Severe Decline in Mir-20a and Mir-92a in the Context of the Mir-17-92 Cluster: Ideal Biomarkers of Various COPD Subtypes

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Abstract - Chronic obstructive pulmonary disease (COPD) is going to be the third leading cause of death by 2020. Circulating miRNAs are among the most beneficial feasible non-aggressive biomarkers for diagnosis and treatment of many diseases. Among members of the significant miR-17-92 cluster, miR-20a and miR-92a are greatly involved in both inflammation and hypoxia (known as the main reasons for COPD comorbidities). Thus, the expression of these miRNAs was evaluated in the serum of 26 patients and 19 controls using the sensitive stem-loop RT-qPCR approach. The results revealed a significant reduction of these miRNAs in patients relative to controls (P<0.001). Decreased expression of miR-20a in a patient might reflect a progressive stage of the disease. MiR-92a might be used as an early-detection biomarker of COPD. These miRNAs can be used as therapeutic targets specifically in the context of the miR-17-92 cluster to address the various clinicopathological aspects of the disease.

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Keywords: Chronic obstructive pulmonary disease; Diagnosis; MicroRNA; Serum

Introduction

Chronic obstructive pulmonary disease (COPD) is a predicted third prominent cause of death after cancer and cardiovascular diseases by 2020 (1). Common COPD comorbidities, such as diabetes, and so on are to a certain extent caused by systemic inflammation and tissue hypoxia (2,3).

The method typically used to diagnose COPD is the evaluation of pulmonary function by spirometry. Spirometric results are interpreted on the basis of the grading criteria presented by the Global Initiative for Chronic Obstructive Lung Disease (GOLD). In relation to the disease, the criteria measure forced expiratory volume in the first second (FEV1) and forced vital capacity (FVC) (3). A decreased FEV1 and increased functional residual capacity (FRC) and total lung capacity (TLC) is correlated with breathlessness in COPD (4). Despite the value presented by spirometry, however, it suffers from many drawbacks as an approach to describing the severity of COPD. It is a time-consuming and difficult test for both patients and technicians. Given these challenges, more than 75% of COPD patients are usually undiagnosed until the last severe nonreversible stages of the disease (5) -a neglect that may be one of the reasons for the increasing mortality rate of COPD patients around the world (in contrast to the decreasing mortality rates of many other diseases). This problem has prompted scientists to search for new feasible non-aggressive genetic and/or epigenetic biomarkers of COPD.

Although a genetic background that renders COPD patients strongly susceptible to smoking is an important biomarker of the disease (6), epigenetic changes [including variations in microRNA (miRNA) levels] followed by exposure to environmental factors, such as cigarette smoke, are equally compelling factors for examination (7,8). miRNAs are single-stranded and highly conserved noncoding RNAs with an approximate length of 19 to 25 nucleotides. The major function of microRNAs is to inhibit the expression of the genes at mRNA levels by the means of two processes: 1-degradation of messenger RNAs (mRNAs) or 2-inhibition of protein translation (9).

Almost all the biological processes of cells and organisms can be regulated with miRNAs (9), in which any pathological changes are closely associated with many diseases, including COPD and asthma (7,10). For this reason, researchers widely use these molecules for the diagnosis, prognosis, and treatment of illnesses,
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evenly cancer (11).
This study focused on the miR-17-92 cluster and its paralogues because of their key elemental functions that exhibit a strong potential correlation with respiratory diseases, such as COPD (12). Almost all members of the significant miR-17-92 cluster have been demonstrated to be down regulated in respiratory diseases (13). Some studies, however, found elevated levels of miR-20a in COPD patients (14). This inconsistency in results may be due to sample and/or technical differences. On the other hand, the selection of miRNA was based on hypoxia and inflammatory processes that, as discussed earlier, are well-associated with COPD comorbidities (2). Both miRNAs are significantly involved in current processes (15-19).

This study was conducted to provide beneficial and accurate data on the exact role of miR-20a and miR-92a in the pathogenesis of COPD and to shed more light on the differential diagnosis of COPD to contrast it with other lung diseases) and new effective methods of treating the disorder.

Materials and Methods

Patient selection and serum collection
Twenty-six COPD patients (cases) who were admitted to the lung clinic of Baqiyatallah Hospital from 1st March 2013 to 1st July 2014 and nineteen normal participants (controls) were appointed to this study. All patients had a documented consumption of at least twenty boxes of cigarettes as well as confirmation with spirometry values and a prior clinical examination by a proficient lung physician. Patients with other respiratory diseases or those who were exposed to any other chemicals except the cigarette smoke were excluded from study. For each case and control, the information including gender, age, weight and spirometry values including forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) were gathered (Table 1).

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### Table 1. Clinical characteristics of COPD patients. Spirometry data are stated as percentage

The severity of COPD disease was categorized based on FEV1 values. Subjects with FEV1 > 80% were classified as “mild”, “moderate” (50% ≤ FEV1 < 80%), and “severe” (30% ≤ FEV1 < 50%) (20). Approved by the Ethics Committee of Baqiyatallah University, a written informed consent was obtained from all participants of this study. 5 mL venous blood was collected in gel containing Venoject glass tubes and incubated at room temperature for one hour to be coagulated. Serum was separated by centrifugation at 2500 g for 20 min at room temperature and stored at −80° C until RNA extraction.

RNA extraction and DNase 1 treatment
25 μL Proteinase k (~20 mg/ml, Thermo Scientific, USA) was added to 250 μL serum and incubated for one hour at 56°C. For RNA extraction, 1 ml Rima ZoLES (Zist Abzar Pajhoohan, Iran) was applied to the 250 μL of the treated serum for homogenization and incubated for 5 min at room temperature. After 15 seconds of vigorous shaking with 250 μL chloroform, the serum sample was incubated for 3 min at room temperature and centrifuged for 15 min at 12 000 g at 4° C.

An equal volume of Isopropanol was added to the aqueous phase and incubated for 10 min at room temperature. The sample was then centrifuged for 10 min at 12 000 g. After supernatant removal, the pellet was added with 1 ml 70% ethanol and centrifuged at 6 000 g for 5 min. The ethanol supernatant was disposed and dissolved in 25 μL RNase-free water for 10 min, at
room temperature, after partial drying.

For likely Genomic DNA removal, 1 µL RNase-free DNase I enzyme (Thermo Scientific, USA) in conjunction with 1 µL of its buffer was added to 1 µg RNA in a final volume of 10 µL and incubated for 30 min at 37°C. Then, 0.5 µL 50 mM EDTA was added and incubated for 10 min at 65°C. RNA was quantified using a Nano Drop (MAESTRO GEN, USA).

The cDNA synthesis for stem-loop real-time quantitative reverse transcription PCR (qRT-PCR)

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About 250 ng total RNA was supplemented with 1.5 µL stem loop RT primer (50 nM) in a final volume of 13 µL and the mixture was incubated for 10 min at 70°C in the thermal cycler (Master cycler Gradient PCR, Eppendorf, Germany). Primer sequences are provided elsewhere (21). To circumvent the traditional probe design, the homologous genes of microRNAs—found in miRBase database—were excluded by designing specific stem-loop and forward primers. 4 µL 5X RT buffer (Thermo Scientific, USA), 2 µL dNTP (10 mM, Thermo Scientific, USA), and 1 µL Reverse Transcriptase (200 u/µL, Thermo Scientific, USA) were added to the 13 µL reaction mixture on ice box. cDNA synthesis was carried out in the final 20 µL reactions as follows: 25°C for 15 min, 37°C for 15 min, 42°C for 60 min, and 70°C for 10 min. The synthesized cDNA was stored at -20°C for Real-time PCR.

Real-time qRT-PCR assay was performed in a 13 µL PCR mixture volume composing 6.5 µL 2 X Prime Q-Master Mix with SYBR Green 1 (GeNet Bio), 0.2 µL 50 X ROX dye, 0.2 µM form each forward and reverse primers, 1.5 µL cDNA and 4.4 µL DNase/RNase-Free H2O (22). MiR-16 and miR-423-5p (data not shown) were used as the housekeeping internal controls or reference genes. Amplification was performed in the following conditions: 1 cycle of enzyme activation at 95°C for 10 min and 40 cycles of two amplification steps at 95°C for 30 sec and 60°C for 30 sec by using an ABI7900 real-time quantitative PCR system (Applied Biosystems, USA).

PCR duplicates were used in all real-time reactions.
To make sure that the RNA samples were not contaminated with genomic DNA, a no reverse transcriptase (no RT) control was included in all runs of real-time PCR. To scan the precision of amplifications, a no cDNA negative control was also incorporated in each reaction.

To determine the reaction efficiencies of each primer pair, LinRegPCR, version 12.x software (AMC, Amsterdam) was used.

Statistical analysis

Group-wise comparison between the serums of COPD (case) and control individuals from the aspect of relative miR-20a and miR-92a expression was performed using Relative Expression Software Tool (REST), version 2.0.13 (QIAGEN GmbH All rights reserved). Microsoft Excel was used to illustrate expression levels in cases and controls.

Statistical Program for Social Sciences (SPSS) software, version 17 (SPSS Inc., Chicago, IL, USA) was utilized for data analysis. One sample Kolmogorov-Smirnov (KS) test was carried out to check the normal distribution of samples. The statistical calculation was based on normalized ΔCt values. MiR-16 was considered as reference internal control gene. The relative average fold changes of expression of miRNAs were calculated as described elsewhere (23). Data were reported as mean ± standard deviation (SD) to represent relative average fold changes of miRNAs between COPD and control serums. If KS test was passed, Student’s t-test was used to estimate whether miRNA alterations between patients and healthy subjects are significant. Statistical differences between case and control samples were determined by the non-parametric Mann-Whitney test if KS test was not satisfactory. P of < 0.05 were considered significant.

The Receiver Operating Characteristic (ROC) curve analysis was employed to determine whether expression of miR-20a and miR-92a has the sensitivity and specificity to discriminate between cases and controls.

Results

Twenty-six patients with COPD and 19 normal people were the subjects of this study.
Among 26 COPD patients, 12 cases were placed in GOLD3 grade of the disease (severe), 7 were in GOLD2 (moderate), and 7 in GOLD1 (mild), according to the spirometry data. The age average for the case and control group were 62.5±5.5 and 47.68±6.9 respectively. No significant differences existed between the cases and the control from the vantages of age and weight. All of FEV1, FVC and FEV1/FVC amounts were greatly lower in the case compared to the control group (Table 1).

NanoDrop (Thermo Scientific, Wilmington, DE) was employed to assure quality and quantity of all RNA samples before any downstream applications. To obtain...
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an accurate comparison result, similar amounts of RNA were used for the subsequent stem-loop procedure.

**Stem-loop qRT-PCR**

The relative miR-20a and miR-92a expression for all samples were normalized to that of the reference genes, and the result of comparison of expression between case and control groups were obtained using $2^{\Delta\Delta CT}$ equation.

The efficiencies of the primers were calculated using LinRegPCR software (public domain). Although the specificity of designed primers was confirmed using agarose gel electrophoresis and melting curve analysis, a final confirmation was established by direct sequencing of qRT-PCR products (data not shown). The validity of PCR products was also assessed using analysis of melt curves and amplification plots (data not shown). The latter demonstrated no amplification in negative and no-RT controls.

**miR-20a and miR-92a are significantly down-regulated in the serum of the case compared with the control group**

Analysis of expression data from stem-loop qRT-PCR on all purified RNAs revealed a substantial reduction in the case compared to the control group, with a $P$ of $< 0.001$ for both miR-20a (~ 24-fold reduction) and miR-92a (~ 1000-fold reduction) using non-parametric Mann-Whitney test and Student’s t-test, respectively (Figure 1).

![Figure 1](image1.png)

**Figure 1.** Relative gene expression of miR-20a and miR-92a in the serum of COPD patients compared to that of control. After normalization to that of internal control, the expression level of both genes was calculated using the $2^{\Delta\Delta CT}$ method in COPD compared to the healthy ones ($P$ of $< 0.001$).

At the level of subgroups of the case group, further miR-20a expression analysis also revealed reduction at all of the severity grades of the disease compared to the control with $P$ of 0.055, and $< 0.001$ for mild (~ 2-fold reduction), and moderate (~ 500-fold reduction) and severe (~ 20-fold reduction) grades of the disease, respectively. A similar miR-92a expression analysis also indicated a substantial decrease at all levels of mild (~ 35-fold reduction), moderate (~ 1000-fold reduction), and severe (~ 1000-fold reduction) with $P$ of $< 0.001$ for all of the severity grades. The expression is nearly suppressed at just moderate, and both moderate and severe grades of the disease, for miR-20a and miR-92a, respectively (Figure 2).
Figure 2. Relative gene expression of miR-20a and miR-92a at different grades of the disease, including mild, moderate and severe. The expression level of both miRNAs was significantly downregulated in patients at any of the severity grades of COPD patients related to the healthy controls, using Mann-Whitney and Student’s t-test, respectively.
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miR-20a and miR-92a expression levels have enough diagnostic accuracy to be used as biomarkers. Due to the high AUC (area under the curve) of 0.881 and 0.943 obtained with the ROC curve method for miR-20a and miR-92a, respectively, both miRNAs had the required amount of sensitivity and specificity to be used as biomarkers. In other words, the test can discriminate COPD from healthy individuals (Figure 3).

Figure 3. ROC curve analysis for miR-20a (A) and miR-92 (B) expression revealed both miRNA as suitable biomarker for determining whether COPD is present or absent. The area under the curve (AUC) for miR-20a and miR-92a was 0.881 and 0.943, respectively (P of < 0.001).

Discussion

Understanding the molecular pathogenesis of a disease is the first step toward solving the problems of detection and treatment. Because of relatively poor pathophysiological information on COPD, current treatment options do not totally eradicate the disease, which is currently known as a principal cause of morbidity and mortality around the world. The most important risk factor for COPD is cigarette smoke; air pollution, dust, and fumes are also involved in the disease to some extent (1-3,5).

Owing to the criticality of environmental factors (cigarette smoke) in the pathogenesis of COPD, epigenetic regulatory mechanisms are equally crucial to such pathogenesis. These mechanisms are increased levels of histone acetylation and DNA methylation and reduced levels of miRNAs. Studies on miRNAs as essential epigenetic factors would therefore be of great value (7,8).

There are three COPD subtypes: (1) chronic bronchitis; (2) emphysema; and (3) small-airway disease (4). In fact, the most important pathophysiological features of COPD are: (1) airway remodeling including fibrosis (caused by fibroblast proliferation) and emphysema [caused by epithelial apoptosis or secretion of Matrix metalloproteinases (MMPs) (24)]; (2) malfunctioning extracellular matrix (ECM) turnover; and (3) chronic bronchitis (caused by mucus hypersecretion). Chronic lung inflammation and oxidative stress (25) can exacerbate or contribute to all pathophysiological features (4). The molecular clock (circadian rhythm) is also disrupted in COPD pathogenesis (26).

The most important signaling pathways involved in these features are Notch (27), mitogen-activated protein kinase (MAPK) (28), adenosine (29), unfolded protein response (UPR) (30), wingless/integrase-1 (Wnt) and TGF-β/Smad3 signaling pathways (31). Almost all of these pathways can be determined by using DIANA miRPath tools: Protein processing in the ER, cell cycle, cancer, circadian rhythm, MAPKs, TGF-β and Wnt signaling pathways are sequentially considered among the most important pathways targeted by miR-20a. On the other hand, ECM–receptor interaction, central carbon metabolism in cancer, and bacterial invasion of...
epithelial cells are sequentially targeted by miR-92a. This means that miR-20a and miR-92a are involved in COPD pathogenesis through the exertion of their effects on the signaling pathways that are involved in such pathogenesis.

A comprehensive review compared the results of seven reports on differential miRNA expression and announced little similarity between studies mainly due to tissue sources (that are lung tissue, whole blood, serum, plasma) (32). Researchers concluded that those miRNAs involved in more than one of COPD features (that are let-7C, miR-1, miR-7, miR-15b, miR-34a, miR-34b, miR-199a-5p and miR-218) are excellent target candidates for COPD therapeutics.

In a recent study, miR-31, miR-155, miR-218 and let-7c demonstrated consistent expression in lung tissue and cell-free bronchoalveolar lavage (BAL) supernatant of both of cigarette smoke (CS)-exposed mice and COPD patients using stem-loop RT-qPCR (33).

An analogous study in 2017 evaluated a comparative expression study of miRNA-145 and miRNA-338 by qRT-PCR in COPD, asthma and asthma-COPD overlap syndrome (ACOS) in the serum and sputum of the patients (34). This study determined sputum miRNAs have the potential application to discriminate patients with ACOS, asthma, and COPD as biomarkers.

A similar validation work by qRT-PCR in 2017, revealed 19 differentially expressed miRNAs in peripheral leukocytes of COPD patients of which miR-3177-3p and miR-183-5p were downregulated and miR-106b-5p, miR-125a-5p, miR-183-5p, and miR-100-5p were identified as important miRNAs in progression of COPD (35).

Evaluating the gene expression analysis of the miR-17-92 cluster and its paralogues is a crucial task in research (36). The cluster has an important role in homeostasis of lung epithelial cells (37) and lung development (38). A recent report investigated the character of the cluster in idiopathic pulmonary fibrosis (IPF) in which the expression of the cluster and DNA methyltransferase (DNMT)-1 was decreased and elevated in fibrotic lungs, respectively and treatment with 5’-aza-2’-deoxycytidine attenuated pulmonary fibrosis in animal model of the disease (13).

In the present work, researchers selected miR-20a and miR-92a as two important representative members of the clusters that target hypoxia and inflammatory pathways to obtain beneficial data for the diagnosis and/or treatment of lung diseases, specifically COPD. Both miRNAs were significantly down regulated in the serum of COPD patients compared with that of healthy individuals, as determined using stem-loop qRT-PCR. The decreased expressions of miR-20a and miR-92a were enough to determine whether COPD was present or absent in the patients (Figure 3). The results agree with those of previous studies, in which the significant down regulation of the miRNAs was detected (39, 40). The data also accord with our previous parallel report published on mustard lung (21).

Hif-1A is a target for miR-20a and Hif-2A (EPAS1) is a target for miR-92a. The former is involved in transient and the latter in prolonged hypoxia (41). Researchers obtained a higher reduction level for miR-92a compared with miR-20a as COPD is a typical chronic disease.

The higher level of reduction in miR-92a on treatment options, attention should be paid to the exact tissue niches for targeting because the patterns of pathogenic pathways are apparently reversed in fibroblastic mesenchymal cells as opposed to the patterns in epithelial parenchymal cells (32). Compensating for reduced clusteral levels in fibroblasts
may cause fibrosis. Conversely, compensating for reduced clusteral levels in epithelial cells may reverse the phenotype of emphysema. Thus, epithelial (and not mesenchymal) cells can be satisfactorily introduced as potential candidate targets for miR-17-92 clusteral therapies for COPD. Accordingly, fibroblasts and mucus-producing cells should be excluded to somehow lessen the influence of off-target effects in miRNA-based therapeutics. Nevertheless, considerable caution should be exercised when using oncomiRs as therapeutics to avoid the tumorigenesis of normal cells.

Our study confirmed the findings regarding the down-regulation of miR-20a and miR-92a with high-throughput techniques. miR-20a might have the potential to discriminate between severe and moderate stages of COPD. In other words, detecting decreased expression of miR-20a in a patient may reflect a progressive stage of the disease. miR-92a might detect the disease at the very early stages of its appearance and can serve as an early-detection biomarker for preventive treatment strategies (Figure 2).

This study was aimed at assessing the expression profiles of miR-20a and miR-92a in the serum of COPD patients. Researchers found a significant down-regulation of the miRNAs in the serum of the COPD group compared with that of healthy individuals. From the perspective of airway remodeling, our results are consistent with the most prominently reported feature of COPD, namely, emphysema, in which matrix degradation occurs through epithelial apoptosis. Our report also confirmed the hypoxic conditions observed in COPD situations. The higher rate of decrease in miR-92a (targeting EPAS1) compared to that of miR-20a (targeting Hif-1A) is consistent with the chronic nature of COPD in which EPAS1 has a potentially significant role.

The lower level of reduction in miR-20a compared with miR-92a may, to some extent, explain the increased levels of miR-20a observed in some studies.

The clusteral expression pattern of miR-17-92 reflects an ideal representative of the pathogenesis of COPD disease in terms of its subtypes. Whereas current ineffective clinical treatments focus on a few aspects of this complicated disease, miRNA-based therapies target a wide range of pathways involved in its pathogenesis. As previously indicated, miR-92a can detect the disease at the very early stages of its appearance and serve as an early-detection biomarker, and a progressive stage might be inferred from decreased miR-20a expression. Off-target effects in miRNA therapies should be considered to avoid the toxicity of nonspecific deliveries to normal or unintended cells.

Our future experiments will be directed by investigations into the origins of cells or tissues given that the significant reductions found in this work may have resulted from fibrotic, epithelial, and/or inflammatory cells. This direction precisely identifies the cells that are amenable to directed miRNA-based therapies.

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