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Peripheral Blood Expressions of MicroRNA-146a and MicroRNA-218 in Chronic Obstructive Pulmonary Disease with/without Cigarette Smoke Exposure

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ABSTRACT

Altered expression and dysregulation of microRNAs (miRNAs) have been reported in different samples of chronic obstructive pulmonary disease (COPD) patients. The present study attempted to evaluate the peripheral expressions of miR-146a and miR-218 in COPD patients and sex-matched healthy controls with/without cigarette smoke exposure (CSE).

In this case-control study, blood samples were collected from 60 COPD patients (30 with CSE and 30 non-CSE in each group) and 60 healthy controls. Peripheral expressions of miRNA-146a and miR-218a were measured using qRT-PCR and results were compared between cases and controls as well as within the subgroups of patients.

We found significantly decreased expressions for both miRNAs in the patients compared to healthy controls. Remarkable underexpression of miRNA-146a and miRNA-218 were found in the CSE and non-CSE patients compared to non-CSE healthy controls and even in the CSE versus non-CSE controls. Both groups of patients showed underexpression of two miRNAs in comparison with CSE healthy controls and interestingly, similar decrements were observed in the CSE versus non-CSE patients. Also, ROC curve analysis revealed the significantly diagnostic powers for both miRNAs in discrimination of patients from healthy individuals and CSE-COPD from non-CSE COPD patients.

The underexpression of miR-146a and miR-218 in COPD patients and relation to CSE can be indicative of CSE-induced changes in miRNA expression profile and potential for these biomarkers in COPD risk assessment, particularly in those patients with CSE.

Keywords: Chronic obstructive pulmonary disease; Smoking

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is known as a major cause of chronic morbidity and

mortality that affects more than 200 million people worldwide and leads to approximately 3 million deaths yearly. This heterogeneous disease is defined by airflow limitation that is not the fully reversible and abnormal inflammatory response of the lung to noxious stimuli especially cigarette smoking as the most common risk factor for COPD. Smoking can induce the recruitment of neutrophils into the inflammatory site in the lung.[1,2] However, not all smokers develop COPD which indicates the attribution of genetic factors in the development of this disease.[3] Alternately, the contradictory results on the elucidation of target genes associated with COPD in different studied populations imply a significant role of epigenetic regulations.[4] MicroRNAs (miRNAs) as one of the main regulators for epigenetic alterations regulate the transcriptional activity of numerous genes involved in lung function and inflammation. Hence, these molecules are thought to be involved in the pathogenesis of COPD.[5]

MicroRNAs are a class of small non-coding RNAs with a regulatory effect on gene expression. They are approximately 19-25 nucleotides, single-stranded and highly conserved RNAs that bind to complementary sites in the 3′-untranslated regions (3′UTR) of their target mRNA and function as post-transcriptional repressors of gene expression. In other words, miRNAs bind to target regions in the messenger RNAs and lead to degradation of messenger RNA or inhibition of protein translation.[6] Furthermore, miRNAs can change gene expression levels by targeting transcription factors and DNA methyltransferases. Thus, miRNAs can interact with hundreds of genes simultaneously and regulate several cellular processes including cell cycle, cellular proliferation, differentiation and apoptosis, organ development, different immunity processes, and tumor progression. Several studies have shown that various miRNAs are expressed in the whole lung tissue, serum, and/or sputum of smoking COPD patients compared to smokers without COPD.[7-11]

In this context, studies have shown the altered expression and dysregulation of miRNAs in the peripheral blood (e.g., miR-199a, miR-24-3p, miR-93-5p, miR-320a, miR-320b, and miR-1273-3p),[12-15] lung tissue (e.g., miR-101, miR-146a, miR-15b, miR-144, and miR-199),[16-18] induced sputum (e.g., miR-34c, miR-218, miR-146a, let-7c, and miR-199a)[10] and bronchial airway epithelium (e.g., miR-218) of COPD patients.[19] Because of the possible contribution of CSE in these epigenetic alterations, we decided to evaluate the peripheral expressions of two candidate miRNAs (miR-146a and miR-218) involved in the COPD pathogenesis in a group of COPD patients with and without cigarette smoke exposure. Moreover, two groups of sex-matched healthy controls based on the history of CSE were enrolled for better comparison between patients and controls, and to find out the possible effects of CSE on target miRNAs expression levels.

MATERIALS AND METHODS

Study Participants

Sixty patients including thirty CSE-COPD (29 males and 1 female, mean age of 64.9±11.02 years) and thirty non-CSE COPD cases (3 males and 27 females, mean age of 67.2±10.3 years) who referred to our university hospital between March 2020 and September 2020 were recruited in this case-control study. COPD was diagnosed according to the criteria established by the Global Initiative for Chronic Obstructive Lung Disease (GOLD). The inclusion criteria for COPD cases were dyspnea, chronic cough, sputum production, and/or a history of exposure to risk factors for the disease. Spirometry was performed to confirm the diagnosis of COPD so that, observation of a post-bronchodilator FEV1/FVC ratio<0.70 confirms the presence of irreversible airflow limitation. The exclusion criteria were as follows: 1) diagnosis of any other inflammatory, autoimmune, metabolic, and chronic diseases; 2) infectious diseases within the past six months; 3) congenital disorders; 4) consumption of other drugs besides conventional treatment regimens for COPD and 5) malignancy.

In addition, currently smoking refers to someone who has smoked more than 100 cigarettes in their lifetime and has smoked in the last 28 days before participation in the study, and nonsmokers or never smoked is someone who has not smoked more than 100 cigarettes in their lifetime and does not currently smoke.[20]

The patient's demographics and clinical characteristics such as age, sex, and the mean scores of four spirometry parameters such as FEV1, FVC, FEV1/FVC, and FEF25%-75% were obtained from the medical records. Also, sixty ethnically and sex-matched healthy control subjects including thirty with CSE (29 males and 1 female, mean age of 37.03±9.5 years) and thirty non-CSE subjects (1 male and 29 females, mean age of 35.90±9.8 years) and without any chronic

diseases were included as controls. However, sex matching was adjusted based on the history of CSE among the patients and healthy controls. All participants in this study signed the written informed consents approved by our Institutional Medical Ethics Committee (IR.UMSHA.REC.1398.311) and according to the recommendations of the Helsinki declaration.

RNA Extraction and Expression Analysis of MiRNAs by qRT-PCR

Total RNA was extracted from the peripheral blood samples by using RNX^{TM}-PLUS solution (CinnaGen, Tehran, Iran) based on the manufacturers' instructions. Then, RNA purity and concentration were determined according to the ratio of absorbance at 260/280nm using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), and RNA integrity was determined by gel electrophoresis. In the next step, cDNA synthesis was carried out by using 1µg of total RNA and 1 pM of stem-loop specific primers for miRNAs and housekeeping genes. Reverse transcription reactions were performed at 37ºC for 60 minutes as per the manufacturer's instructions (BiomiR high sensitivity microRNA kit, Anacell, Tehran, Iran).

Thereafter, relative quantifications of miR-146a and miR-218 expression levels were carried out by the qRT-PCR method. All qRT-PCR reactions were prepared in a duplicate manner for each target miRNA by using 2µL of cDNA, 10µM of each specific primer, and 2xQPCR Master Mix SYBR Green I and RT-PCR program comprised of 1 cycle at 95ºC for 15 minutes, 40 two-step cycles at 95ºC for 15 seconds and 55-60ºC for 60 seconds followed by melting curve analysis at 55- 95ºC. (BiomiR high sensitivity microRNA kit, Anacell, Tehran, Iran). The U6 small nuclear RNA (snRNA) was used as a candidate endogenous control miRNA for data normalization. The reactions were run in a LightCycler instrument (LightCycler 96, Roche, Germany) and finally, the results were analyzed using the $2^{-\Delta CT}$ method as described by Livak et al, study.[21]

Statistical Analysis

Data are presented as mean±SD and the normality of the datasets was examined by D'Agostino-Pearson omnibus normality test to determine the normal distribution of datasets. Because of the non-Gaussian distribution of the expression levels, the Wilcoxon matched-pairs signed-rank test was implemented for comparison analysis between the study groups. Also, the Kruskal-Wallis test was used to compare the other quantitative data among groups. The diagnostic and discriminatory power of miRNAs were evaluated by receiver operating characteristic (ROC) curve analysis and the area under the curve (AUC) was calculated by computing sensitivity and specificity. GraphPad Prism version 6.0 was used for all statistical analyses. *p*-value<0.05 was considered statistically significant.

RESULTS

Demographics of the Study Subjects

Shown in Table 1 are the significant differences between CSE and non-CSE COPD patients as well as CSE and non-CSE healthy controls in terms of age and sex variables. In addition, the mean scores of four spirometry parameters such as FEV1, FVC, FEV1/FVC, and FEF25%-75% were significantly higher in the non-CSE healthy controls versus two groups of COPD patients and the non-CSE versus CSE COPD patients.

Table 1. Demographic information of COPD patients and healthy controls

	Study Groups				*p*
	NS-HC (n=30)	S-HC (n=30)	NS-COPD (n=30)	S-COPD (n=30)	
Age (Years)	35.90±9.87	37.03±9.53	67.17±10.30	64.96±11.02	**<0.001**
Sex (M/F)	1/29	29/1	3/27	29/1	**<0.001**
FEV1	-	99.8±89.24	61.24±67.87	43.20±52.29	**<0.001**
FVC	-	99.9±94.46	70.25±52.89	58.23±96.73	**<0.001**
FEV1/FVC	-	81.1±11.79	63.03±7.61	55.68±11.05	**0.005**
FEF25%-75%	-	83.12±61.33	37.23±22.70	21.14±81.48	**<0.001**

NS: non-smoker, S: smoker, HC: healthy controls, COPD: chronic obstructive pulmonary disease

Expression Levels of MiR-146a and MiR-218 in the Patients and Healthy Controls

We found significantly decreased expressions of miRNA-146a and miRNA-218 in COPD patients compared to healthy controls (p=0.0009 and p=0.0002 respectively, Figure 1). Our results revealed that cigarette smoke exposure (CSE) can significantly change the expression patterns for both miRNAs among subgroups of the study. We observed a remarkable underexpression of miRNA-146a in both CSE and non-CSE patients compared to non-CSE healthy controls (P<0.01) and even in the CSE healthy controls compared to non-CSE controls (p=0.03, Figure 2). Moreover, both groups of the patients showed a reduced expression of miRNA-146a in comparison with CSE healthy controls and interestingly, this downregulation was observed in the CSE versus non-CSE COPD patients (p=0.0001, Figure 1).

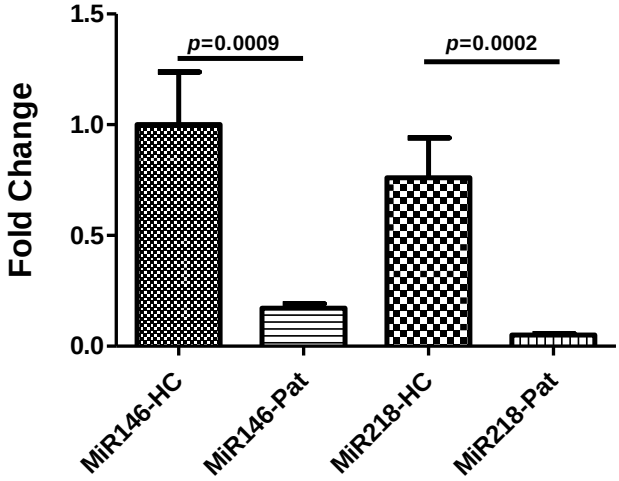

Figure 1. Expressions of miR-146a and miR-218 (Fold changes) in the patients (n=60) and healthy controls (n=60) groups.

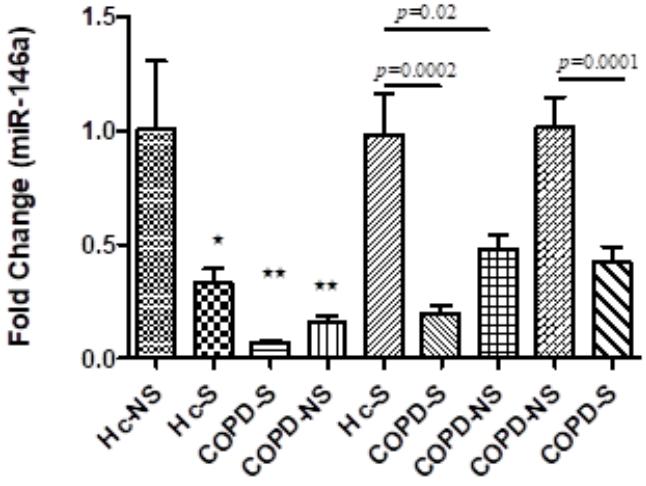

Figure 2. Comparison of the miR-146a expression levels among the study groups. The patients and healthy controls were stratified according to the cigarette smoking exposure (30 Hc-S, 30 Hc-NS, 30 COPD-S, and 30 COPD-NS cases). Significantly decreased expressions were observed in the smokers compared to non-smokers either in the controls or in the patient's groups. S: smokers, NS: non-smokers, Hc; healthy controls, COPD: chronic obstructive pulmonary disease. Data were represented as mean±SD. * p<0.05, ** p<0.01

Similar decrement in the expression of miRNA-218 was observed in the CSE and non-CSE COPD patients compared to non-CSE healthy controls (p=0.03), in the CSE and non-CSE COPD patients versus CSE healthy controls (p<0.05) and even in the CSE compared to non-CSE COPD patients (p=0.04, Figure 3).

Furthermore, ROC curve analysis revealed the significantly diagnostic powers for both miRNAs in the differentiation of patients from healthy individuals regardless of cigarette smoke exposure as well as differentiating CSE COPD from non-CSE COPD patients (Figure 4).

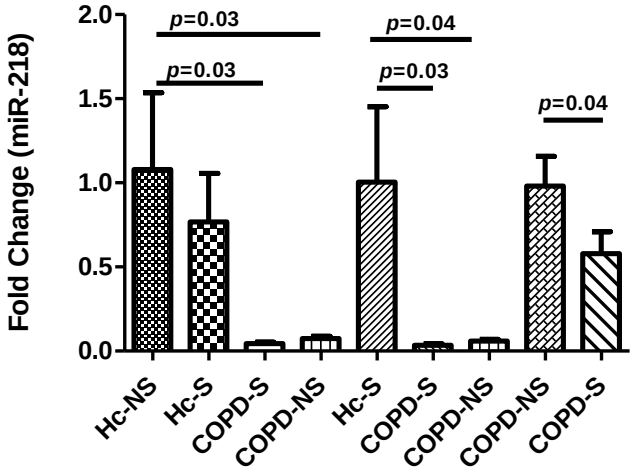

Figure 3. Comparison of the miR-218 expression levels among the study groups. The patients and healthy controls were stratified according to the cigarette smoking exposure (30 Hc-S, 30 Hc-NS, 30 COPD-S, and 30 COPD-NS cases). Significantly decreased expressions were observed in the smokers compared to non-smokers either in the controls or in the patients' groups. S: smokers, NS: non-smokers, Hc; healthy controls, COPD: chronic obstructive pulmonary disease.

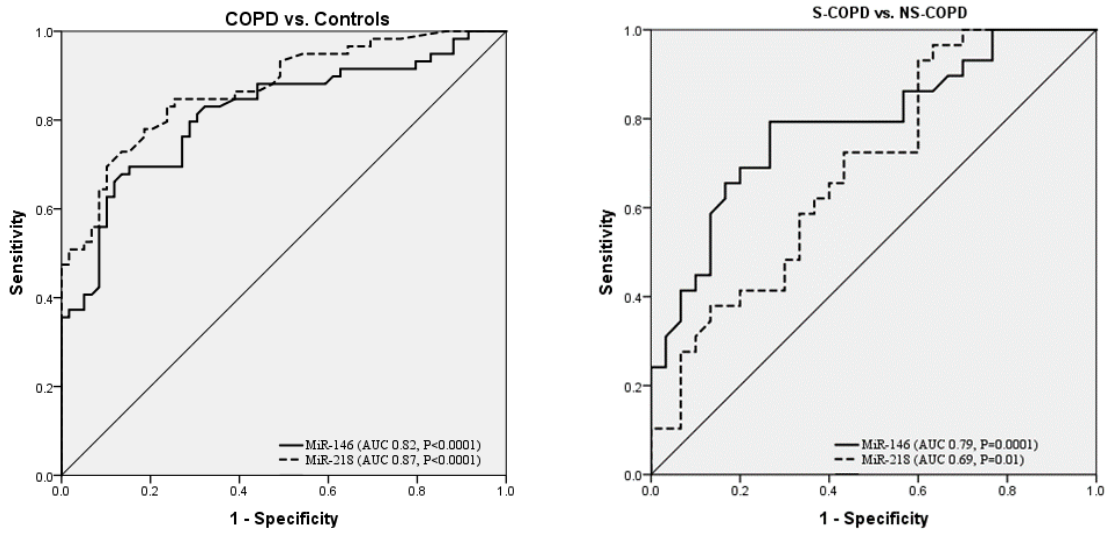

Figure 4. Diagnostic powers of miR-146a and miR-218 in differentiating COPD from healthy controls and discriminatory powers of both miRNAs in differentiating smoker COPD from non-smoker COPD patients utilizing ROC curve analysis. COPD: chronic obstructive pulmonary disease.

DISCUSSION

Although many studies have shown that the COPD pathology is related to systemic inflammatory components, few studies investigated the role of miRNAs in COPD pathogenesis.[22] In the present study we observed reduced expressions of both miRNA-146a and miRNA-218 in COPD patients compared to healthy controls and significantly decreased expressions in the smoker versus non-CSE COPD as well as in the CSE versus non-CSE healthy controls.

Several studies demonstrated the altered expressions and dysregulations of various miRNAs in different clinical samples such as peripheral blood,[14,15] the lung tissue,[16-18] induced sputum[10] and bronchial airway epithelium of COPD patients.[19]

Micro-RNA146a-5p has been proved as a regulator of inflammation. Upon activation of many inflammatory pathways, such as Toll-like receptor (TLR) or IL-1R signaling, miR-146a-5p is stimulated in a nuclear factor (NF)-κB-dependent manner. By targeting key molecules downstream of TLR and IL-1R pathways such as tumor necrosis factor receptor-associated factor-6 (TRAF-6) and IL-1 receptor-associated kinase (IRAK)-1, miR-146a-5p acts as a negative feedback regulator to confine the intensity and duration of inflammatory response.[23-25] Also, the anti-inflammatory role of miR-146a through downregulation of IRAK-1 and consequently IL-8 production has been documented in *vitro* experiments on fibroblasts from COPD patients.[5] In line with these findings, we found a significantly downregulated miR-146a in the COPD patients versus healthy controls and even in the CSE versus non-CSE COPD patients.

Likewise, Sato et al, demonstrated that interleukin (IL)-1β and tumor necrosis factor (TNF)-α, two potent inducers of the NF-κB pathway, induce the expression of miR-146a in both COPD and control fibroblasts but it was less pronounced in COPD fibroblasts. They suggested that this reduced miR-146a expression in COPD fibroblasts resulted in a reduced degradation along with a prolonged half-life of COX-2 mRNA, a predicted target of miR-146a, which ultimately leads to the overproduction of COX-2 protein. COX-2 is a key enzyme for the biosynthesis of prostaglandin E2 (PGE2) that promotes neutrophil recruitment and inhibits the restoration of lung fibroblasts.[10,11,26-30] Moreover, downregulation of miR-146a was shown in the sputum of smoker COPD compared with never-smokers and non-smoker COPD patients.[16] Consistently, we observed downregulation of miR146a in the CSE versus non-CSE COPD patients and the CSE versus non-CSE healthy controls. Moreover, ROC curve analysis in our patients revealed a potential for the discriminatory role of miR-146a and miR-218 expressions for differentiation of patients from healthy individuals and remarkably differentiation of CSE from non-CSE COPD patients.

In support of our results, Smet et al, review study highlighted the downregulation of mir-218 in COPD versus non-smokers as well as decreased expression of both mir-146a and mir-218 in the smokers versus non-smokers.[31] These results may be indicative of the inhibitory role of miR146a for smoke-induced damage in the lung tissue of COPD patients. In contrast to our findings, Ezzie et al, showed that miR146a expression was increased in the lung tissue of patients with COPD in comparison to smokers.[16]

Analysis of miR-218a in our study subjects revealed a decreased expression of miR-218 in two subgroups of the patients compared to either CSE or non-CSE healthy controls. These results are in line with Schembri et al, study that showed a downregulated expression of miR218a in the bronchial epithelium of smokers versus non-smokers, in the induced sputum of current smokers with and without COPD compared to never-smokers and in the bronchoalveolar lavage (BAL) fluid of patients with COPD versus control subjects.[19] MiR218 can regulate multiple genes indirectly in response to CSE by modulating the expression of transcription factor MAFG. CSE-induced changes in miRNA expression in the murine lung are irreversible if a certain threshold is reached, depending on the duration and dose of smoke exposure.[10,19,32,33] In agreement with these findings, we observed a significant underexpression of miR-218 in the CSE versus non-CSE COPD patients.

These findings support the idea that persistent dysregulated gene expression, found in the lungs of smokers and ex-smokers, might be attributable to irreversible changes in miRNA expression induced by exposure to a sufficiently high dose of smoke for a long period.[10,19,33] Regarding the possible role of miR-218 in COPD pathogenesis, Liu et al, demonstrated that miR-218 was downregulated in COPD and CSE-induced BEAS-2B cells, and it was positively associated with FEV1 % in COPD patients. From a mechanistic point of view, overexpression of miR-218 or knockdown of BRD4 mitigated apoptosis and inflammation in BEAS-2B cells induced by CSE. Additionally, overexpression

of BRD4 weakened the miR-218-mediated effects on CSE-induced BEAS-2B cells. On the other hand, overexpression of miR-218 inhibited CSE-induced apoptosis and inflammation in BEAS-2B cells by targeting BRD4 expression.[34] Conickx et al, depicted that miR218-5p was significantly downregulated in smokers without airflow limitation and in COPD patients compared to never-smokers. Notably, the expression of miR218-5p was significantly associated with airway obstruction.[35] Song et al, showed that miR-218-5p was significantly down-regulated in patients with COPD compared to healthy individuals. Also, a mouse model of CS-induced COPD by implementing a miR218-5p inhibitor demonstrated a protective role of miR218-5p in cigarette smoke-induced inflammation and COPD.[36]

In conclusion, we observed the significantly dysregulated miR-146a and miR-218 expressions in COPD patients which were more evident in the presence of cigarette smoke exposure (CSE) not only in the patient groups but also, in the CSE healthy individuals. The underexpressions of two miRNAs in COPD patients and more importantly CSE can be indicative of CSE-induced changes in miRNA expression profile and subsequently altered expressions of COPD-related genes such as those involved in the inflammatory responses, oxidative stress, and apoptosis. Therefore, both miRNAs may have a potential for biomarkers in COPD risk assessment, particularly in those patients with CSE.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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