Original Article

Molecular Detection of *Chlamydia pneumoniae, Haemophilus influenza*, and *Streptococcus pneumoniae* and Expression of miR-146, miR-16, and miR-221 in Patients with Chronic Obstructive Pulmonary Diseases

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) is a debilitating respiratory condition characterized by persistent airflow limitation and chronic inflammation. Microbial infections and dysregulated microribonucleic acid (miRNA) expression have been implicated in COPD pathogenesis. This study aimed to investigate the molecular detection of three respiratory pathogens, *Chlamydia pneumoniae*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*, in the respiratory secretions of COPD patients. In addition, it evaluated the expression levels of miR-146, miR-16, and miR-221 in the peripheral blood of COPD patients. **Methods:** Peripheral blood and respiratory secretions were collected from 40 healthy individuals and 40 COPD patients. The messenger ribonucleic acid expression levels of miR-146, miR-16, and miR-221 were determined using real-time polymerase chain reaction. Statistical analyses, including *t*-test, binomial test, and Pearson correlation, were performed. **Results:** *H. influenzae*, *S. pneumoniae*, and *C. pneumoniae* were detected in the sputum of 12.5%, 17.5%, and 7.5% of COPD patients, respectively. The expression of miR-146, miR-221, and miR-16 was observed in 65%, 15%, and 85% of COPD patients, respectively, compared to 13%, 80%, and 15% of healthy subjects. While miR-221 was downregulated in COPD patients, miR-16 and miR-146 were upregulated. No significant differences were found in the expression of these miRNAs between infected and noninfected COPD patients. **Conclusion:** The molecular detection of respiratory pathogens and the expression profiles of miR-146, miR-16, and miR-221 in COPD patients may have potential diagnostic value. Further research is needed to elucidate the role of these markers in COPD pathogenesis.

Keywords: *Chlamydia pneumoniae*, chronic obstructive pulmonary disease, *Haemophilus influenza*, miR-146, miR-16, miR-221, *Streptococcus pneumoniae*

Introduction

Chronic obstructive pulmonary disease (COPD) is a debilitating respiratory disease associated with various clinical signs such as shortness of breath.[1,2] It is characterized by persistent and progressive airflow limitations and associated with a chronic inflammatory response in the lung and the airways to gases or noxious particles.[3,4] Although it is considered a preventable/treatable disease, it has now become a major concern for global health.[5] It is one of the leading causes of morbidity and mortality in

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the world accounting for approximately 6% of total deaths worldwide.[6]

Micro-ribonucleic acids (miRNAs) play an important role in a wide range of cellular processes, such as apoptosis, cell proliferation, and differentiation.[7,8] miRNAs are small noncoding ribonucleic acid (RNA) molecules, typically ranging from 19 to 25 nucleotides in length.[9] They play a significant role in gene regulation by binding to target messenger RNAs (mRNAs) at various regions.[10,11] Dysregulated miRNA and gene expression have been shown to play in COPD pathogenesis.[1,12] miR-146a has been shown to decrease in primary human lung fibroblasts in COPD and has been linked to COPD pathogenesis.[13] miR-221-3p has been recently shown to alleviate cell apoptosis and inflammatory response in an *in vitro* model of COPD.[14] In addition, miR-16 has been shown to express differentially among allergic and asthmatic and nonallergic and nonasthmatic patients.[12,15]

The lungs are widely exposed to environment-derived microorganisms such as viruses, bacteria, and fungi. COPD is mostly associated with infection caused aforementioned microorganisms.[16] The lung microbiota mostly belongs to the normal flora, which plays a key role in the integrity of pulmonary epithelial, colonization, and the immune system homeostasis in the respiratory tract.[17] *Chlamydia pneumoniae, Haemophilus influenza*, and *Streptococcus pneumoniae* are microorganisms involved in the development and pathogenesis of respiratory infections, specifically in COPD.[18] Therefore, this study was aimed to assess the molecular detection of *C. pneumoniae, H. influenza*, and *S. pneumoniae* in respiratory secretions as well as to measure the expression level of miR-146, miR-16, and miR-221 in the peripheral blood of patients with COPD.

Methods

Ethical consideration

This study was conducted in accordance with the principles of the Declaration of Helsinki. The study protocol was reviewed and approved by the Institutional Review Board of the National Research Institute of Tuberculosis and Lung Diseases (NRITLD) (IR.SBMU.NRITLD.REC.1399.208). All participants provided written informed consent before their enrollment in the study.

Measures were taken to ensure the confidentiality and privacy of the participants' data. Participant information was anonymized, and all data were securely stored in a password-protected database, accessible only to the research team. Participants were informed of their right to withdraw from the study at any time without any consequences. The collection of blood and respiratory samples was performed by trained medical professionals, ensuring minimal discomfort and risk to the participants. Appropriate safety protocols were followed during the handling and processing of the biological samples.

The study design and methods were carefully evaluated to minimize any potential harm or burden to the participants. The potential benefits of the research, in terms of improving the understanding and diagnosis of COPD, were deemed to outweigh the minimal risks associated with the study procedures.

Type of sampling and reasons for selection

Forty respiratory secretions from COPD patients suspected of bacterial infection were collected with the assistance of a pulmonologist. First, the sputum samples were taken to the laboratory quickly in the order they were taken on each day. Before deoxyribonucleic acid (DNA) extraction, there is a preparation and lysis stage of sputum samples, in such a way that the same volume of sputum samples is added to phosphate buffer saline and then for 15 min. It was centrifuged at 2500 rpm. This step is repeated twice, and then the supernatant is discarded. Next, 5 µL of proteinase K was added to the contents of the microtubes and placed at a temperature of 55°C for 3 h and then at a temperature of 95°C for 30 min. Finally, these contents were entered into the (DNA) extraction protocol.[19] The polymerase chain reaction (PCR) product was electrophoresed and the bands were analyzed.

Then, for miRNA detection, 40 peripheral blood samples each were collected from COPD patients and healthy controls. These samples were matched for the age group (22–65 years) and consisted of peripheral blood from diagnosed COPD patients and healthy individuals. Three milliliter of peripheral blood was taken from all participants in the research. The samples were then immediately transported to the laboratory on ice and promptly processed for RNA extraction. After extracting RNA using a special kit and synthesizing complementary DNA (cDNA), a real-time PCR (RT-PCR) test was performed to measure the expression changes of the desired genes.[20]

Patient consent statement

Informed written consent was obtained from the participants before sample collection.

Inclusion criteria

Inclusion criteria for the study included respiratory symptoms such as persistent dry cough, fever, wheezing, shortness of breath, and sputum.

Exclusion criteria

Patients with a history of asthma, allergies, or tuberculosis infection were excluded from the study.

DNA extraction and PCR assay on the respiratory secretions

The respiratory secretions were collected from 40 patients with COPD who were suspected to bacterial infection. Total DNA was extracted using a Genomic DNA Extraction Kit (GeNet Bio). The special primers related to *P6*, *Cps,* and *Omp1* genes for the *H. influenza, S. pneumoniae, and C. pneumoniae,* respectively, were designed with AlleleID software version 7.0 (Premier Biosoft, Palo Alto, CA, USA) as shown in Table 1.

Table 1: The primer sequencing of the microorganisms and microribonucleic acid

	Primer sequencing	Target gene
Microorganism		
Haemophilus influenzae	F: 5'-CCAGCTGCTAAAGTATTAGTAGAAG-3'	P_6
	R: 5'-TTCACCGTAAGATACTGTGCC-3'	
Streptococcus pneumoniae	F: 5'-GCAGTACAGCAGCAGTTTGTTGGACTGACC-3'	*Cps*
	R: 5'-GAATATTTTCATTATCAGTCCCAGTC-3'	
Chlamydia pneumoniae	F: 5'-GTTGTTCATGAAGGCCTACT-3'	*Omp1*
	R: 5'-GTGTCATTCGCCAAGGTTAA-3'	
miRNA		
miR-146	F: 5'-CCGGCAAUUCAGUUUCUACA-3'	
	R: 5'-GGCCCGUUAAGUCAAAGAUGU-3'	
miR-221	F: 5'-GCAGACTCCGCAAATATTCC-3'	
	R: 5′-TGTCCTTCTTCCACTTCATGC-3′	
miR-16	F: 5'-ACACTCCAGCTGGGTAGCAGCACGTAAATATTGGC-3'	
	F: 5'-ACACTCCAGCTGGGCCAGTATTAACTGTGCTGCTG-3'	
U6	F: 5'-CTCGCTTCGGCAGCACA-3'	
	R: 5'-AACGCTTCACGAATTTGCGT-3'	

miRNA: Microribonucleic acid

Each 25−μL, reaction mixture contained 0.3 μL of Taq DNA polymerase (GeNet Bio), 20 μL of PCR Master a 5−μL template DNA. The thermocycler machine (Eppendorf, Germany) was set to provide 2 min at 94°C, followed by 35 cycles each of 30 s at 94°C, 30 s at 55°C, 40 s at 72°C, and 7 min at 72°C. The PCR products were analyzed by 2% agarose gel electrophoresis.

C. pneumoniae detection was performed using a GeneProof RT-PCR kit according to the following program: hold for 2 min at 37°C then 10 min at 95°C, followed by 45 cycles each of 5 s at 65°C, 40 s at 60°C and 2 s at 70°C.

Ribonucleic acid extraction and real-time polymerase chain reaction

Peripheral blood was sampled from a total of 80 individuals, including 40 COPD patients and 40 healthy controls (aged 22–65 years). The total RNA of the samples was extracted using RNeasy Midi Kit (Qiagen Cat No. 75144) according to the manufacturer's protocol. The concentration and purity of RNA were assessed at 260 / 280 nm absorbance, and the isolated RNA with a 260 / 280 ratio of ~2 was used for further experiments. The RNA was reverse transcribed to cDNA using ZIST ROYESH Kit (Tehran, Iran) in triplicate and three vials of each cDNA were synthesized.

The U6 was used as the housekeeping gene or a comparator in parallel to the control. AlleleID7 software was utilized to design the primers used in this study. The sequences of the miRNA primers are shown in Table 1. The expression levels of the dysregulated miRNA-146, miR-221, and miR-16 in the peripheral blood of the patients with COPD were measured using ABI 7300 RT-PCR machine (Applied Biosystem, USA).[19] The RT-PCR was done as follows: 95°C for 15 min followed by 35 cycles of 95°C for 35 s, 55°C for 1 min, and a cycle of melt curve consisting of 95°C for 30 s, 55°C for 1 min, and 95°C for 15 s.

Statistical analysis

The data were analyzed using SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation or median (interquartile range), as appropriate based on the normality of the data distribution. Categorical variables were presented as frequencies and percentages.

Differences in the detection rates of respiratory pathogens between COPD patients and healthy controls were assessed using the Fisher's exact test. The expression levels of miR-146, miR-221, and miR-16 were compared between COPD patients and healthy controls using the Mann–Whitney *U* test, as the data were not normally distributed. To evaluate the relationship between miRNA expression and respiratory pathogen detection, the Mann–Whitney *U* test was used to compare the miRNA expression levels between COPD patients with and without detected pathogen infections. The diagnostic performance of the miRNAs for COPD was assessed using receiver operating characteristic (ROC) curve analysis. The area under the ROC curve (AUC) was calculated, and the optimal cutoff values were determined based on the Youden index. The sensitivity, specificity, positive predictive value, and negative predictive value were calculated for the selected cutoff values.

Results

Molecular findings

Five (12.5%) and 7 (17.5%) of the patients suspected of COPD yielded 273 [Figure 1a] and 227-bp [Figure 1b] products indicative of *H. influenza* and *S. pneumoniae*, respectively. No amplicon was detected in the control samples. In addition, 3 (7.5%) of the patients suspected of COPD were identified as *C. pneumoniae* by RT-PCR. No amplicon was detected in the control samples.

Expression of miR-146

Among 40 COPD patients and 40 healthy subjects, miR-146 was expressed in the peripheral blood of 26 patients (65%) and 5 healthy subjects (13%). The expression of miR-146 in the peripheral blood of COPD patients was significantly different from the healthy subjects ($P < 0.001$) [Figure 2b].

Expression of miR-221

The expression of miR-221 was detected in the peripheral blood of 6 COPD patients (15%) and 32 peripheral blood of healthy subjects (80%). The two-sample binomial analysis test showed a significant difference between peripheral blood of COPD and non-COPD groups in terms of miR-221 expression ($P < 0.001$) [Figure 2c].

Expression of miR-16

The expression of miR-16 was detected in the peripheral blood of 34 COPD patients (85%) and 6 peripheral blood of healthy subjects (15%). The two-sample binomial analysis test showed a significant difference between peripheral blood of COPD and non-COPD groups in terms of miR-16 expression ($P < 0.001$) [Figure 2a].

The expression level of the studied micro-ribonucleic acids using $2^{-\Delta\Delta Ct}$

The expression of miR-16 and miR-146 in the peripheral blood of COPD patients was 2.11 [Figure 2d] and 2.38 [Figure 2e] times higher than the healthy subjects, respectively [Figure 2].

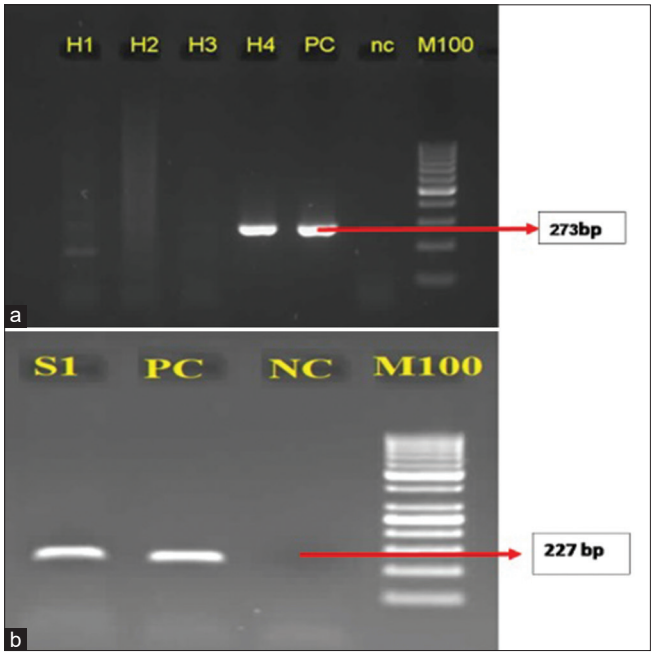

Figure 1: Results of the 2% electrophoresis of the products by polymerase chain reaction-based amplification of DNA extracted from the sputum of chronic obstructive pulmonary disease patients. (a) The 7 lanes contain a molecular-weight ''ladder'' (M100), the products from reference strains (PC), a negative control (NC), and test samples identified as *Haemophilus influenzae* (H4) and (b) the products from reference strains (PC), *Streptococcus pneumoniae* (S1), a negative control (NC), molecular-weight ''ladder'' (M100)

The expression of miR-221 in the peripheral blood of COPD patients was 1.34 times lower than the healthy subjects [Figure 2f].

The statistical relationship between microribonucleic acid and bacterial infection in chronic obstructive pulmonary disease patients

The relationship between the expression of each miR (miR-146, miR-221, and miR-16) and microorganism (*C. pneumoniae, H. influenza*, and *S. pneumoniae*) is shown in Table 2. There was no significant difference between the infected COPD patients and noninfected COPD patients in terms of the expression of studied miRNAs.

Correlation between micro-ribonucleic acids

There was a significant negative correlation between miR-146 with both miR-221 ($P < 0.001$, $r = -0.99$) and miR-16 ($P = 0.03$, $r = -0.96$). There was a significant negative correlation between miR-221 with both miR-16 ($P = 0.01$, $r = -0.97$). ROC curve data showed that under area was 1, 1, and 0.9167 for miR-16, miR-221, and miR-146, respectively [Figure 3].

Discussion

COPD is a common and preventable chronic respiratory and heterogeneous disease resulting from a number of various pathological processes, such as infections.[21] The mortality and morbidity of the disease have continued to increase in the world.[22] The severity of airflow limitation in COPD is related to the degree of infiltration of neutrophils, lymphocytes, and macrophages in lung tissue.[23] Regarding to role of bacterial colonization and miRNAs in the pathogenesis of the disease, in this study, molecular detection of *C. Pneumoniae, H. influenza*, and *S. pneumoniae* in the sputum of COPD patients was first reported. The expressions of miR-146, miR-16, and miR-221 in the peripheral blood of the patients were then evaluated. Finally, the expression levels of the miRs in the peripheral blood of the patients were compared to the healthy subjects. We found that any three microorganisms including *H. influenzae, S. pneumoniae, and C. pneumoniae* are detected in the sputum of COPD patients with frequencies of 12.5%, 17.5%, and 7.5% of the patients suspected of COPD using molecular assay.

Dicker *et al*. studied the changes in the prevalence of airway bacterial pathogens in the respiratory tracts of patients with acute exacerbations and showed the important role of bacteria in the pathogenesis of COPD.[24] Several bacterial pathogens

Table 2: The relationship between the expression of microribonucleic acid and bacterial infection in chronic obstructive pulmonary disease patients

miR	*Chlamydia pneumoniae*	*Haemophilus influenzae*	*Streptococcus pneumoniae*	Total
miR-16	3	3	7	13
miR-221	1	1	0	2
miR-146	3	4	4	11

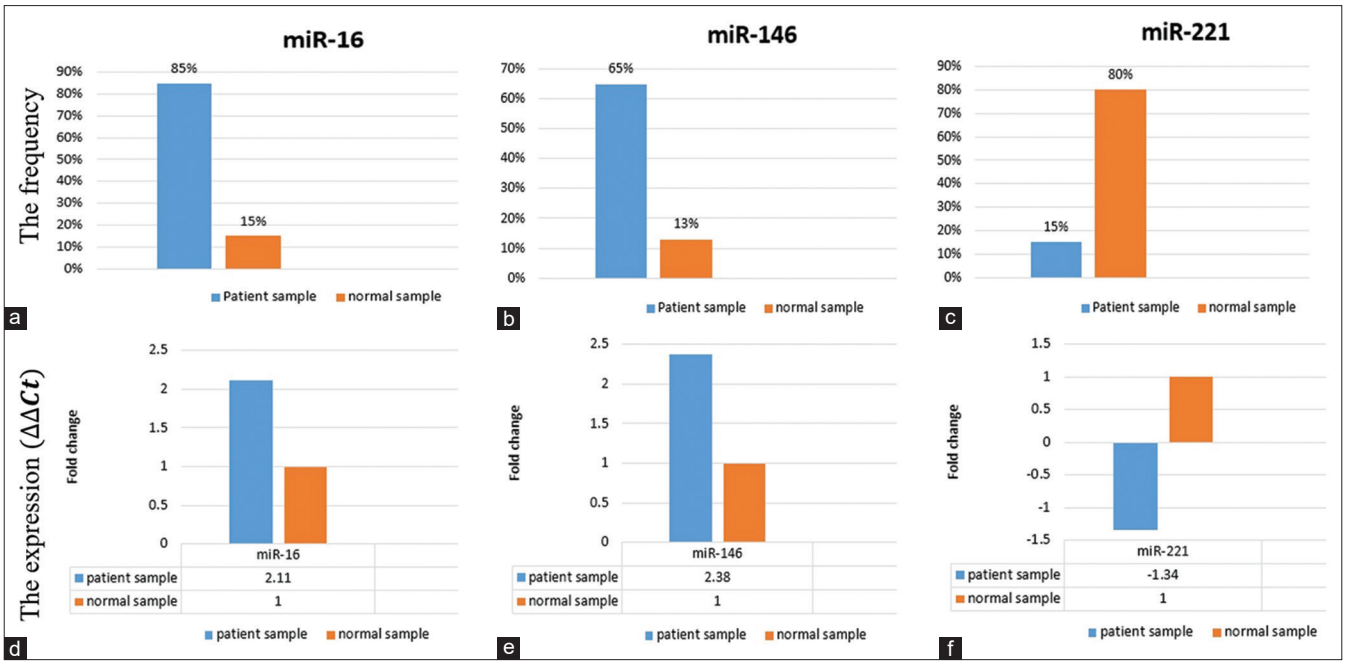

Figure 2: The frequency: (a) miR-16, (b) miR-146, and (c) miR-221in chronic obstructive pulmonary disease patients and healthy subjects as well as the expression level (using $2^{-\Delta\Delta ct}$): (d) miR-16, (e) miR-146, and (f) miR-221 in COPD patients and healthy subjects

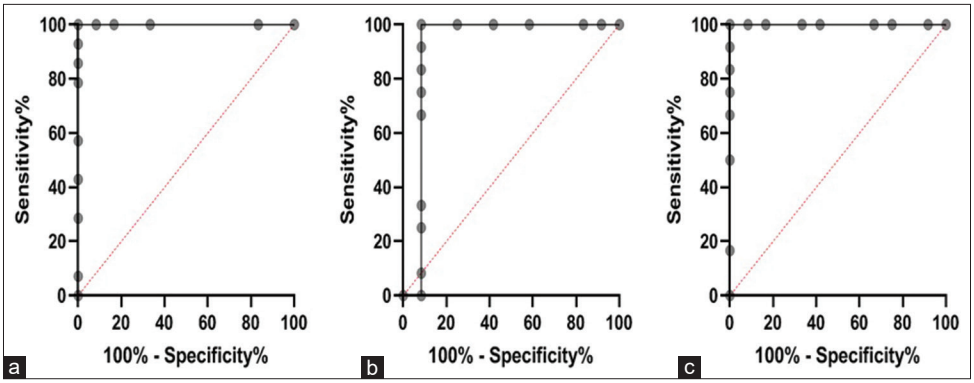

Figure 3: Receiver operating characteristic curves of miR-16 (a), miR-146 (b), and miR-221 (c)

genera, including *Streptococcus*, *Rothia*, *Haemophilus*, *Neisseria*, *Veillonella*, *Granulicatella*, and *Porphyromonas* have been detected from COPD patient sputum samples. However, *Streptococcus* is the most common genus.[14,25]

The difference in frequency of the expression of miRNAs studied in the peripheral blood of COPD patients compared to the normal healthy subjects was one of the main findings of the present study. The miR-146, miR-221, and miR-16 were expressed in peripheral blood of 65%, 15%, and 85% of COPD versus 13%, 80%, and 15% of healthy subjects. In addition, unlike the miR-221 which was downregulated in the peripheral blood of COPD patients compared to the healthy subjects, the miR-16 and miR-146 upregulated. This finding was in accordance with the correlation finding, in which a significant negative correlation between miR-146, miR-221, and miR-16 was identified. Regarding to the AUC, the sensitivity and specificity of miR-16, miR-221, and miR-146 are reported to be acceptable for the diagnosis of COPD.

In agreement with our finding, the increased serum miR-146a-5p level in patients with COPD as compared with normal control subjects has been shown.[26] In addition, circulating miR-146a/b was shown to correlate with inflammatory cytokines, including tumor necrosis factor-α, interleukin (IL-1β, IL-6, and IL-8), and leukotriene E4 in COPD, and predicts the risk of acute exacerbation COPD.[27,28] Proinflammatory cytokines induced expression of both miR-146a and 146-b in airway smooth muscle cells, leading to overexpression of miR-146a in cells derived from patients with asthma.[29] However, the underexpression of miR-146a in the peripheral blood of patients with COPD has recently been shown. The reduced expression of miR-146a in human bronchial epithelial cells has been demonstrated to alter neutrophil migration, leading to the development of asthma. Patients with asthma suffer from

COPD and increased mucus production in the airways.[30] This discrepancy may be caused by exposure to various patient conditions and disease severity. Rodrigo *et al*. included smoking patients in their study. It has been shown that the miR-146a is downregulated in the sputum of smoker COPD patients compared to nonsmoker COPD patients which can be attributed to the inhibitory role of miR146a for smoke-induced damage in the lung tissue of COPD patients.[31]

miR-221 was found to be significantly downregulated in the peripheral blood of COPD patients. The altered miR-221 expression has been documented in airway smooth muscle cells from patients with asthma. The downregulation of miR-221-3p has been shown to alleviate cell apoptosis and inflammatory response in COPD.[14,29] Shen *et al*. suggested that miR-221 may serve as a potential diagnostic biomarker of COPD.[32]

Since the expression of miR-16 in COPD patients attracts researchers' attention, Yu *et al*.. evaluated its expression in the peripheral blood of COPD patients, and the overexpression of miR-16 was found. The miR-16 has been shown to reduce the mRNA and protein expression levels of adrenoreceptor β-2 in a dose-dependent manner in patients with asthma. An inverse correlation between lung function parameters and miR-16 in asthma has also been reported.[33]

As reviewed above, various studies have been carried out independently in the fields of bacteria or miRNAs. In this research, the aim is to identify the set of microorganisms and miRNA to investigate their relationship in COPD.

Conclusion

This study identified *C. pneumoniae*, *H. influenza*, and *S. pneumoniae* in respiratory secretions of COPD patients. Furthermore, the expression levels of miR-146, miR-16, and miR-221 in the peripheral blood are measured. The positivity rate of biomarkers in COPD patients and comparison with healthy people was done by RT-PCR. Furthermore, the expression level of miRNAs was calculated using method. The statistical analysis including *t*-test, two-sample binomial, and Pearson correlation coefficient was performed. Furthermore, the sensitivity and specificity of these miRs are reported to be acceptable for the diagnosis of COPD. For future work, more research in the field of bacterial detection and the role of miRNA in lung patients is needed to provide a useful strategy for disease control.

Outcomes of study

Pathogen detection in respiratory secretions

The molecular detection of respiratory pathogens in the sputum samples of COPD patients and healthy controls revealed the following:

- *H. influenzae* was detected in 12.5% (5 / 40) of COPD patients, compared to 0% (0 / 40) of healthy controls ($P = 0.028$)
- *S. pneumoniae* was detected in 17.5% (7 / 40) of COPD patients, compared to 0% (0 / 40) of healthy controls ($P = 0.006$)
- *C. pneumoniae* was detected in 7.5% (3 / 40) of COPD patients, compared to 0% (0 / 40) of healthy controls ($P = 0.119$).

Expression of miRNAs in peripheral blood

- The expression of miR-146, miR-221, and miR-16 in the peripheral blood samples was analyzed and compared between COPD patients and healthy controls
- miR-146 was expressed in 65% (26 / 40) of COPD patients, compared to 13% (5 / 40) of healthy controls ($P < 0.001$)
- miR-221 was expressed in 15% (6 / 40) of COPD patients, compared to 80% (32 / 40) of healthy controls ($P < 0.001$)
- miR-16 was expressed in 85% (34 / 40) of COPD patients, compared to 15% (6 / 40) of healthy controls ($P < 0.001$).

Compared to healthy controls, COPD patients showed a significant upregulation of miR-146 and miR-16 and a significant downregulation of miR-221 ($P < 0.001$ for all comparisons).

Correlation with pathogen infection

There was no significant difference in the expression of miR-146, miR-221, and miR-16 between COPD patients with and without detected respiratory pathogen infections ($P > 0.05$ for all comparisons).

Diagnostic Performance of miRNAs

The sensitivity and specificity of miR-16, miR-221, and miR-146 for the diagnosis of COPD were determined to be within acceptable ranges, suggesting their potential utility as diagnostic biomarkers.

Rational of the study

COPD is a major public health concern, characterized by persistent airflow limitation and chronic respiratory inflammation. The pathogenesis of COPD is multifactorial, with both environmental and genetic factors contributing to its development and progression. Two key elements implicated in COPD pathogenesis are microbial infections and dysregulation of miRNA expression. Respiratory infections, particularly with pathogens such as *C. pneumoniae*, *H. influenzae*, and *S. pneumoniae*, have been associated with the development and exacerbation of COPD. These pathogens can induce inflammatory responses and contribute to airway remodeling, thereby worsening the clinical outcomes of COPD patients. However, the prevalence and role of these specific respiratory pathogens in COPD have not been extensively investigated. In addition, emerging evidence suggests that aberrant expression of miRNAs, which are small, noncoding RNA molecules that regulate gene expression, may play a crucial role in the pathogenesis of COPD. Specific miRNAs, such as miR-146, miR-16, and miR-221, have been implicated in the regulation of inflammatory pathways, oxidative stress, and airway remodeling processes associated with COPD. Understanding the expression profiles of these miRNAs in COPD patients may provide valuable insights into the molecular mechanisms underlying the disease.

This study aimed to investigate the molecular detection of three common respiratory pathogens (*C. pneumoniae*, *H. influenzae*, and *S. pneumoniae*) in the respiratory secretions of COPD patients, and to evaluate the expression levels of miR-146, miR-16, and miR-221 in the peripheral blood of COPD patients. The findings from this study may contribute to a better understanding of the role of microbial infections and miRNA dysregulation in COPD pathogenesis, and potentially identify novel diagnostic or prognostic biomarkers for this debilitating respiratory condition.

Limitations of the study

Smaller sample sizes might not fully represent the diversity of cases observed in larger studies. In addition, resource availability, equipment limitations, and variations in patient demographics can influence the findings.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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