

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

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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 using qPCR.

Results: Both the *miR-146a-5p* and *miR-196a-2* were significantly down-regulated in the PBMCs of NSCLC patients in comparison with 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: Non-small-cell lung, Carcinoma, *miR-146a*, *miR-196a-2*

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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 evolutionary conserved, short non-coding RNAs, which bind with target mRNAs 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 4° 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™

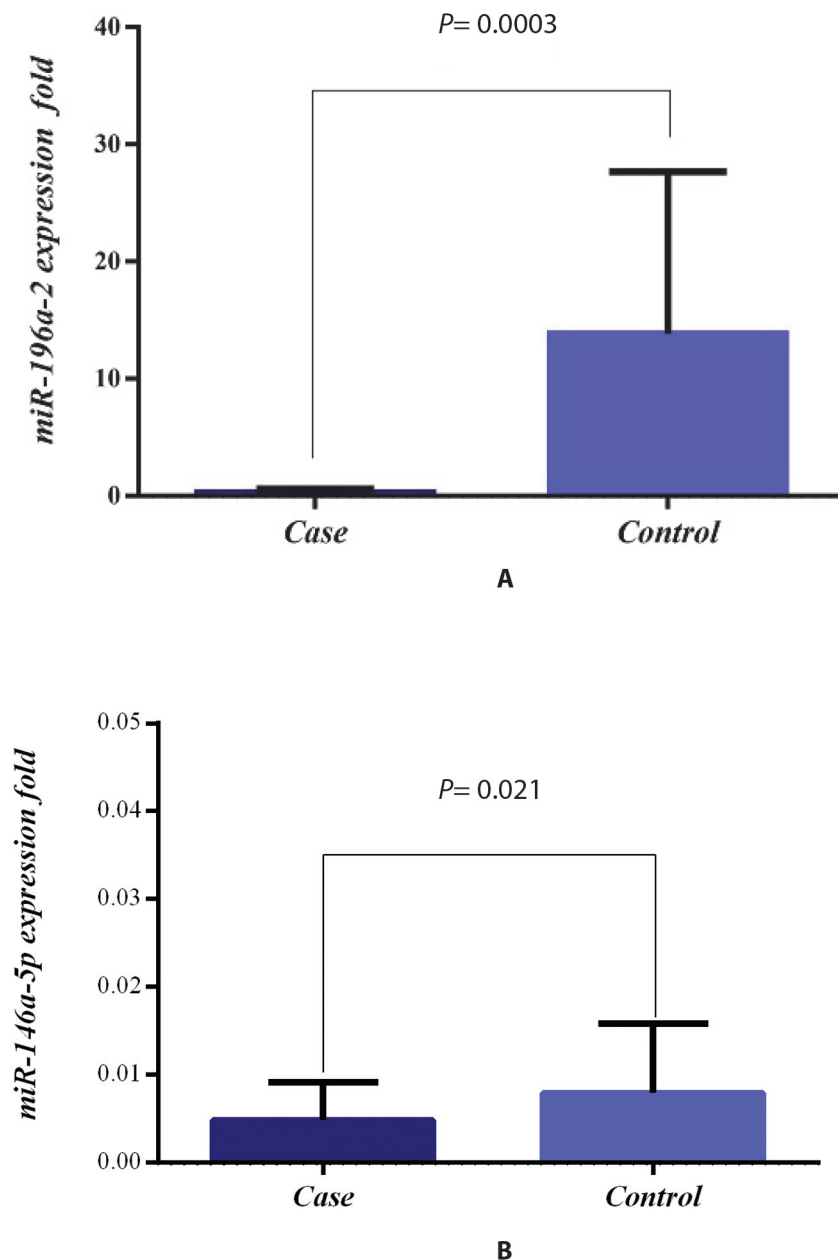


Figure 1. This figure shows the expression levels of *miR-196a-2* (A) and *miR-146a* (B) in PBMCs of NSCLC patients in comparison to healthy control group.

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: 42°C for 60 min, 95°C for 5 min in order to inactivate the

reverse transcriptase enzyme, and immediate cooling and storing at -20°C.

qRT-PCR

We analyzed the microRNAs expression levels using a real-time PCR instrument (Applied Biosystem Step one, USA) and Real Q Plus 2x Master mix Green High ROX (Ampliqon, Denmark). We employed miScript Primer Assay (Qiagen, USA) for each miRNA (miRNA 146a primer

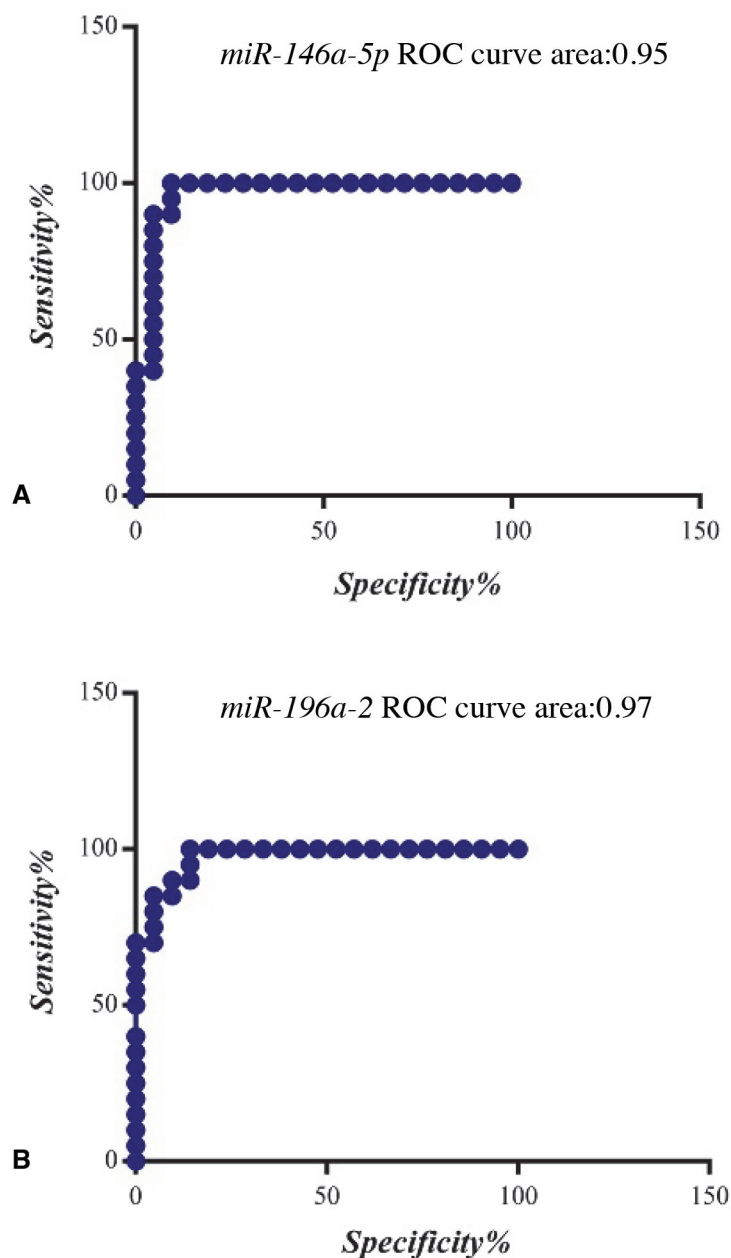


Figure 2. This figure shows the receiver operating characteristic (ROC curve) for *miR-196a-2* (A) and *miR-146a* (B).

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 μ L (10 μ L of master mix, 2 μ L of cDNA (5ng/ml), 1 μ L of primer, and 7 μ L of deionized water). The qRT-PCR amplification condition was 94°C for 10 min, followed by 45 cycles of 94°C for 10 sec, and 60°C for 60 sec in 20 μ L reaction volume for Snord48 and *miR-196a-2*; and 94°C for 10 min, followed by 50 cycles of 94°C for 10 sec and 60°C for 60 sec in 20 μ L 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 Kolmogorov-Smirnov'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 \pm 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 with 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 with 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,¹³⁻¹⁴ and reported their up/down-regulated levels in different human cancers.¹⁵ 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.¹⁶⁻¹⁹ While previous studies showed the higher expression levels of *miR-196b* in lung cancer

Table 1. General characteristics of study participants

Characteristics	Case (n=22)	Control (n=22)
Age (Mean ± SD)		
Male	63.86 ± 8.93	61.80 ± 5.56
Female	61.0 ± 12.18	62.46 ± 5.11
Sex (%)		
Male	63.6	65.4
Female	36.4	35.1
Type of tumors (%)		
Adenocarcinoma	36.4	
Squamous cell cancer	54.5	
Large cell carcinoma	9.1	

tissues and cells, the current study exhibited the down-regulated levels of *miR-196a-2* gene expression in PBMCs of NSCLC patients in comparison with 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.²⁰ 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.²¹⁻²² Similarly, Tian et al. demonstrated the association between CC homozygote variant of *miR-196a-2 rs11614913* and increased risk of lung cancer.²³

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.^{7, 24-27} However, the *miR-146a* was found to be overexpressed in the serum of NSCLC patients.²⁸ *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.²⁹ Interestingly, the study on the prostate cancer has indicated that *miR-146a-5p* could promote apoptosis via targeting Rock1.³⁰ 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;¹⁴ for instance, it acted as a tumor suppressor in hepatocellular carcinoma and inhibited cell proliferation and progression in NSCLC.^{9, 31} 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.³²

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-5p* 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 in measuring target miRNAs in PBMC is non-invasive; therefore, it can be used as a biomarker for diagnosis or prognosis.

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Conflicts of Interest

None declared.

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