Diagnostic value of CD206⁺CD14⁺ macrophages in diagnosis of lung cancer originated malignant pleural effusion

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Background: Pleural effusions are common complications of various diseases. Patients with malignant pleural effusion (MPE) usually face poor prognosis and short life expectancy. Discriminating between MPE and benign pleural effusion remains to be difficult. The aim of our current study was to evaluate whether CD206⁺CD14⁺ macrophages could be a diagnostic biomarker for MPE.

Methods: The percentages of CD14⁺, CD86⁺CD14⁺ and CD206⁺CD14⁺ macrophages in pleural effusions were determined by flow cytometry in 34 patients with MPE and 26 with benign pleural effusion, and their diagnostic performances were evaluated by receiver operating characteristic (ROC) curves.

Results: The percentages of CD14⁺, CD86⁺CD14⁺ and CD206⁺CD14⁺ macrophages were remarkably higher in MPE than in benign pleural effusion (all P<0.05). With a cutoff value of 39.8%, a high sensitivity of 88.2% and high specificity of 100.0% were found in CD206⁺CD14⁺ macrophages to diagnose MPE. The area under the curve, positive predictive value and negative predictive value of CD206⁺CD14⁺ macrophages were 0.980 (95% CI, 0.905 to 0.999), 100.0 (88.4 to 100.0) and 86.7 (69.3 to 96.2), respectively. The diagnostic performance of CD206⁺CD14⁺ macrophages was more accurate than those of CD14⁺ and CD86⁺CD14⁺ macrophages.

Conclusions: CD206⁺CD14⁺ macrophages could be used to discriminate MPE from benign pleural effusion.

Keywords: Biomarker; diagnosis; macrophages; malignant pleural effusion (MPE)

Introduction

Pleural effusions are common clinical complications, with the accumulation of fluid in the pleural space. They can be caused by various diseases, including cancer, inflammation and viral infection (1). Malignant pleural effusion (MPE) occurs in 50% of metastatic malignancy and indicates poor prognosis and short life expectancy (2,3). Differentiating MPE from benign pleural effusion remains a severe challenge. Microbiological and cytological examinations are routine methods to diagnose MPE, however, their diagnostic values vary from 40% to 80% (4). Therefore, novel and efficient biomarkers are required for the diagnosis of MPE.

The cellular environment is complex in MPE, including immune cells, lymphocytes and bone marrow-derived inflammatory cells. As a pivotal component of host immune cells, macrophages play a vital role in immune response. According to different physiological conditions, macrophages can be polarized into a classically activated (M1) phenotype or an alternatively activated (M2) phenotype. M1 macrophages express CD86, CD80,

HLA-DR and promote T helper type 1 (Th1) response by secreting pro-inflammatory cytokines, whereas M2 macrophages promote T helper type 2 (Th2) response by secreting anti-inflammatory cytokines and support tumor development by promoting tumor growth, metastasis, and angiogenesis (5,6). Macrophages existing in tumor microenvironment are always known as tumor-associated macrophages, which are similar to M2 phenotype.

The macrophage mannose receptor CD206 is commonly used as a pan marker of M2 macrophages both *in vivo* and *in vitro*. Previous studies have demonstrated that CD206+ macrophages could function as a hallmark in several types of cancer. Dong and his colleagues reported that CD206+ macrophages was a good prognostic indicator for hepatocellular carcinoma (7). However, the significance of CD206+ macrophages in MPE has not been described yet. In this study, we analyzed the phenotypes of macrophages in both MPE and benign pleural effusion. CD14 was used as a pan marker for macrophages, while CD86 and CD206 were used as markers for M1 and M2, respectively. We aim to figure out whether CD206+CD14+ macrophages could be used as a reliable marker for diagnosing MPE.

Methods

Study populations

The study protocol was approved by the ethics committee of Beijing Chao-Yang Hospital. From January 2018 to December 2018, a total of 60 patients with definite diagnosis of pleural effusion were enrolled in our study, and they all signed the informed consent according to the approved guideline.

For the MPE group, MPE was obtained from 34 patients (24 male, 10 female, and aged between 38 to 87 years) with lung cancer. The diagnosis of MPE was established by the appearance of cancer cells in pleural effusion and/or on closed pleural biopsy samples. Hematoxylin and eosin (H&E) staining of pleural effusions was performed, and the results were evaluated by microscopy to confirm MPE (*Figure S1*). Histologically, 21 subjects were adenocarcinoma, 4 were squamous cell carcinoma, 2 were small cell lung cancer, and 7 were undetermined lung cancer.

Twenty-six patients (16 male, 10 female, aged between 21 and 85 years) with pleural effusion caused by tuberculosis (n=17), pneumonia (n=6), or heart failure (n=3) were defined as the benign pleural effusion group. Patients were not recruited in our study if they were subjected to

thoracic injury or if there were any invasive operations performed into the thoracic cavity within 3 months prior to their hospitalization. All patients had no history of prior treatment for tuberculosis or cancer. The subjects included in the study were all HIV-negative, with no history of corticosteroids usage or any other drugs known to affect the immunological condition.

Sample collection and processing

Pleural fluid sample (100–150 mL) from each subject was collected in tubes containing heparin when the diagnostic thoracentesis was performed within 24 hours after hospitalization. Peripheral blood sample (4 mL) was drawn at the same time. Pleural effusion and peripheral blood samples were immersed into ice and then centrifuged at 400 g for 6 minutes at 4 ℃. After centrifugation, the supernatants were stored at –80 ℃ and the cell pellets were then resuspended in phosphate buffer saline (PBS). Mononuclear cells were isolated using Lymphocyte Separation Medium (MP Biomedicals) according to the manufacturer's instructions.

Flow cytometry analysis

Red blood cells were lysed, and the isolated mononuclear cells were incubated with fluorochrome-conjugated antibodies specific for human for 20 min in the dark. These antibodies, including anti-CD45, anti-CD14, anti-CD206, and anti-CD86 mAbs, were purchased from Thermo Fisher Scientific (Waltham, MA, USA) or BD Biosciences (San Diego, CA, USA). Cells were then fixed in 4% paraformaldehyde. All experiments were analyzed by flow cytometry on a FACS Canto II (BD Biosciences). The analyses were performed with FCS Express 5 software (De Novo Software, Los Angeles, CA, USA).

Statistical analysis

Data were expressed as mean ± SEM. The significance of differences of data between the two groups was analyzed by using Student's t test. Receiver operating characteristic (ROC) analysis were analyzed, and areas under the ROC curve (AUC) were calculated to evaluate the diagnostic value of CD14+, CD86+CD14+ and CD206+CD14+ macrophages to discriminate between MPE and benign pleural effusion. Sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio

Table 1 Clinical and demographic parameters in pleural effusions

Variable	MPE (n=34)	BPE (n=26)	P value
Sex, male/female, n	24/10	16/10	
Age, year	65.7±1.9	54.8±4.3	0.015
Total cell counts, $\times 10^9$/L	2.3±0.2	2.9±0.4	0.177
Protein, g/L	42.7±1.8	45.6±2.2	0.312
Glucose, mmol/L	5.5±0.4	5.6±0.3	0.853
Lactate dehydrogenase, IU/L	566.8±92.4	364.9±50.3	0.083

Data are presented as mean ± SEM. Comparisons of data between MPE and BPE were analyzed by using Student's *t*-test. MPE, malignant pleural effusion; BPE, benign pleural effusion.

Table 2 Percentages of $CD14^+$, $CD86^+CD14^+$ and $CD206^+CD14^+$ macrophages in pleural effusions and peripheral blood

Variable	MPE (n=34)	BPE (n=26)	P value
Pleural effusion			
$CD14^+$ cells, %	20.2±2.9	6.8±1.4	<0.001
$CD86^+CD14^+$ cells, %	16.7±1.5	11.3±1.1	0.008
$CD206^+CD14^+$ cells, %	62.1±2.9	19.5±2.1	<0.001
Blood			
$CD14^+$ cells, %	15.4±1.3	14.7±1.4	0.704
$CD86^+CD14^+$ cells, %	1.6±0.2	1.1±0.1	0.091
$CD206^+CD14^+$ cells, %	0.2±0.1	0.3±0.1	0.311

Data are presented as mean ± SEM. Comparisons of data between MPE and BPE were analyzed by using Student's *t*-test. Comparisons of data in pleural effusion and in the corresponding blood were analyzed by using paired *t*-test. MPE, malignant pleural effusion; BPE, benign pleural effusion.

and negative likelihood ratio were also analyzed (8). These parameters and their 95% confidence intervals (CIs) were also evaluated together in this study. The optimal cutoff points were established on their maximum Youden's index. The Statistical Program for Social Science (SPSS) 19.0 and MedCalc software were used to conduct the analyses, and differences were assumed significant when P<0.05.

Results

Clinical and demographic parameters of patients with pleural effusions

Some clinic pathological characteristics of patients with MPE and benign pleural effusion are summarized in *Table 1*. In this study, patients with MPE were elder than those with benign pleural effusion (P=0.015). There is no difference in the cytological, clinical and demographic characteristics of

pleural effusions between the two groups (all P>0.05).

Numbers of $CD14^+$, $CD86^+CD14^+$ and $CD206^+CD14^+$ macrophages in pleural effusions

As shown in *Table 2* and *Figure 1*, the percentages of $CD14^+$, $CD86^+CD14^+$ and $CD206^+CD14^+$ macrophages in MPE were statistically higher than those in benign pleural effusion (95% CIs for the differences were 6.2% to 20.5%, 1.4% to 9.3% and 35.0% to 50.1%, respectively; all P<0.05). In addition, CD86 and CD206 were barely detected in the peripheral blood monocytes. The numbers of $CD86^+CD14^+$ and $CD206^+CD14^+$ cells in MPE were statistically higher than their corresponding ones in blood (95% CIs for the differences were 12.0% to 18.1% and 56.1% to 67.6%, respectively; both P<0.001). The percentages of $CD86^+CD14^+$ and $CD206^+CD14^+$ cells in
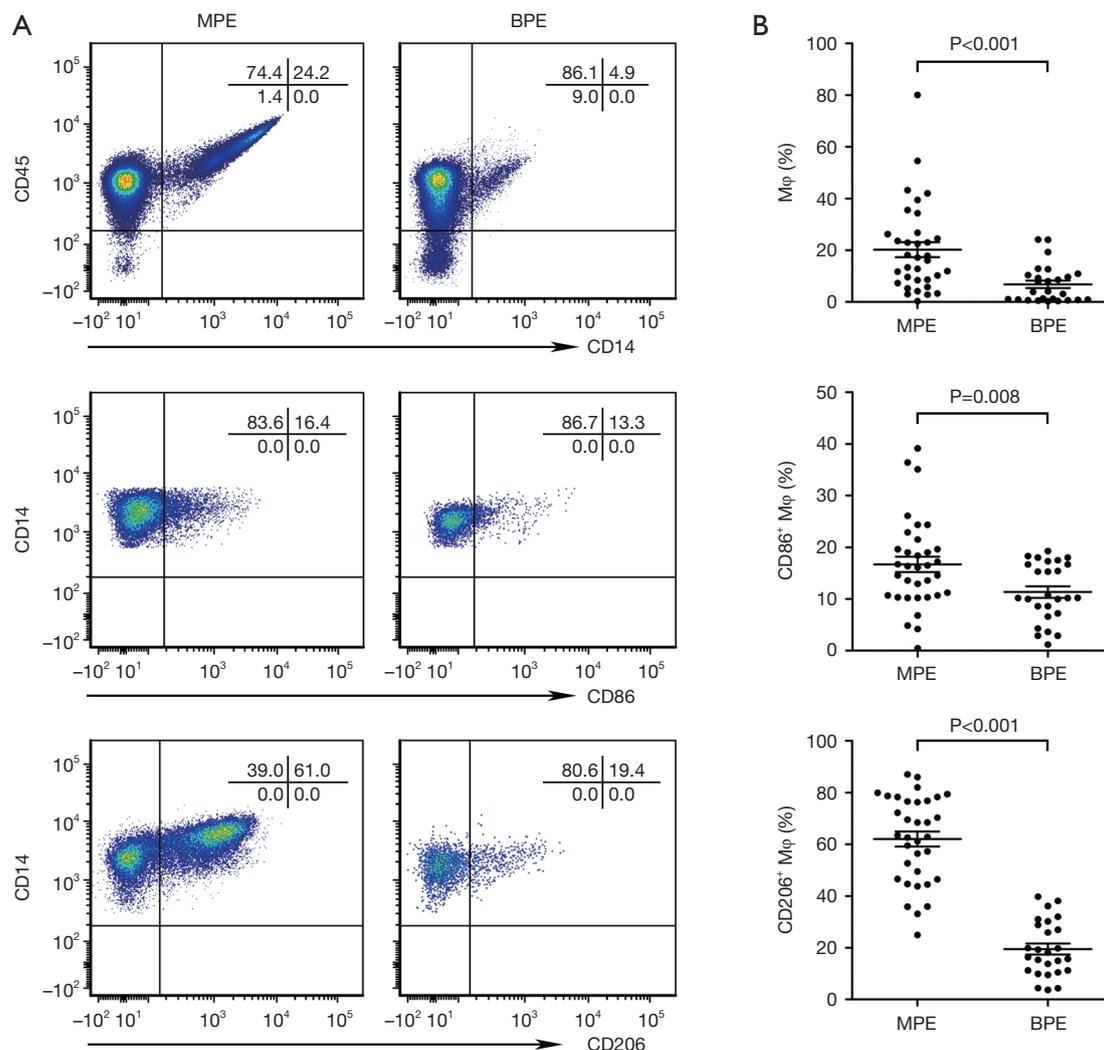
Figure 1 Characterizations of different subsets of macrophages in MPE and BPE. (A) Representative flow cytometry dot plots of Mφ (CD45⁺CD14⁺), CD86⁺ and CD206⁺ Mφ (gated in CD45⁺CD14⁺ cells) frequency in MPE and BPE. (B) Comparisons of percentages of Mφ, CD86⁺ and CD206⁺ Mφ in MPE and those in BPE. Data are presented as mean ± SEM. Comparisons of data between MPE and BPE were analyzed by using Student's *t*-test. MPE, malignant pleural effusion; BPE, benign pleural effusion.

benign pleural effusion were also much higher than those in blood (95% CIs for the differences were 7.9% to 12.4% and 15.0% to 23.4%, respectively; both P<0.001).

Diagnostic values of CD14⁺, CD86⁺CD14⁺, and CD206⁺CD14⁺ macrophages in pleural effusions

The high levels of CD14⁺, CD86⁺CD14⁺ and CD206⁺CD14⁺ macrophages in MPE indicated that they may serve as potential biomarkers to diagnose MPE. Thus, their diagnostic values to discriminate malignant

from benign pleural effusion were analyzed with the ROC analyses. The AUC of CD14⁺ macrophages in the diagnosis of MPE was 0.788 at a cutoff level of 10.9% (sensitivity, 64.7%; specificity, 80.8%). The AUC of CD86⁺CD14⁺ macrophages to diagnose MPE was 0.698 with a cutoff level of 10.2% (sensitivity, 88.2%; specificity, 50.0%). The highest diagnostic accuracy was achieved in CD206⁺CD14⁺ macrophages with the AUC of 0.980 at the cutoff level of 39.8% (sensitivity, 88.2%; specificity, 100.0%) (*Figure 2*).

The diagnostic parameters of CD14⁺, CD86⁺CD14⁺ and CD206⁺CD14⁺ macrophages in pleural effusions,
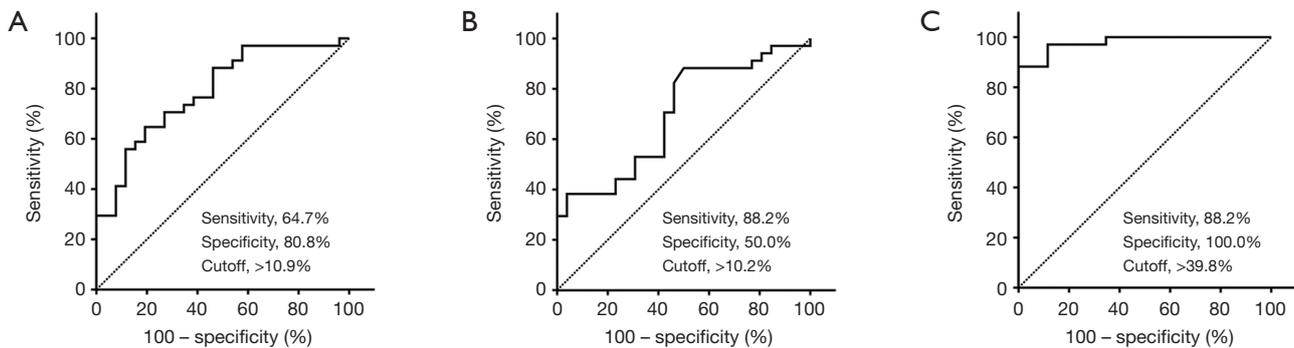
Figure 2 Diagnostic values of CD14$^+$, CD86$^+$CD14$^+$ and CD206$^+$CD14$^+$ macrophages in diagnosing patients with malignant pleural effusion (MPE) from those with benign pleural effusion (BPE). (A) ROC analysis of CD14$^+$ macrophages for the detection of MPE with AUC of 0.788. (B) ROC analysis of CD86$^+$CD14$^+$ macrophages for the detection of MPE with AUC of 0.698. (C) ROC analysis of CD206$^+$CD14$^+$ macrophages for the detection of MPE with AUC of 0.980. ROC, receiver operating characteristic; AUC, area under curve.

Table 3 Diagnostic values of CD14$^+$, CD86$^+$CD14$^+$ and CD206$^+$CD14$^+$ macrophages in discriminating malignant pleural effusion from benign pleural effusion

Variable	Cutoff (%)	AUC (95% CI)	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)	PLR (95% CI)	NLR (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)
CD14$^+$ cells	10.9	0.788 (0.664 to 0.883)	64.7 (46.5 to 80.3)	80.8 (60.6 to 93.4)	3.4 (1.5 to 7.7)	0.4 (0.3 to 0.7)	81.5 (61.9 to 93.7)	63.6 (45.1 to 79.6)
CD86$^+$CD14$^+$ cells	10.2	0.698 (0.566 to 0.810)	88.2 (72.5 to 96.7)	50.0 (29.9 to 70.1)	1.8 (1.2 to 2.6)	0.2 (0.1 to 0.6)	69.8 (53.9 to 82.8)	76.5 (50.1 to 93.2)
CD206$^+$CD14$^+$ cells	39.8	0.98 (0.905 to 0.999)	88.2 (72.5 to 96.7)	100.0 (86.8 to 100.0)	–	0.1 (0.1 to 0.3)	100.0 (88.4 to 100.0)	86.7 (69.3 to 96.2)

PPV, positive predictive value; NPV, negative predictive value; PLR, positive likelihood ratio; NLR, negative likelihood ratio; AUC, area under the curve.

including sensitivity, specificity, positive likelihood ratio, negative likelihood ratio, positive predictive value and negative predictive value, are presented in *Table 3*. Based on these parameters, it was distinguished that CD206$^+$CD14$^+$ macrophages had the highest diagnostic performance compared with CD14$^+$ and CD86$^+$CD14$^+$ macrophages.

Discussion

MPE is a distressing condition which occurs at advanced stages of various malignancies. In the United States, almost 40,000 people are affected by MPE every year, and patients suffering from MPE usually face worse life quality and poor prognosis (2). The diagnosis between malignant and benign pleural effusion remains a difficult problem, since a large variation in diagnostic accuracy of pleural fluid cytology ranges from 60% to 90% (9,10). Tumor markers are not suitable for diagnosing MPE, because their sensitivity and

specificity are relatively low (11,12). Medical thoracoscopy is considered to be a gold-standard method in diagnosing MPE. However, as an invasive technique, it may cause complications and increase morbidity (13-15). Therefore, novel tests and markers are called for discriminating between MPE and benign pleural effusion. As far as we know, our current study is the first attempt to evaluate the diagnostic value of CD206$^+$CD14$^+$ macrophages in MPE.

There are plentiful studies demonstrated the clinical significance of CD86$^+$ and CD206$^+$ macrophages in various disease. In colorectal cancer, CD86$^+$ macrophages were used to indicate a better prognosis (16). In hepatocellular carcinoma, CD206$^+$ macrophages in primary tumor sites were associated with poor prognosis (17). In renal cell carcinoma, high percentage of CD206$^+$ macrophages could be used as a promising biomarker for poor survival (18). However, the role of CD86$^+$ and CD206$^+$ macrophages in MPE is poorly understood. Therefore, we present the

first data on the percentages of CD14$^+$, CD86$^+$CD14$^+$ and CD206$^+$CD14$^+$ macrophages in malignant and benign pleural effusions and analyze their diagnostic values for the diagnosis of MPE.

Previously, Chen and his colleagues reported that combinations of VEGF mRNA and endostatin mRNA provided a high-diagnostic value (sensitivity, 95.7%; specificity, 88.9%) for diagnosis of MPE (19). Pleural fluid cf-DNA integrity index was predicted as a promising biomarker for MPE with a sensitivity of 92% and a specificity of 92.6% (20). Wang *et al.* reported that CD163$^+$CD14$^+$ macrophages were indicated as a helpful marker for MPE with a sensitivity of 81.2% and a specificity of 100% (21). Our study showed that the percentages of CD14$^+$, CD86$^+$CD14$^+$ and CD206$^+$CD14$^+$ macrophages in MPE were statistically higher than those in benign pleural effusion. At a cutoff level of 10.9%, the sensitivity and specificity of CD14$^+$ macrophages to diagnose MPE were 64.7% and 80.8%, respectively. At a cutoff value of 10.2%, the sensitivity and specificity of CD86$^+$CD14$^+$ macrophages in discriminating MPE from benign pleural effusion were 88.2% and 50.0%, respectively. At a cutoff level of 39.8%, CD206$^+$CD14$^+$ macrophages had the highest diagnostic value to diagnose MPE, with a high sensitivity of 88.2% and a high specificity of 100%.

The parameters including positive predictive value, negative predictive value, positive likelihood ratio and negative likelihood ratio are also important for diagnostic analysis. The positive likelihood ratio value of CD206$^+$CD14$^+$ macrophages is infinite, which means that if CD206$^+$CD14$^+$ macrophages determination is positive, there was no possibility that the patient had benign pleural effusion. The negative likelihood ratio value is 0.1, which means that if CD206$^+$CD14$^+$ macrophages determination is negative, the chance that this patient has MPE is 10%. What's more, CD206$^+$CD14$^+$ macrophages also have a high positive predictive value of 100.0% and a high negative predictive value of 86.7%, respectively. Our current data indicate that CD206$^+$CD14$^+$ macrophages can be used as a specific marker to rule out MPE from benign pleural effusion.

It should be aware that the detection of CD206$^+$CD14$^+$ macrophages based on flow cytometry is simple and rapid. Based on the high sensitivity and high specificity of CD206$^+$CD14$^+$ macrophages for MPE, it is recommended that the detection of CD206$^+$CD14$^+$ macrophages is performed in routine clinical practice. We will further analyze the relationship between the high percentage of CD206$^+$CD14$^+$ macrophages and the overall survival of patients with MPE. Targeting tumor-associated macrophages have been reported to be a new and reasonable anticancer therapy (22-26). It is especially important to figure out the phenotypes and subsets of macrophages in MPE, and CD206$^+$CD14$^+$ macrophages may be a novel therapeutic target for treating patients with MPE.

Our study had some limitations. First, the sample size enrolled in our study was relatively small, with 34 patients with MPE and 26 with benign pleural effusion. Therefore, larger populations are required to confirm the value of this approach. Second, MPE analyzed in our study were all derived from lung cancer. Benign pleural effusions were derived from tuberculosis, pneumonia and heart failure. Pleural effusion induced by other types of malignant and benign disease will be analyzed and the diagnostic value of CD206$^+$CD14$^+$ macrophages for MPE will be confirmed in our further studies.

Taken together, our data presented that the percentages of CD14$^+$, CD86$^+$CD14$^+$ and CD206$^+$CD14$^+$ macrophages are significantly increased in MPE compared with those in benign pleural effusion. They can be used as diagnostic indicators to discriminate MPE from benign pleural effusion. In addition, the diagnostic performance of CD206$^+$CD14$^+$ macrophages is more accurate than those of CD14$^+$ and CD86$^+$CD14$^+$ macrophages.

Acknowledgments

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study protocol was approved by the ethics committee of Beijing Chao-Yang Hospital (No. 2018-Ke-195), and all patients signed

the informed consent according to the approved guideline.

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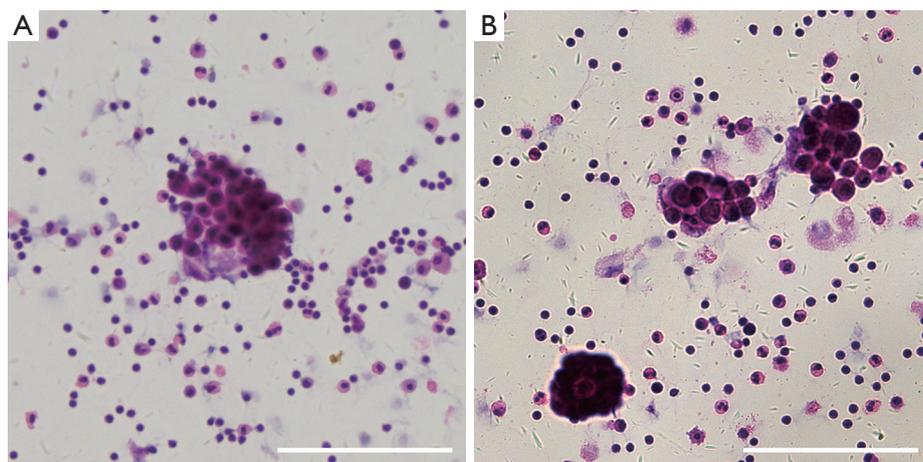
Figure S1 Cytological analysis of the MPEs. (A,B) Immune cells, mesothelial cells and cancer cells with deep-stained large nuclei were visualized in MPE derived from lung cancer. Representative images are shown; 200× magnification; scale bar, 100 μm. MPE, malignant pleural effusion.