

Expression Pattern Study of miR-200a and XIAP Gene in the Non-small Cell Lung Cancer Patients' Blood

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ABSTRACT

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Background: In NSCLC, miR-200a plays a significant role in apoptosis. One of the genes involved in this pathway is *XIAP*, which has been shown anti-apoptotic activity. Research has indicated a significant association between miR-200a and the *XIAP* gene in this pathway. The present study investigated the expression profiles of miR-200a and the *XIAP* gene in NSCLC patients compared to normal individuals, as well as cancer cells compared to normal and apoptosis-inducing conditions.

Methods: In this study, 40 blood specimens were collected from NSCLC patients and 40 from healthy individuals. After isolating plasma and PBMC from these samples, we analyzed the miR-200a and *XIAP* gene expression levels using real-time PCR. Subsequently, normal and lung cancer cells were treated with paclitaxel as a model of apoptosis. The antiproliferative effects and induction of apoptosis were then evaluated using the MTT and flow cytometry assays, respectively. Finally, the expression patterns of miR-200a and the *XIAP* gene were investigated through a real-time PCR method.

Results: Results indicated that the miR-200a expression level was lower in NSCLC patients than in healthy ones, while the expression level of *XIAP* gene increased in the NSCLC Patients' blood. The MTT and flow cytometry results demonstrated a decreased proliferation and increased apoptosis rates in two lung line cells (A549 and MRC5) treated with paclitaxel. *XIAP* expression level also decreased in A549 cells treated with paclitaxel compared to untreated A549 cells.

Conclusion: MiR-200a may be associated with the *XIAP* gene expression and the induction of the apoptosis pathway in NSCLC. **DOI: 10.61882/ibj.4354**

Keywords: Apoptosis, Gene expression, MicroRNAs, Non-small cell lung carcinoma

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INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide. According to GLOBOCAN 2020, lung cancer remains the predominant cause of cancer deaths, accounting for 1.8 million deaths

(18%). Moreover, about 2.3 million (11.7%) new cases of lung cancer were diagnosed among all cancers globally in 2020. Asians also continue to have the highest incidence of lung cancer, representing 61% of all cancer deaths^[1]. Small-cell lung carcinoma and

List of Abbreviations:

cDNA: complementary DNA; **NSCLC:** non-small cell lung cancer; **MTT:** 2,5-diphenyl-2H-tetrazolium bromide; **PBMC:** peripheral blood mononuclear cell; **miRNA:** microRNA; **XIAP:** X-linked inhibitor of apoptosis protein; **IAP:** inhibitor of apoptosis protein; **BCL-2:** the B-cell lymphoma 2 gene; **cDNA:** complementary DNA; **ROC:** receiver operating characteristic; **AUC:** area under the curve

NSCLC are the two primary subtypes of lung cancer, comprising 15% and 85% of lung cancers, respectively^[2]. NSCLC is further categorized into squamous, adenocarcinoma, and large cell carcinoma^[2]. The NSCLC development is associated with molecular, genetic, and epigenetic modifications, along with additional morphological changes that transform benign bronchial epithelium into neoplastic tissue^[3].

MiRNA is a class of small noncoding RNA typically ranging from 21 to 25 nucleotides in length. They can inhibit mRNA translation and promote mRNA degradation by binding to complementary sites on target mRNAs. A single miRNA can target multiple mRNAs; conversely, multiple miRNAs can regulate the same target. As a result, miRNAs are involved in various biological processes, including apoptosis and the maintenance of cell differentiation. Based on estimates, miRNAs regulate one-third of human genes, highlighting their critical role in genomic and epigenomic interactions^[4]. In cancer, various miRNAs can act as oncogenes or tumor suppressors. Furthermore, the expression levels of certain miRNAs can serve as prognostic indicators for patients. The regulation of apoptosis by miRNAs is a crucial mechanism that could contribute to the inhibition of apoptosis and development of resistance to current cancer therapies. Currently, more than 80 miRNAs have been identified to be involved in the regulation or dysregulation of apoptosis, which influences cancer initiation, progression, invasion, metastasis, and resistance to anticancer treatment^[5].

The gene *XIAP*, along with its counterparts *cIAP1* and *cIAP2*, are members of the IAP family. To date, a total of eight IAPs—*NAIP* (*BIRC1*), *BIRC2* (*cIAP1*), *BIRC3* (*cIAP2*), *XIAP* (*BIRC4*), *BIRC5* (*Survivin*), *BIRC6* (*Apollon*), *BIRC7* (*Livin*), and *BIRC8*—have been identified^[6]. The main function of *XIAP* and other IAP family members is to suppress apoptosis. Notably, elevated levels of *XIAP* expression have been observed in various human cancers^[7]. In NSCLC, many miRNAs with abnormal expression patterns have been detected^[8]. For instance, the miR200 family, which consists of five members (miR-200a, miR-200b, miR-200c, miR-429, and miR-141) is involved in human cancers due to its tumor suppressive properties^[9]. Studies have suggested that the suppressive effect of miR-200 on two anti-apoptotic factors, the *BCL-2* and *XIAP* genes, may contribute, in part, to the induction of apoptosis^[9]. These miRNAs are derived from two distinct gene clusters: miR-200a, -200b, and -429 from chromosome 1 (1p36.33) and miR-200c and -141 from chromosome 12. (12p13.31)^[10]. Altogether, in some types of cancer, miR-200a promotes apoptosis, while in cervical cancer, it inhibits apoptosis. However, there are varying

findings regarding the role and function of miR-200a in the modulation of NSCLC^[11]. In this context, an earlier study has demonstrated that miR-200a overexpression can downregulate hepatocyte growth factors and reduce the migration and invasion of NSCLC cells. Likewise, it can promote apoptosis and decrease cell survival in the A549 and H1299 cell lines when exposed to ionizing radiation^[12]. Another investigation has suggested that lncRNA-ATB induces apoptosis in NSCLC cells by downregulating the expression of miR-200a while simultaneously enhancing the expression of β -catenin^[13]. Furthermore, miR-200c can inhibit bladder cancer invasion by directly targeting the 3'UTR region of *XIAP*^[14]. In bladder cancer a novel regulates cancer cell anchorage-independent growth by upregulating of *EGFR* gene. This regulation occurs through the inhibition of miR-200a transcription via the Rac1/PP2A/MAPKK/ MAPK/c-Jun pathway. In other words, the expression of miR-200a is suppressed by the *XIAP* BIR domain^[15]. A previous study has identified that miR-200a inhibits the growth of two NSCLC cell lines and promotes cell apoptosis^[16]. Another study has found that five miRNAs (miR-141, miR-200b, miR-193b, miR-200c, and miR-106b) are overexpressed in the serum of NSCLC patients, as well as in primary tumors of lung adenocarcinoma and lung squamous cell carcinoma^[17]. It has also been indicated that miRNA-200c downregulates the *XIAP* expression level, which contributes to the reduced growth of triple-negative breast cancer cells and induction of apoptosis^[18]. Research has shown that miRNAs such as miR-155, miR-146a, miR-125b, miR-30a, miR-29a, and miR-Let7 exhibit significant expression changes in NSCLC and can be utilized as prognostic and diagnostic biomarkers for this disease^[19]. In addition, there is a likely substantial connection among miR-200a, apoptosis, and the *XIAP* gene expression in NSCLC cells. However, the precise role of miR-200a in NSCLC remains unclear.

Scientists have always been looking for ways to reduce the resistance of cancer cells to anticancer drugs. One that has been approved by medical professionals, is paclitaxel, which is used in the treatment of various cancers, including lung cancer. Paclitaxel is the primary inducer of apoptosis with targeting tubulin^[20]. According to the TargetScan database, both miR-200b/c/429 and miR-200a-3p are predicted to target *XIAP*, and miR-200a potentially plays a crucial role in linking apoptosis to the *XIAP* gene in NSCLC cells ([Fig. S1](#)). The present study investigated the expression pattern of miR-200a in the blood of patients diagnosed with NSCLC, highlighting its relationship with the *XIAP* gene expression, a crucial player in the apoptotic process. Additionally, we treated lung cancer cells with

paclitaxel (Taxol), a widely used anticancer drug known for its apoptotic effects, to evaluate the expression profiles of miR-200a and the XIAP gene in vitro.

MATERIALS AND METHODS

Sample collection

A total of 40 blood specimens were collected from new cases of NSCLC at Dr. Masih Daneshvari Hospital (Tehran, Iran). Also, 40 normal volunteers provided blood samples for comparison. The criteria for including and excluding participants in this study were carefully defined. Inclusion criteria included males and females aged between 25 and 75, with no other cancers, human immunodeficiency virus, or hepatitis. It was also essential that all patients were the newly diagnosed cases. The exclusion criteria involved a history of surgery, prior treatment with anticancer drugs, or chemotherapy. After collecting the blood samples, centrifugation was conducted to separate the plasma samples and PBMC from the whole blood at a speed of 7,000 ×g for 10 minutes. The samples were then stored in laboratory refrigerators at -70 °C.

RNA extraction and cDNA synthesis

To extract RNA, we obtained the RNX-Plus solution for total RNA isolation from the SinaClon Company (Iran). The initial step of the protocol involved adding 1 mL of super-cold RNX-TM-PLUS solution to 100 µL of homogenized samples (plasma and PBMC). Before performing the cDNA synthesis and RT-qPCR, we assessed the purification and concentration of the extracted RNAs using a NanoDrop spectrophotometer (Thermo Scientific, USA). Purification was determined by measuring the A260/280 absorbance ratio, while RNA concentration was qualified in nanograms per microliter. To initiate cDNA synthesis, the stem-loop primer was first heated at 85 °C for 5 minutes, then immediately placed on ice for two minutes to stabilize its linear form. Subsequently, the extracted RNAs were

used to synthesize cDNA using a kit purchased from Pars Toos Company (Iran).

Real-time PCR

Total RNA was extracted using the TRIzol reagent. To perform RT-PCR, we synthesized cDNAs using stem-loop primers. Afterward, RT-PCR was performed with forward and reverse primers for miR-200a and XIAP, utilizing the SYBER Green Master Mix. Also, miR-16 and β-actin were employed as internal controls. The primer sequences are listed in Table 1.

Cell culture

For cell culturing, the NSCLC cell line (A549) and normal lung cells (MRC-5) were obtained from the Cell Bank of the Pasteur Institute of Iran (Tehran). The cells were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Drug treatment, MTT, and flow cytometry assays

The drug concentration used for treatment was 20 µg/mL^[21,22]. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well, and 20 µL of the MTT solution was added to each well. After incubation, 150 µL of DMSO was also added to each well. Following a 15-minute incubation period, cell viability was measured using a spectrophotometer (ELISA) at a wavelength of 570 nm. Flow cytometry was employed to measure the percentage of cell apoptosis using the Annexin-V-Fluos staining method.

Statistical analysis

The statistical analysis of the blood miRNA and gene expression in both patients and normal individuals was carried out using the t-test in GraphPad Prism software version 9.0.0, employing the ΔΔCT method (San Diego, California, USA). Statistical analysis for dose-response assessment in each group and cell line was also conducted using the One-way ANOVA method. *P*-values less than 0.05 were considered statistically significant.

Table 1. Primers used in this study

Primer	Sequence (5' to 3')
miR-200a stem-loop	F: CCGGAGTAGCAGCACGTAAT
	R: ATCCAGTGCAGGGTCCGA
	F: GTGCAGGGTCCGAGGT
	R: GTATACCATCTTACCGGACAG
	F: GTCGTATCCAGTGCAGGGTCCGAGG
	R: TATTCGCACTGGATACGACTCCAGC
	F: ACTCTACTACACAGGTATT
	R: TCAGAACTCACAGCATCAG
	F: AGACGCAGGATGGCATGGG
	R: GAGACCTCCAACACCCAAGCC

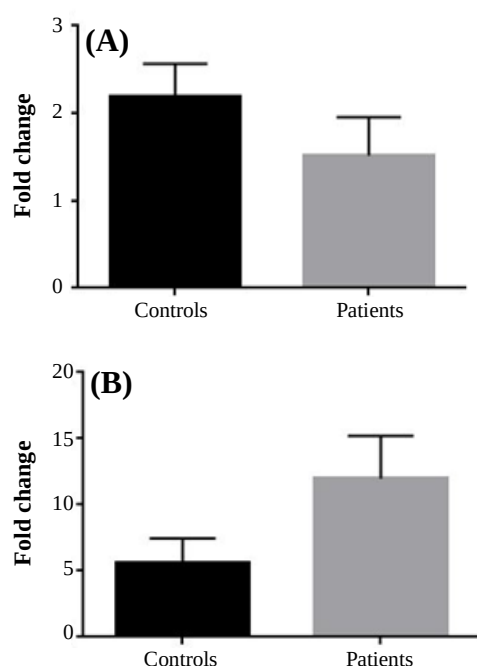


Fig. 1. The expression levels of (A) miR-200a and (B) XIAP in NSCLC patients and normal controls. A significant decrease was observed in the expression level of miR-200a ($p = 0.0426$) in NSCLC patients compared to normal individuals. In addition, the expression level of XIAP gene increased compared to healthy controls.

RESULTS

Results of expression pattern for MiR-200a and XIAP

In patients with NSCLC, a significant decrease was observed in the expression level of miR-200a ($p = 0.0426$) compared to normal individuals. In contrast, the expression level of XIAP was found to be increased compared to healthy controls (Fig. 1). Treatment with paclitaxel resulted in a significant increase in miR-200a expression in A549 cells compared to untreated cells. However, this elevated expression in the drug-treated MRC-5 cells did not reach statistical significance when compared to untreated MRC-5 cells (Fig. 2). Our results demonstrated a significant decreased expression level of the XIAP gene in A549 cells after treatment with paclitaxel compared to untreated A549 cells.

MTT assay, flow cytometry, and ROC Curve analysis

After 48 of treatment, the IC_{50} was 3.31 $\mu\text{g/mL}$ in A549 cells and 1.88 $\mu\text{g/mL}$ in MRC-5 cells. Induction of apoptosis was performed using paclitaxel-treated cells following concentration of 8 $\mu\text{g/mL}$. Propidium iodide and Annexin-V-Fluos staining were used for both A549 and MRC-5 cells. Figure 3A and 3B illustrate the

percentage of apoptosis in A549 cells before (A) and after (B) treatment with paclitaxel. Figures 3C and 3D display the flow cytometry analysis of MRC-5 cells, which served as control samples, before and after treatment. We observed a significant increase in the population of apoptotic cells in quadrants Q2 and Q3 of Figures 3A and 3B, which represent late and early apoptosis (0.962% and 2.51% to 4.71% and 34.4%, respectively). Figure 4 presents the result of the ROC curve analysis. The corresponding AUC for miR200a and XIAP was approximately 0.7, indicating an acceptable level of accuracy.

Patients' demographic information

A total of 80 blood samples (cancer patients and healthy controls) were collected for this research. Among the 80 subjects, 40 were healthy, and 40 had

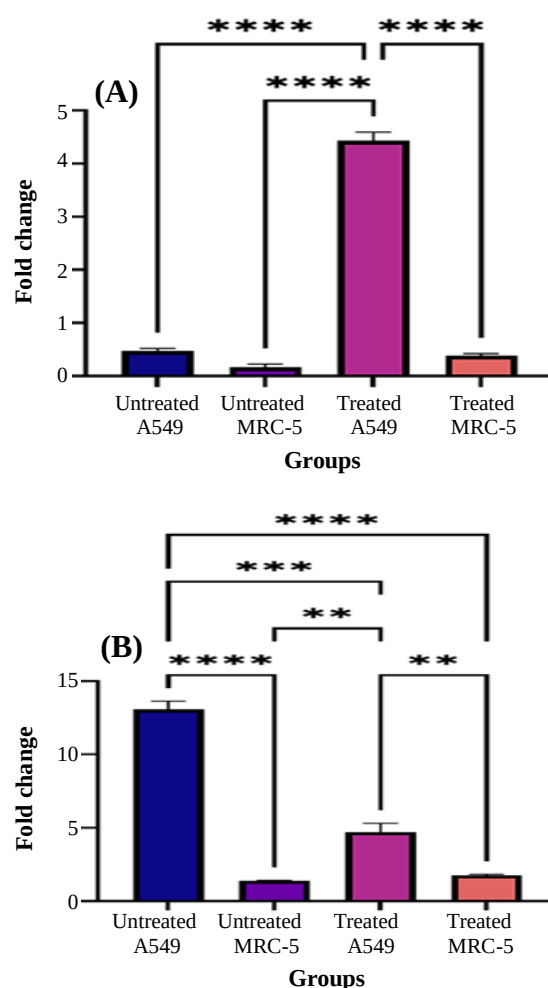


Fig. 2. Comparison of (A) miR-200a and (B) XIAP expression levels in A549 and MRC-5 cells before and after treatment. The XIAP expression level was higher in A549 cells than in healthy MRC-5 cells. Also, treated A549 cells exhibited a significantly higher expression of miR-200a compared to the untreated A549 cells (** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

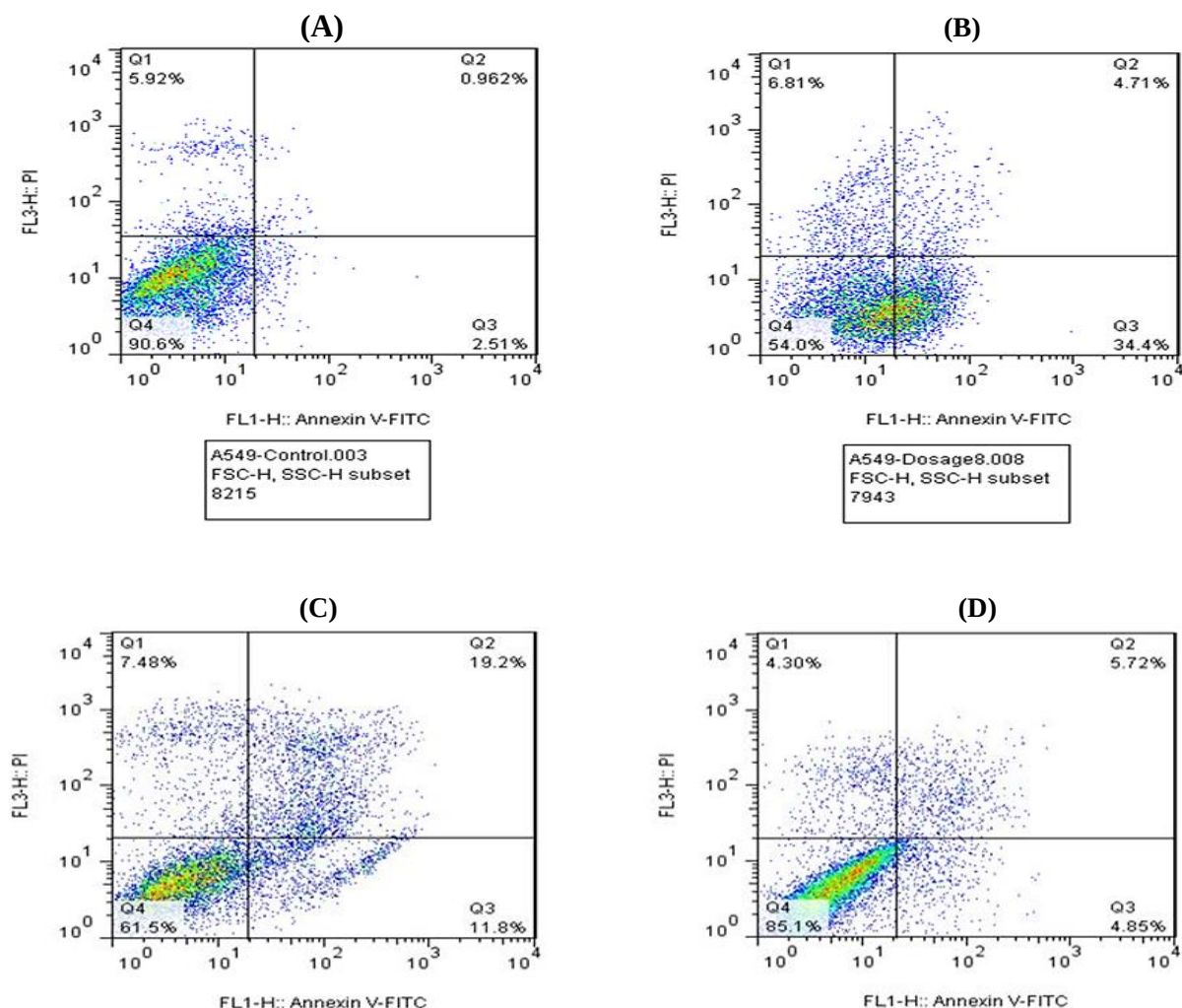


Fig. 3. Apoptosis analysis of A549 cells before (A) and after (B) treating with paclitaxel (8 µg/mL) for 48 hours and MRC-5 cells (C and D) as controls. The results show an increase in the incidence of apoptosis in cells due to the drug treatment.

NSCLC. The types of lung cancer was adenocarcinoma in 22 patients (55.0%), large cell carcinoma in 6 (15.0%), and squamous cell carcinoma in 12 (30.0%). Among the patients, 9 were female, and 31 were male. The results of the chi-square test revealed a significant relationship between sex and health status ($\chi^2 = 6.54$; $p < 0.01$), indicating that men were more affected by cancer than women. Also, there was a likely relationship between age and health status ($\chi^2 = 46.19$; $p < 0.000$), and the individuals aged 61-70 years were more affected by cancer. The analyses also indicated a significant relationship between smoking status and health status ($\chi^2 = 13.21$; $p < 0.001$), showing that cancer patients were significantly more likely to be smokers compared to healthy individuals. Additional data regarding the demographic information of patients are shown in Table 2.

DISCUSSION

Extensive literature highlights the significant alterations in the expression of miR-200 family members in NSCLC. Zhu et al. investigated the crucial role of miR-200b, miR-200c, and miR-429 in the resistance of the lung cancer cell line A549 to cisplatin. They found that the miR-200b/200c/429 cluster was downregulated in cisplatin-resistant A549 cells. However, the protein levels of BCL2 and XIAP were overexpressed in this cell line. Their studies demonstrated that miR-200b, miR-200c, and miR-429 directly regulated two apoptotic proteins, BCL2 and XIAP. The researchers hypothesized that the miR-200b/200c/429 cluster may contribute to the development of cisplatin resistance in lung cancer cells by targeting the expression and regulation of BCL2 or XIAP within the apoptosis pathway^[23].

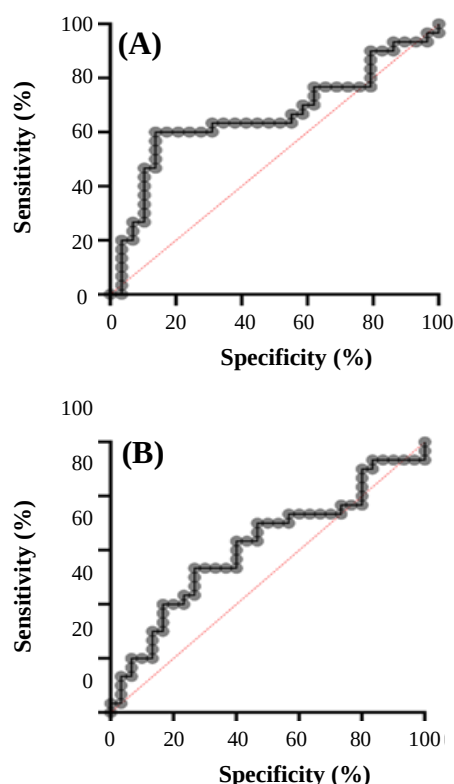


Fig. 4. ROC curve diagram for (A) miR200a and (B) *XIAP*.

In a previous investigation, Qin and colleagues conducted real-time PCR and Western blot analysis on primary tumor samples from 26 patients with NSCLC who underwent lung surgery. The authors observed that *XIAP* protein expression level was significantly higher in NSCLC tissues compared to the matched normal lung tissue. Additionally, *XIAP* protein level was markedly elevated in lung cancer tissue compared to normal tissue. Furthermore, the overexpression of *Smac* increased apoptosis induced by indomethacin^[24].

There is limited information regarding the role of miR-200a in NSCLC. However, a recent study has demonstrated that miR-200a has a tumor-suppressive function in NSCLC. Moreover, tissue samples of NSCLC patients demonstrated lower level of miR-200a compared to the matched normal lung tissues. Importantly, the overexpression of miR-200a inhibited NSCLC cell proliferation and promoted apoptosis^[16].

In the last few years, there has been a significant interest in the relationship between *XIAP* and miR-200a in various types of cancers. One study has reported the crucial role of the BIR domain in the inhibitory effect of *XIAP* on miR-200a expression in bladder cancer^[15]. Another study explored that *XIAP* inhibits miR-200a, which in turn stabilizes c-Myc and also interacts with E2F1, facilitating anchorage-independent growth^[6]. A

recent study analyzed the expression profiles of two common miRNAs (miRNA-155 and miRNA-146a) in NSCLC and tuberculosis. The researchers examined the expression levels of the two miRNAs in the cells obtained from healthy individuals and those diagnosed with TB and lung cancer. The results indicated an elevated expression level of miR-155 in TB and lung cancer patients compared to healthy individuals. However, the expression level of miR-146a decreased in these patients. Hence, both miRNA-155 and miRNA-146a can serve as potential biomarkers for diagnosing lung cancer and TB. Recently, Huang *et al.* have demonstrated decreased levels of miR-200a in NSCLC tissue samples compared to normal lung tissue. They have also shown that overexpression of miR-200a could inhibit NSCLC cell proliferation and promote apoptosis^[16]. The inhibition of miR-200a by *XIAP* has been also reported in multiple studies^[15,6]. Given that *XIAP* protein level in lung cancer tissue is significantly higher than in normal tissue^[24], investigating the potential relationship between miR-200a and *XIAP* in NSCLC is warranted. A follow-up study involving the same patients after treatment with paclitaxel would be highly beneficial. It would allow for the assessment of differences in the expression profiles of the *XIAP* and miR-200a before and after paclitaxel administration in NSCLC patients.

CONCLUSION

This study evaluated the expression patterns of miR-200a and the *XIAP* gene in the blood of patients with NSCLC compared to healthy individuals. We also investigated the potential relationship between miR-200a and its target gene, *XIAP*. Our findings indicate a significant association between the expression levels of the *XIAP* gene and miR-200a in NSCLC patients, highlighting differences in the expression profiles of this miRNA and its target gene between healthy individuals and patients. The high sensitivity and detection efficiency of real-time PCR method for the expression pattern study of miR-200a suggest that miR-200a has the potential to serve as a valuable biomarker for diagnostic and therapeutic strategies related to NSCLC. Additional studies, including luciferase assays, are essential to further corroborate these findings.

DECLARATIONS

Acknowledgments

No artificial intelligence services were used for the preparation of this manuscript.

Table 2. Characteristics of NSCLC patients and healthy individuals

Characteristics		Patients individuals (n = 4)	Healthy individuals (n = 40)	χ^2	Sig
Cancer Type	Healthy	0	40	-	-
	Adenocarcinoma	22	0		
	Large cell carcinoma	6	0		
	Squamous cell carcinoma	12	0		
Gender	Female	9	20		
	Male	31	20		
Age	Up to 30	0	11		
	31-40	2	9		
	41-50	5	11		
	51-60	12	8		
	61-70	15	1		
	Above 70	6	0		
Smoking status	Non-smokers	17	28		
	Current smokers	23	9		
	Former smokers	0	3		
Duration of smoking	Does not consume	17	28		
	1-20	6	8		
	>20	17	4		
Addiction	Not Addicted	26	39		
	Addicted	14	1		
Duration of Addiction	Not Addicted/not mentioned	35	39		
	1-20	2	1		
	>20	3	0		
Special disease	Without special disease	34	39		
	With special disease	6	1		
Special disease type	Neuropsychiatric illness	1	0		
	Diabetes	2	0		
	Cardiovascular disease	1	0		
	Antibiotic sensitivity	1	0		
	Hydrosol	1	0		
	Seasonal allergies	0	1		
Medicine	No drug consumption	34	40		
	drug consumption	6	0		
Medicine type	Tension pills	1	0		
	Vitamin pills	1	0		
	Pressure pills	1	0		
	Morphine	1	0		
	Metoral pills	2	0		

Ethical approval

All the experimental procedures in this study were conducted in accordance with the Pasteur Institute of Iran and approved by the University of Tehran (ethical code: IR.PIL.REC.1400.016).

Consent to participate

All the subjects voluntarily agreed to participate in this research and gave their written informed.

Consent for publication

All authors reviewed the results and approved the final version of the manuscript.

Authors' contributions

TF: investigation and writing original draft; SJZ: supervision, review and editing; SS: methodology and validation; MS: supervision, project administration, methodology, validation, writing-review, and editing.

Data availability

All relevant data can be found within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Supplementary information

The online version contains supplementary material.

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