidans*	2	2	0
Ochrobactrum anthropi	1	*No identification*	1	0	1
Staphylococcus epidermidis	4	*Staphylococcus hominis*	2	2	0
		Staphylococcus capitis	1	1	0
		Staphylococcus haemolyticus	1	1	0
Staphylococcus hominis	1	*Staphylococcus epidermidis*	1	1	0
Streptococcus anginosus	1	*Streptococcus dysgalactiae*	1	1	0
Streptococcus constellatus	1	*Streptococcus anginosus*	1	1	0
Streptococcus dysgalactiae	4	*Streptococcus anginosus*	2	2	0
		Streptococcus pyogenes	2	2	0
Streptococcus pyogenes	1	*Streptococcus dysgalactiae*	1	1	0
Streptococcus pneumoniae	1	*no identification*	1	0	1
Microbacterium aurum	1	*Microbacterium flavescens*	1	1	0
Total	46		46	39 (84.8)	7 (15.2)

*, the identification result identified by *16S rRNA* gene sequencing is regarded as a reference ID for almost all the bacteria except for salmonella and Shigella, both of which refer to serum agglutination test.

microbiology laboratory, an abundant collection of literature (4,5) has highlighted many benefits that result from the use of this technology. In addition, the capability of MALDI-TOF MS to accurately identify bacteria to both the genus and species levels favors its potential application in almost all gram-positive bacteria and gram-negative bacteria in clinical practice (6,7). In our research, 94.7% isolates were correctly identified to the species level by MALDI-TOF MS. Our findings were consistent with a similar 92% correct identification ratio in 980 isolates studied by van Veen, *et al.* (8).

MS was remarkably better than the automated microbiology system in the identification of *Staphylococcus spp*. (P=0.000), especially for Coagulase negative staphylococci (CoNS), achieving 76.0% and 97.7% correct identification rates, respectively. CoNS strains were correctly identified (P=0.000) as similarly reported by of Dubois *et al.* (7). However, literatures estimating the value of Phoenix for identifying *Staphylococcus aureus* are still not in great number and differ significantly. Fahr *et al.* (9) had ever compared the Phoenix, Vitek MS and API ID32 Staph systems at the same time, which reached a concordance rate of 97.1%. On the contrary, Layer *et al.* (10) reported 27 reference isolates of *Staphylococcus aureus* for identification by MS. 18 isolates (66.67%) were correctly identified with a concordance rate of 66.67%, compared with a 76.19% concordance rate of the identification results for Staphylococcus epidermidis from clinical resource, which was in agreement with our finding. In Heikens's study of 47 CoNS isolates (11), 17 were misidentified and 2 failed to be identified, which showed a lower concordance rate.

The CoNS strains obtained in our study were all isolated from blood so it was particularly important to get accurate identification due the severity in clinical practice. Currently, CoNS strains are the most frequently detected strains in positive blood cultures, and there is no gold standard to

determine whether CoNS isolated from blood is pathogenic or contaminated. The U.S. Center for Disease Control and Prevention favors that it might present blood infection caused by CoNS if same strains were isolated from multiple bottles. Therefore, rapid and accurate methods able to identify CoNS to the species level can minimize unnecessary antibiotic regimens and reduce costs.

The occurrence of drug resistance of *Enterococcus faecalis* and *Enterococcus faecium* are rather different because the latter has a much higher drug resistance rate. As a result, correct identification to the species level plays a dominant role in monitoring therapy. Results from our study show that all the *Enterococcus spp.* were correctly identified by Vitek MS while only 17 (54.8%) isolates were correctly identified by the Phoenix system. For *Enterococcus faecalis* and *Enterococcus faecium*, using Vitek MS clearly offers a significant impact on the success of empiric antibiotic therapy and accuracy of statistical monitoring of bacterial resistance as compared to conventional methods.

In the context of difficult identification results of *Streptococcus spp.* with conventional methods, Phoenix has designed a dedicated plate to identify almost 30 strains of *Streptococcus*. Compared with 88.6% isolates of species-level identified by Vitek MS, the Phoenix system identified 68.6% in our study. Owing to its slow growing and small colonies, biochemical methods usually require more mature colonies, which subsequently leads to more time culturing purified colonies. However, a big advantage of Vitek MS, only one to two colonies are enough to do identification using Vitek MS, which allows it to offer accurate results to the clinician with a relatively rapid turn around time.

For the species-level identification of *Enterobacter cloacae* and *Enterobacter asburiae*, MALDI-TOF MS proved no real advantage over conventional methods, as also reported by others (12). Moreover, *Shigella*, belonging to *Escherichia coli* in genetics, has special virulence to humans (13). Thus, to avoid erroneous judgments, specimens of suspected *Shigella* and *Salmonella* infections, particularly from feces, need to be implemented by traditional biochemical methods and serum agglutination tests to obtain finalized results.

In addition to bacteria routinely found in our laboratory, we also identified some unusual ones which were not shown in *Table 1*, including *Haemophilus influenza*, *Legionella pneumophila*, *Clostridium difficile*, *Bacteroides fragilis*, and *Bacillus perfringens*. Among these isolates, the identification results of six *Haemophilus influenza* by Vitek MS accorded with the results of culture added in X and V factors. Several *Clostridium difficile* isolates and two *Legionella pneumophila* isolates identified by Vitek MS were all in agreement with results from standard methods. Therefore, we believe that Vitek MS might also be an effective identification method for some unusual bacteria. There are limitations of using Vitek MS, however. Mucoid bacteria, such as *Streptococcus pneumoniae* frequently failed to be identified. The most plausible reason for failed identifications for these species is the fact that it is quite easy to smear too much sample on the target spot.

In conclusion, the Vitek MS system represents a very powerful, high-throughput microbial identification technology that is efficient, rapid, relatively cheap, and easy to use. Compared with conventional methods, it can reduce workload and significantly shorten turn around times before delivering the formal reports to the clinicaians.

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Necrotizing pneumonia and empyema caused by *Neisseria flavescens* infection

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Abstract: *Neisseria flavescens* is an uncommon pathogen of human infection, pneumonia and empyema caused by *N. flavescens* is rarely reported. Herein, we report a 56-year-old diabetic patient presenting necrotising pneumonia and empyema due to *N. flavescens* infection. The main clinical manifestation of this patient was high fever, sticky pus and gradually aggravating dyspnea. The chest computed tomography (CT) scan showed there are mass of high density areas around hilus of the left lung, hollow sign with inflammation also appeared. A biopsy specimen was taken from the left principal bronchus by lung puncture biopsy and showed necrosis and inflammation. Microscopic examination of direct smear and culture of sticky pus, much more gram-negative diplococcus was present, pathogen was further identified by Vitek NH card, Vitek MS and confirmed as *N. flavescens* by 16S rRNA gene sequencing finally. Anti-infection therapy following the antimicrobial susceptibility test results was effectively. To our knowledge, this is the first report of pulmonary infection caused by *N. flavescens*.

Keywords: *Neisseria flavescens*; pneumonia; empyema; MALDI-TOF MS; 16S rRNA gene sequencing

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Introduction

Neisseria spp. are part of the commensal flora of mucosal membranes of humans and some animals, and are generally considered non-pathogenic except for *N. gonorrhoea* and *N. meningitidis*. *N. flavescens* often be found in the upper respiratory tract and the oropharynx of humans, and are rarely associated with infectious processes (1). However, when patients in special or immunocompromised conditions, *N. flavescens* can be isolated from blood or cerebrospinal fluid (CSF) occasionally (2-8), but never been isolated from lower respiratory tract.

Herein, we reported a case of a 58-year-old diabetic patient with fatal necrotising pneumonia and empyema due to *N. flavescens* infection. To our knowledge, this is the first report that *N. flavescens* as the pathogen of severe low respiratory tract infection.

Case report

A 58-year-old man was admitted to the hospital because of necrotizing pneumonia and empyema in October 2013. He had experienced nausea, vomiting and little cough ten days before admission, after anti-infection therapy with some cephalosporin in local clinic, the symptoms once getting better, but two days before admission, the patient felt anhelation and dyspnea, then presented to the emergency department of our hospital, non symptomatic remission after dealing with cefodizime and methylprednisolone through intravenous injection temporary, then transferred to the

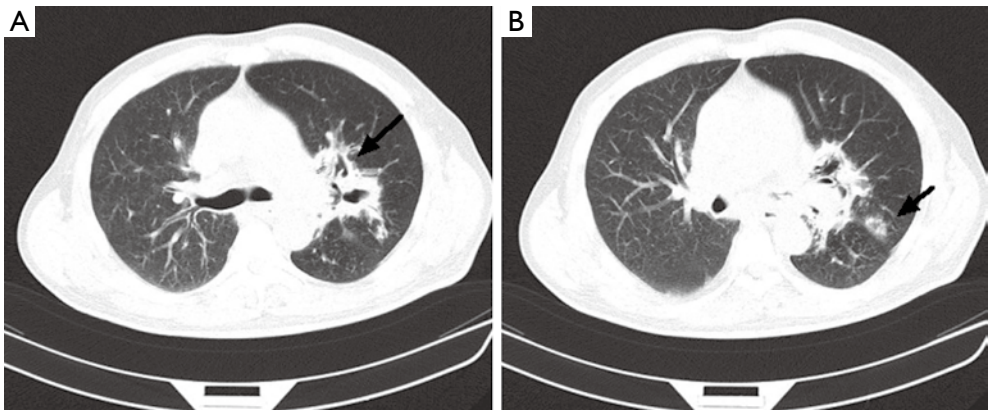

Figure 1 CT scan of the chest. (A) High-density shadow around the hilus of left lung (black arrow); (B) It appears that there is a hollow sign (black arrow) in the peripheral pulmonary. CT, computed tomography.

department of respiration with symptoms of high grade fever (highest temperature is 39.9 ℃/103.82 F), chilling and severe cough with productive of yellow sputum finally.

He has hypertension for four years and controlled well. Four year history of type 2 diabetes and treated with melbine (DMBG) as well as Glipizide, but curative effect is not ideal for fasting blood-glucose more than 10 mmol/L. He also has a smoking history of 20 cigarettes per day for 40 years.

A chest computed tomogram (CT) showed high-density shadow around the hilus of left lung (*Figure 1A* as signed by black arrow), a hollow sign (*Figure 1B* as signed by black arrow) also exists in the left peripheral pulmonary. Initial laboratory tests showed the white blood cell (WBC) count was 36.04×10^9/L (reference level, 4.0×10^9-10.0×10^9/L), the neutrophil cell count and ratio was 33.3×10^9/L (92.4%), the erythrocyte sedimentation rate (ESR) was 115 mm/H, the C-reactive protein (CRP) was 54.1 mg/L (reference level, <5 mg/L).

A transthoracic pulmonary fine-needle aspiration was performed when transferred to the department of respiration. Approximately 2 mL of purulent secretion was obtained and sent for microbiology tests. Direct smear Gram stain was performed and gram-negative diplococci and lots of polymorphonuclear leukocytes can be observed under microscope (*Figure 2A*), acid fast stain was also done and got negative results. The same material was inoculated onto chocolate agar and 5% sheep blood agar (bioMérieux, Shanghai, China). The agar media were incubated at 35 ℃ for 48 h, middle size, bluish grey round opaque colonies were observed. Gram-stain of the pure culture colony was also gram negative cocci. Elementary biochemical properties of this strain were oxidase positive, catalase positive while deoxyribonuclease (DNAse) was negative. The organism was identified with Vitek NH card and Vitek MS successively, but inconsistent results were got, Vitek NH (Ref. V1308 database) identified as *N. flavescens* (99% probability) while Vitek MS (Ref. V2.0 database) identified as *N. subflava* (89.70% probability). Finally, we confirmed this identification as *N. flavescens* (99% probability) by 16S rRNA gene sequencing.

In vitro susceptibility test with agar dilution method was done following the method mentioned in CLSI M45 for *Moraxella catarrhalis*. It is susceptible to penicillin, ampicillin/sulbactam, amikacin, ceftazidime, ciprofloxacin, Trimethoprim-sulfamethoxazole and piperacillin-tazobatam. After one week anti-infection therapy combined piperacillin-tazobatam and Trimethoprim-sulfamethoxazole, the gram negative diplococcic was almost disappeared (*Figure 2B*). But the empyema was not released because of the inflamation and necrosis of cartilagines tracheales (*Figure 3A*,*B*). Necrosis of cartilagines tracheales lead to tracheal collapse and purulent secretion drainage very uneffective. Finally, the patient was got well after tracheal scaffold implantation and further anti-infective therapy for three weeks.

Discussion

Neisseria is a large genus of commensal bacteria that inhibit mucous membrane surfaces of warm-blooded hosts. There are 11 species that colonize humans include *N. gonorrhoeae*, *N. meningitides*, *N. lactamica*, *N. flavescens*, *N. sicca*, *N. subflava*, *N. mucosa*, *N. cinerea*, *N. elongata*, *N. glycolytica and N. nitroreducens.* Most of these *Neisseria* species are normal inhabitants of the upper respiratory tract and are not considered pathogens (1,9). Up to date, only *N. meningitides*, *N. gonorrhoeae*, *N. mucosa* and *N. sicca* have been reported as causative agents of pneumonia,

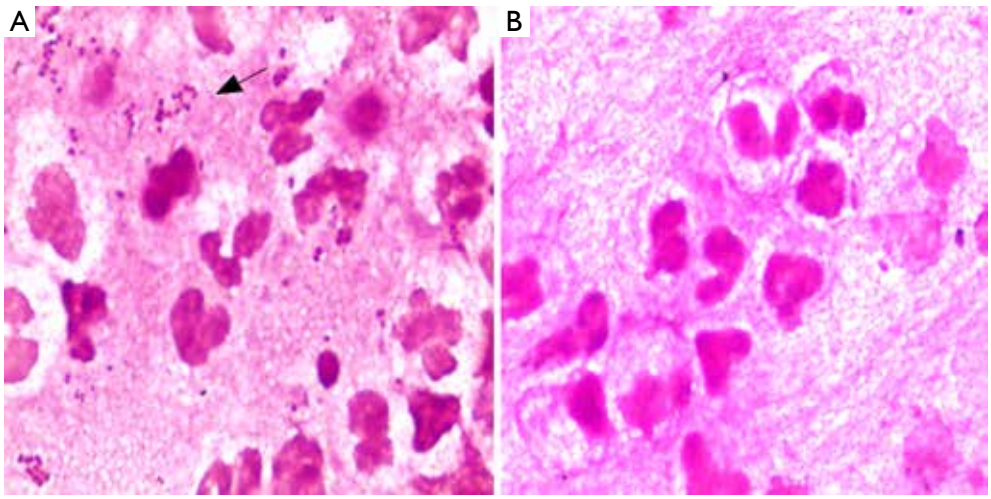

Figure 2 Direct smear Gram stain before and after anti-infection. (A) Direct smear Gram stain of pyogenic fluids before treatment. There are lots of gram-negative diplococcus as well as pyocyte infiltration; (B) Direct smear Gram stain of pyogenic fluids after effective treatment, the diplococcus disappeared. (Gram stain,1,000×).

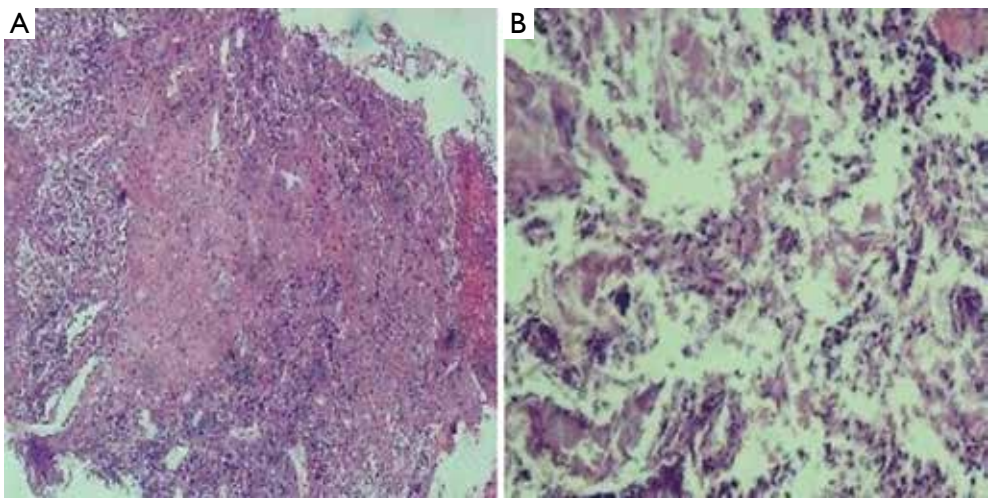

Figure 3 Biospy of principal bronchus mucous membrane. (A) Biospy of distal end of left principal bronchus mucous membrane with deeply acidophilic and fibrinoid necrosis; (B) Biospy of distal end of left principal bronchus cartilage with deeply acidophilic, fibrinoid necrosis and exudation. (H&E stain, 200×).

empyema, bronchopneumonia or bronchiectasis (10-17). Necrotizing pneumonia with empyema caused by *N. flavescens* is the first time reported as we known. Besides as causative agent of pneumonia and empyema *N. flavescens* have else been published as pathogens of septicaemia, meningitis and endocarditis (4-8,18-20).

The clinical symptom and lab tests properties of this case are high fever rate, empyema, elevated WBC, increased CRP value and distinctive imaging changes, all these often lead to a fatal infection as reported infection caused by *N. flavescens* in the other systems (4,6,7,19). We reviewed the literatures and analysed the possible reason may be included the following issues: *N. flavescens* is among the commensal flora of human upper respiratory tract, seldom cause human infection. Most of *N. flavescens* infected patients have severe basic diseases, for example, immunodeficiency and diabetes (2); There are remote causes like dental surgery history, vomiting, chemotherapy and co-infection with HIV or pseudomonas aeruginosa (18,21); Initial experienced clinical application of penicillin and cefixime often failed to cure

Table 1 Supplemental tests which permit differentiation among common gram negative diplococcus (GND)

GND	Oxidase test	Catalase reaction	DNAse test	Nitrate reduction	Acid from				Colistin susceptibility
					G	M	L	S	
N. flavescens	+	Weak	–	–	–	–	–	–	S
N. gonorrhoeae	+	Srong	–	–	+	–	–	–	R
N. meningitides	+	Strong	–	–	+	+	–	–	R
M. catarrhalis	+	Variable	+	+	–	–	–	–	R

Abbreviations: +, most strains positive; –, most strains negative; R, strains grow well on selective medium for *N. gonorrhoeae* and/or show no inhibition around a colistin disk (ten micrograms); acid from G (glucose), M (maltose), L (lactose), S (sucrose).

the *N. flavescens* infection for beta-lactamase producing and *penA* resistant gene expression (5,22-30); severe virulence and inflammatory response caused by lipooligosaccharide of Neisseria lead to septic shock and fibrinoid necrosis and exudation (31). In conclusion, we should pay more attention to human infection caused by *N. flavescens*.

Due to *N. flavescens* may cause severe infection, rapid and accurate identify this organism is more important. As described in this paper, Vitek NH card can be used for accurate identification, but Vitek MS V2.0 database doesn't include *N. flavescens* and should be developed in the future. Among the gram negative diplococcus often cause pulmonary infection, *N. flavescens* can be differentiated from *Moraxella catarrhalis* with DNAse test, differentiated from *N. gonorrhoeae* and *N. meningitides* with rapid acid detection tests and Colistin-susceptible test as summarized in *Table 1*.

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Medical partnerships for improved patients' outcomes—are they working?

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Necessary medical partnerships

To improve patients' outcomes, ICC believes that there must be effective partnerships between the four key medical groups whose efforts are needed to benefit patients (*Figure 1*). The first group includes patient organizations. Patients should let people know what they need, and they need to be represented by patient organizations and patient advocates when decisions are made that affect patients. We should remember that the main reason that medical companies and organizations exist is to improve the welfare of patients. It is a point that is sometimes neglected.

The second group in the patient partnership is health care professionals. They are the ones who can provide care for patients, and their partnership and commitment to patient health is essential. The third group—suppliers—includes the medicine, device, and health care management companies. They work to improve patients' lives by providing the resources they need. Finally, governments and their health ministries, which are responsible for the health of patients and oversee the health care delivery systems in their countries, must be supportive partners for patients.

ICC believes that establishing successful partnerships with each of these groups is a priority for every country's COPD patient organizations, and we believe that each of the medical groups should demonstrate an active commitment to patients and their outcomes. This commitment should not be dominated by the financial interests of these groups in the care of patients.

The most important priority for patient organizations is to promote the best possible care for patients and to call attention when appropriate care is not being provided and attempt to improve it. To assess the success of respiratory patient groups and their partners in health care, ICC surveyed its 125 member organizations and asked how well the health care professionals who cared for COPD patients in their countries were doing in providing needed care. *Figure 2* shows a luke-warm response to this question worldwide. According to COPD patient group leaders and patients worldwide, physician carers for COPD patients are doing an average job, neither good nor bad.

We also asked the ICC member organizations about how well they thought their national health ministries were doing in providing for COPD care in their countries. The response (*Figure 3*) was similar to the responses for physician care: there is room for considerable improvement in the opinion of patient organizations. In the case of the efforts of health ministries on behalf of patients, the rating in developing countries was significantly more negative than in developed countries (*Figure 4*). It appears that the global relationship between respiratory patients and their provider and governmental partners in health care is not perceived as satisfactory.

ICC believes that when a COPD patient needs care, governments should have a system by which they can receive it. Suppliers should work to make their drugs, devices, and services available to those who need them at a price they can afford. Finally, health care professionals should make sure that health care is, in fact, available to COPD patients who need it. When physicians lose their sense of responsibility toward their patients, health care is bound to be bad.

Patients or profits?

A great battle that concerns all patients is occurring

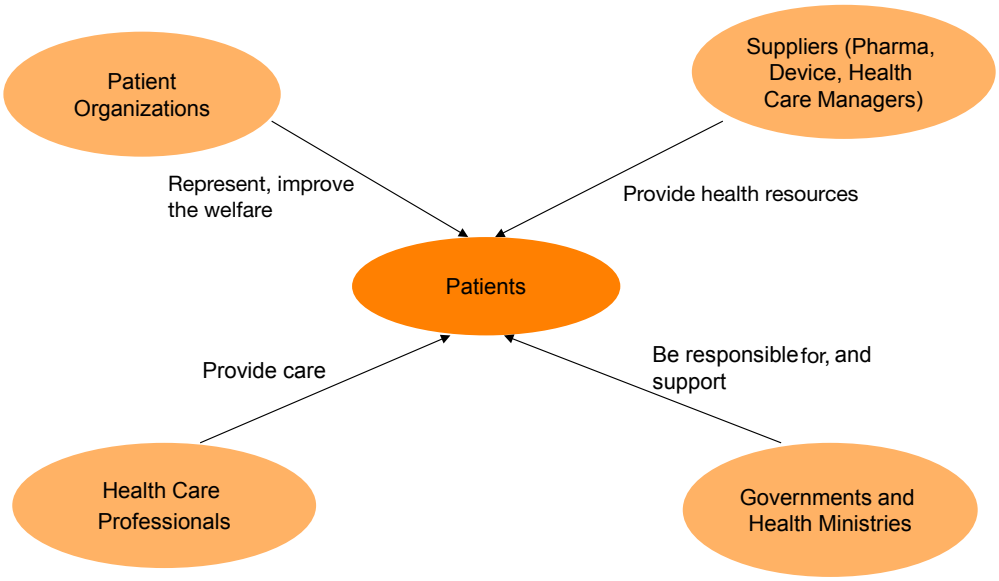

Figure 1 Partnership of four key respiratory organizations.

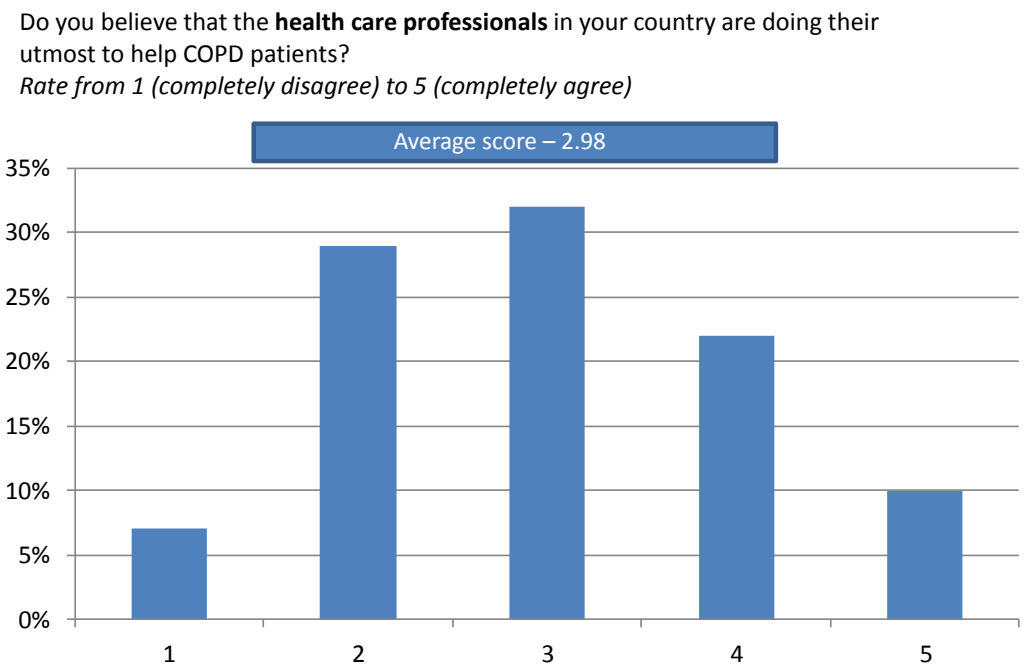

Figure 2 Rating of HCPs.

globally in medicine, and it dramatically affects the relationship of patients and their advocacy groups with their health care partners. At issue is whether or not basic health care is a human right. Obviously, ICC believes that it is, as do most of the populations of both developed and developing countries of the world, which are working to achieve universal health care. Because of limited resources and, in some cases, lack of concern about patients, many countries have not yet achieved this goal.

Opposing the desire for universal health care is the global rise of for-profit health care providers and insurers for whom profit is the primary goal and patient benefit and patient outcomes are a secondary or even a non-existent concern (1).

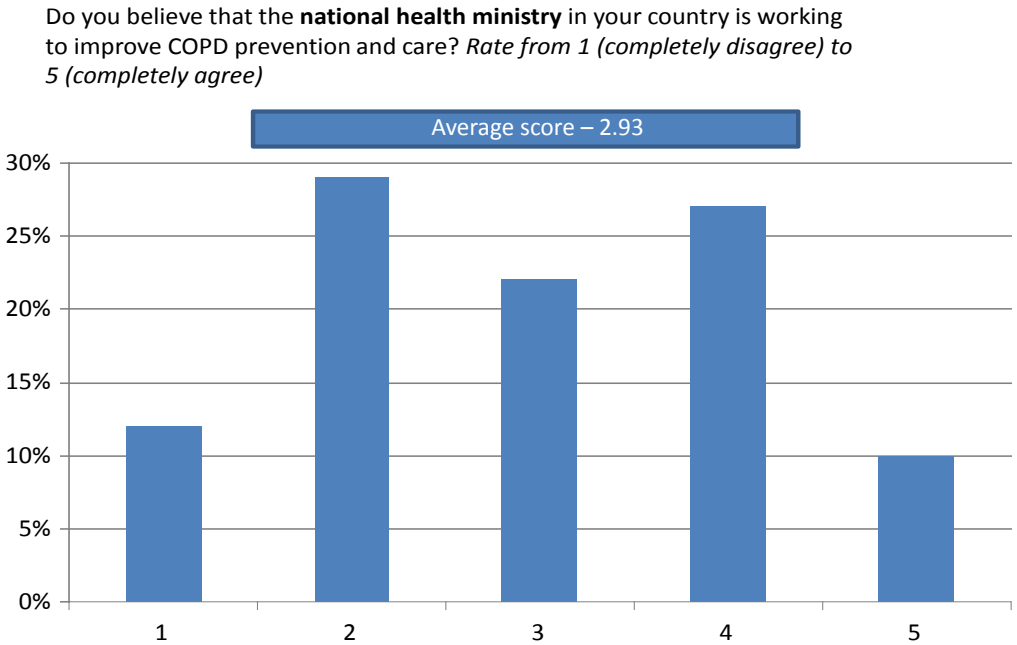

Figure 3 Rating of health ministries in all countries.

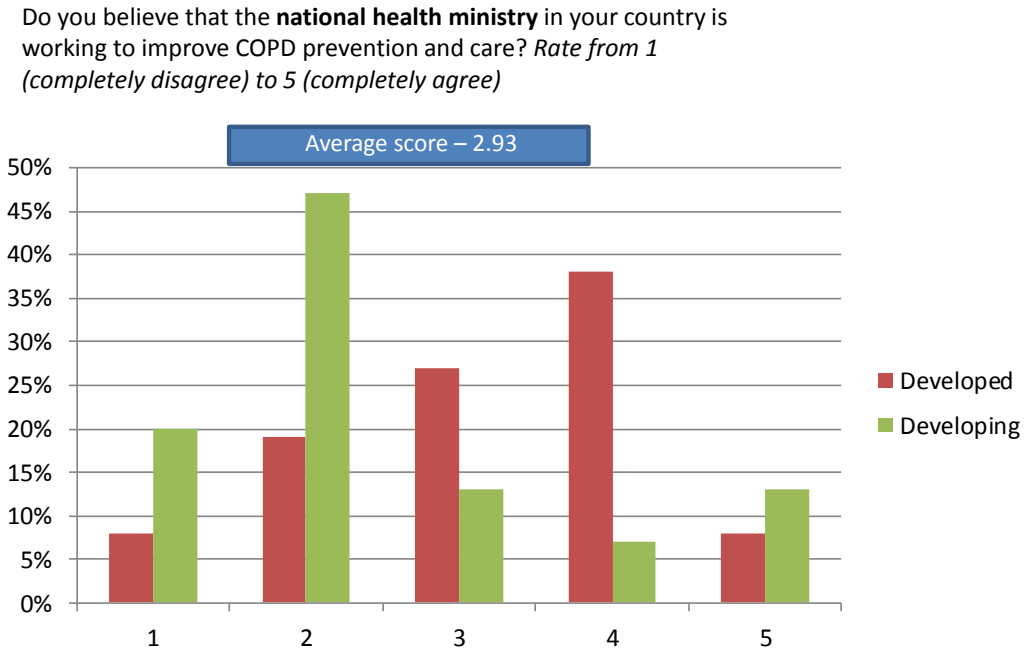

Figure 4 Rating of health ministries in developed and developing countries.

Physicians' ethical principles

Physicians and other health care professionals, ICC believes, have the most important role in ensuring that patients receive care. Since the time of Hippocrates, physicians have taken an oath to commit to the ethical principles of medicine. It is what makes them professionals. There are four major medical ethical principles: primum non nocere-the duty of health care providers not to harm patients; beneficence-the obligation of health care providers to help

people in need; autonomy-the right of patients to make choices regarding their own health care, and justice-the concept of treating all people in a fair manner. To deprive patients of health care violates all the ethical rules of the medical profession. Corporations cannot be held to these rules. They will violate them whenever they can. Neither can governments, but health care professionals and patient groups should be committed to these ethical principles for patients and should fight for them.

Worldwide, patients experience serious problems in obtaining health care because of the combination of overpricing, overtreatment, and inappropriate treatment by for-profit healthcare providing companies, health insurance plans, and pharmaceutical companies. The situation is better in countries with universal health care, but with the current economic problems in many parts of the world, needed health benefits are being withdrawn even in countries with universal health care programs.

Criminal abuses of patients' rights

There are many examples of criminal abuses of patients' rights as well as other actions on the part of health care providers, service organizations, and health ministries to maximize their profit in the health care business to the detriment of patients' welfare and outcomes. A recent example of such criminal abuses in China involves pharmaceutical companies giving bribes to physicians to prescribe their expensive, and often unneeded, medications (2). In addition, there is an increasing concern that clinical trials conducted by pharmaceutical companies to submit to national and regional regulatory agencies in order to gain approval of their products for licensing are falsified by the companies (3). Many companies' failures to allow the release of patient-level data from the clinical trials to be used by regulatory bodies to decide on whether or not their drugs can be approved, indicates that favorable results with new drugs in some cases are based on falsified data, and that negative results are concealed (4). Since trials of this sort are seldom repeated by independent investigators because of the expense, it is likely that falsified trials will not be discovered and that serious unreported side effects and lack of efficacy of the treatments are damaging patients.

In one instance of falsified clinical trial data in Japan, Novartis employees provided falsified data that made it appear that their antihypertensive agent Diovan was beneficial for strokes and heart attacks. However, the actual data did not support these conclusions (3). As a result of this fraud, Novartis was expelled from membership in the association of ethical pharmaceutical companies in Japan. In China, Novartis representatives reportedly paid physicians bribes to prescribe more than five dosages of medications such as Sandostatin. Some observers of these illegal activities recommended that the company change its name from Novartis (New Arts) to Noveritas (No Truth) because of their activities in these scandals. Authorities in China are also investigating bribes paid by Sanofi for prescribing their products.

Influence peddling damages patients

Payments by pharmaceutical companies to expert physicians to influence their recommendations for their products in clinical practice guidelines and for their recommendations in advisory committees of regulatory agencies have led to inappropriate recommendations for expensive new drugs in place of well-established medicines that are less expensive (5).

Because wealthy pharmaceutical, health insurance, and private health provider companies make large donations to political parties and politicians in countries where they market, the laws that they pass and the regulatory authorities' actions favor the welfare of the companies and allow them to financially exploit patients (6). As a result of these and other conflicts of interest that harm patients, in most countries the amount of confidence that people have in their national governments is very low and sinking. In the US, only about 30% of the population has confidence in their government; the people in most European countries have the same or slightly higher confidence levels. The highest confidence globally of people in their national government is in China (75%) and one of the lowest is Japan (15%) (7).

US pro-profit and anti-patient laws may spread globally

The US is bad model for how countries should provide health care, but with many countries expanding their for-profit industries and others with financial problems it is likely that they will develop the same policies that harm patients that are seen in the US. As early as 2004, the US had health care costs that were several times higher than most other developed countries while having markedly inferior mean years of healthy life for their citizens (*Figure 5*) (8). The situation in the US has only gotten worse since that time as its percentage of GDP resulting from health care expense

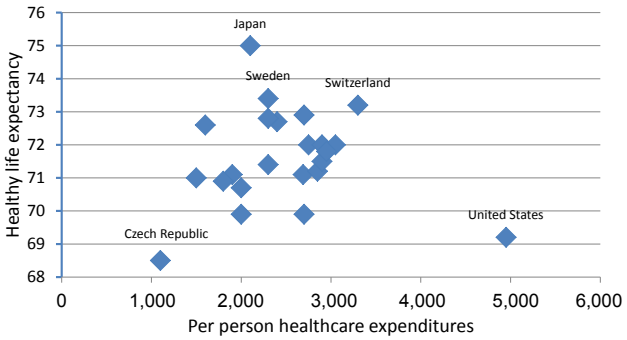

Figure 5 Healthy life expectancy and per person medical expenditure for 23 OECD countries.

has increased and the years of healthy life for its citizens has decreased.

In the US, the windfall profits for health care providing companies, health insurance plans, and supplier organizations have led to deficient, unmanaged health care throughout most of the country, and the high costs of health care have damaged many businesses and have driven many middle-class workers into poverty and led to their deaths.

In the US, more than 57,000 people die each year because they do not have access to basic health care. A total of 32,000 people die in hospitals as a result of preventable medical errors. A total of 20,000 people die unnecessarily each year as a result of high hospital mortality rates that result from deficient care in many hospitals. Millions of other patients suffer unnecessarily because of limited access to health care and from staggering health care costs (9). This is a disgraceful situation for one of the wealthiest countries in the world. The turmoil over the Affordable Care Act in the US as well as over budgetary deficits in the US is in large part the result of the battle over whether or not health care should be available and how it can be paid. A recent article in *The New England Journal of Medicine* profiles a typical case in which the lack of access to health care led directly to a patient's tragic death, a situation that occurs hundreds of times each day in the US (10). Health care is not just another commodity in the market place where one has a choice of whether or not to buy.

Until the US alters its health care system and adopts universal health care and curtails the unrestrained profit taking by physicians, hospitals, health insurance companies, healthcare management companies, and pharmaceutical and device companies, the US economy and population will continue to suffer. As an example, physician salaries are extremely high in the US for most of the procedure-dominated specialties, with an average of about $400,000-500,000 per year and many of the procedures that are performed are overpriced compared to other developed countries (11). In the developing world, few physicians make more than the equivalent of $20,000-30,000 per year (2). Opportunities for high salaries for physicians in the US have prompted substantial immigration of foreign-born and foreign-trained physicians who now make up about one quarter of practicing US physicians. This "brain-drain" of physicians can be detrimental to the public health of countries that train the physicians.

In spite of more expensive health care in the US, many patients are no better off than they were 40 years ago. For example, the care given 40 years ago for COPD was not much different than today's therapy. Short-acting beta agonists, long-acting bronchodilators, antibiotics, oxygen, and corticosteroids were used, as was respiratory therapy with IPPB delivery of medications. Why haven't there been more fundamental improvements in COPD therapy in the last 40 years? The cost of health care for COPD over these past 40 years has tripled in the US but with no evidence for improved outcomes (12). In fact, patients have more YLDs (years lived with disability) (13). Instead of improving the understanding of the pathophysiology of COPD, only the health care system's profit for COPD care has improved!

The out-of-control cost of health care in the US has become so destructive that articles in medical journals are demanding that excessive cost be included in the side effects profiles of drugs since it can do more damage than most biological side effects to patients and their families (14)!

For elderly patients in the US who incur a large part of their lives' health care costs in end-of-life care, the medicalization of death often deprives patients of a peaceful death in their homes with their family without providing any extension of healthy life but definitely resulting in massive expenses. All this overtreatment achieves is to confiscate the life savings of patients. Instead of passing on an inheritance to their children, they are bankrupted by health care costs and are forced to become dependent on their children's financial and custodial assistance! The high cost of health care in the US is contributing to poverty among its elderly population and diminishing resources for lower and middle class people.

In US urban areas such as New York City, those people whose income is in the top 1% receive more than 40% of all the wealth that is generated in the country (15). This increasing concentration of wealth damages the freedom, access to education, and employment opportunities of

most Americans. Many people believe that the US is no longer a democracy, but an oligarchy in which the elected politicians are puppets controlled by wealthy individuals and companies. The enormous healthcare costs in the US fuel these political realities.

There are few voices of morality in the US, which is increasingly secular rather than religious. It is of interest that the Roman Catholic Pope Francis has recently attacked the "idolatry of money" that results in countries with unrestrained capitalist economic systems. He pointed out that these "economies kill" people by depriving them of work, education, and health care by their exclusion and inequality (16). Patients, with their illnesses and inability to protect themselves, are the ideal prey for predatory and unethical health care systems in such countries.

The conflicts of interest that suppliers, providers, and governments have in maximizing their own revenues at the expense of patients have proved too powerful to resist in the US. Hopefully, patients and patient organizations in other countries can learn from this situation and prevent it from occurring in their countries.

The partnerships of patient organizations in the US with providers, service organizations and governments are not effective. Other countries' patient organizations are more successful in working with their partners. Patient organizations worldwide need to protest and advocate against these abuses against humanity when they occur.

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Publishing in open access era: focus on respiratory journals

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Abstract: We have entered an open access publishing era. The impact and significance of open access is still under debate after two decades of evolution. Open access journals benefit researchers and the general public by promoting visibility, sharing and communicating. Non-mainstream journals should turn the challenge of open access into opportunity of presenting best research articles to the global readership. Open access journals need to optimize their business models to promote the healthy and continuous development.

Keywords: Open access; respiratory journal; copyright; peer review; processing fee; business model

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More than 1.8 million articles are published in 28,000 active peer reviewed journals every year. But the huge publishing need still has not been fully met. Open access journals publish peer-reviewed scholarly articles which are online available to readers, in digital form, generally without charge or copyright restriction. Readers can search databases without paying fee and can lawfully read, print, cite and distribute the full text of copyrighted articles. Open access journals have gained momentum and global acceptance. Medical journal publishers need to be fully aware of the impact of open access on health care today since open access means a lot to the medical community.

Open access is imperative and debatable

Open access publishing originally appeared in the second half of the 20th century and gained momentum with the appearance of the internet (1). The first free open access peer-reviewed journals and free scientific online archives appeared in the late 1980s and early 1990s. Almost two decades later, the impact and significance of open access is still under debate, with its organization and implementation questioned.

Still the speed of growth of open access journals has exceeded conventional subscription-based journals. A study in 2013 demonstrated that 7.9% of all peer reviewed scientific articles were published in open access journals (2). In the area of medicine, the impact and quality of open access journals have become comparable to traditional journals.

There is ongoing debate whether open access journals are actually beneficial to scientific community (3). One of possible disadvantage of open access is that journals publish articles with no peer review or low level of quality control in the pursuit of profit.

Through promoting visibility and sharing, researchers and the general public actually benefit from widely available knowledge that had been restricted in traditional publishing times. Open access leads to increase in citations, improvement of article quality, and acceleration of research progress, productivity, and knowledge translation.

In medicine practice, the potential and benefit of open access publishing is more significant. Family practitioners and specialists in remote geographical area who can't get recent research findings through institution-paid subscriptions thus can reach new scientific knowledge and translate into good practice (4-6).

Journals need to face the challenge

The publishing world realm is changing and the premier journals are facing challenges and competition. For instance, more than 5,000 open-access journals have been launched since October 2010. These journals are attracting a growing share of submissions.

Most non-mainstream journals turned the challenge of digitization and open access into opportunity of presenting best research papers to the global readership (7). Making full-text of its articles freely available online, getting archived in PubMed Central can be the drivers for the scientific quality and impact of the journals.

Among 187 Korean open access medical journals indexed in the KoreaMed database, about one-third of the journals are archived in PubMed Central owing to the improved quality of editing and editorial policies encouraging open access (8). A recent study demonstrated that the move toward open access is favored and sponsored by universities and professional societies in Latin American, Eastern European, and Asian countries (9).

In developed countries, the open-access policy is also strongly supported primarily by large commercial publishers using various business models. The Directory of Open Access Journals (DOAJ) database lists more than 8,000 open-access journals, the United States ranks first with its 1,302 open-access journals (10).

In UK, national strategy has been adopted in 2012, allocating a grand proportion of research funds to open-access publishing and archiving papers in publicly accessible digital repositories. The Welcome Trust, one of the major research funders in UK, has been allocating funds for open access since 2007. The lancet journals have endorsed the strategy and given options for open access.

The BMJ Group launched *British Medical Journal* (*BMJ*) Open journal in 2011, which got indexed by PubMed and Web of Science, archived more than one thousand quality items in PubMed Central, and received its first journal impact factor of 1.58 in 2013. *PLOS One*, an open-access journal published by Public Library of Science, has published more than 69,000 papers, archived in PubMed Central with the latest IF of 3.73 and total cites of 133,246 (11).

These successful examples illustrate the viability and importance of the new publishing model (12,13). Open access provides any users online access to peer-reviewed articles and permits free distribution, indexing, copying and lawful reuse without violating the copyrights. We need to embrace the evolution of publishing mode and face the challenges that open access publishing era has brought us and turn them to opportunities of development and quality improvement.

There are diversiform models of open access

Depending on the uttermost provider of articles, OA is divided into green, gold and hybrid OA and is furthermore categorized into subtypes by sources of funding. Open access journals need to secure funds alternative to subscription and pay-per-view fess, maintain high technical quality and online readability of the output, and permanent archiving by prestigious bibliographic databases.

Green open access offers free access to articles by authors publishing their research findings on institutional or central repositories like PubMed, or simply depositing peer reviewed post-prints on other open access websites this is what is called self-archiving.

Journals which have institutional or government funding can afford to implement diamond or platinum open access model from the very beginning, not charging fees to authors or readers and providing no compensation to expert reviewers.

In 2009, 21.7% of medical publications were openly accessible, 13.9% of them were published through open access journals (14). Medical journal publishers prefer gold open access. Gold open access provides immediate free access to articles without embargo period.

Some publishers successfully implemented the gold open access model through mandating payments for publishing and opening access without undermining the quality of published papers. Lowering publishing standards attempting to increase processed payments may jeopardize the quality of this kind of gold open access journals.

Journals published by professional societies generate revenue from advertisements and subscriptions. Hybrid open access is the most suitable publishing model for this kind of journals. Paid open access and traditional subscriptions composite the revenue (15).

Most large publishers of traditional journals, such as Oxford University Press, Springer, Wiley, Elsevier, implement the hybrid open access model (16). Researchers who are publicly funded can pay for open access to their papers, while those who do not have fund to pay for open access can choose traditional publishing mode. The possibility of unethical prioritization of paid open-access papers and rejection or delay of non-paid papers can't be eliminated.

Optimize the business models of open access publishing

When journals provide full accessibility of articles without charging the processing fees, then who pays for the published articles. Besides depending on institutional and

governmental funding, other source of raising fund deserves exploration and consideration.

The cost of publication of open access journals is not reduced even if paper and postage costs are eliminated, the costs are replaced by costs associated with online submission-and-review systems and hosting platforms (17).

There are emerging business models for open access journals. One third of open access journals charge publishing fees, while others receive institutional, governmental, or third-party funding (18-20).

The costs of publication of gold open access journals are covered by author processing or publication charge, and the costs of green open access journals are covered through subscription fees before the embargo period.

Some libraries cancelled subscriptions to small journals in niche areas and those from independent association or university publishers. If institutions are not mandated to pay high subscription fees for non-access journals, they may have spared funds to pay publishing fees and cost of dissemination of their research findings.

When it comes to respiratory journals

Nowadays, there are more and more respiratory journals published worldwide. Open access journals constitute the majority of them. We analyzed the open access mode, publisher, acceptance rate, impact factor, time between submission and final publication, business model of open access respiratory journals published globally. We aimed to investigate whether open access and being published by professional publishing groups are actually beneficial for respiratory journals.

We found that open access respiratory journals have relative higher acceptance rate and less time between submission and final publication than conventionally published journals. For example, *Journal of Respiratory Medicine*, a peer-reviewed, open access journal which publishes original research articles, review articles, and clinical studies in all areas of respiratory medicine, currently has an acceptance rate of 24%. The average time between submission and final decision is 57 days and the average time between acceptance and final publication is 44 days.

Authors have broader options for open access journals publishing. Respiratory Medicine is published by Elsevier, and it offers authors option to publish their research. Authors can choose to pay the open access publication fee to allow their articles freely available to wider public, or do not pay any fee only allowing their articles available to subscribers as well as developing countries and patient groups through access programs of Elsevier. As to *Primary Care Respiratory Journal*, for all accepted Protocol Summary papers, there will be a one-off author charge of £500, payable on manuscript acceptance.

Respiratory journals published by large publishing groups have some advantage over those published by societies or universities. For example, the research published in Biomed Central open access journals is free, not only on the journal's website but also via PubMed Central, the National Institutes of Health's electronic archive of full text articles, UK PubMed Central and other national archives, which means anyone with Internet access can read the research. Average full text downloads are 250 per article per month. All research articles published in BioMed Central's journals are included in PubMed.

Professional societies also actively seek cooperation with publishing groups to popularize their publications. *European Respiratory Journal* (*ERJ*) is the official journal of European respiratory society (ERS) and is distributed by Maney publishing. European Respiratory Society offers an optional service *ERJ* Open that enables authors to ensure that their final published contribution is made available for anyone to access online.

Primary Care Respiratory Journal announced that it will be published by Nature Publishing Group from April 1st. Accepted summaries of the journal will be listed on Medline/PubMed and indexed by Thomson Reuters ISI. They will be available to readers as an open access full text manuscript free of charge.

To sum up, scholarly publishing and information obtaining in developing countries is still dominated by traditional print format. Print format journal publishing is expensive and inconvenient to distribute. Optimized open access and publishing model have enhanced the impact and visibility of scientific peer-reviewed respiratory journals. There is urgent need to build infrastructure in publishing top-tier, open access respiratory journals.

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The use of noun phrases in biomedical research papers written by Chinese scholars

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Grammatically, the simplest kinds of clause in English usually consist of nouns and verbs, for example:

Helen saw Bill.
(noun) (verb) (noun)

However, both in spoken and written languages, the positions before and after the verb are rarely occupied by just a single noun. More usually, they are filled by groups of two or more words. These groups of words are called noun phrases (NP), for example:

My friend saw Bill who comes from Leeds.
(NP) (NP)

NPs consist of a head noun plus one or two of the optional elements. These optional elements fit into four predetermined slots in the NP:

NP = (____ ____ ____ ____)
1 2 3 4

1= determiner and/or enumerator (e.g., the, a, first, his)
2= pre-head modifier (e.g., red, washed, painting, steel)
3= head noun
4= post-head modifier (e.g., in Leeds, which I showed you)

Because of the optional nature of the slots 1, 2 and 4, NPs have a highly variable length, ranging from just single words to passages of text (1).

Specifically in biomedical research papers, NPs are commonly long containing large quantities of information, which are also the common reason for long and complex sentences, a prominent linguistic feature of research papers in English. Some examplar sentences are as the following:

Example 1

NP (The age-matched LETO rats that are developed from the same colony of OLETF rats but do not show DM and pancreatic fibrosis) *were used as a normal control* (2).

The subject of this sentence is a long NP, with the head noun of *rats* and slots 1, 2 and 4. The slot 4, as the post-head modifier, consists of two clauses, which is the main reason for the length and complexity of the sentence.

Example 2

These rats were maintained in ***NP (a temperature- and humidity- controlled room with a 12:12 h light-dark cycle)*** (2).

After the preposition *in*, there is an NP with the head noun of *room*. The slots 1, 2 and 4 define the number and conditions of the room. The comparatively complex NP enlarges the quantity of information contained in just one simple sentence.

Such long and complex NPs are common in biomedical research papers, whose use constitutes challenge to Chinese scholars due to the differences in habitual way of thinking and language expressing between Chinese and English. Linguistically, it is known as the negative transfer of the mother tongue (3). In this paper, I categorize the inappropriate use of NP in biomedical research papers written by Chinese scholars into four types as the following.

Absence of determiner, especially third personal possessive pronouns such as *its, their*

Example 3: *the occurrence of hypertension has* ***NP (its causes)*** *and consequences.*

Example 4: *in autumns and winters, many people perform moxibustion at home to preserve* ***NP (their health)***.

By contrast of the above two examples between Chinese and English, it can be found that Chinese do not use such determiners as *its* and *their* as the references and the cohesive

ties of the discourse (4). Therefore, such determiners are commonly absent in English research papers written by Chinese scholars.

Inappropriate use of pre-head modifier

Example 5: *the risk factors of hypertension include* ***NP1 (insufficient physical activities)***, ***NP2 (excessive intake of the sodium salt)***, …
Example 6: *the symptoms of hypoglycemia are various, including* ***NP1 (rapid heartbeat)***, ***NP2 (blurry vision)***, …
Example 7: ***NP (More than half Chinese women at the child bearing age)*** *are frequently exposed to second hand smoking.*

In Examples 5 and 6, there are totally four marked NPs. In Chinese, they have the structure of "Noun + Adjective". However, they are expressed in English in such a reverse order of "Adjective + Noun". Due to the negative transfer of their mother tongue and limited adjectives within their command of English, Chinese scholars tend to use the pre-head modifiers inappropriately, such as "lack physical activities", "less physical activities", "over taking the sodium salt", "heartbeat that is accelerated", "dim vision", …

And in Example 7, the subject is a comparatively long NP with the head noun of *women*. In Chinese, the head noun is the last word in the NP with many pre-modifiers. However, in English, some pre-modifiers in Chinese (like *Chinese* and *more than half*) are kept as the pre-modifiers and some function as the post-modifier (such as *at the child bearing age*). Due to the negative transfer of their mother tongue, Chinese scholars tend to confuse the pre-head modifiers and the post-head modifiers in such cases and their inappropriate uses include "child bearing age women in China" or "Chinese child bearing age women".

Difficulty choosing the head noun

Example 8: *the experts recommended people receive qualified physical assessment for* ***NP (appropriateness of moxibustion)*** *and seek professional guidance for proper position of moxibustion.*

In Example 8, the marked NP in English has no corresponding NP in Chinese. In such cases, the choice of the head noun for an NP presents great challenge to Chinese scholars. Instead of the noun *appropriateness*, Chinese scholars tend to use a clause to express in English, such as "whether moxibustion is appropriate". As shown in linguistic studies, the packaging of a clause into NP is customary in English research papers (5).

Unnecessary clause as the post-head modifier

Example 9: *type II diabetes is commonly related to* ***NP (overweight induced hyperglycemia)***.
Example 10: *a recent survey demonstrated that* ***NP (more than 80% university graduates majoring in clinical medicine or nursing)*** *are working in hospitals.*

From the structural analysis of NPs, we know that the verbs in either form of -ing or -ed can be used in both pre-head modifier and post-head modifier. However, Chinese scholars tend to put verbs in clauses and therefore overuse clauses as the post-head modifier. The above two marked NPs in the Examples 9 and 10 are commonly found to be inappropriately expressed as "hyperglycemia that can be induced by overweight" and "more than 80% university graduates who majored in clinical medicine or nursing".

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Gomers

The Dean was a gentle, bearded man, a pipe-smoking neurologist. He loved to sail in the San Juan Islands. He was a far-seeing person not given to anger. The failures, large and small, of his medical students were duly noted, but the Dean always saw past these failures to the future successes of his embryo physicians. The only time I ever saw the Dean angry was when he heard someone using the term "gomer".

Many say that this term is an acronym for "Get out of my emergency room", a phrase frequently yelled at gomers by emergency-room physicians. The gomers themselves were down-and-outers. They were the alcoholics, the addicts, and the senile, raging geriatrics. The term was ubiquitous among the students and house staff at the hospitals in our university system. Underground pamphlets circulated that described the classification of gomers, competitions of legendary gomers, even gomer olympics. Elaborate point systems were invented to differentiate the ordinary gomer from the super gomer. The title super gomer was awarded for accumulating 150 gomer points. Ward rounds on these devastated human beings were always turbulent.

Gomers always had every possible complication, and treating them was terribly frustrating. The house staff was surprised at the vehemence with which the Dean attacked the term gomer, but they were not deterred in its use. We intimated among ourselves that the Dean was obviously far removed from clinical medicine and had forgotten what things were like on the wards of the county hospital.

On the pediatric service a few years later, I supervised the care of a four-year-old boy named Allan, dying of hepatic failure. The medical details aren't relevant, but it would be difficult to imagine a more depressing case. Bleeding complications rather than hepatic coma threatened to become the terminal event, and the child was in pain.

I noticed that the house staff always became paradoxically whimsical as we discussed Allan's case. They indulged in what I considered to be "medical fantasy", that is, going off on tangents related to remote and horrendous complications that might occur. There was a great deal of inappropriate levity and hilarity. I knew that this bonhomie was not malicious. Individually, they were as upset about the illness and their inability to affect it as I was. It reminded me of the way we had talked about gomers during my training and that disturbed me.

One particularly grim day we made rounds on Allan the morning after a serious hemorrhage. His parents had finally gone home to rest, and he was sitting alone on his hospital bed. As we were leaving the room he suddenly asked, "*Will you read me for a few whiles?*".

Our silence was deafening, and the looks of sheer panic on the faces of my colleagues surprised me. They immediately left for their day's duties. I realized that all of the inappropriate hilarity, the medical fantasy, and the talk of gomers among the house staff were defenses against the terrible fear of failure and death. I realized how I had also hidden behind the jesting, cynical demeanor and brusque, busy professionalism. It dawned on me that all those years of gomer talk simply revealed the callow inexperience and inhumanity in me and in my colleagues.

I sat down with Allan. I read to him *The Cat in the Hat* twice, followed by *The Golden Book of Dinosaurs* and was about a third of the way into *Selected Mother Goose* when my little patient fell asleep.

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Case Report

A 7.3×5.3×3.5-cm heterotopic thyroid in the posterior mediastinum in a patient with situs inversus totalis

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Abstract: Ectopic posterior mediastinal thyroid is a very rare clinical disease entity. It accounts for less than 1% of all mediastinal tumors. The mass is difficult to diagnose due to its rarity and non-specific clinical symptoms and thus remains asymptomatic for many years until it becomes larger. Since the mass is considerably large at presentation, it should be distinguished from metastatic thyroid carcinoma. We present herein the case of a 62-year-old woman with situs inversus totalis who complained of atypical chest pain and dysphagia and had a huge posterior mediastinal ectopic thyroid. The patient underwent complete resection through thoracotomy, with relief of symptoms. In summary, although ectopic posterior mediastinal thyroid is a rare disease entity, it should be considered in the differential diagnosis of posterior mediastinal tumors. Surgery is the treatment of choice for posterior ectopic posterior thyroid and its prognosis is excellent following complete resection.

Keywords: Thyroid; mediastinum; situs inversus totalis

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Introduction

Although ectopic thyroid is clinically benign, it is successfully treated with surgical resection. Ectopic thyroid is at risk of malignant transformation and they can cause serious clinical conditions due to severe bleeding or compression of the adjacent vital organs during progressive enlargement (1,2). Thoracotomy has been widely used to remove ectopic thyroid is because this procedure allows for complete tumor resection in a wide surgical field and has low postoperative mortality and morbidity. After the ectopic thyroid is completely removed, the disease can be completely cured without any additional treatments (1,3).

Case report

A 62-year-old woman presented at our department due to abnormal findings in chest X-rays. She had undergone a left hemithyroidectomy at a regional hospital 30 years prior to this presentation because of a tiny thyroid nodule in the left thyroid gland associated with hyperthyroidism. Thereafter she was regularly followed up with and her thyroid function test results were normal. At the time of the operation, preoperative evaluation revealed situs inversus totalis without any symptoms and signs. Six years prior to this presentation, the patient underwent brain imaging studies, including MRI, which revealed no abnormal findings. He was taking medications for hyperlipidemia. She was a house wife and had no remarkable family history. She was a non-smoker and a non-drinker. At presentation, her vital signs were stable. Physical examination revealed no specific findings except for a previous thyroidectomy scar. Chest radiographs exhibited situs inversus totalis and a left mediastinal mass.

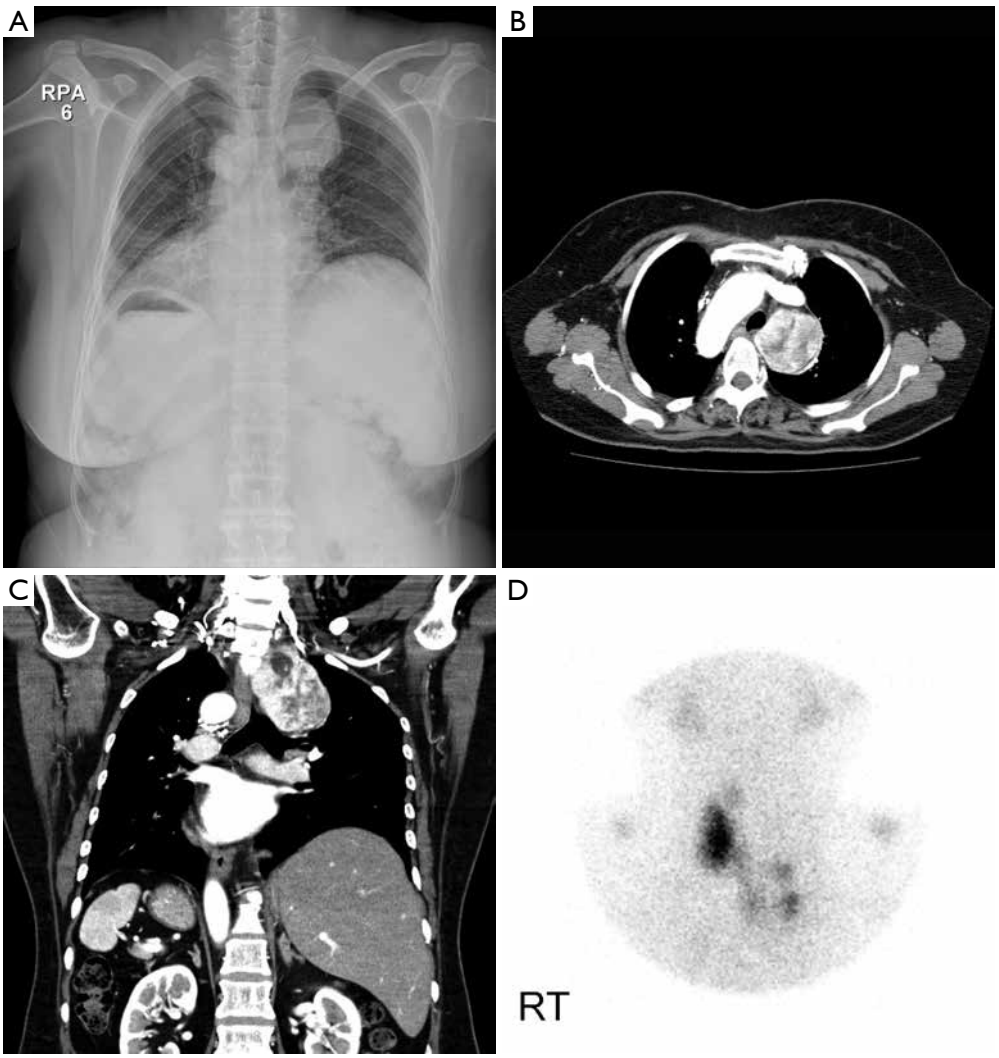

Figure 1 (A-C) Preoperative anteroposterior view of the chest, transverse computed tomography and saggital computed tomography reveal situs inversus totalis and a well-encapsulated mass located in the left posterior mediastinum; (D) Preoperative thyroid scan reveals the remaining right thyroid lobe following a previous left thyroid lobectomy and mildy increased uptake in the left mediastinal mass. RPA means the right posteroanterior view and RT means the right side.

Chest CT revealed a huge well-encapsulated mass in the posterior mediastinum which had no invasion of the trachea, esophagus, or the adjacent lymph nodes suggestive of malignancy. Thyroid scan with radioactive iodine only showed the right thyroid grand alone and a slightly increased iodine uptake in the left mediastinal mass (*Figure 1*). There were no specific findings in laboratory tests, including the thyroid function test. Esophagogastroscopy revealed no abnormal findings.

Under general anesthesia, a standard left posterolateral incision was made. The surgical approach was difficult due to the mirror image of the anatomical structures. The mass was bordered by the azygos vein inferiorly, the superior vena cava anteromedially, and the trachea and esophagus medioposteriorly. The mass was removed carefully not to damage the adjacent tissues and blood vessels. There was no direct invasion of the superior vena cava, trachea, or esophagus. The resected mass was well-encapsulated and measured 7.3 cm × 5.3 cm × 3.5 cm. Pathologic examination confirmed benign ectopic thyroid tissue with no invasion of the adjacent lymph nodes (*Figure 2*). Her dysphagia and atypical chest pain improved. Her hospital course was uneventful and she was discharged from the hospital on the 10th postoperative day. She has been followed up with on a

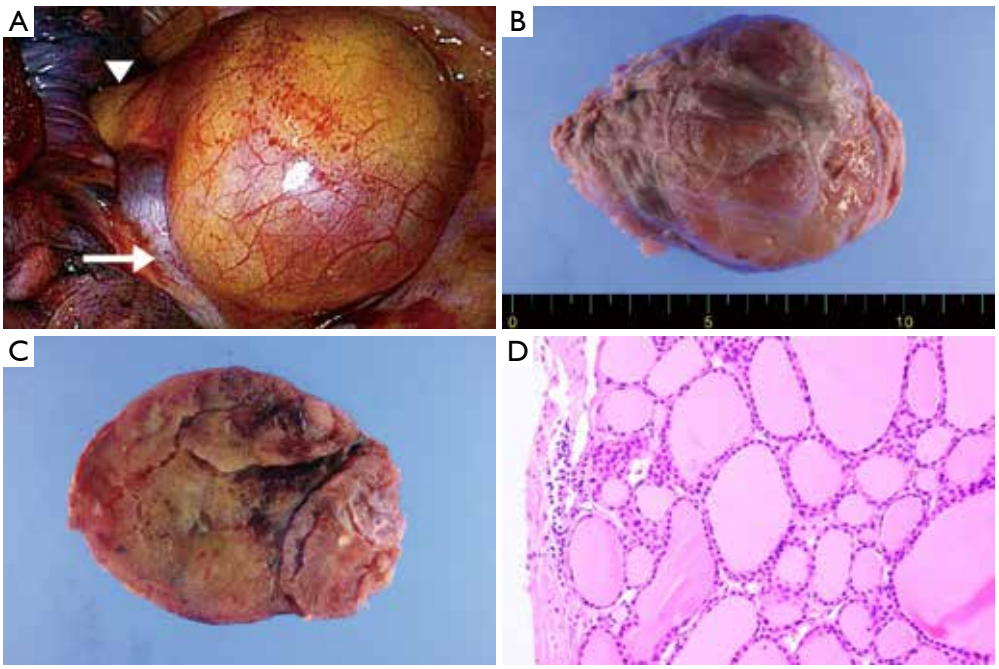

Figure 2 (A) Intraoperative view of a mass adjacent to the azygos vein (white arrow) and the superior vena cava (white arrow head); (B,C) Macroscopic examination of the outer surface and the cut surface of the posterior mediastinal tumor shows a multilobular mass surrounded with a capsule; (D) Histologic examination shows a peripheral rim of preserved thyroid follicles around a central paucicellular fibrotic area (H&E stain, original magnification ×100).

regular basis at our outpatient clinic.

Discussion

Ectopic thyroid is a rare clinical disease entity. The thyroid gland develops at the gestational age of 24 days and starts to migrate at the gestational age of five weeks, during which ectopic thyroid occurs due to abnormal embryological development or thyroglossal duct remnant (4). Thus, ectopic thyroid is usually located on the midline of the anterior neck and inferior to the normal position of the thyroid gland. The most common presentation of the ectopic thyroid is the lingual thyroid which is located along the thyroglossal duct remnant (5).

There have been several reports on unusual locations of ectopic thyroid, such as the axilla, trachea, adrenal gland, and porta hepatis. The most common non-cervical location of ectopic thyroid has been reported to be the thoracic cavity (6). The true primary ectopic thyroid is retrosternal goiter, which is usually located in the anterior mediastinum. However, Madjar *et al*. (7) reported that 10-15% of all retrosternal goiters were located in the posterior mediastinum. Chin *et al*. (8) demonstrated that 5 of the 190 patients with goiters had masses in the posterior mediastium on CT scans. Tumors that originate in the posterior mediastinum include neurogenic tumors, Castleman disease, bronchogenic cysts, Bochdalek's hernia, and mesenchymal tumors. There have been few reports on thyroid tumors arising in the posterior mediastinum. The incidence of such thyroid tumors is relatively low even when primary ectopic goiters and invasion of thyroid carcinomas are included. Though ectopic thyroid rarely develops in the posterior mediastinum, it should be considered in the differential diagnosis of posterior mediastinal tumors (6). Demirhan *et al*. (3) reported a 62-year-old man who was admitted to the emergency department with atypical chest pain and dysphagia, and was diagnosed as having a posterior mediastinal mass causing esophageal compression. The mass was completely encapsulated and totally localized in the posterior mediastinum as an isolated intrathoracic tumor causing dysphagia, and pathologic sections confirmed the lesion as ectopic thyroid with neither connection with normal thyroid glands nor functional impairment. Karapolat and Bulut (1) reported a 74-year-old Caucasian male with a mass lesion in the right posterior mediastinum, which was successfully resected and confirmed as ectopic

thyroid gland with neither connection with normal thyroid glands nor functional impairment. Mace *et al*. (4) reported an 80-year-old female who presented with an incidental retrosternal mass on magnetic resonance imaging which is consistent with a benign ectopic sequestered thyroid nodule without evidence of malignancy.

True primary ectopic thyroid accounts for less than 1% all goiters, but its accurate incidence has not yet been reported. There have been numerous mechanisms behind the pathogenesis of ectopic thyroid, but the precise mechanisms remain to be elucidated. To the best of our knowledge, this is the first case of a patient with situs inversus totalis who had ectopic thyroid in the posterior mediastinum. The relationship between ectopic thyroid and situs inversus totalis is unclear. Ectopic posterior mediastinal thyroid is generally known to be benign. Thus, it should be distinguished from metastatic lymph nodes of well-differentiated thyroid carcinoma. Most patients with ectopic thyroid show clinical features of euthyroid, while a few patients show clinical features of hyperthyroidism. Thyroid function tests are very useful for preoperative diagnoses as well as postoperative follow-ups and thyroid scan with radioactive iodine is also helpful in making a correct diagnosis and evaluating clinical patterns. However, since in some cases, thyroid scan with radioactive iodine shows negative findings, this diagnostic modality cannot completely rule out ectopic thyroid. Chest computed tomography is the most useful radiological diagnostic modality and provides important information for a safe surgical approach. Although some investigators have demonstrated that magnetic resonance imaging is helpful in the differential diagnosis and treatment of ectopic thyroid, there is still controversy regarding this issue (1,3). Most patients with ectopic thyroid are asymptomatic, whereas a few patients have clinical symptoms and signs due to compression of the adjacent organs, such as cough, dyspnea, wheezing, dysphagia, and obstruction of the superior vena cava (1). It seems likely that our patient complained of intermittent dysphagia due to compression of the adjacent organ by the enlarged ectopic thyroid.

Although ectopic thyroid is clinically benign, it is successfully treated with surgical resection Ectopic thyroid is at risk of malignant transformation and they can cause serious clinical conditions due to severe bleeding or compression of the adjacent vital organs during progressive enlargement (1,2). Thoracotomy has been widely used to remove ectopic thyroid is because this procedure allows for complete tumor resection in a wide surgical field and has low postoperative mortality and morbidity. After the ectopic thyroid is completely removed, the disease can be completely cured without any additional treatments (1,3).

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Case Report

Flail chest stabilization with Nuss operation in presence of multiple myeloma

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Abstract: Nowadays the Nuss operation has been widely adopted as a minimally invasive procedure and standard surgical choice in pectus excavatum. However, much debate and concern have been raised regarding its applicability in adults with pectus excavatum flail chest and other thoratic wall deformities, as compared with younger patients, in terms of complications after surgery. To stabilize the segment of paradoxical chest wall movement we performed the Nuss operation on a patient with multiple myeloma who sustained blunt thoracic trauma. The patient presented with paradoxical movement of the thoracic wall and sternum instability due to multiple myeloma, which led to severe dyspnea, hypoxemia, hypercapnea, and bedridden state. His condition progressed to acute respiratory distress syndrome and did not respond to conservative treatment. We performed the Nuss operation on the patient, and his clinical symptoms were relieved after surgery. The patient regained the ability to walk unassisted and was discharged from the hospital without any specific events.

Keywords: Flail chest; multiple myeloma; Nuss operation

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Introduction

Although skeletal involvement is common in multiple myeloma, flail chest with paradoxical thoracic wall movement and sternum instability is very rare. There have been only a few cases of flail chest complicating multiple myeloma. Fleegler *et al.* (1) reported a case of pathological flail chest complicating multiple myeloma. Abisheganaden *et al.* (2) described the use of bilevel positive airway pressure ventilatory support for pathological flail chest complicating multiple myeloma. However, there have has been no standard treatment method for this clinical condition. Surgical stabilization of flail chest is controversial. It has been shown that select patients benefit from this method. Several operative techniques using Kirshner wires, Judet's struts, polypropylene mesh, and malleable metal plates have been developed; however, these methods are inadequate for patients with comorbid medical conditions. The Nuss operation described first in 1998 by Donald Nuss is a safe and minimally invasive one for pectus excavatum (3). We describe a male patient with multiple myeloma who presented with paradoxical movement of chest wall, chronic pain, and dyspnea on exertion as well as severe respiratory failure following blunt trauma and was successfully treated by the Nuss operation for internal stabilization.

Case report

A 35-year-old male patient was referred from a regional hospital to our department for further management of chest pain, dyspnea, and oliguria. Two months ago, he had sustained mild blunt thoracic trauma at a traffic accident with the safety belt being fastened around the waist. At the regional hospital, the sternum fracture had been managed

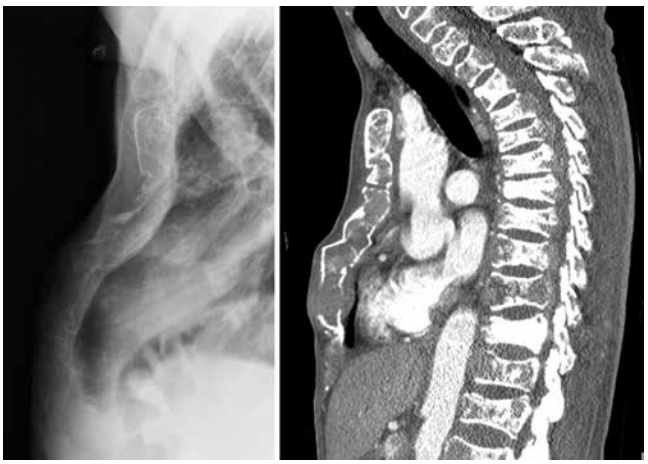

Figure 1 Preoperative plain lateral X-ray and chest computed tomography (CT) saggital view show severe depression of the upper half of the sternum in the flail segment.

conservatively, and the compression fracture of the 11th thoracic vertebral body was treated by vertebraoplasty. The patient had been bedridden for approximately two months. At presentation, he had no underlying disease except sternum fracture and compression fracture of the 11th thoracic vertebral body. He had a white-collar job by occupation and did not have any specific family history, including the cancer. His blood pressure was 110/70 mmHg, pulse rate 82 beats/min, respiratory rate 16/min, and body temperature 36.7 ℃. Physical examination revealed a 4-cm subcutaneous bulging mass in the left 10th rib and mild tenderness around the mass. Paradoxical movement of the right upper chest wall and sternum was observed, and crackles and inspiratory friction rubs were audible in the entire lung field. Complete blood counts revealed white blood cells, 14,800/μL; hemoglobin, 9.0 g/dL; platelets 130,000/μL; and erythrocyte sedimentation rate 42 mm/h. Blood chemical test results were as follows: albumin, 3.6 g/dL; blood urea nitrogen, 163.9 mg/dL; creatinine, 13.3 mg/dL; calcium, 11.4 mg/dL; C-reactive protein, 3.59 mg/dL; and uric acid, 15.3 mg/dL. Urine protein was 2+ (100 mg/dL) on urinalysis. Arterial blood gas analysis (ABGA) revealed pH, 7.39; PaO_2, 77.6 mmHg; $PaCO_2$, 34.0 mmHg; and oxygen saturation (SpO_2), 95.5%. Plain chest radiographs, including the sternum lateral view and both rib oblique views, exhibited diffuse osteopenia, multiple rib fractures, sternum fracture and left the 10th rib destruction. Chest computed tomography (CT) revealed extensive osteoclastic lesions and bony destruction of multiple the sternum, spine ribs, as well as a 3×4-cm soft tissue mass in the left 10th rib (*Figure 1*). Further evaluation by whole body bone scintigraphy revealed multifocal increased or decreased uptakes in the whole axial and proximal appendicular skeletons, diffusely increased whole periarticular uptakes, and slightly increased uptakes in the sternum and ribs, especially in the mass of the left 10th rib with a bone to soft tissue ratio of 2.56:1. For the management of acute renal failure, hemodialysis was started immediately, and further workups were continued. Measurement of serum immunoglobulin (Ig) and immunoelectrophoresis revealed the following results: increased beta2-microglobulin level, 14.38 ug/mL (normal range, 1.0-3.0 ug/mL); decreased IgM, 12.0 mg/dL (normal range, 40-230 mg/dL); IgG, 388.0 mg/dL (normal range, 700-1,600 mg/dL); IgA, 15.0 mg/dL (normal range, 70-400 mg/dL), and IgD, <0.45 mg/dL (normal range, 0.77-13.21 mg/dL). Serum protein immunochemistry results were as follows: the serum decreased kappa-light chain, 87.1 mg/dL (normal range, 170-370 mg/dL) and increased lambda-light chain, 489.0 mg/dL (normal range, 90-210 mg/dL) (*Table 1*). Immunofixation electrophoresis of the serum and urine showed abnormal bands in the lambda lane. Under local anesthesia, incisional biopsy of the mass in the left 10th rib was performed, and it showed atypical plasma cells having centrally or eccentrically located ovoid nuclei and eosinophilic cytoplasm. It had been proved that this mass was consisted with a relatively well circumscribed tumor, and composed of monomoprhic plasmacytoid cells (Hematoxylin and Eosin, ×200). The tumor cells show diffuse and strong immunoreactivity against CD138 antibody (×400) (*Figure 2*). Immunohistochemical studies of the specimen showed positive lambda-chains and negative kappa-chains. This histologic finding indicates a plasma cell neoplasm suggestive of plasmacytoma of the rib. Histologic findings obtained from bone marrow biopsy were suggestive of plasma cell myeloma. Immunohistochimical staining of the specimen was positive for the cluster of differentiation (CD) 138 and for the lambda with monotypic lambda Ig expression, but negative for CD3, CD20, and kappa.

The patient was diagnosed with multiple myeloma with rib and sternum involvement based on laboratory test results and histological findings. His final diagnosis was Ig D-lambda-type multiple myeloma with Durie-Salmon stage IIIB and International staging system IIIB, so he was transferred to the Department of Hemato-oncology and started to receive chemotheraphy with the VAD regimen (vincristine + doxorubicin + dexamethasone). However, he complained of more aggravated thoracic wall pain,

Table 1 Summarization of preoperative evaluation

Studies	Results	Variation	Normal range	Units
Complete blood counts				
White blood cells	14,800	▲	4,000-10,000	/μL
Red blood cells	3.24	▼	4.1-5.5	$\times 10^6$/μL
Hemoglobin	9.0	▼	13.3-17.2	g/dL
Platelets	130,000	▼	131,000-400,000	/μL
Blood chemical test				
Albumin	3.6		3.5-4.8	g/dL
BUN	163.9	▲	8-26	mg/dL
Creatinine	13.3	▲	0.7-1.2	mg/dL
Calcium	11.4	▲	8.2-10.2	mg/dL
Ionized calcium	1.45	▲	1.12-1.42	mmol/L
Phosphate	8.3	▲	2.5-5.5	mg/dL
CRP	3.59	▲	0-0.75	mg/dL
Uric acid	15.3	▲	3.5-7.0	mg/dL
CK	413	▲	53-348	U/L
LDH	954	▲	263-450	IU/L
Urinalysis				
Urine protein	++	▲	0	mg/dL
Electrolyte				
Sodium	132	▼	135-145	mmol/L
Potassium	4.1		3.5-5.5	mmol/L
Chloride	95	▼	98-110	mmol/L
ABGA				
pH	7.39		7.35-7.45	
$PaCO_2$	34.0	▼	35-45	mmHg
PaO_2	77.6	▼	80-100	mmHg
Oxygen saturation	95.5		95-100	%
Bicarbonate	18.6	▼	21.0-27.0	mmol/L
Base excess	–4.0	▼	–2.0-2.0	mmol/L
Immunochemistry				
beta2-microglobulin	14.38	▲	1.0-3.0	ug/mL
IgM	12.0	▼	40-230	mg/dL
IgG	388.0	▼	700-1,600	mg/dL
IgA	15.0	▼	70-400	mg/dL
IgD	<0.45	▼	0.77-13.21	mg/dL
Serum kappa-light chain	87.1	▼	170-370	mg/dL
Serum lambda-light chain	489.0	▲	90-210	mg/dL

Abbreviations: ▲, increased level; ▼, decreased level; Ig, immunoglobulin; ABGA, arterial blood gas analysis; BUN, blood urea nitrogen; CRP, C-reactive protein; CK, creatine kinase; LDH, lactate dehydrogenase; ++, two positive.

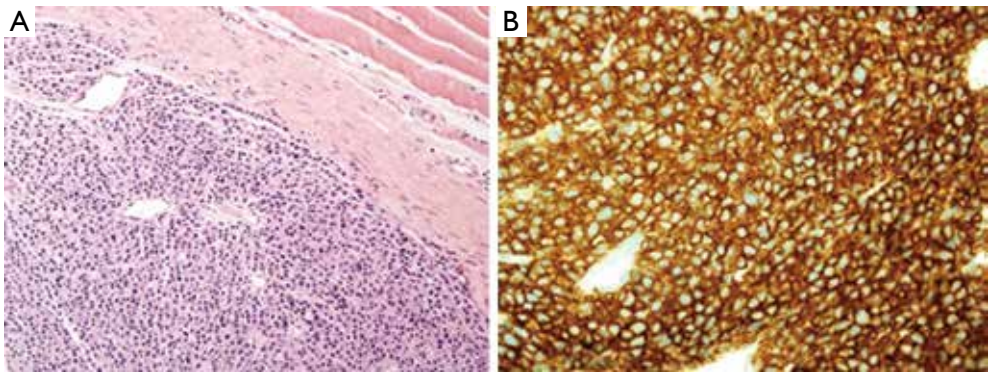

Figure 2 (A) Histologic finding shows that the tumor is a relatively well circumscribed, and composed of monomoprhic plasmacytoid cells (Hematoxylin and Eosin, ×200); (B) The tumor cells show diffuse and strong immunoreactivity against CD138 antibody (×400).

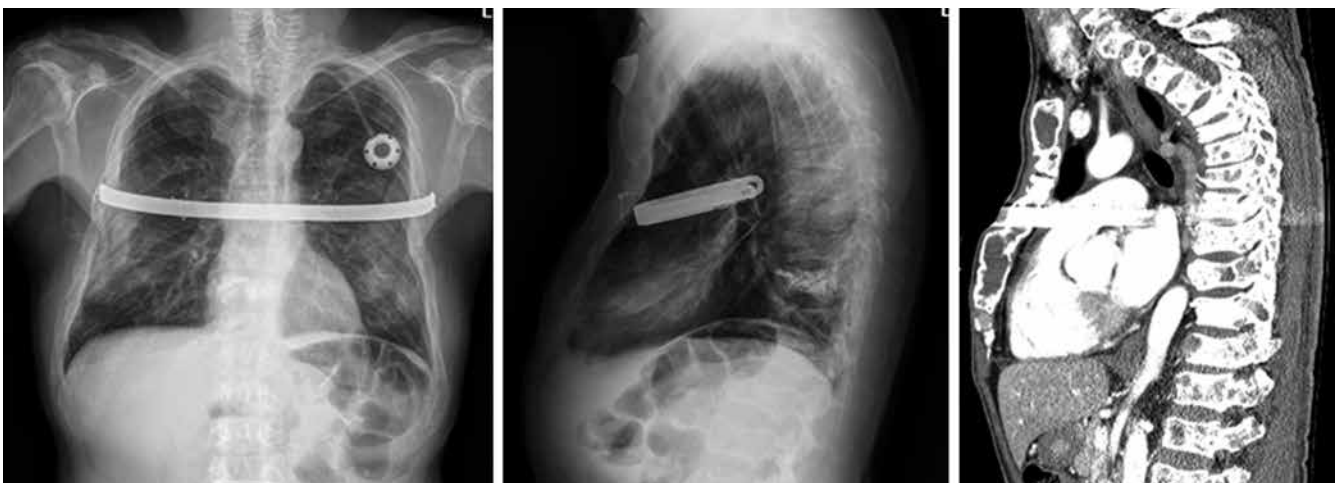

Figure 3 Postoperative plain posteroanterior/lateral X-ray and chest computed tomography (CT) saggital view show Nuss bars and restored thoracic wall.

dyspnea, and paradoxical movement of the chest wall. ABGA results were still abnormal: pH, 7.24; PaO_2, 62 mmHg; $PaCO_2$, 46.0 mmHg; and SpO_2, 85%. We considered further management of flail chest with paradoxical thoracic wall movement, including mechanical ventilation and surgical stabilization with an external fixator. Nonetheless, since less invasive but more effective management was required due to underlying multiple myeloma, we decided to perform the Nuss operation 107 days after blunt thoracic trauma. Under general anesthesia, the patient was placed in the supine position with both arms abducted. Two small incisions were made bilaterally in the center of flail segment on the mid-axillary line. After creation of subcutaneous tunnels to the hinge points, a specially designed transilluminated introducer was passed through the mediastinum under the depressed sternum instead of a pectus clamp. A 28-F chest tube was used as a guide to pull through the bent metal bar (13-inch pectus bar, MX-bar^{TM}, MedixAlign Tech, Seoul, Korea). By rotating the bar, the convexity of the bar lifted the depressed portion of the flail segment. Both ends of the bar and one hingepoint were fixed to adjacent ribs with wires (Surgical steel, EthiconTM, Inc. Somerville, NJ, USA).

The patient's postoperative course was uneventful, restored with thoracic stability (*Figure 3*). He was relieved from paradoxical movement of thoracic wall and pain, and ABGA results returned to normal. On the first postoperative day, the ABGA results without O_2 supply was improved: pH, 7.39; PaO_2, 98.8 mmHg; $PaCO_2$, 34.2 mmHg; and SpO_2, 99%. He was consulted to the Department of Rehabilitation Medicine for the further managements, including ambulation, and started ambulation with aids after bed side physical therapy on the 14th postoperative day. His Eastern Cooperative Oncology Group Performance Status Scale

was changed from grade 4 to 2 on the 50th postoperative day after the third session of VAD chemotherapy. On the 97th postoperative day, he was discharged from the hospital and followed up at the Department of Hemato-oncology, without further dyspnea for two years.

Discussion

Medical or surgical stabilization of the segment of paradoxical chest wall movement is important for patients with flail chest. Currently, general acceptance of surgical stabilization has been gradually increased through improved surgical stabilization of flail chest with the development of variable rib fixation prostheses, e.g., anatomic rib plates, intramedullary splints, and absorbable nails (4,5). Several previous studies have demonstrated that surgical stabilization can significantly reduce the duration of mechanical ventilation, the incidence of pulmonary complications, the duration of ICU stays/hospitalization, and the incidence of chest deformities compared to internal pneumatic stabilization by using ventilator appliances (6). Nevertheless, surgical fixation is nearly impossible in patients with metastatic bone disease, such as multiple myeloma, which weakens the rib and sternum and in patients with compromised general medical conditions which induce high perioperative mortality and morbidity. To date, flail chest associated with multiple myeloma has been treated conservatively with mechanical ventilation or bilevel positive airway pressure ventilator support (2). Recently, several less invasive surgical fixation techniques, including percutaneous osteoplasty and intramedullary rib splints, have been proposed (7,8). Pacheco *et al.* (9) has reported the Nuss operation in a massive flail chest induced in a snowmobile accident and asserted that Nuss bars can be used to stabilize severe flail chest injuries, when chest reconstruction is necessary but fractured segment fixation is infeasible due to adjacent chest wall instability. In our case, since we were accustomed to the Nuss operation for the correction of pectus excavatum, we decided to perform the Nuss operation, a minimally invasive technique, because lateral thoracic structures were intact and flail segment was just the upper part of the anterior chest wall, including the upper half sternum and the second to fourth ribs.

Though not all patients with flail chest can be treated with the Nuss operation, especially in patients with the lack of intact lateral bony structures, the Nuss operation may be a useful for stabilizing of paradoxical movement of the chest wall, in select patients with the advantage of being minimally invasive. A case series would be required despite the rare occurance to determine if the Nuss operation can be a useful tool in stabilization of flail chest in these select patients.

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Case Report

Emergence of linezolid resistance in a clinical *Staphylococcus capitis* isolate from Jiangsu Province of China in 2012

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Abstract: Linezolid (LZD) is an important antimicrobial agent for the treatment of infections caused by Gram-positive organisms, including methicillin-resistant *Staphylococci*. And until now, LZD resistance in clinical is still rare. Here we reported the first case of LZD resistance *Staphylococcus capitis* in Jiangsu, China. This strain was isolated from a 92-year old female who received long-term and repeatedly antibiotics treatment because of recurrent pulmonary infections in August 2012. Isolated from blood, the *Staphylococcus capitis* showed a resistance to LZD with a minimal inhibitory concentration (MIC) of 64 μg/mL, and the followed gene detection showed that the isolates existed C2190T and C2561Y point mutations in the 23S rRNA. Moreover, the isolation was also found carrying the cfr gene.

Keywords: Linezolid-resistance (LZD-resistance); *Staphylococcus*; pulmonary infections

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Introduction

Linezolid (LZD) is an oxazolidinone antibacterial agent approved by FDA of America in the year of 2000 for the treatment of infection with gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (1), glycopeptide-intermediate *S. aureus* and vancomycin-resistant *Enterococci*. It exerts its antibacterial activity by acting on the early stage of the protein synthesis process; it binds to the ribosomal 23S portion of the 50S subunt of target bacteria and thereby inhibits the formation of the 70S initiation complex (2). LZD resistance occurs by mutations in the LZD 23S rRNA binding site, the ribosomal proteins L3 and/or L4 of the peptide translocation centre of the ribosome or by acquisition of a plasmid-borne ribosomal methyltransferase gene, *cfr* (3,4). In 2001, the first LZD-resistant *S. aureus* was reported in a US patient who had received a 1 month LZD treatment for dialysis-associated peritonitis (5). Since then, cases of LZD-resistant *Staphylococcus* have been reported worldwide in America (5-7), Europe (8-10) and Asia (11,12). In China, LZD was approved into clinical use in 2007 and several clinical cases of LZD-resistant *Staphylococcus capitis* have emerged in Zhejiang Province and the city of Beijing (13,14), but this is the first report, to our knowledge, of LZD-resistant *Staphylococcus capitis* in Jiangsu of China. The strain was isolated from blood sample of a patient with severe pulmonary infection. It showed an apparent resistance to LZD and was proved to have C2190T and C2561Y point mutations in the 23S rRNA and carry the *cfr* gene.

Case report

In August 2012, a 92-year old female who suffered recurrent pulmonary infections in recent two years was admitted to the geriatrics department because of the onset of cough and sputum for two days. The following CT scan showed that she got pneumonia (*Figure 1*). She was started with

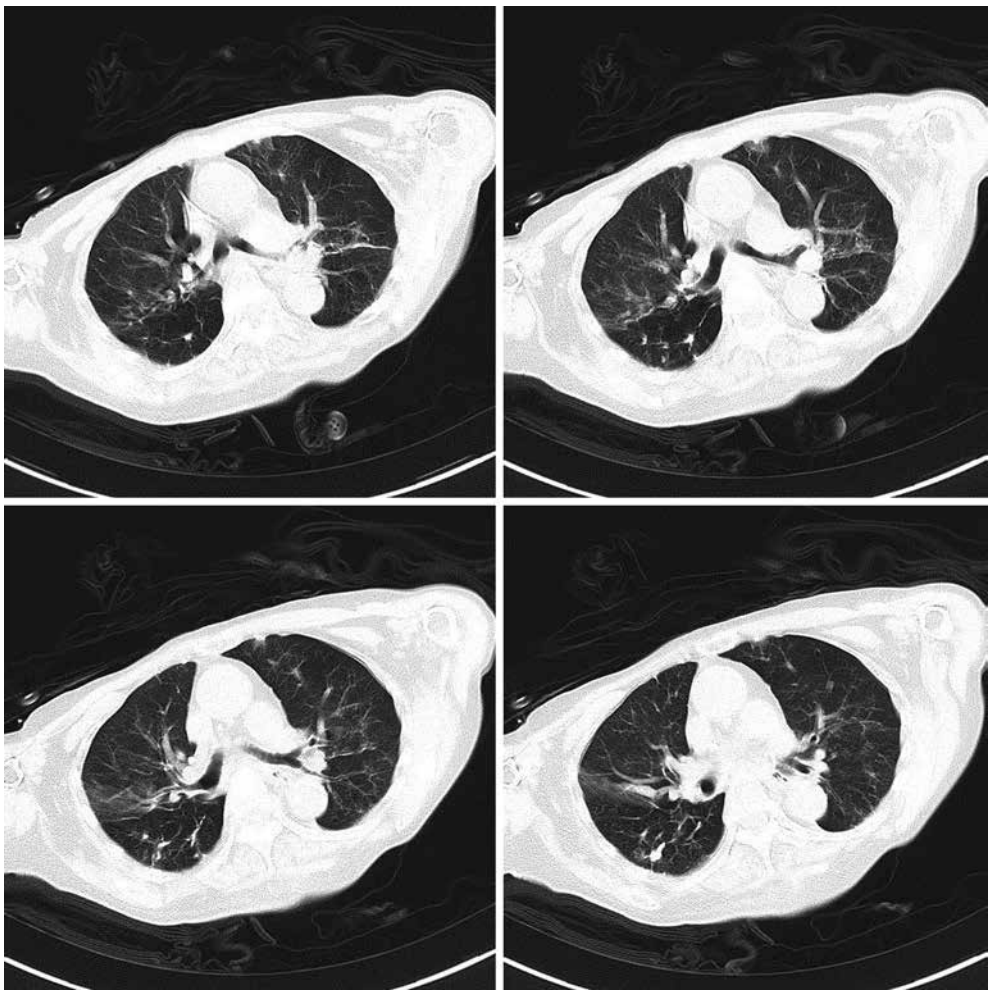

Figure 1 Chest CT scans shown signs of pneumonia.

ceftazidime on an empirical treatment with moxifloxacin but showed no response to the treatment and developed a fever (39.0 ℃) two days after admission. The subsequent bacterial cultures showed the coexistence of *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Aspergillus* in her sputum sample and *Enterococcus avium* in the urine sample. Then we used diflucan to resist the fungal and ceftazidime, cefoperazone/sulbactam, imipenem, fosfomycin and tigecycline successively to combat the bacterial infection. To resist the gram-positive *cocci*, LZD was used on the 20th day after the patient's admission with a dosage of 1,200 mg/d, and then the drug was discontinued 11 days later. Though the count of white blood cells (WBC) came to a transient decrease (drop from 19.5×10^9/L to 11.8×10^9/L) after the use of the antibiotics above, the patient eventually died of a sudden onset of ventricular tachycardia on the 35th day after admission. Patient in this case had long-term hospitalization history because of her recurrent pulmonary infection and had accepted lots antibiotics treatments. During her last hospitalization, the patient accepted airway intubation because of typeII respiratory failure and femoral vein catheterization to conduct hemodialysis for her renal failure. Because of her continued fever (varied between 37.0 and 39.0 ℃), we conducted bilateral double bottles for blood cultures repeatedly to confirm the existence of bacteremia. We performed blood culture for three times, the first two (sampling on the 15th and 21th days after admission, respectively) both showed negative results whereas the last one (sampling on the 28th day after admission) showed a positive result. A strain of *Staphylococcus capitis* was isolated in last blood culture by using Viteck 2 compact (*Figure 2A,B*), and the strain showed resistance to LZD using the K-B method (*Figure 2C*) and also show high resistance with a MIC of 64 μg/mL using

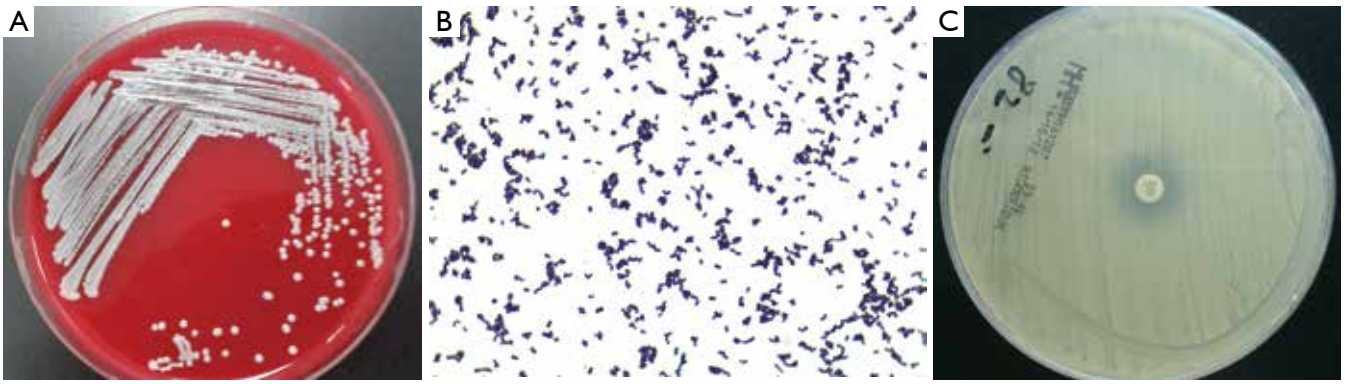

Figure 2 Microbiological examinations. (A) Isolation of the *S. capitis* from the blood using blood agar; (B) *Morphology of S. capitis under Microscope using* Gram stain; (C) *S. capitis* shown resistance to LZD by K-B method. LZD, Linezolid.

the broth dilution method. The *Staphylococcus capitis* also showed resistance to penicillin, pipercillin/tazobactam, cefepime, amikacin, levofloxacin, clindamycin while it was susceptible to cotrimoxazole, vancomycin and teicoplanin. Antimicrobial sensitivity test was conducted by the K-B disc diffusion method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (15). Domain V region of the *23S rRNA* gene spanning 2001 to 2597bp (E. coli numbering) was amplified. Oligonucleotide premiers 5'-TGG GCA CTG TCT CAA CGA-3' and 5'-GGA TAG GGA CCG AAC TGT CTC-3' were used to amplify a 596bp fragment. Polymerase chain reaction (PCR) conditions were 30 cycles consisting of 94 ℃ for 1 min, for 30 s at 50 ℃ sec, and 72 ℃ for 1 min. The PCR fragments (596 bp) were purified and sequenced and the strain was found to have C2190T and C2561Y point mutations in the 23S rRNA. We also confirmed the existence of *cfr* gene in the *Staphylococcus capitis* using the method of PCR which was described in previous reports (16,17).

Disccusion

LZD is used for the treatment of infection with gram-positive bacteria, and had curative effect constantly since it was approved in 2000. Data from the USA and global surveillance studies report of LZD resistance were <1% of *Staphylococcus aureus* and 2% of coagulase-negative *Staphylococcus* (10,18-21).

A systematic review has shown that the mean time of LZD therapy reported prior to isolation of LZD-resistance coagulase-negative was 20 months, significant longer than case of LZD-resistance *Staphylococcus* (11 days) (22). In our case, the LZD resistance *Staphylococcus capitis* was isolated from the patient's blood sample only eight days after the use of LZD, which suggested that short-term use of LZD may also induce the LZD-resistance coagulase-negative *Staphylococcus. T*he *cfr* gene which is capable of transmitting horizontally between species is also an important factor leading to the LZD resistance. So we suggest that effective infection control measures should be enhanced to prevent the spread of multi-drug resistant strains.

The LZD resistance *S. capitis* in this case was isolated from a blood sample, which was documented in the study of Gu B *et al.* (22) that the most common samples cultured the LZD resistance coagulase-negative *Staphylococcus.* So when patients undergo conditions similar to this case: suffering a symptom of high fever, receiving invasive operations such as airway intubation, deep vein catheterization and so on, it is important for the clinicians to conduct blood culture to confirm whether there is a *Staphylococcus* induced blood stream infection. Timely detection of LZD resistance *Staphylococcus* is of great significance in the rational use of antibiotics and avoiding the emergence of multi-drug resistance bacteria.

Regarding to the mechanism of LZD resistance, the main explanation is mutations in the domain V region of the *23S rRNA* gene. While the C2190T mutation was reported in several isolates of *S. homin* (23) , this is the first report, to our knowledge, of C2190T and C2561Y mutations in *Staphylococcus capitis*. The isolate was also PCR-positive for the *cfr*, a gene located on a transferable element, which indicated a potential to disseminate horizontally among Gram-positive pathogenic strains.

This article systematically reviews the published literature for case reports of LZD-resistant coagulase-negative Staphylococcus (LRCoNS) (*Table 1*). In all reported cases, strains including *S. cohnii*, *S. epidermidis*, *S. lugdunensis*, *S. hominis* and *S. kloosii* were isolated from aseptic sample,

Table 1 Clinical information and mechanisms of LZD-resistant coagulase-negative Staphylococcus

Author (Reference)	Strains	Sample type	Isolated time	Location	Method	Susceptible drugs *in vitro*	Treatment and outcome	LNZ use before LRS	Resistant mechanism
Mendes *et al*., 2012 (24)	*S. cohnii, S. epidermidis*	Blood, abdominal fluid	Aug-Oct 2009	Mexico	Broth microdilution	Tigecycline, teicoplanin, doxycycline, daptomycin, vancomycin	ND		L3/L4 mutation
Lincopan *et al*., 2009 (25)	*S. epidermidis*	Catheter tip	Mar 2008	Brazil	DD, Etest, MicroScan	ND	LZD, died		G2603T
Liakopoulos *et al*., 2010 (26)	*S. epidermidis*	Blood	May 2008	Greece	Vitek 2, Etest, broth microdilution	Daptomycin, erythromycin, teicoplanin, tigecycline, vancomycin	ND		T2504A
Kalawat *et al*., 2011 (27)	*S. lugdunensis, S. hominis*	Catheter tip	Jun 2010	India	DD, Hi-Combi strips	Vancomycin, teicoplanin	ND	ND	ND
Peer *et al*., 2011 (28)	*S. cohnii, S. kloosii*	Blood	Jul 2009, Feb 2010	India	Etest, broth microdilution	Vancomycin, teicoplanin, ciprofloxacin, amikacin	ND	1: LZD; 1: ND	ND
Gupta *et al*., 2012 (29)	*S. haemolyticus*	Pus	2011	India	KB, microdilution	Amikacin, teicoplanin, clindamycin	ND	LZD	A2503
Feßler *et al*., 2014 (30)	*S. haemolyticus*	Bronchoalveolar lavage fluid	ND	Germany	Vitek, broth microdilution	Co-trimoxazole, glycopeptides	LZD, died	LZD	cfr

Abbreviations: ND, not data; DD, disc diffusion; LZD, Linezolid.

which included blood, pus, bronchoalveolar lavage fluid. Nevertheless blood sample was the vast majority. LRCoNS were reported worldwide, including North America, South America, European and Asia. All these strains reported were conducted the susceptibility test by broth microdilution. The mechanisms for LZD resistance were L3/L4 mutation, G2603T, T2504A, A2503 mutations in the 23S rRNA and the presence of a transmissible *cfr* ribosomal methyltransferase. The outcome of the LRCoNS infected patients is obscure, however, two cases mentioned the patients were died in the end, which had the same outcome in our report.

In conclusion, though the LZD-resistant *Staphylococcus* is still sporadic now, the prolonged hospital stays, frequent interventions and abuse of antibiotics may accelerate the dissemination of LZD resistance *Staphylococcus*. Judicious use of LZD and surveillance of resistance in staphylococci are necessary to preserve the therapeutic efficacy of this important antimicrobial.

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Effusion cytology: an effective method for the diagnosis of pulmonary lymphangioleiomyomatosis

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Abstract: Lymphangioleiomyomatosis (LAM) is a rare progressive lung disease. Chylous effusion is one of the most common clinical manifestations of LAM. Herein we present a 39-year-old female who presented progressive dyspnea on exertion and chylothorax. The chest computed tomography showed multiple thin-walled cysts in both lungs which suggesting LAM. The pleural effusion cytology plus with further immunocytochemistry confirmed the computed tomography diagnosis. Therefore, the LAM can be diagnosed by cytologic examination combined with conventional chest computed tomography and clinical manifestations, which can help some patients to avoid an invasive biopsy.

Keywords: Lymphangioleiomyomatosis (LAM); immunocytochemistry; cytology; diagnosis

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Introduction

Lymphangioleiomyomatosis (LAM), a rare progressive lung neoplasm belonging to the family of perivascular epitheloid cell tumors, mainly affects females of childbearing age. It occurs sporadically or in association with tuberous sclerosis complex (TSC) (1-3). LAM is characterized by the neoplastic smooth muscle-like LAM cells and the diffuse cystic destruction of the lung parenchyma (4,5). Patients with LAM usually present progressive dyspnea on exertion, recurrent pneumothorax, chylous effusions and occasional haemoptysis (1,6). According to the recent European Respiratory Society (ERS) guidelines for the diagnosis and management of LAM, a definite diagnosis of LAM can be established by lung biopsy or high-resolution computed tomography (HRCT) combined with typical clinical features (7). In fact, the cytologic diagnosis of LAM for pleural, ascitic or pericardial fluid samples has already proven feasible and dependable (8-12). However, this effective method was not raised in the ERS guidelines. Here we present an additional case of LAM diagnosed by effusion cytology which can help the patients to avoid an invasive biopsy.

Case report

A 39-year-old female nonsmoker was admitted to our hospital because of progressive dyspnea on exertion. A chest CT scan was performed in another hospital where she was treated previously, revealing right pleural effusion. Thereafter, a chest drainage tube was placed. The fluid yielded was milky and processed for cytologic examination. Further chest CT in our hospital showed numerous cystic lesions in bilateral lungs (*Figure 1*). The cysts are round and thin-walled. Both ThinPrep cytological test (TCT) and cell block analysis for the pleural effusion were performed to detect if there were malignant cells. On TCT slides, some round spool like clusters with a 3-dimensional structure were detected in an inflammatory background interspersed with lymphocytes, granulocytes, macrophages, and some mesothelial cells (*Figure 2A*). In a close-up view, the clusters included two different kinds of cells (*Figure 2A*). The inner cells were similar in size and presented a lightly higher nuclear/cytoplasmic ratio, however, mitotic figures were not recognized. The surface of the clusters was composed of flattened cells like lymphatic endothelial cells. Neither tumor diathesis nor solitary LAM cells could be detected on

the TCT slides. On the cell block sections, the above clusters were also identified (*Figure 2B*). To make a better clarification, a panel of immunocytochemistry stainings were performed on cell block sections. The inner cells demonstrated diffuse reactivity for smooth muscle actin (SMA) (*Figure 3A*) and HMB-45 (*Figure 3B*). The outer cells were positive for D2-40 (*Figure 3C*). But the two kinds of cells were negative for Melan-A, estrogen receptor (ER), progestrone receptor (PR), thyroid transcription factor (TTF)-1, AE1/AE3, cytokeratin (CK)7, Calretinin and CK5/6.

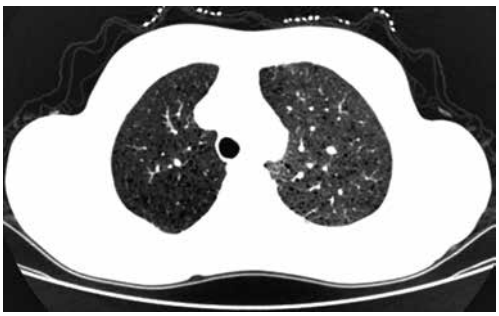

Figure 1 Chest CT revealing numerous thin-walled cystic lesions in both lung fields.

Considering the age and gender of the patient, the clinical symptom of dyspnea and chylothorax and the multicystic manifestation of chest CT, a diagnosis of LAM was highly suggested. The cytologic examination of the pleural effusion confirmed what we suspected. An abdomino-pelvic CT revealed there was no evidence of angiomyolipoma and other abdominal lesions.

Discussion

LAM is a rare and slowly progressive systemic disease, and it often causes the ignorance and delay in clinical diagnosis (6,13,14). Based on the study of Taylor *et al.*, the interval between initial symptoms and diagnosis was 44 months (14). It results in the patient losing right clinical treatment including refraining from smoking, inhaled bronchodilators, progesterone and others (7). The ERS

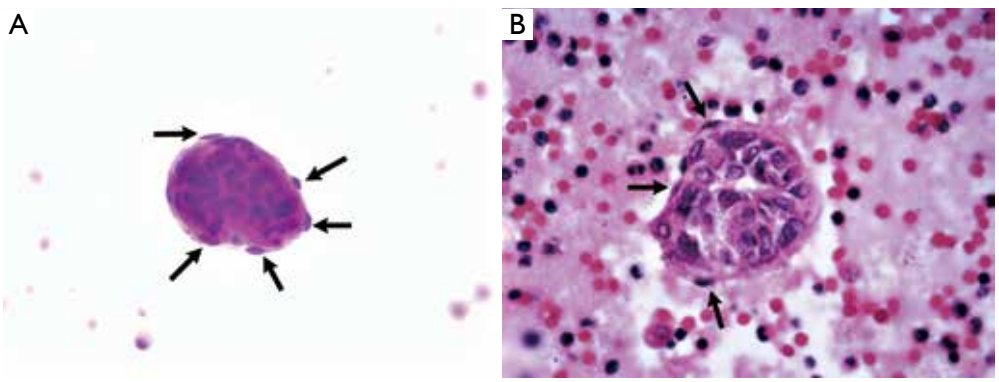

Figure 2 Hematoxylin and eosin (HE) staining (original magnification ×400). (A) On the TCT slide, LCCs were detected. Note the outer flattened cells (arrow); (B) LCCs were also identified on the cell block section. Note the outer flattened cells (arrow).

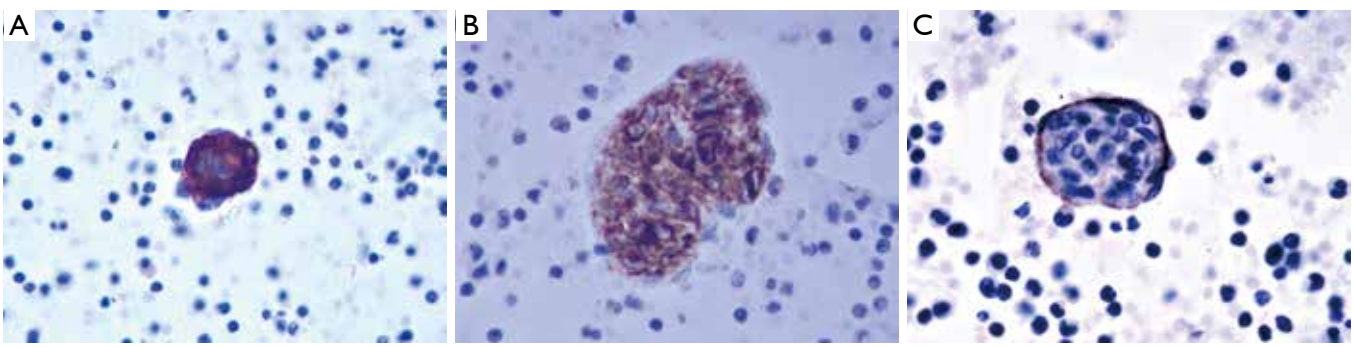

Figure 3 Immunocytochemical staining (original magnification ×400). (A) The inner cells with strong positivity for SMA; (B) The inner cells with strong positivity for HMB-45; (C) The superficial cells showing immunoreactivity for D2-40.

guidelines for the diagnosis of LAM were issued in 2010. They emphasize the role of pathological examination based on biopsy, HRCT and clinical manifestation (7). However, cytologic examination based on chylous effusion was not mentioned in the guidelines. Actually, the LAM cell clusters (LCCs) can exist in the chylous effusion has been confirmed by Itami *et al.* in 1997 (8). What is really exciting is that recognizable LCCs appear to be always detected in chylous effusion, including hydrothorax, ascites and pericardial effusion (12).

A few researchers have previously described the cytologic findings of LAM (8-12,15). The reported cases have common cytologic characters as we described. LCCs, specific feature for LAM, present 3-dimensional spheres on the smear. They are consistently composed of two different cellular components: inner cells and outer cells. The inner cells are oval or spindle, and they have similar size in appearance. The outer layer of cells is scattered, flat and looks like endothelial cells. Histologically, proliferating smooth muscle cells lead to the formation of cysts in pulmonary alveolus and interstitium around the blood vessels and small airways. Chylous effusion might be observed in the thoracic cavity when smooth muscle cells around lymphatic vessels obstructed lymphatic pathways (9,16,17). Immunocytochemical analysis is helpful for confirming the diagnosis of LAM. The inner cells usually show immunoreactivity for SMA and HMB45. In some cases they can also be positive for Melan-A, ER or PR. The superficial cells frequently express D2-40 (10,12,17,18).

In fact, chylous fluid is one of the most frequent complications of LAM (6,13,14). In a large series reported by Chu SC *et al.*, chylothorax was observed in 23% of cases during the course of the disease. Other effusions were ascites (11%) and pericardial effusion (6%) (13). Therefore, in theory, quite a number of LAM cases can be confirmed by cytologic examination. The cell block method was 15% more than the routine conventional smear method in cellularity and additional yield for malignancy (19). In addition, it can be used to perform specific pathology detections, such as molecular testing or immunocytochemistry.

The ERS diagnostic criteria for LAM highlights the function of HRCT but ignores that of conventional CT (7). Characteristic lung HRCT of LAM is multiple (>10) thin-walled round well-defined air-filled cysts throughout the lungs. And the cysts are mostly 2 mm to 2 cm in size (20). However it is difficult to popularize HRCT in every medical center, especially in developing countries. In this case, multiple cysts can also be seen clearly in both lung fields on a conventional chest CT. We believe conventional chest CT can still provide some useful information for pulmonary LAM, although its resolution is lower.

In conclusion, the diagnosis of LAM can be confirmed by cytologic examination based on chylous effusion in conjunction with conventional chest CT and clinical manifestations. This effective method can help some patients to avoid an invasive biopsy.

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Letter to the Editor

Non-invasive mechanical ventilation in postoperative esophagectomy. Is a safe and efficacy indication always?

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Dear Editor,

Acute respiratory distress syndrome (ARDS) in postoperative esophagectomy surgery associated poor outcomes (1). In this context, measurements to prevent reintubation as early use of non-invasive positive pressure ventilation (NIPPV) could reduce prevalence of complications (2). However, NIV in postoperative ARDS is still a controversial task (3).

Yu KY *et al*. (4) evaluated efficacy of NPPV in the treatment of ARDS following esophagectomy for esophageal cancer. This study opens a great contribution for management of postoperative ARDS and prevents associate complications. However, although authors described favourable effects on gas exchange after NIV at 24 hours and minor surgery-related complications, there are some key practical aspects to need comments regarding use of NIV.

First, in subgroup of ARDS patients associated with excessive airways secretions authors applied systematic use of bronchoscopy to remove secretions (5). Although, this combination of NIV-bronchoscopy is a well-known association, it is not clear if could be systematic indications for all patients and if it could be deleterious in severe high risk hypoxemic patients (6). This is not properly reflected by the authors.

Second, NPPV in early esophageal surgery may have a relative contraindication for application of positive pressure at high level (7) with and risk of loss of integrity esophageal sutures (8). This aspect is not adequately taken into account and secondary esophageal perforation is plausible.

Third, information regarding, positive pressure and respiratory parameters as tidal volume were not reported. This is interesting, because previous studies consider that limit of 15 cm H_2O for inspiratory pressure support and 5 cm H_2O for positive end-expiratory pressure are safe. What range of positive pressure was applied and recommend by authors?

In this context, achieve a more favourable outcomes in postoperative esophagectomy ARDS patients, but further clinical studies are necessary to define methodology and some key practical aspects of NIPPV.

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Erratum

Prolonged pleural catheters in the management of pleural effusions due to breast cancer: Erratum

Editorial Team, Journal of Thoracic Disease

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Erratum to: J Thorac Dis 2014;6:74-8

In the article (1) that appeared on Pages 74-78 of the February 2014 Issue of the *Journal of Thoracic Disease*, there were errors in the authors' affiliations. The correct affiliation information is as follows:

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The publisher regrets these errors.

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1. Demirhan O, Ordu C, Toker A. Prolonged pleural catheters in the management of pleural effusions due to breast cancer. J Thorac Dis 2014;6:74-8.

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