Peer review file

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Reviewer A

In the present study, the authors assessed the association between serum tumor biomarkers (CEA and CYFRA 21-1), and CT characteristics with tumor cell proliferation (Ki-67) in 182 patients with early stage (Ia and Ib) of lung adenocarcinoma. They found that several clinicopathologic features, CT evaluation and serum biomarkers were associated with Ki-67 index. Specifically, tumor shadow disappearance rate (TDR) was negatively correlated with Ki-67 and multivariate logistic regression analysis demonstrated that gender, differentiation grade, TDR and attenuation types were independent factors associated with Ki-67 expression.
Although the work is interesting for readers, some points of concern with the methodology need to be better explained.

Comment 1:

Ki-67 labelling index is a very important biomarker for clinical use in breast cancer (Editorial Comment. Is the Ki-67 labelling index ready for clinical use? Annals of Oncology 22: 500–502, 2011 editorial doi:10.1093/annonc/mdq732). However, in lung cancer usually its indecency of expression is evaluated in neuroendocrine tumors (NETs and NECs), more specifically in Neuroendocrine carcinomas of high degree (Small cell and Large cell), and to differentiate between typical and atypical carcinoid tumor (NETs). In 2013, Maki Y and colleagues published an article with the impact of GLUT1 and Ki-67 expression on early‐stage lung adenocarcinoma diagnosed according to a new international multidisciplinary classification IASLC / ATS / ERS (Travis WD, Brambilla E, Noguchi M, et al: International association for the study of lung cancer / american thoracic society / european respiratory society international multidisciplinary classification of lung adenocarcinoma. J Thorac Oncol. 6: 244–285. 2011.). These authors suggested that GLUT1 and Ki-67 play important roles in acquiring biological malignant potential in early-stage lung adenocarcinoma. After that no more important articles were published according to Ki-67 index in lung adenocarcinoma. In the present manuscript the two references included by the authors to justify the cut-off of Ki-67 index are from 2002 and 2009!

Reply 1:

Thank you for your detailed interpretation of Ki-67 research progress and constructive comment. We fully agree with your viewpoints. We acknowledged that the prognostic role of Ki-67 in lung adenocarcinoma is still in the exploratory stage. Besides, the consensus on the prognostic value of Ki-67 in lung cancer has not been reached because of the controversial results. But there were still some studies to confirm the value of Ki-67 as a potential prognostic factor in lung adenocarcinoma, which inspired us to conduct this work. A recent meta-analysis including 108 studies based on 14831 patients highlighted the prognostic value of Ki-67 expression for lung cancer (PMID: 30103737). Furthermore, some previous studies demonstrated that the high Ki-67 labeling index had an adverse prognostic impact on lung adenocarcinoma (PMID: 23076555, PMID: 32813926, PMID: 25051406). Notably, a more recent literature published in February 2021 demonstrated that Ki-67 expression was the independent prognostic factor associated with either overall survival or disease-free survival for lung adenocarcinoma (PMID: 33626488). Consistently, in terms of our unpublished results in another study, Ki-67 was an indicator independently associated with disease-free recurrence in early-stage lung adenocarcinoma and the Ki-67-based nomogram performed well in identifying high-risk patients to initiate adjuvant chemotherapy. Consequently, though the prognostic role of Ki-67 in lung adenocarcinoma is currently under exploration, we believe Ki-67 expression harbors potential in survival estimation and risk stratification in lung adenocarcinoma, which is worth investigating.

Changes in the text:

We have made the following changes in the revised manuscript as suggested.

(1) we have updated the references with the following latest literature (see Page 22, line 465-477):

"Wei DM, Chen WJ, Meng RM, et al. Augmented expression of Ki-67 is correlated with clinicopathological characteristics and prognosis for lung cancer patients: an up-dated systematic review and meta-analysis with 108 studies and 14,732 patients. Respir Res 2018;19:150.

Maki Y, Soh J, Ichimura K, et al. Impact of GLUT1 and Ki-67 expression on early‐stage lung adenocarcinoma diagnosed according to a new international multidisciplinary classification.

Oncol Rep 2013;29:133-40.

Li Z, Li F, Pan C, et al. Tumor cell proliferation (Ki-67) expression and its prognostic significance in histological subtypes of lung adenocarcinoma. Lung Cancer 2021;154:69-75.

Warth A, Cortis J, Soltermann A, et al. Tumour cell proliferation (Ki-67) in non-small cell lung cancer: a critical reappraisal of its prognostic role. Br J Cancer 2014;111:1222-9."

(2) We have modified our text as advised with the following sentences to explain the prognostic role of Ki-67 in lung adenocarcinoma based on the above references in the introduction section (see Page 6-7, line 132-136):

"A recent meta-analysis including 108 studies based on 14831 patients highlighted the prognostic value of Ki-67 expression for lung cancer (9). Furthermore, some previous studies demonstrated that high Ki-67 labeling index (LI) had an adverse prognostic impact on ADC (10-12)."

Comment 2:

Here, the first concern arises in relation to the experimental design of the work. The new WHO edition that will be published now in 2021 divides adenocarcinomas into:

1.3 Epithelial Tumours

1.3.3 Precursor Glandular Lesions

1. Atypical Adenomatous Hyperplasia (AAH)
2. Adenocarcinoma in Situ (AIS)

1.3.4 Adenocarcinomas

1. Minimally invasive adenocarcinoma
2. Invasive non-mucinous adenocarcinoma
3. Invasive mucinous adenocarcinoma
4. Colloid adenocarcinoma of the lung
5. Fetal adenocarcinoma of the lung
6. Enteric-type adenocarcinoma of the lung

Of note:

- **Invasion is defined as follows:**
  - **A recognized histological pattern other than lepidic**
  - **Myofibroblastic stroma associated with invasive tumour cells (neofibroplasia)**
  - **Spread in air spaces (STAS), lymphatic, vascular, pleural**

In situ adenocarcinoma (AIS) and minimally invasive adenocarcinoma (MIA): lesions ≤ 30mm (ground glass, sub-solid, solid) The authors should include in the M&M section the size and characteristics of AIS and MIA on CT.

Reply 2:

Thank you for your constructive comment. The patients with adenocarcinoma in situ and minimally invasive adenocarcinoma have excellent prognoses with the recurrence-free survival rate of nearly 100% 5 years after radical surgical resection (PMID: 29748007, PMID: 23242438). Thus, this study excluded those patients and exclusively focused on the five histological subtypes of invasive lung adenocarcinoma (lepidic, acinar, papillary, micropapillary, and solid predominant adenocarcinoma).

Changes in the text:

We have added the following exclusion criterion in the Methods section (see Page 8, line 170-171):

"(a) patients with adenocarcinoma in situ and minimally invasive adenocarcinoma, which have excellent prognoses after radical surgical resection;"

Comment 3:

Invasive non-mucinous adenocarcinoma:

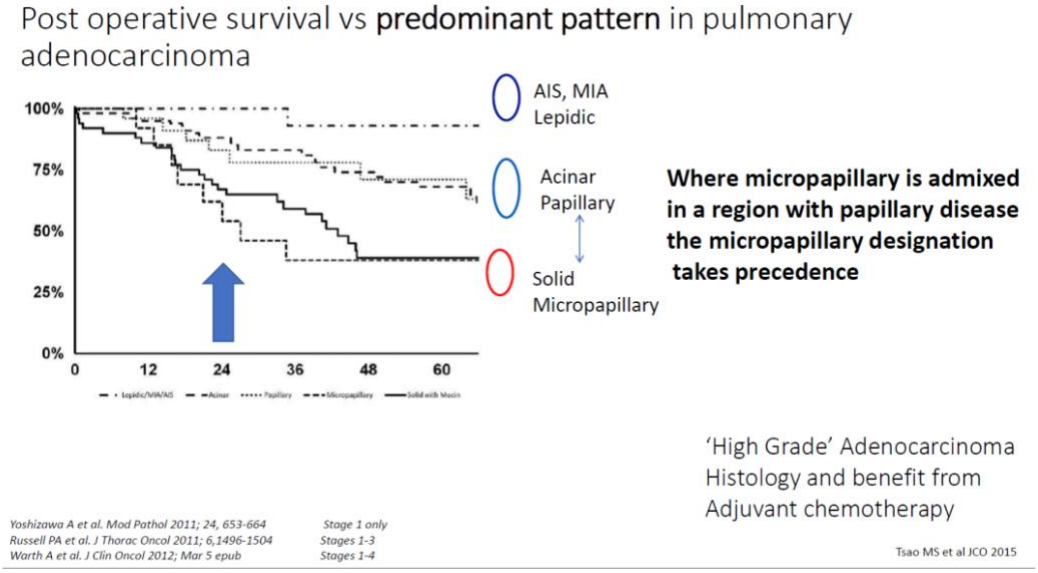
Definition

Invasive non-mucinous adenocarcinomas are non-small cell lung carcinomas with morphological or immunohistochemical evidence of glandular differentiation, with greater than 5mm of invasion and not fulfilling criteria of other adenocarcinoma types.

Essential:

Malignant epithelial tumour with

- glandular differentiation by architecture (lepidic, acinar, papillary, micropapillary, cribriform), or
- in a non-small cell carcinoma with a pure solid pattern with:
- a) immunohistochemical expression of pneumocyte markers associated with adenocarcinoma (eg TTF1 or Napsin A) or
- b) histochemical demonstration of intracytoplasmic mucin (eg DPAS) in a solid tumour in at least 5 tumour cells in each of two high power fields (approximately 0.4 mm^2).



The authors did not classify the lung adenocarcinomas according to the predominant pattern (lepidic, acinar, papillary, micropapillary, cribriform and solid), which present different behavior as shown by the KM survival curve. This indicates that Ki-67 index certainly should be different (and not prominent in Lepidic Lesions) among the non-mucinous and mucinous lung adenocarcinoma. Without this classification it is difficult to conclude or to suggest that Ki-67 presents implications on malignant behavior of early lung adenocarcinoma and that is associated with CT image and serum markers.

Reply 3:

Thank you for your detailed interpretation of histological classification for lung adenocarcinoma and your constructive comment. We have classified invasive lung adenocarcinoma into five subtypes (lepidic, acinar, papillary, micropapillary and solid adenocarcinoma) according to the predominant pattern. Strikingly, we indeed found that Ki-67

expression differed significantly among histological subtypes with an increasing trend across lepidic, acinar, papillary, micropapillary and solid adenocarcinoma, as you expected. Consistently, tumors with micropapillary and/or solid components had higher Ki-67 expression than those without micropapillary and/or solid components. We're sorry that an analysis on the Ki-67 difference between non-mucinous and mucinous lung adenocarcinoma is unavailable, because no mucinous lung adenocarcinoma was included in this small sample-size study. Further large sample-size work should be conducted to make up for this drawback.

Changes in the text:

(1) We have added the following sentences to explain the histological subtypes and grading of invasive lung adenocarcinoma in the Introduction section (see Page 6, line 123-129):

"The International Association for the Study of Lung Cancer (IASLC), American Thoracic Society (ATS), and European Respiratory Society (ERS) have proposed a classification system for ADC in 2011 to classify invasive adenocarcinoma into different subtypes according to the predominant histological pattern (5). Recently, a new grading system for invasive ADC was endorsed by the IASLC pathology committee, which took the proportion of high-grade patterns into consideration and revealed a superior performance in prognostic classification (6)."

(2) We have added the following paragraph to explain the histological evaluation and grading of invasive lung adenocarcinoma in the Methods section (see Page 9, line 177-186):

*"**Histological evaluation and grading***

Invasive ADC was classified into five subtypes of lepidic (LPA), acinar (APA), papillary (PPA), micropapillary (MPA) and solid predominant adenocarcinoma (SPA) based on histological patterns (5). According to the new grading system endorsed by the IASLC pathology committee in 2020 (6), invasive ADC was categorized into three histological grades: well-differentiated (LPA with no or less than 20% of high-grade patterns); moderately-differentiated (APA or PPA with no or less than 20% of high-grade patterns); and poorly-differentiated ADC (any tumor with 20% or more of high-grade patterns). The high-grade patterns included solid, micropapillary, and complex glandular patterns."

(3) We have added the following paragraph to reveal the Ki-67 expression across histological subtypes of invasive lung adenocarcinoma in the Results section (see Page 12-13, line 261-269):

"As revealed in Table 3 and Fig. 3A, SPA (60[40, 60]) had the highest Ki-67 LI, followed by MPA (30[10, 40]), PPA (10[8, 20]), and APA (10[5, 27.5]), while LPA (5[3, 10]) had the lowest Ki-67 LI ($P < 0.001$). The Ki-67 subgroup of < 10% was composed of APA (40.9%), LPA (39.4%) and PPA (19.7%) with no MPA and SPA, while the Ki-67 subgroup of ≥ 50% mainly consisted of SPA (65%) and APA (25%); APA and PPA were the primary histological subtypes both in Ki-67 subgroups of 10-25% and 25-50% ($P < 0.001$; Table 3 and Fig. 3B). Accordingly, Ki-67 LI in ADC with micropapillary and/or solid components (30[10, 50]) was significantly higher than that in ADC without micropapillary and/or solid components (8[5, 15], $P < 0.001$; Fig. 3C)."

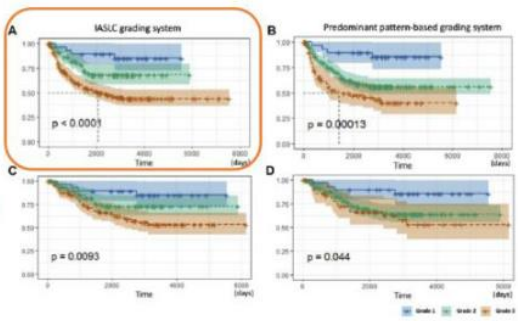
Comment 4:

Another methodologic problem with the present study concerns to the graduation system that the authors employed. The new graduation system for lung adenocarcinomas was endorsed by IASLC and should be categorized according to the criteria below.

A Grading System for Invasive Pulmonary Adenocarcinoma: A Proposal From the International Association for the Study of Lung Cancer Pathology Committee

Best model by ROC analysis is predominant pattern PLUS high-grade pattern (20% cut off)

Grade 1 – Lepidic predominant with absence or high grade disease no more than 20% of lesion
Grade 2 – Acinar or papillary predominant with absence or high grade disease no more than 20% of lesion
Grade 3 – Any tumour with 20% or more micropapillary, solid or cribriform/complex-glandular pattern adenocarcinoma



Moreira AL et l. J Thorac Oncol 2020

In my opinion, without these adjustments the results do not justify the conclusions drawn by the authors.

Reply 4:

Thank you for your comment. The new grading system was endorsed by the International Association for the Study of Lung Cancer (IASLC) pathology committee in 2020, which is based on the predominant architectures and the proportion of high-grade histological patterns. This new grading system considered tumor histological heterogeneity and offered a superior prognostic grouping than the previous grading system based on the predominant pattern only. As suggested, we have categorized invasive lung adenocarcinoma into three histological grades: well-differentiated (lepidic predominant tumors with no or less than 20% of high-grade patterns); moderately-differentiated (acinar or papillary predominant tumors with no or less than 20% of high-grade patterns); and poorly-differentiated lung adenocarcinoma (any tumor with 20% or more of high-grade patterns). The high-grade patterns included solid, micro papillary, and complex glandular patterns. According to the multivariate regression analysis, histological grade was the independent factor associated with Ki-67 expression. Moreover, we wondered whether differential expressions of Ki-67 across histological subtypes of lung adenocarcinoma could contribute to the known prognostic differences among them, which was also the underlying basis of the grading system. A recent literature indicated that the survival difference between lepidic/acinar/papillary and micropapillary/solid predominant adenocarcinoma became insignificant when Ki-67 expression was comparable, which was in favor of our assumption. However, more well-designed studies are warranted to verify these observations.

Changes in the text:

(1) We have added the following sentences to explain the histological grading of invasive lung adenocarcinoma in the Introduction section (see Page 6, line 126-129):

"*Recently, a new grading system for invasive ADC was endorsed by the IASLC pathology committee, which took the proportion of high-grade patterns into consideration and revealed a superior performance in prognostic classification (6).*"

(2) We have added the following paragraph to explain the histological estimation and grouping

of invasive lung adenocarcinoma in the Method section (see Page 9, line 177-186):

*"**Histological evaluation and grading***

Invasive ADC was classified into five subtypes of lepidic (LPA), acinar (APA), papillary (PPA), micropapillary (MPA) and solid predominant adenocarcinoma (SPA) based on histological patterns (5). According to the new grading system endorsed by the IASLC pathology committee in 2020 (6), invasive ADC was categorized into three histological grades: well-differentiated (LPA with no or less than 20% of high-grade patterns); moderately-differentiated (APA or PPA with no or less than 20% of high-grade patterns); and poorly-differentiated ADC (any tumor with 20% or more of high-grade patterns). The high-grade patterns included solid, micropapillary, and complex glandular patterns."

(3) We have added the following paragraph to explain the association between the histological grade and Ki-67 expression in the Discussion section (see Page 15-16, line 325-339):

"In this study, the new grading system proposed by IASLC pathology committee was adopted to determine the histological grade, which is based on the predominant architecture and the proportion of high-grade histological patterns instead of the predominant pattern only (6). The IASLC grading system considered the histological heterogeneity and served as a strong prognostic classifier of invasive ADC. Surprisingly, we also found that Ki-67 expression differed significantly among ADC histological subtypes with an increasing trend across LPA, APA, PPA, MPA and SPA. Furthermore, tumors with micropapillary and/or solid components had higher Ki-67 expression than those without micropapillary and/or solid components. It was figured out whether differential expressions of Ki-67 across ADC histological subtypes could contribute to the known prognostic differences among them. As we expected, a recent literature indicated that the survival difference between LPA/APA/PPA and MPA/ SPA became insignificant when Ki-67 expression was comparable (11), which was in favor of our assumption. More well-designed studies are warranted to verify these observations."

Reviewer B

Comment: This study evaluated a single center experience in the relationships among serum tumor marker index (TMI), morphological computer tomography features, and a well-established prognosticator cell proliferation (Ki-67) in stage Ⅰ adenocarcinoma.

Authors concluded that high Ki-67 expression is independently associated with male patients of stage I ADC with worse differentiation, lower TDR and solid tumors, which might be of prognostic value for poor prognosis in stage I ADC.

While this is an interesting topic, I am not persuaded that this manuscript adds new information to the immunohistochemical and radiological features of ADC.

The worse prognosis of high Ki-67 expression and/or solid component of adenocarcinoma already have been established in the published literatures.

The histological subtypes of adenocarcinoma should be added to evaluate oncological features in this study.

Reply:

Thank you for your constructive comments. We're sorry that this study failed to add new information to the immunohistochemical and radiological feature of lung adenocarcinoma due to the inherent limitations of study design. According to your suggestion and reviewer A's opinions, we have added histological subtypes of invasive lung adenocarcinoma to evaluate the Ki-67 expression across different histological subtypes and the association between Ki-67 expression and histological grade. We believe that based on your highly insightful comments, the quality of our manuscript will be substantially improved after careful revision. We would be glad to respond to any further questions and comments that you may have.

The detailed explanations and conclusions were as followed:

(1) We have classified invasive lung adenocarcinoma into five subtypes (lepidic, acinar, papillary, micropapillary and solid adenocarcinoma) according to the predominant pattern. Strikingly, we found that Ki-67 expression differed significantly among histological subtypes with an increasing trend across lepidic, acinar, papillary, micropapillary and solid adenocarcinoma. Consistently, tumors with micropapillary and/or solid components had higher Ki-67 expression than those without micropapillary and/or solid components.

(2) A new grading system for invasive lung adenocarcinoma was proposed by the International Association for the Study of Lung Cancer (IASLC) pathology committee in 2020, which was based on the predominant architectures and the proportion of high-grade histological patterns instead of the predominant pattern only. The IASLC grading system considered tumor

histological heterogeneity and offered a superior prognostic grouping than the previous grading system. Thus, we have categorized invasive lung adenocarcinoma into three grades according to the IASLC grading system: well-differentiated (lepidic predominant tumors with no or less than 20% of high-grade patterns); moderately-differentiated (acinar or papillary predominant tumors with no or less than 20% of high-grade patterns); and poorly-differentiated lung adenocarcinoma (any tumor with 20% or more of high-grade patterns). The high-grade patterns included solid, micropapillary, and complex glandular patterns.

(3) According to the multivariate regression analysis, histological grade was the independent factor associated with Ki-67 expression. We wondered whether differential expressions of Ki-67 across histological subtypes of lung adenocarcinoma could contribute to the known prognostic differences among them, which was also the underlying basis of the grading system. A recent literature indicated that the survival difference between lepidic/acinar/papillary and micropapillary/solid predominant adenocarcinoma became insignificant when Ki-67 expression was comparable, which was in favor of our assumption. However, more well-designed studies are warranted to verify these observations.

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contribute to the known prognostic differences among them. As we expected, a recent literature indicated that the survival difference between LPA/APA/PPA and MPA/ SPA became insignificant when Ki-67 expression was comparable (11), which was in favor of our assumption. More well-designed studies are warranted to verify these observations."