Original Article

Running Title: Expression Level of Involved microRNAs in NSCLC Patients

Received: August 11, 2019; Accepted: April 04, 2020

Expression Changes of miR-146a-5p and miR-196a-2 in Non-small Cell Lung Cancer Patients: A Case-control Study


* Department of Biochemistry, higher education institute of Rab-e Rashidi, Tabriz, Iran
** Tuberculosis and Lung Disease Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
*** Tamworth Hospital, Hunter New England Health District, Tamworth, NSW, Australia
**** Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

a) Vafadar S and Zafari V have contributed equally to this work

♦ Corresponding Author
Ensiyeh Seyedrezazadeh, PhD
Tuberculosis and Lung Disease Research Center,
Pashmineh Research Complex, Daneshgah Street,
Tabriz, Iran
Tel/Fax: +98 41 33378093
Email: e.s.rezazadeh@gmail.com

Abstract
Background: Recent evidence underscores to the important regulatory roles of microRNAs as a biomarker for diagnosis of non-small cell lung cancer (NSCLC); this disease often has poor prognosis, and it is the most prevalent cause of cancer-related mortality worldwide. This study investigated the levels of miR-146a-5p and miR-196a-2 in peripheral blood mononuclear cells (PBMC).

Methods: In the present case-control research, we collected the PBMCs through isolating blood from 22 NSCLC patients and 22 healthy individuals. Following the extraction of total RNA and cDNA synthesis, we studied the expression levels of miR-146a and miR-196a-2 by use of qPCR.

Results: Both the miR-146a-5p and miR-196a-2 were significantly down-regulated in the PBMCs of NSCLC patients in comparison to normal healthy ones (P=0.002 and P<0.001, respectively). There was an association between the expression levels of microRNAs and the types of tumors, which was significant for miR-146a-5p (P=0.02). Furthermore, in NSCLC cases, a significant positive correlation existed between miR-196a-2 and miR-146a-5p expression levels (r=0.71, P=0.002).

Conclusion: According to the study results, miR-146a-5p and miR-196a-2 that were down-regulated in the PBMCs of NSCLC patients might serve as potential biomarkers for diagnosis if confirmed in future studies.

Keywords: NSCLC, miR-146a, miR-196a-2
Introduction

Lung cancer (LC), classified into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), accounts for approximately 1.8 million diagnosed cases and 1.6 million cancer-related deaths around the world; making up for about 85% of these mortalities, NSCLC is the most prevalent cause of cancer death.\(^1\)\(^-\)\(^4\) Despite the decreasing rate of LC incidence in developed countries, the rates are growing in developing countries due to less severe smoking regulations; smoking is the main risk factor for LC which increases with the quantity and duration of smoking.\(^3\) Even though screening and diagnosis methods of LC have significantly progressed over the recent decades, most cases are detected in advanced stages due to the asymptomatic nature and feature of this disease; therefore, the five-year survival of these patients is only 17%.\(^5\) Therefore, it is highly necessary to identify novel biomarkers as a reliable test for an earlier detection and diagnosis of LC.

MicroRNAs (miRNAs) are a subset of evolutionarily conserved, short non-coding RNAs which bind with target miRNAs and post-transcriptionally regulate the expression of target genes. The regulatory function of miRNAs has been recognized not only in the vital processes of cell cycles such as cell differentiation, proliferation, apoptosis, and developmental balance, but also in some pathogenic processes and carcinogenesis.\(^6\) Accordingly, dysregulation of a number of their activities prompt homeostasis disruption and pathologies condition, including lung cancer, which are manifested by oncogenic and suppressor activities. MiR-146 family with two different members (miR-146a and miR-146b), transcribe from two different loci but with the same seed region.\(^7\) miR-146a-5p acts as a tumor suppressor factor in various cancers. Some in vitro evidence exhibited its inhibition role in the NSCLC cell cycle proliferation and progression; in contrast, one study revealed its stimulation role in squamous carcinoma cells through promoting Epithelial-Mesenchymal Transition (EMT) by Notch2 as a target gene.\(^8\) Additionally, owing to its down-regulation in hepatocellular carcinoma, it was reported as a tumor suppressor.\(^9\) Moreover, miR-196s with different genes (miR-196a-1, miR-196a-2 and miR-196b) is located on chromosomes 17q21, 12q13, and 7p15, respectively.\(^10\) According to some studies, although this miRNA stimulated the proliferation and migration of NSCLC progression via activating PI3K/AKT signaling, the transcription factor implicated on EMT process in NSCLC exhibited a potent metastatic suppressor activity by inhibiting the expression of HOXC8.\(^11\)\(^-\)\(^12\)

The identification of the miRNA expression differences between tumor and normal cells might be a promising approach for an early detection of lung cancer and a better prognosis of patients. Therefore, this study evaluated the PBMC expression levels of miR-196a-2 and miR-146a-5p, as the low invasive and repeatable way for sampling, in NSCLC patients.

Materials and Methods

Clinical samples

In this case-control study, during a six-month period in Emam-Reza hospital, we collected whole blood samples from 22 cancer patients whose cancer had been diagnosed by an oncologist according to the histopathological result of their bronchoscopy, a routine approach for the diagnosis of pulmonary malignancies. The control group included 22 healthy volunteers selected by matching sex and age variables and adjusting smoking as a confounder factor. We calculated the sample size based on the outcome variable estimated by standard deviation (SD) in a similar study.
We excluded the patients with inflammatory or autoimmune diseases. As a basic rule, we immediately transferred the collected samples to the laboratory for PBMC extraction from the whole blood. All participants signed written informed consent prior to the study.

**Extraction of PBMCs**

We collected 2.5 ml of fresh peripheral blood of participants into heparinized CBC tubes. We used a standard procedure using Ficoll (Lymphodex, Germany). Briefly, the whole blood was diluted with an equal volume of phosphate buffered saline (PBS). The diluted blood was then deliberately layered over the equal volume of Ficoll solution; with a rate of 2000rpm, the sample was centrifuged for 20 minutes at 4º C. Afterwards, the upper layer was discarded, and the mid-layer, containing mononuclear cells, was attentively transferred to a new 15 ml tube and washed two or three times with PBS. Next, the total RNA was extracted from the final pallet of the previous step.

**RNA extraction from PBMCs and reverse transcription**

We used the All-In-One DNA/RNA/Protein Mini-Preps kit (Bio Basic Inc, Canada) to extract the total RNA from PBMCs according to the manufacturer’s protocol. After that, we employed the Thermo Fisher Scientific Nanodrop™ One/One C Micro-volume Spectrophotometer to assess the quality and quantity of the extracted RNA.

In the next step, we used the Mircury LNA Universal RT microRNA PCR kit (Exiqon, USA) to generate complementary DNA from the extracted RNA in accordance with the manufacturer’s guidelines. The reverse transcription incubation protocol included: 42º C for 60 min, 95º C for 5 min in order to inactivate the reverse transcriptase enzyme, and immediate cooling and storing at -20º C.

**qRT-PCR**

We analyzed the microRNAs expression levels using a real-time PCR instrument (Applied Bio-system Step one, USA) and Real Q Plus 2x Master mix Green High ROX (Ampliqon, Denmark). We employed miScript Primer Assay (Qiagene, USA) for each miRNA (miRNA 146a primer catalogue number: MS00003535, miRNA 196a catalogue number: MS00031563). To calibrate the PCR reaction, we built a dilution series of human genomic standards. hgDNA was serially diluted from 1/10 to 1/10000. Each reaction mixture contained a total volume of 20 µl (10 µl of master mix, 2 µl of cDNA (5ng/ml), 1 µl of primer, and 7 µl of deionized water). The qRT-PCR amplification condition was 94ºC for 10 min, followed by 45 cycles of 94ºC for 10 sec, and 60ºC for 60 sec in 20µl reaction volume for Snord48 and miR-196a-2; and 94ºC for 10 min, followed by 50 cycles of 94ºC for 10 sec and 60ºC for 60 sec in 20µl reaction volume for MiR-146a. We performed each sample in triplicate according to the manufacturer’s protocol. For both miRNAs, we considered SNORD48 as the reference gene.

**Statistical analysis**

Statistical analysis was conducted using the Graph Pad Prism 6 (Graph Pad Software Inc. San Diego, CA, USA). To evaluate the normality of the data, we applied Kolmogrov-Smithnov’s test. Unpaired t-test was performed to compare the expression level of target genes between the case and control groups. We expressed all results as mean±SD and considered 0.05 as the significance level for all P values.

The Ethics Committee of Tabriz University of Medical Sciences approved this study (confirmation code: IR.TBZMED.REC.1397.881).

**Results**

Table 1 shows the general characteristics of our patients.
We analyzed the expression levels of target miRNAs in the PBMCs of the lung cancer patients and compared them between the groups; the expression levels of both miR-196a-2 (P=0.001) and miR-146a-5q (P=0.02) were significantly down-regulated in cancer patients in comparison to the control group (Figure 1A and B).

A significant positive correlation existed between miR-196a-2 and miR-146a-5q expression level (r= 0.71, P=0.002) in NSCLC patients but not in the healthy group. Also, there was a significant association between the expression levels of both miRNAs and type of tumors regarding miR-146a (P=0.02) but not miR-196a-2 (P=0.16). However, for tumor stages evaluation, the non-significant correlation was exhibited in comparison to both of miRNAs. Moreover, we plotted the Receiver Operating Characteristic (ROC curve) for both microRNAs. Later, we computed the area under the curve (AUC) to determine their specificity and sensitivity for lung cancer prediction. miR-196a-2 had a ROC area of 0.97, 95% CI: 0.93-1.01, P< 0.05 (Figure 2 A), and the ROC area for miR-146a was 0.96, 95% CI: 0.92-1.02, P< 0.05 (Figure 2 B).

Discussion

This study exhibited the significant down-regulated levels of miR-146a and miR-196a-2 expression in the PBMCs of different subtypes of NSCLC patients in comparison to healthy subjects. The analysis of the possible relationship between the microRNAs expression levels and the tumor types showed meaningful outcomes for miR-146a-5p.

Previous studies frequently investigated the role of microRNAs in the carcinogenesis process, and reported their up/down-regulated levels in different human cancers. Most investigations have shown that miRNAs potentially target a wide range of mRNAs as minor changes in their expression could affect the expression of a large number of proteins implicated in neoplasia. Therefore, the study of their molecular role, changes in expression level, and the possibility of their use as a diagnostic or prognostic marker can be beneficial for future studies, diagnosis improvement, and treatment or even prevention approaches.

Some of the previous studies reported the role of miR-196a as an oncomiR with aberrant expression in various cancers, including head and neck squamous cell carcinoma, laryngeal cancer, pancreatic cancer, and gastric cancer. While previous studies showed the higher expression levels of miR-196b in lung cancer tissues and cells, the current study exhibited the down-regulated levels of miR-196a-2 gene expression in PBMCs of NSCLC patients in comparison to healthy control participants. The reason for such contradiction might be the type of cancer or cells studied in these projects. The role of miR-196a-2 in NSCLC is similar to its activity in breast cancer whose down-regulation suppressed metastasis. Moreover, according to new evidence, genotype variation in miR-196a-2 is another factor influencing the risk of lung cancer and the survival of patients. Similarly, Tian et al. demonstrated the association between CC homozygote variant of miR-196a-2 rs11614913 and increased risk of lung cancer.

The results of the current study also showed the down-regulated levels of miR-146a-5p. Some previous studies reported its under-expression level in prostate cancer, esophageal squamous cell cancer, and papillary thyroid carcinoma. Differently, a high expression was displayed in cervical carcinoma. However, the miR-146a was found to be overexpressed in the serum of NSCLC patients miR-146a directly acts
as an inhibitor on COX2 and 5-lipoxygenase activating protein (FLAP) and consequently controlling PGE2 and LTB4 production in NSCLC cell lines. Accordingly, in this study, the decreasing expression of miR-146a-5p could be attributed to the increased production levels of these factors in different types of tumors in NSCLC. Interestingly, the study on the prostate cancer has indicated that miR-146a-5p could promote apoptosis via targeting Rock1. Furthermore, through targeting a wide range of distinct genes in various cellular pathways, miR-146a-5p plays diverse roles in the regulation of cell phenotypes in cancers; for instance, it acted as a tumor suppressor in hepatocellular carcinoma and inhibited cell proliferation and progression in NSCLC. There are also some pieces of evidence indicating that miR-146a-5p has a role in inflammation pathways, hence the necessity to exclude patients suffering from inflammatory and autoimmune diseases.

In the interpretation of ROC curves, both miR-146a-5p and miR-196a-2 potentially had high scores for use as a biomarker for NSCLC. Another promising result of this study is using PBMC with the non-invasive property for evaluating such biomarkers which is easy and repeatable for obtaining specimens.

The limitation of this study was the lack of lung cancer tissues of participants to compare the differences between tissue and PBMC target miRNAs expression levels.

**Conclusion**

Fluctuations in the expression level of molecular markers during the genesis and progression of carcinogenic processes usually occur much earlier than the apparent characteristics and symptoms. Developing such biomarkers for early diagnosis can render the treatment approaches more effective. In this research, we showed that the expressions of miR-196a-2 and miR-146a-p5 were down-regulated in the PBMCs of NSCLC patients. Previous studies reported the down-regulation of these microRNAs in the cells and tissues of NSCLC patients. However, our approach to measuring target miRNAs in PBMC is non-invasive; therefore, it can be used as a biomarker for diagnosis or prognosis.

**Acknowledgment**
The authors would like to acknowledge the Research Center for Tuberculosis and Lung Disease, Tabriz University of Medical Sciences, for the financial support.

**Conflicts of Interest**
None declared.

**References**


Table 1. General characteristics of study participants

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Case (n=22)</th>
<th>Control (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (Mean ± SD)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>63.86 ± 8.93</td>
<td>61.80 ± 5.56</td>
</tr>
<tr>
<td>Female</td>
<td>61.0 ± 12.18</td>
<td>62.46 ± 5.11</td>
</tr>
<tr>
<td><strong>Sex (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>63.6</td>
<td>65.4</td>
</tr>
<tr>
<td>Female</td>
<td>36.4</td>
<td>35.1</td>
</tr>
<tr>
<td><strong>Type of tumors (%)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>36.4</td>
<td></td>
</tr>
<tr>
<td>Squamous cell cancer</td>
<td>54.5</td>
<td></td>
</tr>
<tr>
<td>Large cell carcinoma</td>
<td>9.1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Expression levels of miR-196a-2 (A) and miR-146a (B) in PBMCs of NSCLC patients in comparison to healthy control group.
Figure 2. The Receiver Operating Characteristic (ROC curve) for miR-196a-2 (A) and miR-146a (B).