Clinical relevance of plasma miR-106b levels in patients with chronic obstructive pulmonary disease

SEIKO SOEDA[1], JUNKO H. OHYASHIKI[2], KAZUSHIGE OHTSUKI[2], TOMOHIRO UMEZU[3], YASUHIRO SETOGUCHI[1] and KAZUMA OHYASHIKI[3,4]

[1]Division of Respiratory Medicine, Tokyo Medical University; [2]Institute of Medical Science, Tokyo Medical University; Departments of [3]Molecular Science, [4]Hematology, Tokyo Medical University, Tokyo, Japan

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Abstract. Chronic obstructive pulmonary disease (COPD) is characterized by both chronic inflammation in the airway and systemic inflammation; however, the molecular mechanism of COPD has not been fully elucidated. By measuring microRNA (miRNA) expression in the plasma of COPD subjects, we aimed to identify the clinical relevance of plasma miRNA levels in these patients. Blood samples were obtained from COPD patients and age-matched normal controls. We initially produced plasma miRNA expression profiles using TaqMan low-density array screening. For further validation, individual qRT-PCRs were performed in 40 COPD patients and 20 healthy subjects. TaqMan low-density array screening showed that 9 miRNAs (miR-29b, miR-483-5p, miR-152, miR-629, miR-26b, miR-101, miR-106b, miR-532-5p and miR-133b) were significantly downregulated in the plasma from COPD patients when compared with normal smokers. Among these miRNAs, we focused on miR-106b. A reduction in the plasma miR-106b levels was evident in COPD ex-smokers and COPD current smokers compared with levels in smokers. There was a negative correlation between the plasma miR-106b level and the duration of disease since diagnosis in COPD ex-smokers and the duration of smoking in COPD current smokers. These findings support the concept that progressive reduction in the plasma miR-106b level may reflect persistent and systemic changes even after the discontinuation of smoking in COPD patients. miR-106b may therefore play an important role in the pathogenesis of COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease characterized by persistent airflow limitation, as well as extrapulmonary dysfunction such as skeletal muscle dysfunction, increased risk of cardiovascular disease, osteoporosis and depression (1,2). Diagnosis and assessment of the severity of COPD are based on the degree of airflow limitation by spirometry. However, the forced expiratory volume in 1 sec (FEV_1) does not directly reflect systemic manifestations in patients with COPD (3). Although both chronic inflammation in the airway and systemic inflammation have been attributed to the pathogenesis of COPD, the origin of systemic inflammation in COPD is not well understood (4-6).

microRNAs (miRNAs) are small noncoding RNAs, ranging from 18 to 25 nucleotides in length, that post-transcriptionally regulate gene expression. Recent studies have shown that miRNAs control a wide range of biological functions such as cellular proliferation, differentiation and apoptosis (7,8). Dysregulation of miRNAs has been implicated in the pathogenesis of several diseases, and their expression patterns in tumor tissues and body fluid serve as biomarkers (9-11). Several studies have examined the role of miRNAs in lung tissue of COPD patients and in airway epithelial cells from smokers versus nonsmokers (12,13). miRNAs exist stably in serum and plasma (11,14), and one recent study revealed the presence of circulating miRNAs within microvesicles (14). Since routine examination using lung epithelium or sputum is sometimes difficult in COPD patients, we sought for altered plasma miRNA expression levels in COPD patients in order to develop a screening protocol for the disease.

In the present study, we profiled levels of plasma miRNAs in COPD patients and identified those that were differentially expressed in COPD. We also assessed clinical characteristics such as smoking history, duration of disease since diagnosis, and the Global Initiative for Obstructive Lung Disease (GOLD) stages of the COPD patients.

Materials and methods

Patients and samples. A total of 70 consecutive subjects who did not meet any exclusion criteria were invited to participate, and 60 agreed to take part in the study. Participants were classified into 4 groups: those who had never smoked (hereafter 'nonsmokers') without COPD (n=10), current smokers without COPD (n=10), ex-smokers with COPD (n=20), and current smokers with COPD (n=20). Age and gender-matched healthy

Correspondence to: Dr Seiko Soeda, Division of Respiratory Medicine, Tokyo Medical University, 6-7-1 Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan
E-mail: skume@tokyo-med.ac.jp

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Table I. Characteristics of controls and COPD patients.

	Non-smoker without COPD	Smoker without COPD	COPD ex-smoker	COPD current smoker	P-value
No. of subjects	10	10	20	20	
Age (years)	65.0±11.5	62.8±14.6	64.6±7.4	64.7±7.5	0.95
Male (%)	80	100	85	95	0.42
Pack-years of smoking	0	47.6±34.8	69.3±42.8	77.4±72.7	0.39

Data are expressed as the means ± SD (standard deviation). P-values were determined by the ANOVA test. COPD, chronic obstructive pulmonary disease.

subjects were also enrolled in this study (Table I). Current smokers were defined as those who still smoked at the time of participation in the study. Subjects were classified in the COPD group (n=40) if they had a post-bronchodilator ratio of FEV_1 to forced vital capacity (FVC) of <0.70. All patients with COPD had stable disease; patients with symptoms or clinical signs of COPD exacerbation within 2 months prior to the study were excluded. Other exclusion criteria included a diagnosis of asthma, bronchiectasis, lung cancer, or upper or lower respiratory tract infection in the preceding 4 weeks. Among the 40 COPD patients, there were 21 patients in GOLD stage I, 9 in stage II, 8 in stage III, and 2 in stage IV. This study was approved by our institutional review board (no. 930, 24 June, 2008), and written informed consent was obtained from all patients prior to collection of specimens, according to the Declaration of Helsinki.

RNA isolation. Isolation of plasma miRNAs for miRNA profiling or quantification of individual miRNAs was performed using the mirVana PARIS kit (Ambion, Austin, TX, USA), diluting 500 μl of plasma with 500 μl of binding solution. After a 5-min incubation, 1 μl of 1 nM ath-miR-159 (Hokkaido System Science, Hokkaido, Japan) was added to each aliquot as a spike control for losses in preparation, and the solution was then vortexed for 30 sec and incubated on ice for 10 min. The subsequent phenol extraction and filter cartridge steps were carried out according to the manufacturer's instructions (15,16).

miRNA expression profile. To assess the levels of specific miRNAs in plasma samples, a fixed volume of 3 μl of RNA solution from the 50-μl elute was used as input in each reverse transcription (RT) reaction. The RT reaction and pre-amplification step were set up according to the manufacturer's recommendations. miRNAs were reverse transcribed using the Megaplex™ Primer Pools (Human Pools A v2.1). RT reaction products from the plasma sample were further amplified with Megaplex™ PreAmp Primers (Primers A v2.1). The expression profile of miRNAs was determined using the Human TaqMan miRNA Array card A (all were from Applied Biosystems, Bedford, MA, USA). This array enables quantification of 377 human miRNAs and 3 endogenous controls (RNU6B, RNU44 and RNU48). Ath-miR-159 was also included as an external reference. qRT-PCR was carried out on an Applied Biosystems 7900HT thermal cycler using the manufacturer's recommended program (GeneSifter; VizX Labs, Seattle, WA, USA).

Quantification of individual miRNAs. To confirm the results obtained from the TaqMan miRNA arrays, we measured expression levels by TaqMan miRNA assays (hsa-miR-106b; 000442; Applied Biosystems). The input of each RT reaction consisted of 10 ng of the total RNA. Using SDS2.2 software (Applied Biosystems), plasma samples were run in duplicates (15,16). Since we could not detect RNU6B in plasma, which is commonly used as an internal standard for miRNA expression analysis in cells, the plasma miRNA expression was calculated based on the Ct values normalized by those of ath-miR-159, which was spiked in each qRT-PCR aliquot.

Transforming growth factor-β_1 (TGF-β_1) measurement. Plasma collected from the 40 COPD patients (20 ex-smokers and 20 current smokers) and 20 healthy controls (10 nonsmokers and 10 current smokers) was used for the measurement of TGF-β_1. The biologically active TGF-β_1 concentration was determined using a commercially available ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA).

Statistical analysis. GraphPad 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis. The Mann-Whitney test was used to determine statistical significance between 2 groups, and one-way analysis of variance was used to compare 3 or more groups. A P-value of <0.05 was considered to indicate a statistically significant difference. The receiver operating characteristic (ROC) curve and the area under the ROC curve (AUC) were used to assess the feasibility of using plasma miR-106b levels for the diagnosis of COPD. We used the Youden index for identification of the optimal cut-off point.

Results

COPD patient characteristics. There were no significant differences in age, body mass index (BMI), FEV_1 (% predicted), FEV_1/FVC, duration of smoking and pack-years between COPD patients who were ex-smokers or current smokers. The duration of disease since diagnosis in ex-smokers with COPD was significantly longer than that in current smokers with COPD (Table II).

Identification of differentially expressed plasma miRNAs in COPD patients and healthy controls. We first compared miRNA expression levels in plasma obtained from 3 randomly

Table II. Characteristics of COPD patients.

	COPD		
	Ex-smoker	Current smoker	P-value
No. of patients	20	20	
BMI	22.2±3.5	22.5±3.6	0.76
FEV_1/FVC (%)	52.8±14.2	58.8±10.6	0.14
FEV_1 (% of predicted)	71.5±25.4	79.7±24.1	0.30
GOLD stages			
Stage I (n)	9	12	0.40
Stage II (n)	5	4	
Stage III (n)	5	3	
Stage IV (n)	1	1	
Duration of smoking (years)	37.50±7.78	41.95±6.24	0.053
Duration of smoking cessation (months)	59.64±52.17	0	
Duration of disease since diagnosis (months)	36.80±25.60	25.60±27.29	0.028[a]
Inhaled corticosteroid use, yes/no	3/17	1/19	0.61
No. of peripheral neutrophils (/μl)	3761±1322	3987±1232	0.58
Plasma TGF-β_1 level (ng/ml)	36.44±6.53	35.58±9.23	0.74

Data are expressed as means ± standard deviation. P-values were determined by the Mann-Whitney test, Fisher exact test, or Chi-square test. [a]P<0.05. BMI, body mass index. COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 sec. FVC, forced vital capacity; GOLD, Global Initiative for Obstructive Lung Disease; TGF-β_1, transforming growth factor-β_1.

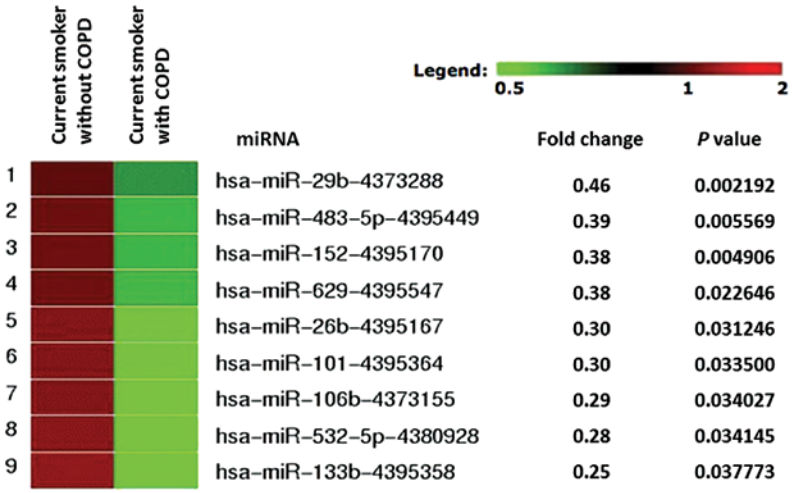

Figure 1. TaqMan low-density array screening of differentially expressed microRNAs in plasma. We compared miRNA expression levels in plasma from current smokers without COPD (n=3) with those from current smokers with COPD (n=3). Nine miRNAs (miR-29b, miR-483-5p, miR-152, miR-629, miR-26b, miR-101, miR-106b, miR-532-5p and miR-133b) were significantly downregulated in plasma from COPD patients.

selected nonsmokers and 3 randomly selected current smokers without COPD. Using the TaqMan low-density array, we found that 6 miRNAs (miR-499-5p, miR-486-5p, miR-19a, miR-92a, miR486-3p and miR-133b) appeared to be downregulated in current smokers without COPD. However, the fold decrease was not significant; those miRNAs were downregulated <1.5-fold (data not shown). We next compared miRNA expression in plasma from randomly chosen current smokers without COPD (n=3) with that of current smokers with COPD (n=3). According to TaqMan low-density array screening (card A), 214 of the 381 miRNAs were expressed in all plasma samples. Of these, 205 miRNAs showed no particular difference in expression level between these 2 groups. Although we did not find any miRNA that was preferentially upregulated in COPD samples, we did find 9 miRNAs (miR-29b, miR-483-5p, miR-152, miR-629, miR-26b, miR-101, miR-106b, miR-532-5p

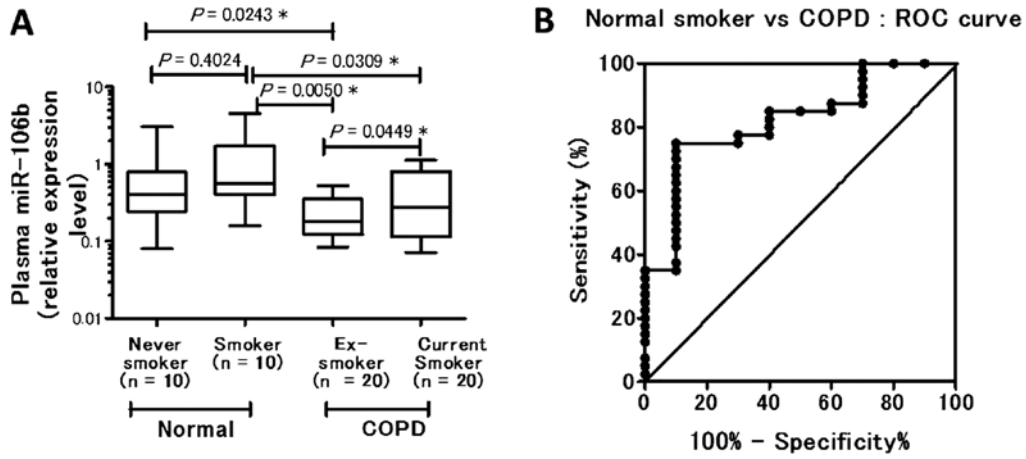

Figure 2. Expression of plasma miR-106b levels in patients with COPD and controls. (A) Expression of miR-106b was determined by qRT-PCR. The plasma miR-106b level in ex-smokers with COPD was significantly downregulated compared with that in current smokers without COPD (P=0.0050). The plasma miR-106b level in current smokers with COPD was significantly lower than that in current smokers without COPD (P=0.0309). Among the COPD subjects, the level of miR-106b in ex-smokers was significantly lower than that in current smokers (P=0.0449). (B) The receiver operating characteristic curve showed that the cut-off level of plasma miR-106b in all COPD patients at diagnosis was 0.4005; the sensitivity was 75.00% (95% CI, 58.80-87.31) and the specificity was 90.00% (95% CI, 55.50-99.75).

and miR-133b) that were significantly downregulated in plasma from COPD patients, with a fold change threshold of 2.0 or more, using GeneSifter software (Fig. 1).

Plasma miR-106b levels are significantly downregulated in patients with COPD. Based on array fold change, P-value, and the biological relevance of the predicted target by database, such as TargetScan (Table III), the TGF-β receptor was thought to be a possible predictive target for miR-106b; therefore, we chose miR-106b for further analysis. Moreover, the level of plasma miR-106b in COPD patients (n=40) was found to be significantly lower than that in normal controls (n=20; P=0.00243) (data not shown). Among the control subjects, no significant difference in plasma miR-106b level was evident between nonsmokers and smokers (P=0.4024). The plasma miR-106b level was significantly downregulated in COPD ex-smokers (P=0.0050) and in COPD current smokers (P=0.0309) compared with the smokers without COPD. Among the COPD patients, COPD ex-smokers had a significantly lower plasma miR-106b level than COPD current smokers (P=0.0449) (Fig. 2A). Based on the results obtained from individual qRT-PCRs of miR-106b, we performed further statistical analysis while combining the data of nonsmokers and current smokers without COPD as controls.

Receiver operating characteristic curve. A receiver operating characteristic curve was generated using the relative expression level compared with normal smoker subjects. The AUC was 0.8200, indicating 75.00% sensitivity (95% CI, 58.80-87.31) and 90.00% specificity (95% CI, 55.50-99.75) when the cut-off level of plasma miR-106b in COPD patients at diagnosis was 0.4005 (Fig. 2B).

There were significant differences in age and duration of smoking between COPD patients with plasma miR-106b <0.4005 (cut-off level) and those with plasma miR-106b >0.4005 (Table IV). COPD patients with plasma miR-106b <0.4005 were older (66.3±6.0 vs. 59.7±9.2 years; P=0.0121) and had smoked for a longer duration (41.2±7.2 vs. 35.2±6.0 years; P=0.0219).

Table III. Predicted targest for miR-106b.

Ankyrin repeat domain	ANKRD
Bone morphogenetic protein receptor, type 2	BMPR2
Fibrinogen-like 2	FGL2
Integrin β8	ITGB8
Protocadherin 1-protocadherin 13	PCDHA1-13
Transforming growth factor, β receptor 2	TGFBR2

Inverse correlations between plasma miR-106b levels and duration of disease since diagnosis and duration of smoking. The plasma miR-106b level was inversely correlated with duration of disease since diagnosis in ex-smokers with COPD (r=-0.4611, P=0.0407; Fig. 3A), although there was no relationship between the plasma miR-106b level and duration of smoking or duration of smoking cessation in COPD ex-smokers (Table V). The plasma miR-106b level in COPD current smokers was inversely correlated with duration of smoking (r=-0.5391, P=0.0142; Fig. 3B), while there was no relationship between the plasma miR-106b level and duration of disease since diagnosis in COPD current smokers. Plasma miR-106b levels showed no relationship with FEV_1 (% of predicted), FEV_1/FVC, or GOLD classification in patients with COPD, suggesting that no relationship existed between plasma miR-106b and the severity of airflow limitation (Table V).

Plasma miR-106b and plasma TGF-β_1. The plasma TGF-β_1 level was not significantly elevated in COPD patients compared with healthy controls. However, the plasma TGF-β_1 level was inversely correlated with duration of smoking cessation in ex-smokers with COPD (r=-0.5019, P=0.0241) and with FEV_1 (% of predicted) in current smokers with COPD (r=-0.6333,

Table IV. Background characteristics of patients with COPD when subgrouped according to the miR-106b cut-off level.

	Plasma miR-106b <0.4005	Plasma miR-106b >0.4005	P-value
No. of patients	30	10	
Age (years)	66.3±5.99	59.7±9.23	0.0121[a]
BMI	22.0±3.25	23.4±4.35	0.298
Pack-years of smoking	76.3±63.9	64.6±42.6	0.595
FEV_1/FVC (%)	54.8±14.0	58.6±7.62	0.421
FEV_1 (% of predicted)	74.5±26.3	78.9±20.5	0.635
Duration of smoking (years)	41.2±7.16	35.2±6.03	0.0219[a]
Duration of smoking cessation (months)	36.6±52.2	9.60±17.7	0.120
Duration of disease since diagnosis (months)	30.7±27.9	17.20±26.2	0.186
No. of peripheral neutrophils (/μl)	3866±1172	3884±1582	0.969
Plasma TGF-β_1 level (ng/ml)	35.7±8.19	37.0±7.29	0.660

Data are expressed as means ± standard deviation. COPD, chronic obstructive pulmonary disease; BMI, body mass index; FEV_1, forced expiratory volume in 1 sec; FVC, forced vital capacity; TGF-β_1, transforming growth factor-β_1. P-values were determined by the Mann-Whitney test. [a]P<0.05.

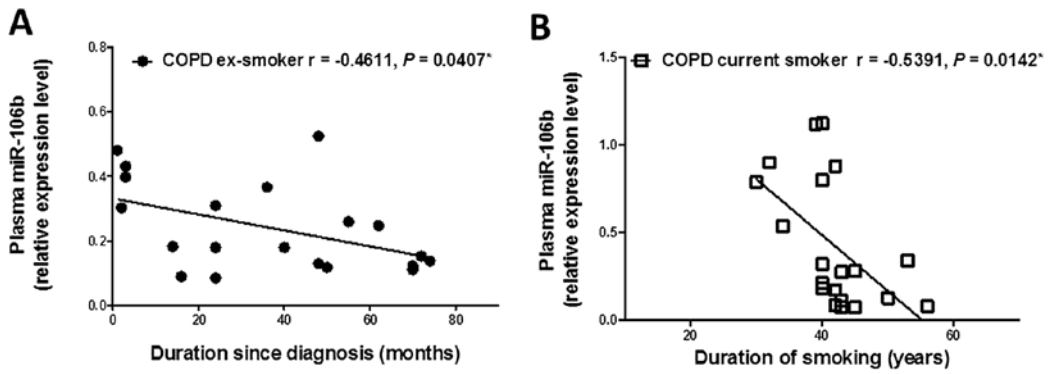

Figure 3. Relationship of plasma miR-106b expression in COPD patients and smoking history. (A) Correlation between the levels of plasma miR-106b and duration of disease since diagnosis in ex-smokers with COPD. Pearson's correlation coefficient (r) is provided. (B) Correlation between the levels of plasma miR-106b and duration of smoking in current smokers with COPD.

Table V. Pearson correlation of miR-106b and clinical characteristics in patients with COPD.

	COPD			
	Ex-smoker		Current smoker	
	Pearson *r*	P-value	Pearson *r*	P-value
Age (years)	-0.4031	0.0781	-0.2845	0.2241
BMI	0.1359	0.5679	-0.0716	0.7643
Pack-years of smoking	-0.0262	0.9126	-0.1772	0.4547
FEV_1/FVC (%)	0.2260	0.3379	0.0842	0.7241
FEV_1 (% of predicted)	0.1009	0.6721	-0.0911	0.7025
Duration of smoking (years)	-0.3147	0.1766	-0.5391	0.0142[a]
Duration of smoking cessation (months)	-0.2133	0.3665	-	-
Duration of disease since diagnosis (months)	-0.4611	0.0407[a]	0.0911	0.7026
No. of peripheral neutrophils (/μl)	-0.3182	0.1716	0.3157	0.1879
Plasma TGF-β_1 level (ng/ml)	0.2794	0.2329	-0.0423	0.8595

COPD, chronic obstructive pulmonary disease; BMI, body mass index; FEV_1, forced expiratory volume in 1 sec; FVC, forced vital capacity; TGF-β_1, transforming growth factor-β_1. [a]P<0.05.

Table VI. Pearson correlation of TGF-β1 and clinical characteristics in patients with COPD.

	COPD			
	Ex-smoker		Current smoker	
	Pearson *r*	P-value	Pearson *r*	P-value
Age (years)	-0.3781	0.1003	-0.1938	0.4128
BMI	0.3943	0.0854	0.1399	0.5562
Pack-years of smoking	0.2225	0.3458	0.2640	0.2606
FEV_1/FVC (%)	0.0674	0.7778	-0.3686	0.1098
FEV_1 (% of predicted)	-0.0469	0.8444	-0.6333	0.0027[a]
Duration of smoking (years)	-0.2431	0.3018	0.0468	0.8447
Duration of smoking cessation (months)	-0.5019	0.0241[a]	-	-
Duration of disease since diagnosis (months)	-0.1316	0.5803	-0.2445	0.2988
No. of peripheral neutrophils (/μl)	0.1447	0.5428	0.4229	0.0712

COPD, chronic obstructive pulmonary disease; BMI, body mass index; FEV_1, forced expiratory volume in 1 sec; FVC, forced vital capacity; TGF-β_1, transforming growth factor-β_1. [a]P<0.05.

P=0.0027). The plasma TGF-β_1 level was not correlated with the plasma miR-106 level or other clinical parameters (Table VI).

Discussion

To the best of our knowledge, this is the first report to substantiate the clinical relevance of plasma miRNAs in patients with COPD. There is an urgent need to clarify the molecular pathogenesis of COPD to improve our understanding of the heterogeneity of COPD patients and the therapeutic efficacy of various treatments (17-19).

This study focused on plasma miRNAs, samples of which can be easily collected, thereby providing a less invasive systematic assessment. Plasma miRNAs were profiled using TaqMan low-density array screening, and miR-106b was selected as a candidate miRNA. We found that the level of plasma miR-106b in COPD subjects was lower than that in smokers without COPD. Our findings indicate that the plasma miR-106b level is related to duration since diagnosis of COPD and duration of smoking.

In a previous study, airway epithelium was used for miRNA profiling between smokers and nonsmokers (13). miRNA expression in COPD patients was also extensively studied in various samples, including sputum (20), fibroblasts (21), muscle (22) and lung tissue (12). In addition, miRNA profiling of lung tissues has been performed using cigarette smoke-exposed rats and a mouse model of lung fibrosis (23,24). Akbas *et al* (25) currently reported alteration of serum miRNAs in COPD patients; their results were different from our data, possibly due to the different technology, sample materials and race of patients.

The most significant finding of this study was that the plasma miR-106b levels in the current smoker and ex-smoker COPD groups were decreased significantly compared with that of normal smokers. Furthermore, the miR-106b level in the COPD ex-smokers decreased significantly compared with the level in the COPD current smokers. This clearly indicates that miR-106b was progressively downregulated after discontinuation of smoking. This suggests that this alteration could be linked to a systemic reaction even after the cessation of smoking, which is characteristic of COPD patients (26,27). Although it may be difficult to estimate the exact onset of COPD, the plasma miR-106b level was inversely correlated with duration of disease since diagnosis, but not with smoking history or duration of smoking cessation. These findings suggest a relationship between the progressive reduction in plasma miR-106b levels and the deterioration of the COPD condition, even after the discontinuation of smoking.

In silico analysis by microRNA.org (targets and expression), Targetscan 5.2, and PicTar revealed several predicted targets of miR-106b, including ankyrin repeat domain, bone morphogenetic protein receptor type 2, fibrinogen-like 2, integrin β8, protocadherins 1-13 and TGF-β receptor 2 (Table III). The crosstalk between integrins and TGF-β_1 signaling has been proposed to induce the differentiation of airway fibroblasts to myofibroblasts, resulting in the thickening of small airways in COPD patients (28). Another study found that the TGF-β_1 level of airway epithelial cells was elevated in COPD patients (29), although there were conflicting findings concerning plasma TGF-β_1 levels in patients with COPD (30,31). Although we did not perform a functional analysis to prove a relationship between miR-106b and target genes, miR-106b may be involved in TGF-β_1 signaling, since miR-106b regulates the cyclin-dependent kinase inhibitor p21/CDKN1A, which is downstream of TGF-β_1 (32,33).

In this study, we found a significant improvement in the plasma TGF-β_1 level in relation to the length of the period of smoking cessation in COPD patients. In other words, an elevated TGF-β_1 level was associated with the progressive decline of FEV_1 (i.e., airway limitation), and the TGF-β_1 level decreased depending on the duration of smoking cessation. In contrast, the plasma miR-106b level progressively decreased, even after the COPD patients stopped smoking. These observations suggest that the plasma TGF-β_1 level was linked to

airway limitation due to current smoking, whereas the plasma miR-106b level was linked to the mechanism underlying persistent and systemic changes in COPD patients. Therefore, the plasma miR-106b level could be an important clinical indicator for COPD.

Although the number of patients studied was quite small to draw a definitive conclusion, our findings suggest that progressive reduction in the plasma miR-106b level may reflect persistent and systemic changes even after the discontinuation of smoking in COPD patients. Although the biological implications of molecules regulated by miR-106b need to be clarified in future research, the measurement of the plasma miR-106b level could provide important information concerning COPD patients in clinical practice.

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