

ORIGINAL ARTICLE

BCL2, *IRS1*, *AKT3*, *PTEN*, and *HIF1A* expression levels in non-small cell lung cancer patients

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Abstract

Background: Lung cancer is the leading cause of cancer-related deaths worldwide. MicroRNAs (miRNAs) are small noncoding molecules that play critical roles in cell proliferation, apoptosis, invasion, and metastasis, and they can target multiple genes at the mRNA level.

Materials and methods: Some online tools like TargetScan, miRDIP, miRmap, and miRanda were used to evaluate the validated target genes. Before choosing target genes, we took advantage of some bioinformatics tools including STRING, GeneMANIA, and TRED to predict the target genes. Finally, the expression levels of the target genes were measured in non-small cell lung cancer (NSCLC) tumor and their adjacent normal tissues via SYBR Green-based quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR).

Results: According to bioinformatics tools, *BCL2* and *AKT3* were selected as target genes for miR-15/16, and *BCL2* was shown to demonstrate a robust negative correlation with miR-15a in our previous analysis of NSCLC tumor samples. Furthermore, we found a significant correlation between *BCL2* expression level and stage III ($p = 0.04$). *PTEN* was assumed as a validated target gene of miR-21 that presented a significant decrease in tumor tissues compared to adjacent normal tissues. *IRS1* was assigned as a target gene of miR-126/miR-128, and finally, *HIF1A* was selected as the target gene of miR-210. There was a significant negative association between *IRS1* expression level and miR-126/miR-128, but a positive correlation was demonstrated between miR-210 and *HIF1A* at mRNA level.

Conclusion: Restoration of miR-15/16, miR-126, and miR-128 in NSCLC might be therapeutic candidates to control cell proliferation and apoptosis.

KEYWORDS

AKT, *BCL2*, *HIF1A*, *IRS1*, miRNAs, non-small cell lung cancer, *PTEN*

INTRODUCTION

Lung cancer has been the main cause of all cancer-related deaths in the world for several decades. In 2012, The Global Cancer Observatory (GLOBOCAN) estimated approximately 1.8 million new cases, accounting for

12.9% of the total, and 1.59 million deaths, representing 19.4% of the total [1]. This cancer is predominantly diagnosed in men globally, with 1.2 million cases, representing 16.7% of the total, whereas the incidence rates are generally lower in women [1]. Non-small cell lung cancer (NSCLC) is the most common type of lung

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cancer, accounting for 80%–85% of all cases [2]. This disease has had a grim prognosis and the 5-year overall survival is below 15% [3]. At the time of diagnosis, a significant proportion of patients (70%) already have advanced or unresectable disease [4, 5]. The most common histologic type of NSCLC is adenocarcinoma, and its incidence is increasing worldwide [6]. Patients suffering from this type of lung cancer experience treatment failure due to its highly invasive and metastatic nature [7]. The second common histological subtype is lung squamous cell carcinoma (SCC), which accounts for approximately 30% of all NSCLCs and is more frequently observed in smokers and males [8, 9]. The third histological subtype is large cell lung carcinoma (LCC), which accounts for approximately 5%–10% of all NSCLC cases, with a close relationship with smoking [10]. Small cell lung cancer is another type of lung cancer that has been decreasing in frequency over the last two decades [2].

MicroRNAs (miRNAs) are small (~22 nt) noncoding RNAs that play important roles in posttranscriptional regulation of coding genes [11]. Previously, we tested the expression levels of several miRNAs including miR-15a/16, miR-21, miR-34a, miR-126, miR-128, and miR-210 in NSCLC tissues, and significant dysregulation of the aforementioned miRNAs was observed in NSCLC tissue samples relative to adjacent normal tissues [12]. To find out the most important target genes for each miRNA, we used a protein-protein interaction (PPI) network-based approach and literature-based information. PPI networks have emerged as an applicable tool for identifying the key hub genes of a given gene list, especially in cancer studies [13]. Several genes including B-cell-lymphoma-2 (*BCL2*, as miR-15a/16 and miR-34a target gene), phosphatase and tensin homolog (*PTEN*, as miR-21 target gene), *AKT3* (as miR-15a/16 target gene), insulin receptor substrate 1 (*IRS1*, as miR-126 and miR-128 target gene), and hypoxia-inducible factor-1 (*HIF1A*, as miR-210 target gene) were then found as the candidate targets from hundreds of possible target genes for each miRNA.

BCL-2, an antiapoptotic protein, plays a critical role in apoptosis regulation. In the majority of the studies, high expression of *BCL-2* is associated with better prognosis in NSCLC [14]. *PTEN*, a tumor suppressor gene that has a key role in phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway, could affect cell growth, proliferation, and survival by negatively regulating PI3K/AKT signaling pathway [15]. *AKT*, a serine/threonine protein kinase, has different isoforms, *AKT1*, *AKT2*, and *AKT3*, and they have various prognostic roles in NSCLC in a narrow correlation with *PTEN* protein [16]. *IRS1* has a critical role in the insulin-like growth factor signaling pathway. Its tyrosine would be phosphorylated in response to insulin and cytokines, and then some signaling pathways including PI3K, mitogen-activated protein kinase (MAPK), and *AKT* become activated, which can lead to cell growth, differentiation, and survival [17]. *HIF1A* has a key role in different biological processes

including cell proliferation, angiogenesis, and drug resistance [18]. In agreement with the results of PPI graph analysis, recent studies have also reported the regulatory functions of these genes in the initiation and progression of NSCLC [19–23].

In this study, the expression levels of *BCL2*, *PTEN*, *AKT3*, *IRS1*, and *HIF1A* genes were measured in tumor tissues relative to adjacent normal tissues. The results were then correlated with the clinic pathological features of the patients and with the miRNA expression profiles in our previous study.

MATERIALS AND METHODS

Patients and tissue specimens

Tissue samples from 30 untreated patients with NSCLC were studied (Table 1). Surgical resection specimens of primary lung tumor tissues (approximately equivalent to a 3 mm × 3 mm × 3 mm cube) and matched adjacent normal tissues were collected from anonymous patients with untreated NSCLC followed at Masih Daneshvari and Atieh Hospitals, Tehran, Iran, between September 2011 and December 2013. Tissue samples from patients were collected before chemotherapy or radiotherapy and were immediately transferred to liquid nitrogen at -180°C . Then all the tissues were stored at -80°C in Pasteur Institute of Iran. The study was approved by the Local Committee of Ethics and patients' informed consent was taken before sample resection. Before any molecular analysis was performed on the tissue samples, they had been

TABLE 1 The clinic-pathologic features of the NSCLC patients.

Tissue	Cases
Age (year)	
≤ 57	11
> 57	19
Sex	
Male	22
Female	8
TNM	
I or II	21
III	9
Smoking	
Yes	19
No	11
Subtype	
Adenocarcinoma	17
SCC	13

processed by pathologists according to standard diagnostic procedures.

RNA extraction and cDNA synthesis

RNA extraction from NSCLC tumor tissues and matched adjacent normal tissues (15–30 mg) was performed using the miRNeasy Kit according to the manufacturer's instructions (Qiagen, USA). The concentration and purity of extracted RNA were measured by NanoPhotometer. For SYBR Green-based quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis of miRNA targeting genes (Table 2), cDNA was synthesized using a Two-step RT-PCR Kit (Vivantis, USA) according to the manufacturer's instructions. Briefly, 1 µg RNA was reverse-transcribed in a reaction mixture containing PCR buffer, 1 µL of 10 mmol/L dNTP mix, 1 µL Oligo dT, and 100 U of M-MuLV Reverse Transcriptase, with final volume of 20 µL. The reverse transcription reaction was completed at 42°C for 60 min and terminated at 85°C for 5 min. For later analysis, the cDNA was stored at –20°C.

Identifying the key target genes using graph-based studies

To determine the most important target genes, we constructed a PPI network for each miRNA's target genes. The putative interactions between the target genes and their respective neighbors were obtained from STRING (search tool for recurring instances of neighboring genes) [24], GeneMANIA (via the website of GeneMANIA), and TRED [25] databases as well as literature-based information. We considered the validated directed interactions and the important indirect interactions, such as binding and genetic interactions, to construct the PPI networks. The seven resulting PPI networks were analyzed and visualized using Cytoscape software [26]. The Network Analyzer, a plug-in of Cytoscape, was used to measure the topological

properties of each network. Among the nine considered measures, we found that the Betweenness centrality and Degree measures were highly capable of finding the important functional hub genes. Degree measure refers to the number of edges of a node and the nodes with higher degree centrality are considered as biologically important hub genes within biological networks. Betweenness centrality is another centrality measure that represents the number of times each node is visited while traversing all shortest paths and is computed as follows:

$$Cb(n) = \sum_{s \neq n \neq t} (ost(n)/ost),$$

The s and t are nodes in the network different from n , ost indicates the number of the shortest paths from s to t , and $ost(n)$ is the number of the shortest paths from s to t that n lies on.

Finally, we selected the top hub genes and inputted them into the DAVID [27] and KEGG web tools to identify the key lung cancer-related genes.

Real-time PCR

SYBR Green qRT-PCR amplifications were performed in Real-Time PCR Detection System ABI 7300 (Applied Biosystems, USA). PCR primers were designed through Allele ID 7.5 for five candidate miRNA target genes and an internal control (*GAPDH*) which represented different functional categories (Table 2). The reactions were performed in a total volume of 20 µL, containing 10 pmol of each primer and 2 µL of diluted cDNA template, which included 800 ng of cDNA. The thermal cycler conditions were as follows: Step 1, 95°C for 10 min (1 cycle); Step 2, 95°C for 15 s and 60°C for 1 min (45 cycles); Step 3, 95°C for 15 s, 60°C for 20 s, and 72°C for 1 min (1 cycle). 10-fold serial dilutions covering a log transformed dynamic range of the diluted cDNA were carried out and used as templates in SYBR Green qRT-PCR to generate standard curves.

TABLE 2 The sequences of primers for candidate miRNA target genes.

Target gene	Accession number	Forward primer	Reverse primer	Amplicon size (bp)
<i>BCL2</i>	NM_177410.2	5'-CTGCACCTGACGCCCTTACC-3'	5'-CACATGACCCACCGAACTCAAAGA-3'	119
<i>AKT3</i>	NM_005465.4	5'-GTTGGAGAATCTAATGCTGGA-3'	5'-TCATTATCTTCTAACACCTCTGG-3'	147
<i>HIF1A</i>	NM_001530.3	5'-AAGTTCACCTGAGCCTAATAGTC-3'	5'-AGTCTAAATCTGTGTCCTGAGTAG-3'	152
<i>IRS1</i>	NM_005544.2	5'-GAGAGCAGCGGTGGTAAG-3'	5'-GGCAATGAGTAGTAGGAGAGG-3'	149
<i>PTEN</i>	NM_000314.4	5'-CACACGACGGGAAGACAAGTTC-3'	5'-CCTCTGGTCCTGGTATGAAGAATG-3'	161
<i>GAPDH</i>	NM_002046.5	5'-ACACCCACTCCTCCACCTTTG-3'	5'-TCCACCACCCTGTTGCTGTAG-3'	112

Statistical analysis

All obtained data were analyzed by student's *t*-test and graphs were drawn using Graphpad Prism 5.0. To evaluate the correlation between miRNAs and target genes, the correlation coefficient was considered in Graphpad and SPSS.

RESULTS

Bioinformatics tools for miRNA target prediction

To obtain the predicted targets of miRNAs, which were analyzed in the previously published article [12], various databases, including Human TargetScan, RNA22, mirTar, PicTar, mirDIP, and miRmap, were used. Furthermore, the obtained data of the predicted target genes were analyzed using STRING, GeneMANIA, and Cytoscape Servers. The number of nodes and edges in the resulted PPI networks were calculated as follows: hsa-miR-15a network (74 nodes and 226 edges), hsa-miR-16 network (73 nodes and 302 edges), hsa-miR-21 network (75 nodes and 374 edges), hsa-miR-34a network (75 nodes and 393 edges), hsa-miR-126 network (72 nodes and 179 edges), hsa-miR-128 network (77 nodes and 530 edges), and hsa-miR-210 network (79 nodes and 207 edges) (Data not shown). Analysis revealed that the majority of hub genes are functionally associated with cancer initiation and progression. The top 20 hub genes of these three measures were selected for functional annotation studies (Table 3). According to the over-represented functional categories and pathways, we selected the most important lung cancer-associated genes for experimental validation at the mRNA level. In this study, *BCL2*, *AKT3*, *PTEN*, *IRS1*, and *HIF1A* were analyzed as target genes of miR-15/16 and miR-34a, miR-15/16, miR-21, miR-126/128, and miR-210, respectively.

The correlation between *BCL2* gene expression and miR-15/16, as well as between *BCL2* and miR-34a

The expression levels of miR-15/16 and miR-34a were previously analyzed by TaqMan qRT-PCR in 30 tumor tissues compared with data obtained from adjacent normal tissues [12]. Then, the expression level of *BCL2*, as target gene for miR-15/16 and miR-34a, was measured through SYBR Green qRT-PCR in the samples obtained from the same patients. The results showed downregulation of *BCL2* in 19 out of 30 (63.3%) cases. The correlation of *BCL2* expression level with miR-15/16 and miR-34a was compared by one-way ANOVA test in Graphpad Prism 5.0. The

results showed a significant correlation between *BCL2* and the miRNAs ($p < 0.0001$). *BCL2* expression level was associated with stage, however, we could not find any correlation between *BCL2* expression level and sex, histological subtype, or smoking (Figure 1a,b).

The correlations between *AKT3* and miR-15/16, and between *PTEN* and miR-21 expression levels

The expression level of *AKT3* was analyzed by SYBR Green qRT-PCR in 30 tumor tissues compared to adjacent normal tissues. The results showed that *AKT3* was downregulated in 63% of all NSCLC cases. This gene was considered as the target gene of miR-15/16, which showed a negative correlation with miR-15/16, however, this association was not significant. Also, *PTEN*, as a target gene of miR-21, was downregulated in 37% of tumor tissues compared with adjacent normal tissues. In this study, the correlation between *PTEN* and miR-21 was analyzed through *t*-test and we found a negative correlation between miR-21 and *PTEN* expression level in these samples ($p = 0.01$, Figure 2). The correlation between *PTEN* and *AKT3* genes was analyzed, but we could not find any link between these genes at mRNA level.

The correlation between *IRS1* and miR-126, as well as between *IRS1* and miR-128

IRS1 expression level was measured via SYBR Green qRT-PCR in 30 tumor tissues, and the results showed a downregulation in 61% of the cases ($p < 0.05$). According to bioinformatics analysis, *IRS1* was predicted as the target gene of both miR-126 and miR-128, so the correlations between *IRS1* and miR-126, and between *IRS1* and miR-128 were analyzed through Prism 5 (Figure 3). As demonstrated, *IRS1* had a remarkable negative correlation with miR-126 ($p = 0.01$), but there was no correlation between *IRS1* and miR-128 at mRNA level.

The correlation between *HIF1A* and miR-210

According to our previous study, miR-210 was upregulated in 70% of NSCLC tumor tissues versus matched adjacent normal tissues ($p < 0.001$). By using bioinformatics tools, *HIF1A* was chosen as the target gene of miR-210. The expression level of *HIF1A* in 30 NSCLC tumor tissues was compared with matched adjacent normal tissues. *HIF1A* showed a significant downregulation in 92.5% of cases, and was negatively correlated with miR-210 ($p = 0.07$, Figure 4).

TABLE 3 The top 20 hub genes obtained from topological analysis of each PPI network.

hsa-miR-15	hsa-miR-16	hsa-miR-21	hsa-miR-34a	hsa-miR-126	hsa-miR-128	hsa-miR-210
Gene	Gene	Gene	Gene	Gene	Gene	Gene
Value	Value	Value	Value	Value	Value	Value
BCL2	AKT3	P TEN	BCL2	IRS1	PCF11	HIF1A
0.21	0.11	0.21	0.09	0.4	0.05	0.17
RBBP6	MYB	SOX2	FUT8	TBC1D15	SMARCA2	CEND1
0.2	0.09	0.07	0.06	0.13	0.04	0.16
AKT3	BCL2	TGFB1	NRIP3	PII5	FBXO30	ATF4
0.11	0.07	0.05	0.05	0.1	0.04	0.15
MYB	CHEK1	NFIB	ACSL4	SLC10A1	IRS1	TLR1
0.1	0.07	0.04	0.05	0.07	0.04	0.13
PIAS1	NFIB	CHD7	ANK3	SPEN	CHUK	DYRK1A
0.08	0.06	0.04	0.05	0.06	0.04	0.12
TP53	NFIA	TPD52	SF3B1	MDM4	ARMC8	CAMTA1
0.08	0.05	0.04	0.04	0.06	0.03	0.11
CHEK1	RELN	RAP1B	SGPP1	COQ10B	TMTC2	CGREF1
0.05	0.05	0.03	0.04	0.06	0.03	0.1
PCMT1	POLR3F	ALX1	LPHN3	SORBS2	JAG1	MNT
0.04	0.04	0.03	0.03	0.06	0.03	0.1
CUL2	CDC25A	PGM1	NRN1	SPAG16	TRIP12	RNF139
0.04	0.03	0.03	0.03	0.05	0.03	0.09
KRAS	RAG2	PFKM	CD2AP	ZNF85	FMN2	BIRC7
0.04	0.03	0.03	0.03	0.04	0.02	0.09
POLR2D	ZNF143	FN1	SLC12A2	CCDC91	ARHGAP12	ONECUT1
0.03	0.03	0.02	0.03	0.04	0.02	0.08
CAPZA2	NFIX	PDCD4	OGFRL1	RABGAP1	RPS6KA5	GPR17
0.03	0.03	0.02	0.03	0.04	0.02	0.07
SRSF7	ATXN2	TIAM1	STRN3	GABRA4	MYOF	AIFM3
0.03	0.03	0.02	0.03	0.04	0.01	0.06
PPP2R3A	SSB	PCBP1	TAF5	VPS13A	IRS2	GGNBP2
0.03	0.02	0.02	0.03	0.04	0.01	0.06
RAG2	KRAS	STAG2	ATP8A2	KIAA1456	POGZ	F7
0.02	0.02	0.02	0.02	0.03	0.01	0.06
UBE2I	STOX2	GID4	RUSC1	ERGIC2	SOC56	EPB41L1
0.02	0.02	0.02	0.02	0.03	0.01	0.06
VHL	DHRS2	EEF1A1	CACNB3	PCDHGA8	RNF182	KIF17
0.02	0.02	0.01	0.02	0.03	0.01	0.05
STK33	SON	IL12A	PLXNA2	TRIM8	OPA1	PLA2G2D
0.02	0.02	0.01	0.02	0.03	0.01	0.05
TPT1	RGL2	LUM	SLC31A2	MAP3K2	VEGFC	LILRA3
0.02	0.02	0.01	0.02	0.02	0.01	0.05
CCNE1	CCNE1	TUBB2A	KRAS	TFF3	MED23	RAB27B
0.02	0.02	0.01	0.02	0.02	0.01	0.04

Note: The selected hub genes are shown in bold font.

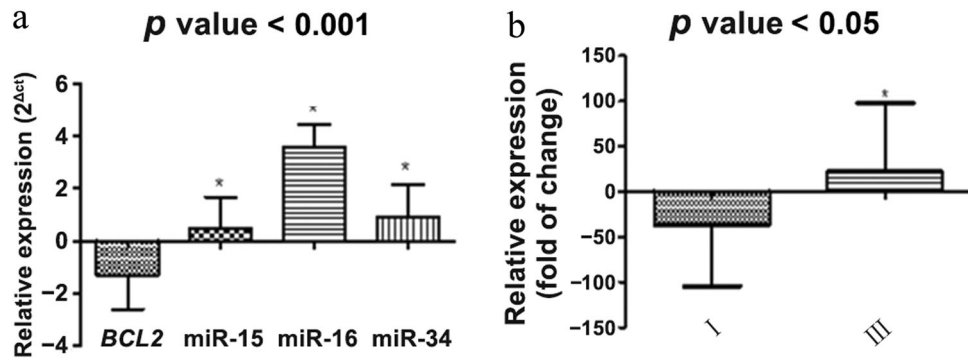


FIGURE 1 The correlations between *BCL2* expression level and miR-15/16, and between *BCL2* and miR-34a in tumor tissues. *BCL2* was upregulated significantly in tumor tissues compared to adjacent normal tissue ($p < 0.0001$). There is a negative correlation between *BCL2* and miR-15/16, as well as between *BCL2* and miR-34a expression at mRNA level.

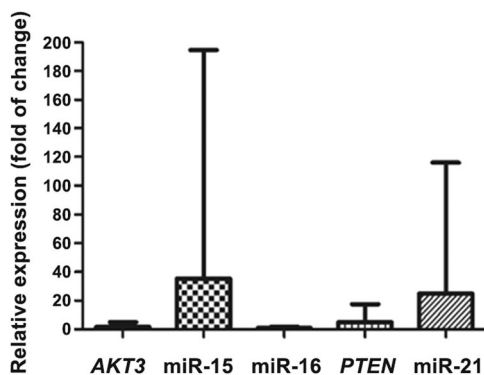


FIGURE 2 The correlations between *AKT3* and miR-15/16, and between *PTEN* and miR-21 at mRNA level.

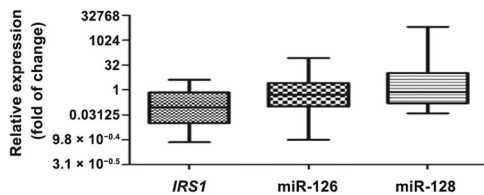


FIGURE 3 The expression level of *IRS1* at mRNA level as the target gene of miR-126 and miR-128 in 30 NSCLC tumor tissues versus adjacent normal tissues.

DISCUSSION

Evasion of apoptotic cell death is one of the hallmarks of cancer. There are many genes that are involved in the regulation of apoptotic pathways, including BCL2 family proteins. This family of proteins is divided into two main groups: the antiapoptotic (e.g., BCL2, Bcl_{xL}, and Mcl-1) and proapoptotic (e.g., Bak, Bax, Bad, and Bid) members [28]. BCL2 protein is overexpressed in different types of cancers, therefore, it can be used as a potential prognostic factor. Also, this protein might have a key role in

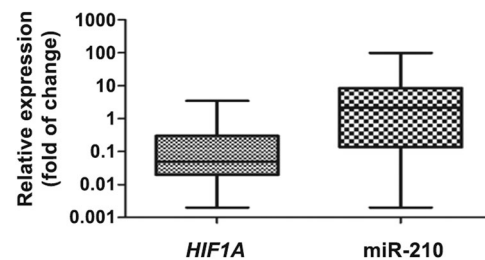


FIGURE 4 The expression levels of miR-210 and *HIF1A* in 30 NSCLC tumor tissues compared with matched adjacent normal tissues.

tumor aggressivity in NSCLC, as well as syndromic and nonsyndromic keratocystic odontogenic tumors [29, 30]. *BCL2* gene, as a member of BCL2 family, is located on chromosome 18, and acts as an antiapoptotic factor in several cancers, including NSCLC [31]. Laudanski et al. demonstrated that BCL2 protein is expressed in 46% (39 out of 84) of NSCLC patients undergoing surgical resection. In his study, 27 out of 46 cases represented squamous cell carcinoma, 7/25 were adenocarcinoma, and 5/13 were LCC [32]. In this study, 66.6% of the patients were characterized by downregulation of *BCL2* gene, while miR-15/16 and miR-34a were downregulated in 50%, 83.3% and 83.3% of the patients, respectively. Moreover, when the expression level of *BCL2* was compared with the expression levels of miR-15a/16 and miR-34a, the correlation coefficient between *BCL2* and miR-15 was negative, with $p = 0.0003$. Therefore, we can conclude that there is a negative correlation between miR-15 and *BCL2*, and *BCL2* is the target gene of miR-15, that is, wherever miR-15 is upregulated in the same sample, *BCL2* is downregulated (13 out of 30). Since BCL2 is an antiapoptotic protein and one of the miRNAs that target *BCL2* is miR-15, so if miR-15 can be overexpressed, the cell might be sensitive to apoptosis. On the other hand, Coutinho-Camillo et al. demonstrated that there is a weak negative correlation between miR-15a

and *BCL2*, as well as between miR-34a and *BCL2* transcript in oral squamous cancer [33]. It was demonstrated that by using miR-16 mimic and upregulation of *BCL2* protein in glioma cells, the sensitivity of the cells to apoptosis was increased [34]. miR-15/16 can be used as potential therapeutic targets in NSCLC through the inhibition of *BCL2* and the activation of apoptosis pathways. Furthermore, miR-21 might target *BCL2* as well. Xu et al. have shown that when miR-21 is downregulated in NSCLC cell lines, *BCL2* is decreased at both mRNA and protein levels [35]. *BCL2* is a target gene for many miRNAs that might have oncogenic or tumor suppressor function. Based on the miRNA expression profile in different kinds of cancers, the role of *BCL2* protein and its impact on apoptosis may vary depending on the type of cancers.

The roles of miR-126 and miR-128 in inhibiting NSCLC progression have been frequently reported; however, the distinct mechanism of their action is poorly understood [36, 37]. Previously, we found that the expression levels of miR-126 and miR-128 are significantly decreased in NSCLC samples when compared to the adjacent normal tissue samples. In this study, we examined the possible molecular association of miR-126 and miR-128 with *IRS1* gene in NSCLC patients. *IRS1*, one of the most important targets of miR-126 and miR-128, acts as an essential signaling intermediate downstream of several cell surface receptors, mediating the regulation of various cellular processes such as cell growth and proliferation induced by insulin, growth hormone, interleukin-4 (IL4), and other cytokines [38]. Phosphorylation of *IRS1* by insulin receptor enables it to regulate several signaling pathways, including PI3K and MAPK pathways [39, 40]. Furthermore, *IRS1* can induce cell cycle progression and tumorigenesis through activating the Wnt/ β -catenin signaling pathway [41]. Dearth et al. have recently reported that *IRS1* functions as an oncogene protein involved in mammary tumorigenesis and metastasis [42]. Studies have noted that overexpression of *IRS1* or *IRS4* is highly associated with tumor growth and proliferation, while overexpression of *IRS2* is most often correlated with cancer invasion and metastasis [43]. Zhang et al. reported that miR-126 inhibits cell cycle progression from G1/G0 to S phase through targeting *IRS1* [44]. Recently, it has been demonstrated that miR-126 can inhibit the migration and invasion of endometrial cancer cells through directly targeting *IRS1* [45]. Additionally, miR-128 is highly capable of suppressing cell growth and metastasis by regulating *IRS1* expression [46]. In agreement with previous studies, we found a negative correlation between miR-126 and *IRS1* ($p < 0.05$). This suggests that the tumor suppressor activity of these miRNAs is obviously mediated through targeting *IRS1*.

An increase in the expression of oncogenic miR-21 was demonstrated in our previous study [12]. One of the target genes of miR-21 was shown to be a tumor suppressor *PTEN* [47]. The *PTEN* protein functions as a lipid

phosphatase, which negatively regulates the antiapoptotic (AKT/*BCL2*) and proliferation pathways (PI3K/AKT) [48]. MiR-21 is one of the most frequently upregulated miRNAs in solid carcinomas and considered to be a typical onco-miR acting by inhibiting the expression of phosphatases, which limit the activity of PI3K/AKT signaling pathway [49]. In our study, *PTEN* was downregulated in 22 samples (74%) of tumor tissues compared with adjacent normal tissues and a significant negative correlation was found between miR-21 and its target gene *PTEN* ($p = 0.01$). Recent studies have shown that the reduction and loss of *PTEN* expression is observed in 70% of NSCLCs, and loss of *PTEN* might lead to cell proliferation and survival [50].

This study demonstrates significant changes in the expression level of *AKT3* in NSCLC tumor tissues compared to adjacent normal tissues at mRNA level. In 86.6% of cases, *AKT3* was downregulated significantly in tumor tissues versus adjacent normal tissues, and there was no significant correlation between miR-15/16 and *AKT3* at mRNA level. Also, we have found the downregulation of miR-15a/16 (50%/83.3%) in tumor samples. Expression of miR-15/16 inhibits cell proliferation and promotes apoptosis of cancer cells, by targeting several oncogenes, including *BCL2* and *AKT3* [51]. Studies have indicated a significant association of miR-15/16 expression with tumor progression in colorectal cancer [52]. In our previous study, we found the remarkable correlation of miR-15a/16 downregulation with NSCLC stages I/II, compared to normal tissues [12].

HIF1A is a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) transcription factors that bind to the hypoxia-response elements (HRE) within the target gene promoters to induce adaptive responses under hypoxia conditions [53]. This gene activates the transcription of over 150 genes, whose products are actively involved in several physiological processes such as cell proliferation, apoptosis, autophagy, erythropoiesis, immune responses, cell metabolism, and angiogenesis [54]. It has been frequently reported that overexpression of *HIF1A* is associated with poor prognosis in patients with NSCLC [19, 55]. Although, miR-210 is known as a hypoxia-induced miRNA involved in cancer progression [56], downregulation of *HIF1A* through a negative feedback regulation by miR-210 has been established previously [57]. In line with this, we found a negative correlation ($p = 0.07$) between miR-210 and *HIF1A* expression. On the other hand, there was a direct association between miR-210 and *HIF1A* in 37% of samples, which means that *HIF1A* might have a negative feedback loop with miR-210.

CONCLUSION

In conclusion, we found significant negative correlations between *BCL2* and miR-15a, between *IRS1* and miR-126, and between *PTEN* and miR-21 among

studied miRNAs and their target genes. These target genes and miRNAs might be potential therapeutic agents in the future and it is required to further investigate the function of candidate genes both in vitro and in vivo.

AUTHOR CONTRIBUTIONS

Elham Tafhiri conceived the study. Mahdi Mohammadi developed the theoretical framework and performed the experiments. Kiana Taheri and Shamim Fooladgar aided in the analysis. Saghar Omidvar Masoumi was responsible for analyzing the qRT-PCR results. Elham Tafhiri supervised the project. All authors discussed the results and contributed to the final manuscript.

ACKNOWLEDGEMENTS

This project was supported by the grants from the Pasteur Institute of Iran (Grant No. #592) and the Tracheal Diseases Research Center, NRITLD of Shahid Beheshti University of Medical Sciences. The authors would also like to thank the patients for their cooperation.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

ETHICS STATEMENT

This article does not contain any studies with human participants performed by any of the authors. Also, this study does not contain any studies with animals performed by any of the authors.

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How to cite this article: Mohammadi M, Taheri K, Fooladgar S, Omidvar Masoumi S, Tafhiri E. *BCL2, IRS1, AKT3, PTEN, and HIF1A* expression levels in non-small cell lung cancer patients. *Malign Spect.* 2025;2(1):37-45. doi:10.1002/msp2.70002