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Analysis of serum micro-RNAs as potential biomarker in chronic obstructive pulmonary disease

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) as a complex disease with genetic and environmental compound is one of the leading causes of death in worldwide. This disease is characterized by lower airway inflammation, and increases risk of lung cancer in smokers. Micro-RNA (miRNA) molecules are key regulators in gene expression that have been widely associated with a several diseases. Differential expression of miRNAs is involved in lung tissue of COPD, but there is no information about biomarker potential of circulating miRNAs in patients. To analyze the miRNA expression profile in COPD, levels of serum miRNAs were profiled by quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) array system. The authors examined 72 miRNAs by qRT-PCR array, in 20 COPD patients and 12 control subjects. U6snRNA was used for normalization of the expression of miRNAs for each sample. According to the results, 5 miRNAs were found to be significantly dysregulated. There was down-regulation of miR-20a, miR-28–3p, miR-34c-5p, and miR-100, and up-regulation of miR-7, compared with the controls. This was the first study in COPD for screening of serum miRNAs for searching for biomarker. These results are preliminary screening data and should be confirmed with large patient groups. If so, these miRNAs are likely being involved in pathogenesis of COPD and may give clues for designing therapeutic strategy.

KEYWORDS gene expression, molecular marker, quantitative RT-PCR

Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the world [1]. Prevalence, morbidity, and mortality of the disease vary and are directly related to the prevalence of tobacco smoking [2, 3]. It is a complex disease with genetic, epigenetic, and environmental influences characterized by progressive airflow limitation, chronic inflammation in the lungs, and associated systemic inflammation. Prevalence is projected to increase due to smoke exposure and the changing age structure of the world population [4]. COPD shows progression with a chronic inflammation of the lower airway, which importantly increases the risk of lung cancer among long-term smokers [5, 6]. Early diagnosis and identification of novel therapeutic targets for this deadly disease have recently gained an interest. Finding of new biomarkers and diagnostic methods for early detection of COPD are essential to reduce the disease morbidity and mortality.

Micro-RNAs (miRNAs) are a class of small noncoding RNAs that work in regulation of gene expression by binding 3' untranslated region of target mRNAs. The miRNAs control cellular activities during cell growth, differentiation, apoptosis, adhesion, and cell death [7]. It is estimated that miRNAs can regulate nearly 30% of human genes that are involved in nearly all cellular functions and pathogenesis of human diseases [8]. Differential expression of miRNAs has been associated with several diseases [9]. Some muscle diseases, cancer types, cardiovascular diseases, and immunity- or inflammation-related diseases were reported to be associated with abnormal expression of miRNAs [10–14], and COPD is also categorized in this group.

In previous studies, miRNA expression profiles obtained from serum were evaluated as potential diagnostic and prognostic biomarkers in several types

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of human cancers as well as lung cancer [15–17]. The miRNAs are remarkably stable in blood and expression patterns seem to be tissue specific. These characteristics make circulating miRNAs a good candidate for noninvasive testing marker. Recently, circulating miRNAs have been reported as diagnostic markers for not only cancerous but also noncancerous diseases, including coronary, hepatic, and renal pathologies [15–20].

In this study, we hypothesized that the serum miRNA profile would be associated with COPD. To test this hypothesis, we used quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) array for scanning differential expression of miRNA in the serum of COPD patients. Finding a serum miRNA expression profile in COPD can potentially assist diagnosis and treatment. By statistical analysis, we found a profile of 9 serum miRNAs, which can potentially serve as a biomarker for COPD detection.

MATERIALS AND METHODS

Study Subjects

In the matched case-control study, we recruited 20 COPD patients aged between 46 and 67 years, average 59 years, with a smoking history of at least 20 pack-years, and 12 healthy control smokers (mean age 55 years) with at least 20 pack-years smoking history and normal lung function. Serum samples of both COPD and control subjects were collected prospectively over the same time period from Yedikule Chest Disease and Surgery Training and Research Hospital 3rd clinic between January 2010 and January 2011. COPD was defined by GOLD (The Global Initiative for Chronic Obstructive Lung Disease) criteria at least stage 2 (forced expiratory volume in 1 second [FEV_1]/forced vital capacity [FVC] = 0.7 with FEV_1 = 80% predicted) [21]. COPD patients and control subjects had no other concomitant diseases, including significant cardiac dysfunction, active infection (hepatitis, tuberculosis, etc.), and neurological or psychiatric disorders at the time when blood was drawn. Subjects have a history of malignancy were excluded.

Ethics Statement

Written informed consent was obtained from all subjects, and this study was approved by the Fatih University Ethics Committee.

RNA Isolation

Venous blood samples (5 mL) were collected from each donor and placed in a serum separator tube. Separation of the serum was accomplished by centrifugation at 800 $\times$ g for 10 minutes at room temperature, followed by a 15-minute centrifugation at 10,000 $\times$ g at room temperature. The supernatant serum was recovered and stored at -80°C until RNA extraction. The frozen serum were thawed and transferred into Eppendorf tubes. For enrichment and increasing of concentration of target serum miRNAs, ExoQuick Exosome precipitation solution (SBI System Biosciences, USA) was used. Total RNA was extracted from serum precipitate using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Typically, after extracting RNA from 250 μL serum precipitate using TRIzol reagent, the RNA concentrations in the yield were between 100 and 200 ng/μL. All serum RNA preparations were quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and then pretreated with RNase-free DNase I (Roche) to eliminate DNA contamination.

qRT-PCR Array Assay

For the expression profiles of serum miRNA, we used real-time quantitative RT-PCR (qRT-PCR) method with miRNA array kit (QuantiMir RT Kit Small RNA Quantitation System; SBI System Biosciences). Seventy-two miRNAs were selected for testing of differential expression analyses (see Table 3). The protocol is based on polyadenine tailing and reverse transcription of mature miRNAs to provide in cDNA form. A universal 3'-tag sequence is incorporated during reverse transcription to enable miRNA expression analysis by quantitative PCR. The reverse primer was universal and provided by the kit. The forward sense primer is designed by using the sequence of the mature miRNA converted into DNA sequence, which was included in the kit.

Real-time PCR amplifications are performed following manufacturer's protocol with 2 μg of RNA using FastStart DNA Master SYBR Green I (Roche Diagnostics, Germany). Real-time PCR was performed in a Stratagene Mx-3000P (Stratagene, La Jolla, CA, USA) thermocycler. Thermal cycling conditions were 10 minutes at 95°C followed by 45 cycles at 95°C for 20 seconds, 60°C for 15 seconds, and 72°C for 20 seconds. Each sample was studied in duplicate.

Expression levels of each individual miRNA were evaluated using comparative threshold cycle (C_t) method. These results were normalized to those of

TABLE 1 Clinical Features of COPD Patients

Patient no.	Age	Height (cm)	Weight (kg)	FVC	FEV_1	FEV_1/FVC	pH	Pco_2	Po_2	HCO_3	$SATo_2$ (%)
1	63	168	70	1.7(47%)	1.3(43%)	73	7.37	41.5	67	24.2	92.5
2	65	169	71	3.2(85%)	2.2(74%)	68	7.42	40.5	74	26.6	95.0
3	62	170	74	2.54(68%)	1.24(42%)	49	7.38	41.9	61	25.2	90.6
4	67	170	65	1.7(45%)	0.9(31%)	54	7.38	40.3	92	24.3	91.1
5	65	160	73	2.1(66%)	1.2(48%)	57	7.4	37	65	23.2	92.6
6	67	173	70	2.3(60)	1.5(50%)	63	7.38	43.2	64	25.6	91.5
7	67	170	72	1.8(46%)	1.0(33%)	55	7.43	37.4	77	25.3	95.8
8	61	170	65	2.4(61%)	1.4(46%)	60	7.39	36.7	71	22.4	94.0
9	56	178	55	1.8(41%)	0.8(24%)	46	7.39	44.4	59	27.1	89.8
10	51	170	76	2.1(51%)	1.3(40%)	63	7.35	48.4	68	24.3	87.1
11	56	182	79	3.9(80%)	2.5(66%)	66	7.37	45.5	58	26.4	88.7
12	61	167	95	1.8(49%)	0.8(27%)	44	7.38	47.1	78	28.1	95.0
13	65	186	65	1.8(37%)	0.6(17%)	36	7.39	44.8	70	27.6	93.6
14	53	163	88	0.8(23%)	0.4(13%)	47	7.39	50.3	59	30.7	89.5
15	60	170	93	3.1(80%)	1.8(60%)	60	7.43	35.1	84	23.5	96.7
16	58	165	74	1.3(39%)	0.8(30%)	62	7.39	40.9	71	24.9	93.9
17	60	165	62	2.2(62%)	1.8(62%)	79	7.39	38.8	74	23.7	94.6
18	58	165	55	1.6(45%)	0.8(28%)	49	7.35	47.3	74	26.1	93.7
19	54	160	55	2.7(76%)	1.7(60%)	65	7.39	41.7	78	25.2	95.3
20	61	166	55	2.66(73%)	1.19(41%)	56	7.35	48.8	77	27.1	94.4

Note. FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; Po_2 = oxygen pressure; Pco_2 = carbon dioxide pressure; HCO_3 = bicarbonate value; $SATo_2$ = saturated oxygen.

U6snRNA as a housekeeping gene for each sample (ΔC_t). The relative amount of each miRNA to U6snRNA was described by the equation $\Delta C_t = (C_t$ miRNA $- C_t$ U6snRNA) [22]. Fold changes between control and patient were calculated as $\Delta\Delta C^t$, as a decrease in 1 C_t value was equivalent to a 2-fold increase in the starting amount of cDNA. The *N*-fold differential expression of the miRNA gene of a COPD sample compared with the control sample was expressed as $2^{-\Delta\Delta CT}$ [23]. In this study, increased mRNA expression was defined as *N*-fold $\geq$2.0. The cutoff value was set at the 40th cycle and a gene was considered not detectable when $C_t > 40$. C_t was defined as 40 for the ΔC_t calculation when the signal was under detectable limits. Mean values of fold difference for COPD patients were compared with mean values obtained from control samples.

Statistical Analysis

The statistical calculation was based on normalized ΔC_t values. For average fold change of miRNAs in COPD patient serum compared with control serum, data were reported as mean $\pm$ standard deviation. Student *t* test was used to compare the serum level of each miRNA between COPD and healthy subjects. *P* values $< .01$ were considered statistically significant. For the confirmation of normal distribution in differentially regulated genes, 1-sample Kolmogorov-Smirnov (KS) test was used.

RESULTS

To determine the miRNA species differently expressed in the COPD, we used the quantitative RT-PCR array (QuantiMir RT Kit; Systems Bioscience) with selected 72 human miRNAs. The median age was 59 $\pm$ 6.6 and 55 $\pm$ 5.2 years for controls and COPD, respectively. There was no significant difference in the distribution of age between the COPD patients and the normal subjects (Table 1). All of the subjects were men, and had a history of tobacco smoking for controls and COPD patients. We observed the lowest values of FEV_1, FVC, and FEV_1/FVC in patients with COPD and these values were statistically significant compared with controls (Table 2).

Total RNA was extracted from the serum samples from patients and controls for qRT-PCR analysis.

TABLE 2 Characteristics and Respiratory Parameters of COPD patients and Controls

Characteristic	COPD patients ($n = 20$)	Controls ($n = 10$)	*P* value*
FEV_1	48.7 $\pm$ 2.0	98.1 $\pm$ 1.8	<.001
FVC	70.8 $\pm$ 2.4	96.7 $\pm$ 1.7	<.001
FEV_1/FVC	55.9 $\pm$ 1.2	93.4 $\pm$ 2.8	<.001
DLCO	70.0 $\pm$ 29.8	89.8 $\pm$ 18.1	<.001

Note. Data are reported as percentage. FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; DLCO = lung diffusion capacity testing.
*P values obtained by Student *t* test.

We first evaluated the reliability and reproducibility of our qRT-PCR assay with U6snRNA primers for measuring serum miRNAs. Our results suggest that miRNA can be efficiently extracted and amplified from serum.

By comparing miRNAs from COPD and healthy controls, a miRNA signature was obtained to discriminate COPD subjects. The miRNAs were down-regulated in COPD compared with the healthy control samples. All samples were normalized to those of U6snRNA as a housekeeping gene according to C_t values. Relative quantification of the data showed that 1 of the 72 miRNAs were significantly up-regulated and 4 of them were significantly down-regulated in the diseased group than in the controls ($P < .001$) (Table 3). Volcano plot shows the distribution of all the miRNAs according to their fold changes and P values (Figure 1). The fold changes of miRNAs in COPD were compared with controls by quantitative RT-PCR array based on ΔC_t values. C_t values of quantifiable results were tested for normal distribution. For the differentially regulated genes, 1-sample Kolmogorov-Smirnov (KS) test was used. The test results confirmed that all of the 5 miRNAs were found in normal distribution (measured C_t values were $P < .05$) (Figure 2).

Unpaired sample t test was used to compare the differential expressions for the P values. Down-regulated miRNA $\Delta\Delta C^t$ values and according fold changes ($2^{-\Delta\Delta CT}$) were as follows ($\Delta\Delta C^t$, P, fold changes): miR-20a (7.95, $P = .006$, 247), miR-28–3p (>8.22, $P = .003$, 298), miR-34c-5p (8.2, $P = .002$, 294), and miR-100 (>7.75, $P = .005$, 215). The values for down-regulated miR-7 were >8.26, $P = .01$, 306 (Figure 3). $\Delta\Delta C^t$ indicated with ">" means at least, because no amplification was detected before the 40th cycle. As a result of the study, serum miRNAs that are expressed in COPD diseases were analyzed using miRNA-based real-time PCR expression profiling. There were 5 miRNAs significantly (P value $< .001$) differentially expressed, and all miRNAs had fold change more than 7.

TABLE 3 Differentially Expressed miRNAs in COPD Serum Samples Compared With Those in Controls

miRNA	$\Delta\Delta C^t$	P value	miRNA	$\Delta\Delta C^t$	P value
hsa-let-7a	−2.17	.521	hsa-miR-29c	3.11	.311
hsa-let-7b	1.10	.706	hsa-miR-30a	−2.39	.073
hsa-let-7c	0.32	.920	hsa-miR-30b	1.9	.571
hsa-let-7d	0.27	.804	hsa-miR-30c	−2.31	.463
hsa-let-7e	1.24	.708	hsa-miR-30d	0.57	.850
hsa-let-7f	4.06	.108	hsa-miR-30e	−0.11	.347
hsa-let-7g	−1.27	.701	hsa-miR-31	2.91	.168
hsa-let-7i	3.75	.214	hsa-miR-32	1.91	.578
hsa-miR-1	1.49	.626	hsa-miR-33a	0.42	.840
hsa-miR- 7*	−7.59	.010	hsa-miR-33b	4.31	0.134
hsa-miR-9	3.14	.227	hsa-miR-34a	2.1	.507
hsa-miR-10a	3.93	.141	hsa-miR-34b	2.89	.230
hsa-miR-10b	0.27	.363	hsa-miR-34c-3p	4.45	.123
hsa-miR-15a	2.17	.479	hsa-miR-34c-5p*	8.81	.002
hsa-miR-15b	−1.84	.134	hsa-miR-92a	1.31	.648
hsa-miR-16	3.47	.220	hsa-miR-92b	3.21	.087
hsa-miR-17	3.73	.203	hsa-miR-93	2.62	.025
hsa-miR-18a	0.38	.859	hsa-miR-95	−1.91	.566
hsa-miR-18b	5.59	.101	hsa-miR-96	4.33	.127
hsa-miR-19a	0.63	.732	hsa-miR-98	1.89	.346
hsa-miR-19b	1.07	.759	hsa-miR-99a	0.83	.343
hsa-miR-20a*	8.18	.006	hsa-miR-99b	0.22	.550
hsa-miR-20b	4.53	.092	hsa-miR-100 *	7.74	.005
hsa-miR-21	2.38	.223	hsa-miR-101	3.35	.102
hsa-miR-22	0.82	.787	hsa-miR-103	−3.44	.108
hsa-miR-23a	1.97	.486	hsa-miR-103-as	3.33	.267
hsa-miR-23b	−0.74	.826	hsa-miR-105	−1.9	.462
hsa-miR-24	−2.65	.169	hsa-miR-106a	0.25	.930
hsa-miR-25	0.41	.913	hsa-miR-106b	0.04	.992
hsa-miR-26a	2.23	.479	hsa-miR-107	3.86	.214
hsa-miR-26b	5	.087	hsa-miR-122	−5.62	.137
hsa-miR-27a	1.91	.363	hsa-miR-124	5.17	.094
hsa-miR-27b	2.52	.274	hsa-miR-125a-3p	1.46	.493
hsa-miR-28–3p*	7.11	.003	hsa-miR-125a-5p	4.57	.142
hsa-miR-28–5p	−3.11	.102	hsa-miR-125b	3.31	.233
hsa-miR-29a	−0.35	.843	hsa-miR-125b	1.63	.220
hsa-miR-29b	−0.1	.969			

Note. $\Delta\Delta C^t$ indicates ΔC_t miRNA − ΔC_t U6snRNA.
*$P < .01$.

DISCUSSION

COPD is predominantly induced by cigarette smoking and chronic exposure to irritants. Symptoms of COPD include a progressive obstruction of the airflow, shortness of breath, chronic inflammation of the lungs, and chronic cough and phlegm production [3]. Chronic inflammation in the airway and systemic inflammatory drive have been attributed to the pathogenesis of COPD [24].

Micro-RNAs play critical roles in developmental, cell death, proliferation, stress response, and stem cell division [25]. Abnormal regulation of miRNA can be caused by genomic amplification, genomic deletion, epigenetic alteration, and abnormal expression or down-regulation of the proteins that regulate miRNA expression [26–28]. Smoking has been linked to pulmonary inflammation, increased risk of cancer, and exacerbations in asthma and COPD [29]. Many miRNAs are involved in activation of the inflammation pathway response after smoking. The expression of miRNA in mouse and rat lungs exposed to cigarette smoke extract has been analyzed [30]. Down-regulation of miRNAs was observed in both animals. This observation is consistent with the miRNA expression analyses in

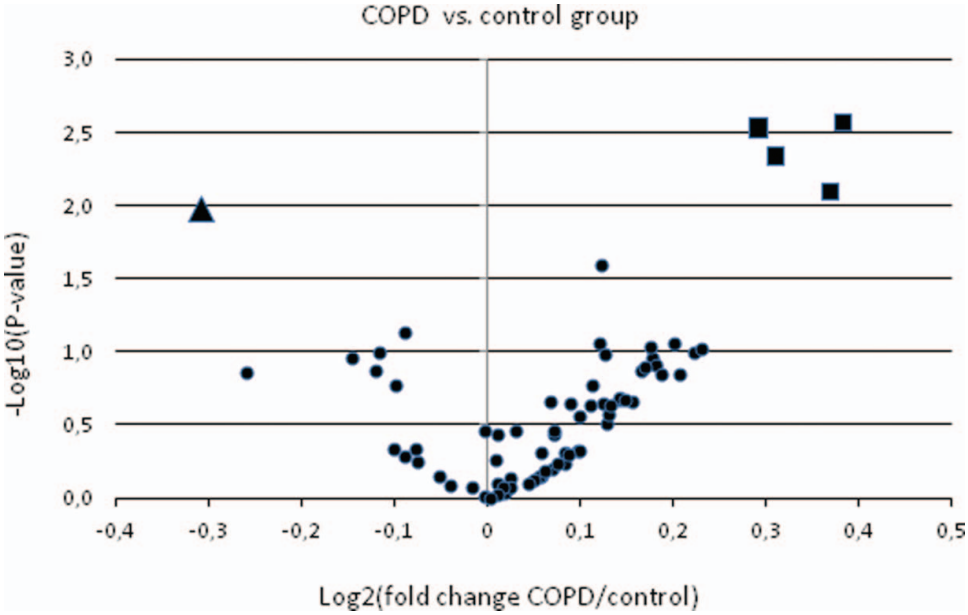

FIGURE 1 The volcano plot displaying circulating miRNAs up- and down-regulation in COPD compared with controls. The change factor values $\log_2$ fold change are plotted on the *x*-axis against the *P* value in logarithmic scale on the *y*-axis. The up-regulated miRNA is indicated as ▲ and down-regulated miRNAs are indicated as ■.

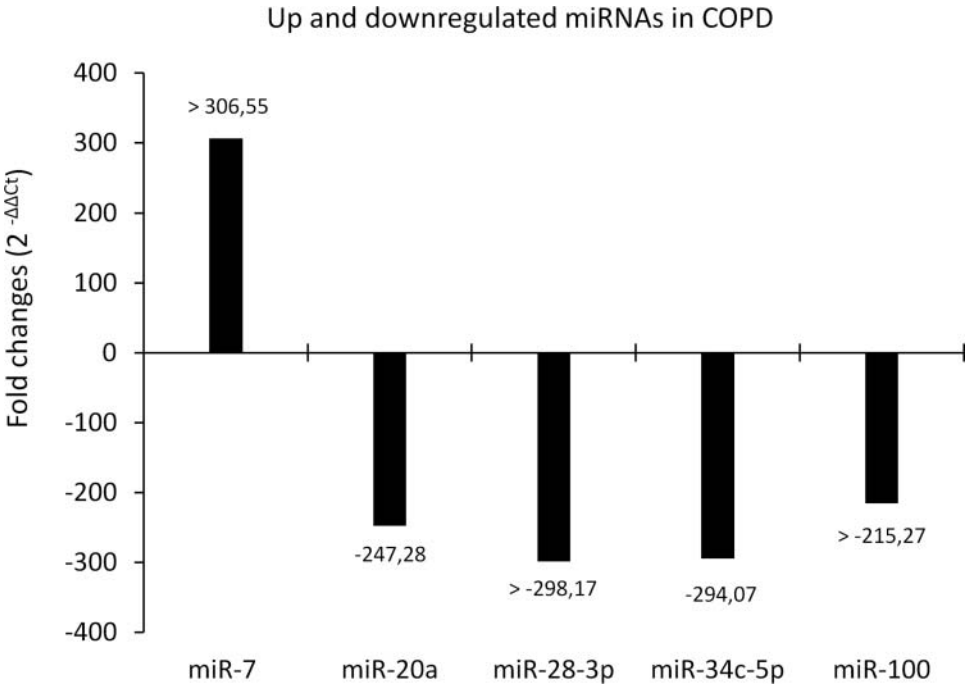

FIGURE 2 Dysregulated serum miRNAs in COPD patients versus healthy controls. According to qRT-PCR results, 4 miRNAs were down-regulated and 1 miRNA was up-regulated. C_t value at 40 adjusted was cutoff point and over this value was evaluated as unexpressed. Expression levels of selected miRNAs were normalized to U6snRNA and are presented as fold changes ($2^{-\Delta\Delta CT}$). Fold change indicated with ">" means at least.

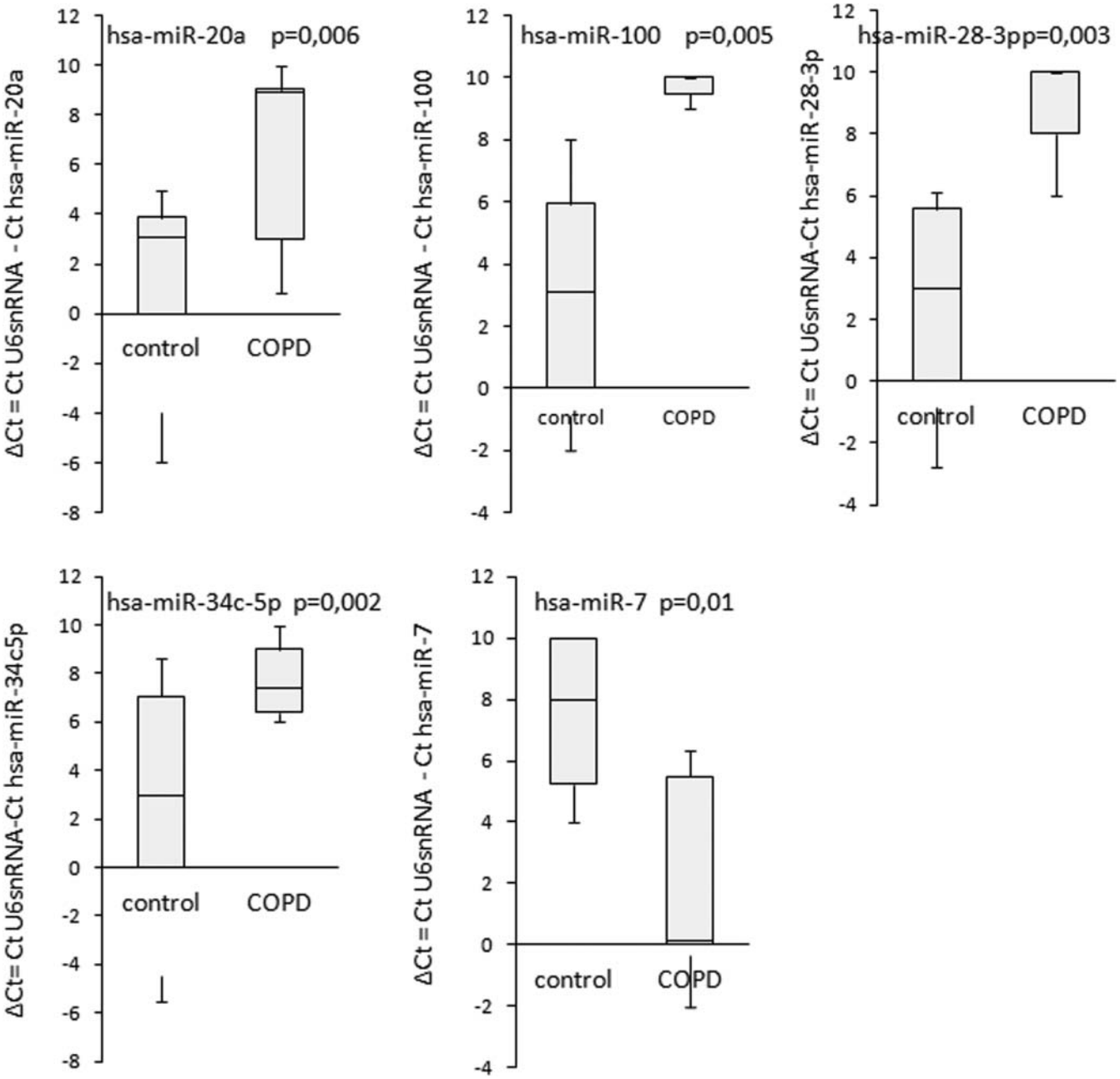

FIGURE 3 Box plot diagrams showing the normalized expression levels of hsa-miR-7, hsa-miR-20a, hsa-miR-28–3p, hsa-miR-34c-5p, and hsa-miR-100 in 20 COPD patients and 12 controls. Expression levels of selected miRNAs were normalized to U6snRNA. The line within the boxes indicates the median expression level. The top edge of the boxes represents the 75th percentile, and the bottom edge, the 25th percentile. Student *t* test was used to determine statistical significance.

human bronchial epithelium of smokers [31]. In silico analysis of miRNAs implies that these molecules are involved in a variety of functions, including development of airway epithelial cells, inflammation, formation of pulmonary surfactant, and stress responses [32]. Recently, specific miRNA expression profiles have been reported as a prognostic factor or a predictive for disease progression in several cancerous or noncancerous diseases [9, 16–18, 33, 34]. The first serum miRNA biomarker discovered was miR-21 [35]. Patients with B-cell lymphoma had up-regulated serum levels of miR-21. Later it was found that serum miR-141 could identify prostate cancer patients, and revealed for the first time that serum miRNAs may be used as a biomarker in diagnosis. It is also demonstrated that specific serum

miRNAs can be identified in serum from patients with lung cancer, and diabetes patients [9].

In some cancer types, increased miRNA levels decreased after removal of the primary tumor tissue, and increased again after the tumor recurred [36]. According to these data, circulating miRNAs are useful for the diagnosis of the disease, and can be used for monitoring and follow-up of cancer patients. There is some evidence that in COPD, lung and blood cells express different amount of miRNAs compared with healthy controls. Differentially expression of miRNAs in blood cells can be used to discriminate lung cancer patients and COPD patients [37]. COPD and aging are associated with significant dysregulation of the immune system, which leads to a chronic inflammatory response. Aging-dependent dysregulation of miRNA shows a parallel expression profile. This type of conditions makes it difficult to describe disease-specific biomarker in serum-based on miRNA expression [38].

COPD-dependent skeletal muscle dysfunction may also contribute to change of serum miRNA profile. Muscle-specific miRNAs are secreted into serum and are evaluated as COPD-related finding [39]. At the lung tissue level, it has been found that several miRNA genes are dysregulated and some of them also are secreted into bloodstream [40–43].

Therefore, we hypothesized that in COPD serum miRNA level can also be changed and those miRNAs can be used as a biomarker. In a cohort of well-characterized COPD subjects, we report 5 miRNAs differentially expressed in the serum. We found that down-regulation of 4 miRNAs and up-regulation of 1 miRNA were significantly associated with COPD. This is the first study using qRT-PCR array method to investigate the association between serum miRNAs and COPD. miR-20a, miR-28–3p, miR-34c-5p, and miR-100 were down-regulated and miR-7 was up-regulated in patients. In previous studies, these miRNAs are closely involved in several cancer diseases, also including lung tissue [44–48]. Evaluation of these up- and down-regulated miRNAs according to their target mRNAs and biological significance gives some clues for their importance in COPD. Up-regulation of miR-7 was significantly associated with COPD, which may be a potential biomarker for diagnosis of the disease. miR-7, which controls regulation of the epidermal growth factor receptor, is frequently overexpressed in cancer. Aberrant increased expression and function of miR-7 has been associated with tumorigenesis and causes breast cancer, lung cancer, and bladder cancer [44, 49, 50], whereas miR-7 is down-regulated in glioblastoma [51].

These results are rather preliminary data that need to be confirmed individually for each gene with quantitative RT-PCR and with Northern hybridization to demonstrate expression of candidate biomarkers in larger patient groups in different populations. These findings also suggest that these miRNAs may have a role in COPD pathogenesis. Our results present potential novel biomarkers for early detection and monitoring of COPD and serve as basis for further studies. However, our patient and control groups are not sufficiently large to claim for characterization of new biomarker. Further studies are needed to elucidate whether a direct relationship exists between our candidate miRNAs and the prognosis of COPD in a larger group of patients.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

[1] Oglesby IK, McElvaney NG, Greene CM: MicroRNAs in inflammatory lung disease-master regulators or target practice? Respir Res. 2010;11:148.

[2] Adcock IM, Caramori G, Barnes PJ: Chronic obstructive pulmonary disease and lung cancer: new molecular insights. Respiration. 2011;81:265–284.

[3] Pauwels RA, Buist AS, Calverley P, Jenkins CR, Hurd SS: Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. Am J Respir Crit Care Med. 2001;163:1256.

[4] Lopez AD, Mathers CD, Ezzati M, Jamison DT, Murray CJL: Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. Lancet. 2006;367:1747–1757.

[5] Punturieri A, Szabo E, Croxton TL, Shapiro SD, Dubinett SM: Lung cancer and chronic obstructive pulmonary disease: needs and opportunities for integrated research. J Natl Cancer Inst. 2009;101:554.

[6] Ben-Zaken Cohen S, Pare PD, Man SF, Sin DD: COPD and lung cancer in women: examining sex differences in cigarette smoke metabolism. Am J Respir Crit Care Med. 2007;176:113–12.

[7] He L, Hannon GJ: MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet. 2004;5:522–331.

[8] Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RHA, Cuppen E: Phylogenetic shadowing and computational identification of human microRNA genes. Cell. 2005;120:21–24.

[9] Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al.: Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res. 2008;18:997–1006.

[10] Liu Z, Li G, Wei S, Niu J, El Naggar A: Genetic variants in selected pre microRNA genes and the risk of squamous cell carcinoma of the head and neck. Cancer. 2010;116:4753–4760.

[11] Perry MM, Moschos SA, Williams AE, Shepherd NJ, Larner-Svensson HM, Lindsay MA: Rapid changes in microRNA-146a expression negatively regulate the IL-1-induced inflammatory response in human lung alveolar epithelial cells. J Immunol. 2008;180:5689.

[12] Stanczyk J, Pedrioli DML, Brentano F, Sanchez Pernaute O, Kolling C, Gay RE, et al.: Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. Arthritis Rheum. 2008;58:1001–1009.
[13] Okubo M, Tahara T, Shibata T, Yamashita H, Nakamura M, Yoshioka D, et al.: Association study of common genetic variants in pre-microRNAs in patients with ulcerative colitis. J Clin Immunol. 2011;31:69–73.
[14] Liu R, Zhang C, Hu Z, Li G, Wang C, Yang C, et al.: A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as a fingerprint for gastric cancer diagnosis. Eur J Cancer. 2011;47:784–791.
[15] Chen X, Hu Z, Wang W, Ba Y, Ma L, Zhang C, et al.: Identification of ten serum microRNAs from a genome wide serum microRNA expression profile as novel non invasive biomarkers for non small cell lung cancer diagnosis. Int J Cancer. 2011. Online ahead of print. DOI: 10.1002/ijc.26177.
[16] Zhang T, Wang Q, Zhao D, Cui Y, Cao B, Guo L, et al.: The oncogenetic role of MicroRNA-31 as a potential biomarker in esophageal squamous cell carcinoma. Clin Sci. 2011;121: 437–447.
[17] Zhao H, Shen J, Medico L, Wang D, Ambrosone CB, Liu S: A pilot study of circulating miRNAs as potential biomarkers of early stage breast cancer. PLoS ONE. 2010;5:e13735.
[18] Santarelli L, Strafella E, Staffolani S, Amati M, Emanuelli M, Sartini D, et al.: Association of MiR-126 with soluble mesothelin-related peptides, a marker for malignant mesothelioma. PLoS ONE. 2011;6:e18232.
[19] Widera C, Gupta SK, Lorenzen JM, Bang C, Bauersachs J, Bethmann K, et al.: Diagnostic and prognostic impact of six circulating MicroRNAs in acute coronary syndrome. J Mol Cell Cardiol. 2011;51:872–875.
[20] Kerr TA, Korenblat KM, Davidson NO: microRNAs and liver disease. Transl Res. 2011;157:241–252.
[21] Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, et al.: Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med. 2007;176:532.
[22] Sui W, Dai Y, Huang YS, Lan H, Yan Q, Huang H: Microarray analysis of MicroRNA expression in acute rejection after renal transplantation. Transplant Immunol. 2008;19:81–85.
[23] Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta] CT method. Methods. 2001;25:402–408.
[24] Decramer M, Rennard S, Troosters T, Mapel DW, Giardino N, Mannino D, et al.: COPD as a lung disease with systemic consequences-clinical impact, mechanisms, and potential for early intervention. COPD J Chron Obstruct Pulm Dis. 2008;5:235–256.
[25] Ambros V: The functions of animal microRNAs. Nature. 2004;431:350–355.
[26] Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, et al.: Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2002;99:15524.
[27] Lum A, Wang B, Li L, Channa N, Bartha G, Wabl M: Retroviral activation of the mir-106a microRNA cistron in T lymphoma. Retrovirology. 2007;4:5.
[28] Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, et al.: Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol Cell. 2007;26:745–752.
[29] Jardim MJ, Fry RC, Jaspers I, Dailey L, Diaz-Sanchez D: Disruption of microRNA expression in human airway cells by diesel exhaust particles is linked to tumorigenesis-associated pathways. Environ Health Perspect. 2009;117:1745.
[30] Izzotti A, Calin GA, Arrigo P, Steele VE, Croce CM, De Flora S: Downregulation of microRNA expression in the lungs of rats exposed to cigarette smoke. FASEB J. 2009;23: 806.
[31] Schembri F, Sridhar S, Perdomo C, Gustafson AM, Zhang X, Ergun A, et al.: MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. Proc Natl Acad Sci U S A. 2009;106:2319.
[32] Izzotti A, Calin GA, Steele VE, Croce CM, De Flora S: Relationships of microRNA expression in mouse lung with age and exposure to cigarette smoke and light. FASEB J. 2009;23:3243.
[33] Ueda T, Volinia S, Okumura H, Shimizu M, Taccioli C, Rossi S, et al.: Relation between microRNA expression and progression and prognosis of gastric cancer: a microRNA expression analysis. Lancet Oncol. 2010;11:136–146.
[34] Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al.: Circulating microRNAs as stable blood-based markers for cancer detection. Proce Natl Acad Sci U S A. 2008;105:10513.
[35] Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, et al.: Detection of elevated levels of tumour associated microRNAs in serum of patients with diffuse large B cell lymphoma. Br J Haematol. 2008;141:672–675.
[36] Kanemaru H, Fukushima S, Yamashita J, Honda N, Masuguchi ROAKS, Ishihara T, et al.: The circulating microRNA-221 level in patients with malignant melanoma as a new tumor marker. J Dermatol Sci. 2011;61:187–193.
[37] Leidinger P, Keller A, Borries A, Huwer H, Rohling M, Huebers J, Lenhof HP, Meese E: Specific peripheral miRNA profiles for distinguishing lung cancer from COPD. Lung Cancer. 2011;74:41–47.
[38] Provinciali M, Cardelli M, Marchegiani F: Inflammation, chronic obstructive pulmonary disease and aging. Curr Opin Pulm Med 2001;17(S1):S3–10.
[39] Lewis A, Riddoch-Contreras J, Natanek SA, Donaldson A, Man WDC, Moxham J, Hopkinson NS, Polkey MI, Kemp PR: Downregulation of the serum response factor/miR-1 axis in the quadriceps of patients with COPD. Thorax. 2011;67:26–34.
[40] Oglesby IK, McElvaney NG, Greene CM: MicroRNAs in inflammatory lung disease–master regulators or target practice? Respir Res. 2010;11:148.
[41] Adcock IM, Caramori G, Barnes PJ: Chronic obstructive pulmonary disease and lung cancer: new molecular insights. Respiration. 2011;81:265–284.
[42] Li LJ, Gao LB, Lv ML, Dong W, Su XW, Liang WB, Zhang L: Association between SNPs in pre-miRNA and risk of chronic obstructive pulmonary disease. Clin Biochem. 2011;44:813–6.
[43] Nana-Sinkam SP, Karsies T, Riscili B, Ezzie M, Piper M: xxx. Exp Rev Respir Med. 2009;3:373–385.
[44] Crawford M, Batte K, Yu L, Wu X, Nuovo GJ, Marsh CB, et al.: MicroRNA 133B targets pro-survival molecules MCL-1 and BCL2L2 in lung cancer. Biochem Biophys Res Commun. 2009;388:483–489.
[45] Connolly E, Melegari M, Landgraf P, Tchaikovskaya T, Tennant BC, Slagle BL, et al.: Elevated expression of the miR-17–92 polycistron and miR-21 in hepadnavirus-associated hepatocellular carcinoma contributes to the malignant phenotype. Am J Pathol. 2008;173:856.
[46] Bommer GT, Gerin I, Feng Y, Kaczorowski AJ, Kuick R, Love RE, et al.: p53-mediated activation of miRNA34 candidate tumor-suppressor genes. Curr Biol. 2007;17: 1298–1307.
[47] Schultz J, Lorenz P, Gross G, Ibrahim S, Kunz M: MicroRNA let-7b targets important cell cycle molecules in malignant melanoma cells and interferes with anchorage-independent growth. Cell Res. 2008;18:549–557.

[48] Leite KRM, Sousa-Canavez JM, Reis ST, Tomiyama AH, Camara-Lopes LH, Saudo A, et al., eds.: Change in expression of miR-let7c, miR-100, and miR-218 from high grade localized prostate cancer to metastasis. Urol Oncol. 2011;29:265–9.

[49] Veerla S, Lindgren D, Kvist A, Frigyesi A, Staaf J, Persson H, et al.: MiRNA expression in urothelial carcinomas: important roles of miR 10a, miR 222, miR 125b, miR 7 and miR 452 for tumor stage and metastasis, and frequent homozygous losses of miR 31. Int J Cancer. 2009;124:2236–2242.

[50] Webster RJ, Giles KM, Price KJ, Zhang PM, Mattick JS, Leedman PJ: Regulation of epidermal growth factor receptor signaling in human cancer cells by microRNA-7. J Biol Chem. 2009;284:5731.

[51] Kefas B, Godlewski J, Comeau L, Li Y, Abounader R, Hawkinson M, et al.: microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. Cancer Res. 2008;68: 3566.