

MicroRNA Profiling in Non-Small Cell Lung Cancer and its Implications for the Disease Pathogenesis

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Abstract

Background: Lung cancer is one of the most common cancers worldwide. Despite the progression in screening and diagnostic methods, the prevalence and mortality rates of this cancer have not decreased in recent decades. Recent evidence has implied the possible roles of miR-212, miR-124a, miR-125b, miR-27a, and miR-133b in carcinogenesis process. Hence, we examined the changes in the expression level of these microRNAs (miRNAs) during carcinogenesis determined the possible application of these factors as diagnostic or prognostic biomarkers for non-small cell lung cancer (NSCLC).

Methods: 50 NSCLC patients participated in this descriptive case-control study. During bronchoscopy, we collected their tumor and adjacent normal tumor-free tissues. We further extracted the total RNA from the cells, synthesized cDNA, and examined the expression level of target miRNAs by quantitative real-time PCR. Subsequently, we analyzed the expression levels of these genes and their correlation with clinico-pathologic features of patients.

Results: The output data of our study showed a statistically significant deregulation in miR-212 ($P=0.002$), miR-124a ($P=0.001$), miR-125b ($P=0.023$), miR-27a ($P=0.012$), and miR-133b ($P=0.05$). Moreover, the expression levels of these miRNAs had significant correlations with metastasis, lymph node involvement, tumor cell differentiation degree, and tumor size of NSCLC patients.

Conclusion: All of the studied miRNAs could potentially be used as diagnostic or prognostic biomarkers.

Keywords: Carcinoma, Non-small-cell lung, MicroRNA, Biomarker

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Introduction

Lung cancer is a major cause of cancer-related deaths worldwide; the

estimated rate of newly diagnosed cases in the United States was 234,030 in 2018, and 154,050 death.

cases will occur due to lung cancer in 2020.¹ Non-small cell lung cancer (NSCLC), with 85% rate, is considered as the most prevalent type of lung cancer.² Diagnostic and screening methods have significantly progressed over the recent decades; however, due to the asymptomatic genesis of lung cancer, most cases are detected in advanced stages;¹ they may experience persistent cough, sputum streaked with blood, chest pain, voice change, worsened shortness of breath and recurrent pneumonia or bronchitis as symptoms;¹ this is when the 5-year survival of these patients is only 17%.¹ Therefore, it is highly necessary to identify novel biomarkers, which can be instrumental in the earlier diagnosis of NSCLC.

MicroRNAs (miRNAs) are short, approximately 20-nucleotide long, non-coding RNAs encoded in specific miRNA gene loci or clusters. Sometimes the gene locus of miRNAs is located within the intron segment of protein coding genes.³ These small RNAs post-transcriptionally regulate cellular genes involved in the basic processes of cells such as cell cycle regulation, cell differentiation, and cell development.^{4,5} miRNAs accomplish this work

mainly through interaction with the 3'-UTR of their targeted mRNA; in rare cases, they can also interact with the 5'-UTR or coding regions of target genes.⁶ This type of binding leads to the inhibition of translation or degeneration of target mRNA. Various studies have shown that a huge number of miRNAs are associated with diverse human diseases such as cancer.^{7,8} These oligonucleotides have particular characteristics that separate them from other RNA types like mRNA. These features have also made them novel biomarkers and potential targets in the future.⁹ The expression profile of miRNAs demonstrates its plausible role in cell processes. Notably, this profile is more beneficial and sensitive to classifying poorly differentiated tumors compared with mRNA profiles.

Numerous data have revealed that miRNAs play a vital role in human carcinogenic processes. It was shown that some miRNAs were significantly deregulated compared with paired normal tissues in various cancers; this underscores the potential of miRNAs as diagnostic and therapeutic markers in cancer. Moreover, miRNAs were found to be correlated with the outcome of the determined cancers. Specifically, these

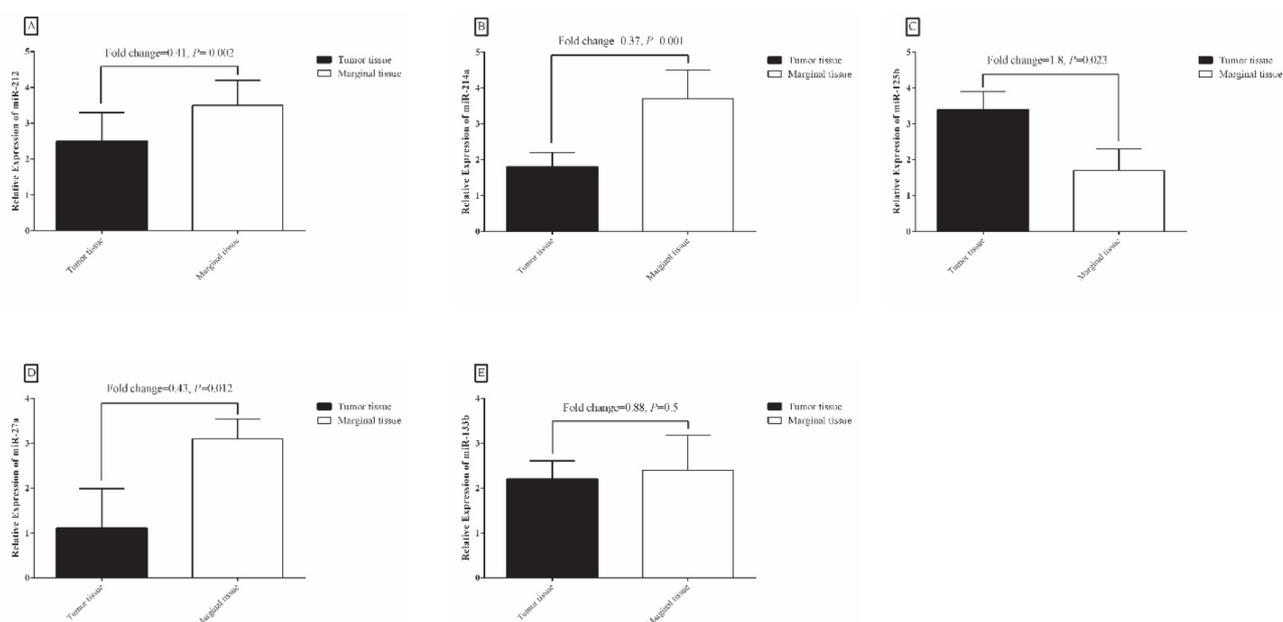


Figure 1. Bar graphs to illustrate the miRNA expression levels in tumor tissues obtained from NSCLC patients and their corresponding marginal tissues.

NSCLC: non-small cell lung cancer

miRNAs exist not only in cells but also in body fluids, including urine, serum, and saliva. This issue promises the new non-invasive screening and diagnostic methods. For this research, we selected these mRNAs based on the findings of previous studies, which investigated the fundamental regulatory role of each microRNA in cancer-related cellular processes such as proliferation differentiation, apoptosis, and cell cycle checkpoints. Deregulation of each important factor leads to cell cycle imbalance and various tumor foundations.¹⁰⁻¹⁴

Accordingly, in the current study, we aimed to investigate the expression level of a number of miRNAs, whose roles in complex carcinogenesis were previously implied. Afterwards, we evaluated the correlation between the expression level of these gene regulators and the clinical features of patients.

Materials and Methods

Study population

In this descriptive case-control study, conducted from March to July 2018, we collected NSCLC tumor and normal marginal tissue specimens from 50 patients, who underwent bronchoscopy in the hospitals of Tabriz University of Medical Sciences. None of the participants had undergone chemotherapy or radiotherapy. Fresh tissues were immediately transferred into RNAase inhibitor solution (QIA Gene Cat No. /ID: 76104) and stored at -80°C until RNA extraction. All participants provided a written informed consent, and the Ethics Committee of Tabriz University of Medical Sciences approved the study (Ethical code is IR.TBZMED.REC.1397.133). Table 1 shows the general characteristics of our patients.

RNA isolation, cDNA synthesis, and real time-PCR

To extract the total RNA from tumoral and normal marginal tissues, we utilized Tripure isolation reagent (Roche, Germany, Cat No. 11667165001) according to the manufacturer's instructions. Next, to evaluate the purity and yield of the extracted RNA, we used a Nanodrop spectrophotometer at 260/280 nm (Nanodrop, Thermo

Table 1. Clinicopathological characteristics of the NSCLC subjects

Characteristic	Value (N=50)
Age	
<60	29 (58%)
>60	21 (42%)
Sex	
Male	34 (68%)
Female	16 (32%)
Smoking	
Yes	39 (62%)
No	11 (38%)
Tumor metastasis	
pM0	42 (78%)
pM1	8 (22%)
Lymph node involvement	
Yes	33 (66%)
No	17 (34%)
Differentiation pattern	
Poor	11 (22%)
Moderate	26 (52%)
Well	13 (26%)

NSCLC: non-small cell lung cancer

Fisher Scientific). We assessed the samples by gel electrophoresis on 1% Agarose gel in order to examine the RNA quality. Eventually, we stored the samples at -80° C till the following steps.

In the first step, the universal cDNA synthesis kit (Exiqon, Cat No. 40023301) was used to reverse transcribe the miRNAs to cDNA templates. Afterwards, we conducted quantitative real-time PCR using SYBER Green Master mix (Exiqon, cat number: 400203421) and microRNAs specific primer sets separately. The cat numbers of primers purchased from Exiqon Company were as follow.

- * miR-212 202061
- * miR-124a 202840
- * miR-125b 202026
- * miR-27a 202011
- * miR-133b 202146

The expression level of U6 gene was used to normalize the expression level of target genes. Subsequently, we calculated the average of the duplicated Ct values and employed Pfaffl formula to specify the relative expression level of miRNAs using comparative Ct method.

Statistical analysis

Table 2. The relation between the expression level of microRNAs and clinicopathological characteristics of patients

	Age	Sex	Smoking	Tumor metastasis	Lymph node involvement	Differentiation pattern	Tumor size
MiR-212	NS	NS	NS	S($P=0.021$)	S($P=0.018$)	NS	NS
MiR-124a	NS	NS	NS	NS	NS	S($P=0.034$)	NS
MiR-125b	NS	NS	NS	NS	NS	NS	NS
MiR-27a	NS	NS	NS	S($P=0.026$)	S($P=0.012$)	S($P=0.009$)	S($P=0.035$)
MiR-133b	NS	NS	NS	NS	NS	NS	NS

NS=non-significant, S=significant

Statistical analysis was done using the GraphPad Prism 6 (GraphPad Software Inc. San Diego, CA, USA). We examined the normality of data via Kolmogorov-Smirnov test. Independent sample t-test was performed to compare the miRNAs expression level between tumoral and normal marginal tissues. We made use of the Pearson's correlation analysis to analyze the relationship between expression level of the miRNAs and the clinicopathological characteristics of the study subjects. Statistical significance level for all P values was less than 0.05, and all data were expressed as mean \pm standard deviation (SD).

Result

Significant down-regulation of MiR-212 in NSCLC

Our experiments revealed the significant down-regulation of miR-212 in tumor tissues in comparison with the marginal tissues (fold change=0.41, $P=0.002$; Figure 1.A). Moreover, the relationship analysis of miR-212 expression with clinicopathological characteristics of patients showed a statistically significant relationship between the expression level of miR-212 and lymph node involvement ($P=0.018$) and metastasis ($P=0.021$).

Significant down-regulation of MiR-124a in NSCLC

Our data indicated the down-regulation of miR-124a in tumor tissue samples compared with matched normal tissues (fold change=0.37, $P=0.001$; Figure 1.B). In the correlation analysis, we discovered a significant correlation between the expression level of miR-124a and cell differentiation degree ($P=0.034$).

Significant up-regulation of MiR-125b in NSCLC

The expression level of miR-125b was up-

regulated in tumor tissues in comparison with the adjacent normal tissues (fold change=1.8, $P=0.023$; Figure 1.C). However, there was no significant relationship between the expression level of this gene and the clinicopathological characteristics of patients.

Significant down-regulation of MiR-27a in NSCLC

The expression level of miR-27a in NSCLC tumor tissues revealed a significant down-regulation compared with the adjacent normal tissues (fold change=0.43, $P=0.012$; Figure 1-D). Additionally, correlation analysis showed a significant relationship between miR-27a expression and tumor size ($P=0.035$), metastasis ($P=0.026$), cell differentiation degree ($P=0.009$), and lymph node involvement ($P=0.012$).

Significant down-regulation of MiR-133b in NSCLC

Our study revealed the down-regulation of miR-133b in the tumor tissues of NSCLC patients in comparison to the marginal issues; however, the difference was not statistically significant (fold change=0.88, $P=0.05$; Figure 1.E). Nonetheless, there existed no significant relationship between microRNA expression and the clinicopathological variables.

Discussion

The results of our study revealed the deregulation of some microRNAs (with reported critical roles in carcinogenesis) in NSCLC tumor tissues in comparison to adjacent tumor-free normal tissues. We also showed the relationship between the alterations of expression levels of miRNAs genes and certain clinicopathological features of NSCLC patients. We observed the down-regulation of miR-212, miR-124a, miR-

27a, and miR-133b, the up-regulation of miR-125b, and an association between metastasis and the transcription level of miR-212 and miR-27a. Moreover, the tumor size of NSCLC patients correlated with the transcription level of miR-27a. Also, the expression levels of miR-27a and miR-124a were associated with the cell differentiation degree of the patients' tumor. Lymph node metastasis of these patients also had a significant relationship with the transcription level of miR-27a and miR-212 (Table 2).

In accordance with the conducted studies, our selected miRNAs indicated both up- and down-regulation in human cancer types. The kind of deregulation and its function as oncogene or tumor suppressive gene can change depending on the type of cancer and the genes they target. So, further studies are required to explore the exact function of this miRNAs.¹⁵

Utilizing advanced technologies such as miRNA oligonucleotide array and quantitative RT-PCR for validation, various studies have revealed a significant association between miRNA expression level and tumor type, grade, response to treatment, and prognosis.¹⁶⁻²¹

According to evidence, miR-125 family is able to control the progression of tumor cells in different levels. For instance, increasing the anti-apoptotic ability of glioma cells accelerated the proliferation of gastric cancer.²²⁻²³

Several studies have shown the deregulation of miR-124a. Based on their findings, this gene plays an important role not only in promoting the apoptosis of some cancer types, such as colorectal cancer, but also in suppressing proliferation, migration, and invasion of tumor cells as in gastric cancer. This microRNA functions via targeting the molecules of vital signaling pathways involved in the foundation and progression of carcinogenesis processes such as STAT3 or Rho-associated protein kinase1.^{10, 24-26}

The aberrant expression of miR-212 is utilized for the diagnosis or prediction of cancer outcomes. For example, it may help identify early-stage breast cancer; moreover, it correlates with tumor grade and disease stage in pancreatic ductal adenocarcinoma.^{14, 27-28}

miR-27a is another important microRNA in cancer biology shown by many studies to be involved in tumorigenesis, proliferation, apoptosis, invasion, migration, angiogenesis, drug sensitivity, treatment of cancer, and patients' prognosis. It acts through targeting the genes of diverse momentous signaling pathways, including MCPH1, MXI1, FBXW7, KRAS, and PAB.²⁸⁻³¹

Myriad studies have exhibited the central role of miR-133b as a regulator of different vital pathways, resulting in control apoptosis, G1 cell cycle arrest, and invasion of tumor cells.³²⁻³⁴

In fact, the purpose of our study and other similar studies is to assess the potential of these specific microRNAs as novel biomarkers, whose up- or down-regulation can be used as a symptom of carcinogenesis initiation or a sign for cancer prognosis. The exact role of each microRNA biomarker, as oncogene or tumor suppressor, depends on the role of its targets.

Our study had some limitations that should be mentioned. Due to budget and time constraint we were not able to collect any more samples and investigate the expression level of the predicted target genes of these microRNAs. This can be the objective of our future studies so as to better understand the significant function of these promising biomarkers.

In conclusion, our research showed the dysregulated expressions of miR-212, miR-124a, miR-125b, miR-27a, and miR-133b, which have not only promising implications for the etiology and the pathogenesis of NSCLC, but also great potentials as biomarkers for NSCLC. Nonetheless, further studies are needed to confirm the effectiveness of these molecules in clinical practice.

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Conflict of Interest

None declared.

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