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Exosomal MicroRNA Expression Profiling in Patients with Lung Adenocarcinoma-associated Malignant Pleural Effusion

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Abstract. *Background/Aim: Cytological analysis for diagnosing malignant pleural effusion associated with lung adenocarcinoma (Ad-MPE) shows limited sensitivity and novel diagnostic biomarkers are needed. The aim of this study was to evaluate the profile of four microRNAs (miRNAs) in exosomes in Ad-MPE and benign (non-neoplastic) pleural effusion (BPE). Materials and Methods: A total of 56 pleural effusion samples from patients with lung adenocarcinoma and benign diseases were collected. Exosomal miRNA expressions were evaluated using real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). Results: The expression levels of miR-182 and miR-210 were significantly higher in the Ad-MPE than in the BPE samples. The receiver operating characteristics curve analyses of miR-182 and miR-210 for diagnosis of Ad-MPE yielded areas under the receiver operating characteristic curves of 0.87 and 0.81, respectively. Conclusion: These miRNA signatures may have a diagnostic potential for differentiating Ad-MPE from BPE.*

Lung cancer is the most common cause of cancer-related deaths worldwide (1). Malignant pleural effusion (MPE) has been reported to be the initial presentation of cancer in 10-50% of patients, and half of all patients with lung cancer develop MPE at some time during their disease (2). Although MPE can occur in patients with all histological types of lung cancer, they are more common in patients with adenocarcinoma (3). MPEs are usually diagnosed by pathological analysis, however, the sensitivity of cytology is 50-70% even when thoracenteses are repeated, and repeated aspiration is only helpful in an additional 10% of cases (4). Therefore, establishment of a novel diagnostic marker of MPE is desired.

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Exosomes are small membrane vesicles of endocytic origin and were initially demonstrated in the peripheral circulation of patients with cancer (5). Tumor-released exosomes in peripheral blood contain microRNAs (miRNAs) (6). Exosomal miRNA reflects the miRNA signature of the parental tumor because the profiles of circulating exosomal miRNAs are similar to those of tumor-derived miRNAs and there appears to be a significant difference in exosomal miRNA levels between patients with cancer and controls (7). Therefore, circulating extracellular miRNAs have been recognized to be minimally-invasive biomarkers for cancer diagnosis (8, 9). There are some reports that describe cell-free DNA or cell-free miRNA expression in MPE associated with lung adenocarcinoma (Ad-MPE) (10-12). However, cell-free DNA and miRNAs occur in short fragments and originate from dying cells, which can further complicate molecular analysis (13). It is unclear to what extent these cells represent the genotype of the prevailing population of malignant cells (14). Exosomal miRNAs may be better diagnostic biomarkers because of their stability in body fluids, which may be explained by the fact that exosomes protect miRNAs from degradation (15). Therefore, analyzing exosomal miRNAs would provide less contaminated and more cancer-specific results than analyzing cell-free DNAs and miRNAs.

Emerging evidence suggests that several miRNAs function as biomarkers for diagnosis of lung cancer. Among those miRNAs, *miR-21, miR-31, miR-182*, and *miR-210* have been reported to be up-regulated in lung adenocarcinoma tissue and peripheral blood (16-18). However, little is known about the diagnostic significance of these miRNAs as exosome content in PE. The aim of the present study was to determine whether these miRNAs in exosome might be diagnostic biomarkers for differentiating Ad-MPE and benign PE (BPE).

Materials and Methods

Patients and sample collection. Pleural effusion samples (n=56: Ad-MPE, n=41; BPE, n=15) from patients with lung adenocarcinoma and other diseases were collected between the July 2014 and June 2018 at the University of Tokyo Hospital.

Matched serum samples were obtained from patients with lung adenocarcinoma (n=20). The time interval between collection of PE and serum samples was 0-48 days (median=1 day). These patients did not receive any systemic treatment such as chemotherapy or surgery between PE and serum sampling (three patients received local radiotherapy during this period). The samples were centrifuged at 900 × *g* for 10 min and the supernatants were obtained and stored at −80˚C for further analysis. The PE was categorized as MPE if the cytology or cell block specimen was positive for malignancy. Patients with negative cytology were also diagnosed as having MPE if they had disseminated malignancy without alternative causes for the effusion. This study was approved by the Institutional Review Board (approval number 10226-3), and written informed consent was obtained from all participants.

Extraction of exosomal miRNA. Total exosome containing miRNA was extracted from samples by using total exosome isolation reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. In brief, frozen samples were thawed at room temperature and then centrifuged at 2,000 × *g* for 30 min to remove cellular debris. A total of 1000 μl for PE and 250 μl for serum was used for downstream analysis. Each sample was combined with 0.2× the volume of total exosome isolation reagent and then mixed well. The samples were incubated at 4˚C for 30 min and then centrifuged at room temperature at 10,000 × *g* for 10 min. The exosome pellet was resuspended in phosphate-buffered saline and then stored at −20˚C.

RNA recovery using total exosome RNA and protein isolation kit. A Total Exosome RNA and Protein Isolation Kit (Invitrogen) was used according to the manufacturer's instructions with slight modifications. In brief, each sample was lysed with denaturing solution, and RNA was extracted with acid-phenol:chloroform and 100% ethanol. After washing, samples were dried by spinning and 50 μl of preheated elution solution was applied once. After centrifugation, the eluate containing the RNA was collected and stored at −20˚C.

Real-time reverse transcription quantitative polymerase chain reaction analysis of RNA sequences isolated from exosomes. A reverse transcription (RT) master mix was prepared for each sample using the TaqMan MicroRNA Reverse Transcription Kit reagents (Applied Biosystems, Foster City, CA, USA) with gene-specific RT primers for each miRNA target according to the manufacturer's instruction with slight modifications. A total of 7 μl of each sample and 3 μl of RT primer was added to 5 μl of master mix. Each tube was placed into a PC-818S thermal cycler (ASTEC, Kasuya, Fukuoka, Japan) and incubated as follows: 16˚C for 30 min, 42˚C for 30 min, and 85˚C for 5 min.

RT-quantitative polymerase chain reaction was performed using an Mx3000P QPCR instrument (Agilent Technologies, Santa Clara, CA, USA) along with TaqMan Universal PCR Master Mix II, each TaqMan small RNA assay (Applied Biosystems), and each RT reaction with the following thermocycler protocol: 50˚C for 2 min, 95˚C for 10 min, and (95˚C for 15 s and 60˚C for 60 s) for 40 cycles. All reactions were performed in triplicate. The comparative cycle threshold method was used to determine the relative expression levels of the target miRNAs with reference gene (*miR-16*).

Table I. *Patient characteristics*.

Characteristic	Value		
	Ad-MPE (n=41)	BPE (n=15)	*p*-Value
Age, years			
Median (range)	68 (49-87)	73 (37-95)	0.51
Gender, n			
Male	28	8	0.35
Female	13	7	
Smoking status, n			
Current/former	26	12	0.34
Never	15	3	
Amyloidosis, n		1	
Parapneumonic effusion, n		1	
Chylothorax, n		1	
Congestive heart failure, n		1	
Benign asbestos-related effusion, n		1	
Hypoalbuminemia, n		2	
Empyema, n		2	
Reactive effusion, n			
Post operative		1	
Inflammation, n		4	
Pneumothorax, n		1	
Metastatic site, n			
Brain	12	NA	
Bone	9		
Liver	3		
Lymph node	4		
CEA, ng/ml			
Median	46.9	1.6	<0.0001

Ad-MPE, Malignant pleural effusion associated with lung adenocarcinoma; BPE, benign pleural effusion; CEA, carcinoembryonic antigen; NA, not applicable.

Immunoassay for carcinoembryonic antigen. Levels of carcinoembryonic antigen (CEA), which is the most commonly used biomarker for lung adenocarcinoma, were measured using a chemiluminescent enzyme immunoassay (SRL, Shinjuku-ku, Tokyo, Japan) for PE and an immune enzymatic assay (Tosoh, Minato-ku, Tokyo, Japan) for serum.

Statistical analysis. Differences in age between the two groups were evaluated by performing a *t*-test. Fisher's exact tests were performed to assess the distribution of sex and smoking status between groups. The Mann–Whitney *U*-test was used to determine statistically significant differences in miRNA expression between independent groups. Spearman's rho coefficient was used to assess correlations between miRNA expression level in PE and serum, and CEA and miRNA expression level in PE. Differences with *p*-values less than 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism, version 5.04 (GraphPad Software, San Diego, CA, USA) and EZR, version 1.36 (Saitama Medical Center, Saitama-shi, Saitama, Japan, and Jichi Medical University, Shimotsuke-shi, Tochigi, Japan) (19), which is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0).

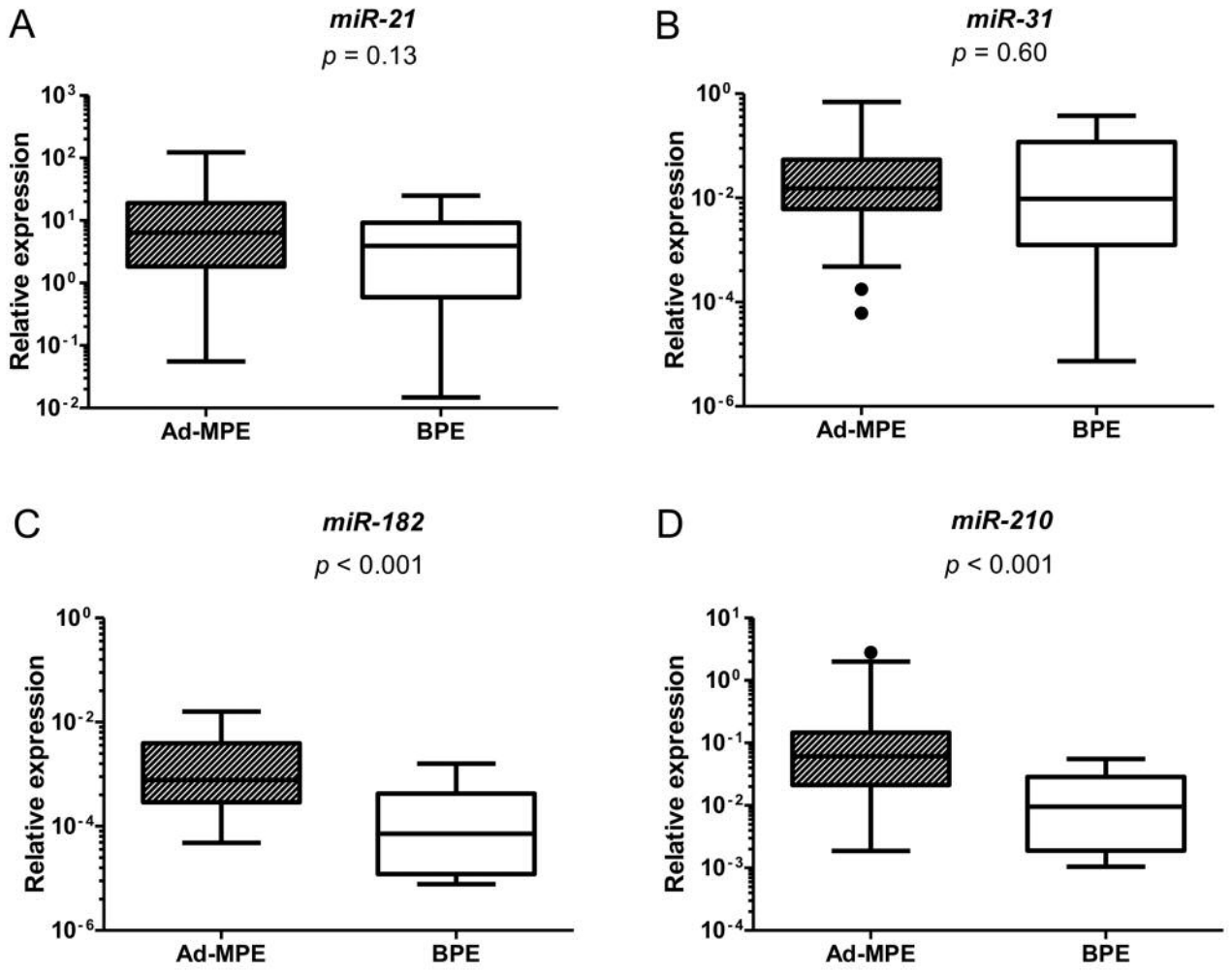

Figure 1. *Expression levels of miR-21 (A), miR-31 (B), miR-182 (C), and miR-210 (D) in malignant pleural effusion associated with lung adenocarcinoma (Ad-MPE) and benign pleural effusion (BPE). The expression levels of miR-182 and miR-210 in the Ad-MPE samples were significantly higher than those in the BPE samples. The horizontal bars indicate the median value. miR-16 was used as a reference gene.*

Results

Patient characteristics. There were no significant differences in the characteristics of the patients with regard to age, sex, and smoking status between the groups (Table I). Among 41 Ad-MPE samples, 39 were cytology-positive.

Expression levels of miRNAs and CEA in PE and serum. The expression levels of *miR-182* and *miR-210* were significantly higher in the Ad-MPE samples than in the BPE samples (Figure 1). In contrast, the expression levels of *miR-21* and *miR-31* were comparable between the two groups. The median CEA level was significantly higher in the Ad-MPE samples than in the BPE samples (46.9 ng/ml *vs*. 1.6 ng/ml, $p<0.0001$). CEA was not significantly correlated with the expression levels of miRNAs in PE (Figure 2). There were no significant differences in miRNA expression levels in PE between patients with extrathoracic metastases of lung adenocarcinoma (n=22) and those without (n=19) (*miR-21*, $p=0.49$; *miR-31*, $p=0.89$; *miR-182*, $p=0.87$; *miR-210*, $p=0.21$). Twenty patients with lung adenocarcinoma underwent serum miRNA testing (matched serum). Among them, no significant correlation was found between PE and serum miRNA expression levels (Figure 3), which seemed to imply that miRNA expression in PE was independent of that in serum.

Diagnostic performance of miRNA for Ad-MPE. The performance of each miRNA for diagnosing Ad-MPE and BPE was evaluated by receiver operating characteristics (ROC) curve analysis. The cut-off value was selected at the point of the

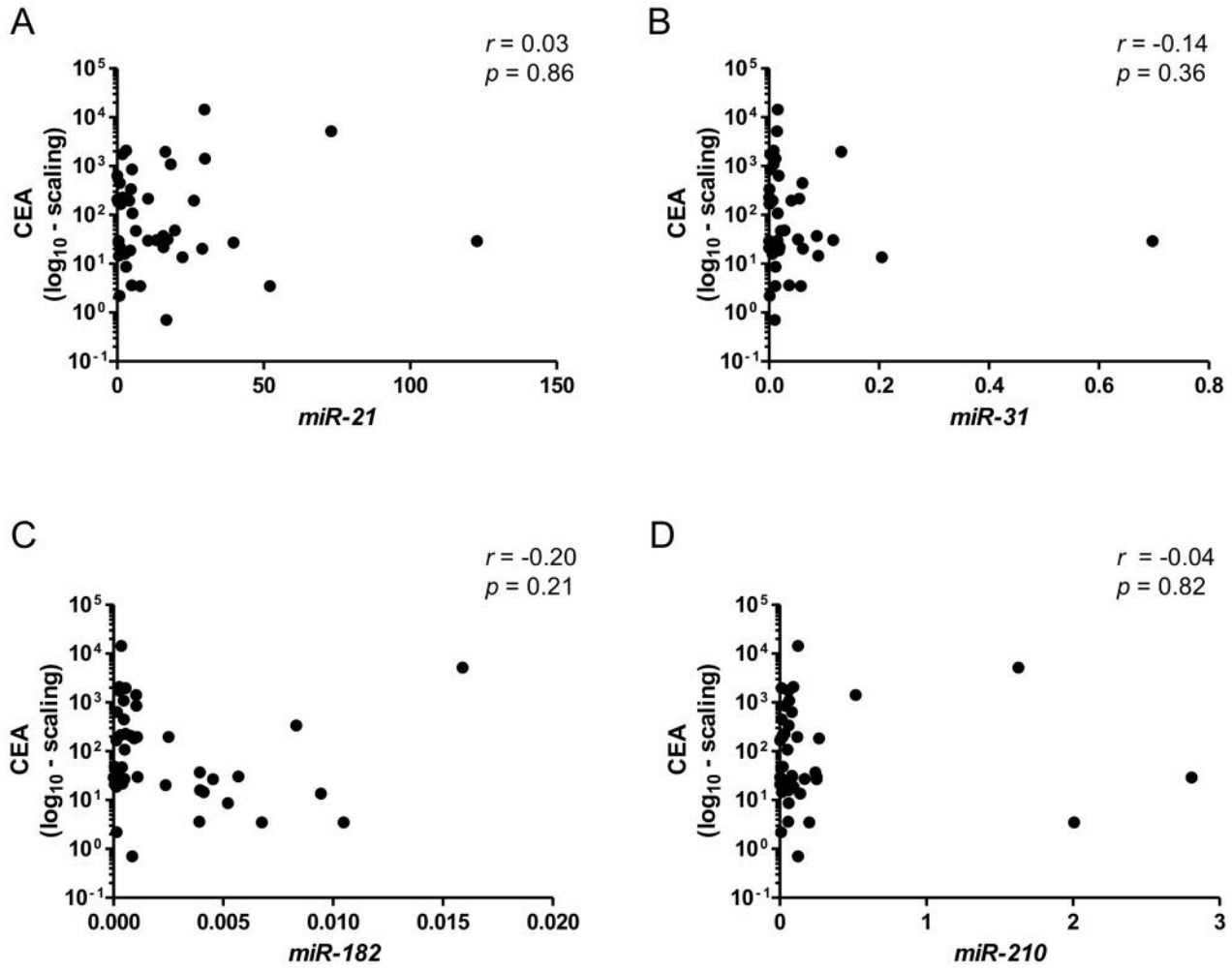

Figure 2. *Correlation between carcinoembryonic antigen (CEA) and miR-21 (A), miR-31 (B), miR-182 (C), and miR-210 (D) in pleural effusion in patients with lung adenocarcinoma. The expression levels of microRNAs and CEA were not significantly correlated. CEA levels were log-transformed.*

highest Youden index. The area under the curve (AUC) values for *miR-182* and *miR-210* for differentiating Ad-MPE from BPE were 0.87 (sensitivity=92.7%; specificity=73.3%) and 0.81 (sensitivity=58.5%; specificity=93.3%), respectively (Figure 4). Combining assessment of these two miRNAs (*miR-182* and *miR-210*) increased the diagnostic performance, yielding an AUC of 0.88 (95% confidence interval=0.78-0.97).

Discussion

Our study demonstrated that Ad-MPE samples had higher expression levels of exosomal *miR-182* and *miR-210* than those of the BPE samples. The ROC curve analyses revealed that *miR-182* and *miR-210* for diagnosis of Ad-MPE yielded AUCs of 0.87 and 0.81, respectively. The AUC for these miRNAs combined was 0.88. These findings indicate that these miRNAs are potential diagnostic markers of MPE in patients with lung adenocarcinoma.

CEA is a commonly used serum biomarker for patients with lung adenocarcinoma, and its diagnostic usefulness is also reported in Ad-MPE (12, 20-23). However, compared with serum CEA, CEA in PE shows a variable cutoff value, and its sensitivity has been found to be poor in meta-analysis (24). Indeed, five Ad-MPE samples had CEA levels below cutoff (5.0 ng/ml) in our study. On the other hand, three of these samples had higher expression levels of both *miR-182* and *miR-210*, and one sample had higher expression level of *miR-182* than the cutoff value. The expression of these miRNAs in PE was not correlated to the CEA level; therefore, the miRNA signature in PE might have complementary value for diagnosing Ad-MPE.

miR-182 is a member of the *miR-183* family, and the *miR-182*-96-183 cluster is located in the same chromosomal region

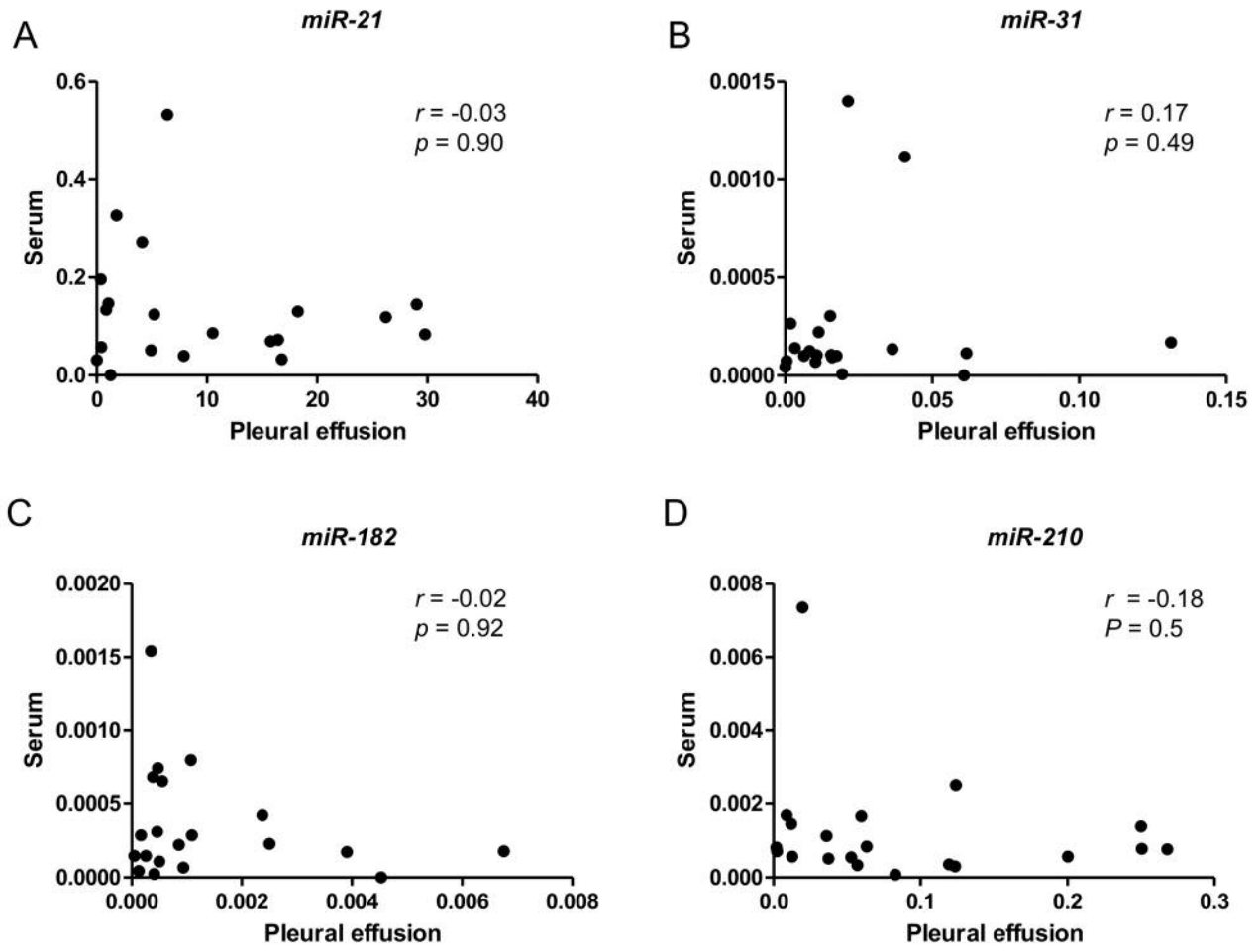

Figure 3. *Correlation between microRNA expression in serum and in pleural effusion in patients with lung adenocarcinoma: miR-21 (A), miR-31 (B), miR-182 (C), and miR-210 (D). No significant correlation was found between pleural effusion and serum microRNA expression levels.*

(7q32.2). The molecule functions as an oncogene *via* promotion of tumor cell growth and migration by targeting the transcription factor early growth response protein 1, by stimulating angiogenesis *via* fibroblast growth factor receptor substrate 2 (25). In addition, Barshack *et al*. reported that *miR-182* was overexpressed in primary lung cancer tissue and concluded that its expression level was useful for distinguishing lung primary tumors from metastases to the lung (26). We investigated the *miR-182* expression levels of MPE samples in seven patients with other metastatic disease (ovarian cancer, n=2; malignant melanoma, n=1; urothelial cancer, n=1; hypopharynx cancer, n=1; malignant lymphoma, n=1; thyroid cancer; n=1), but there were no significant differences in the expression level between Ad-MPE and metastatic MPE (data not shown). To evaluate the diagnostic significance of *miR-182* in PE, further studies are warranted with a larger number of samples.

miR-210 is recognized as an important regulator of the cellular response to hypoxia (27). Intrapleural cavities in healthy individuals exhibit negative pressures (28), and physiological-negative pressure is associated with an oxygen content of less than 4% (29). Furthermore, in malignant disease, hypoxia evolves because of the consumption of oxygen by proliferating cancer cells (30). These conditions might lead to a high expression level of *miR-210* in MPE. Overexpression of *miR-210* in human umbilical vein endothelial cells enhances vascular endothelial growth factor (VEGF) expression and promotes angiogenesis *in vitro* (31). VEGF is recognized as an important cytokine in the formation of MPE (32). High levels of VEGF produced by tumor cells, mesothelial cells, and infiltrating immune cells increased vascular permeability, cancer cell transmigration and angiogenesis (33). Therefore, increased expression of *miR-210* itself would contribute to production of MPE by modulating that of VEGF.

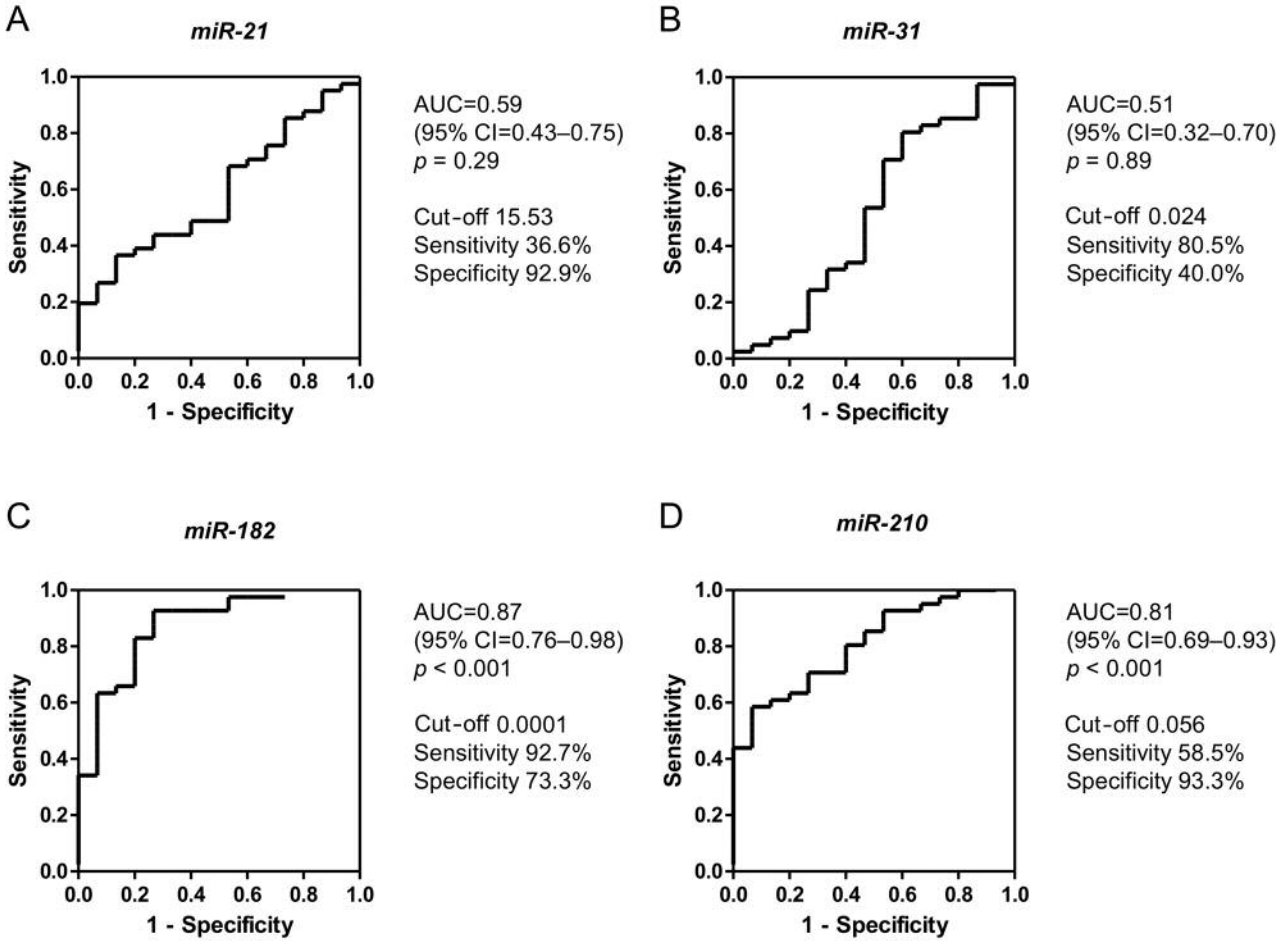

Figure 4. *Receiver operating characteristics curves showing the diagnostic performance of expression of miR-21 (A), miR-31 (B), miR-182 (C), and miR-210 (D) for differentiating malignant pleural effusion associated with lung adenocarcinoma from benign pleural effusion. The area under the curves of miR-182 and miR-210 were significantly diagnostic.*

miR-21 inhibits expression of phosphatase and tensin homolog and stimulates growth and invasion of non-small cell lung cancer cells (34). *miR-31* increases cell migration, invasion, and proliferation in an extracellular signal-regulated kinase 1/2 signaling-dependent manner (35). These miRNAs have been shown to be deregulated in lung cancer in many studies; however, the expression levels of these miRNAs were not significantly different among two groups. The cause for this discrepancy requires further investigation. Our results demonstrated that miRNA expressions in serum and PE were not correlated, and the miRNA profiles were not different between patients with extrathoracic metastases and those without. Although the study sample size was small, these results indicate that miRNAs in PE might be expressed separately from that in circulating blood.

Our study had several limitations. Firstly, small sample sizes were used. Secondly, some of the patients included in this study were not cancer therapy-naïve; therefore, we cannot exclude the possibility that previous treatments may have influenced miRNA expressions in PE and serum. Thirdly, there were no serum samples from patients with BPE, thus miRNA expression levels in PE and serum in patients with benign disease need to be validated.

In conclusion, we demonstrated exosomal miRNA profiles in the Ad-MPE and the BPE samples and showed that *miR-182* and *miR-210* expression could be used as novel diagnostic tools for discriminating Ad-MPE from BPE. We propose that these miRNAs may be useful and minimally-invasive screening biomarkers for diagnostic evaluation of PEs. These results need to be clarified in a larger cohort of patients.

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Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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