Original Article

Expression of *miRNA-601* and *PD-L1* among Iranian Patients with Lung Cancer and Their Relationship with Smoking and *Mycoplasma* Infection

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Received: 06/July/2020, Accepted: 29/May/2021

Abstract

Objective: microRNAs (miRNAs) are highly conserved noncoding RNA molecules that mainly function to regulate gene expressions, and have a significant role in tumourigenesis. Programmed cell death-ligand 1 *(PD-L1*) is a major co-inhibitory checkpoint signal that controls T cell activities, maintains peripheral tolerance and is contribute to the development of cancer. The aim of this study is to examine *miRNA-601* and *PD-L1* gene expression in patients with non-small-cell lung cancer (NSCLC) and its relation with *Mycoplasma* infection.

Materials and Methods: In this case-control study, respiratory secretions and blood samples were collected from 80 healthy people and 80 NSCLC patients. The expression levels of *miRNA-601* and *PD-L1* were evaluated using real-time polymerase chain reaction (qRT-PCR). The presence of *Mycoplasma* species in respiratory secretions was detected by biochemical assays and PCR.

Results: There was no significant difference in the expression level of *miRNA-601* between control and patients with tumour stage I, but *miRNA-601* expression was significantly downregulated in patients with tumour stages II, III, and IV ($P<0.05$). A significant, negative relationship was found between *miRNA-601* expression and tumour stage ($P<0.001$). Overexpression of *PD-L1* was found in all of the disease stages. PCR results showed the presence of *Mycoplasma pneumoniae* (*M. pneumoniae*) in respiratory secretions from patients with stages III and IV NSCLC. We observed that 72% of patients with stages III and IV NSCLC had a positive smoking history and 65.3% were positive for *Mycoplasma*.

Conclusion: Serum miRNA-601 may act as a potential noninvasive biomarker for lung cancer and *Mycoplasma* infection prognosis.

Keywords: Lung Cancer, *Mycoplasma pneumoniae,* Smoking

Cell Journal(Yakhteh), Vol 23, No 7, December 2021, Pages: 723-729

Citation: **Ganjali M, Kheirkhah B, Amini K. Expression of miRNA-601 and PD-L1 among Iranian patients with lung cancer and their relationship with smoking and mycoplasma infection. Cell J. 2021; 23(7): 723-729. doi: 10.22074/cellj.2021.7704.**

Introduction

Worldwide, lung cancer is a common cause of mortality and a major cause of cancer-related deaths due to restricted therapeutic resources. Because of late diagnosis, restricted therapeutic resources, disease relapse and the development of drug resistance, lung cancer prognosis is not promising because of the lack of therapeutic success for its treatment. Lung cancer is divided into two histologic subgroups-small-cell lung cancer (SCLC) and non-SCLC (NSCLC). More than 80% of lung cancer cases are NSCLC, which is subdivided into adenocarcinoma, squamous cell carcinoma (SCC), and large-cell carcinoma types (1). Despite numerous studies, the prognosis of lung cancer is low, and less than 15% of patients survive more than five years after diagnosis (2). The high mortality rate is mostly due to the late presentation of this disease, tumour heterogeneity, restricted knowledge on lung cancer biology, and, more importantly, the development of treatment resistance. High sensitivity and specificity biomarkers are able to detect the disease in its early stages (1). Recently, microRNAs (miRNAs) have received much attention in metastasis-related investigations. It is believed that miRNAs play a key role in the pathogenesis, diagnosis, and prognosis of lung cancer. However, comprehensive studies are necessary to evaluate the impact of miRNA targeted therapy on cancer patients (3).

miRNAs are involved in suppressing mRNA translation or contributing to mRNA degradation. It has been reported that miRNAs are significantly involved in numerous biological processes (inflammation, cell growth, apoptosis, development, differentiation, endocrine homeostasis and cancer). A correlation between the expression patterns of miRNAs and clinicopathological parameters in cancer subtypes suggests the importance of miRNAs as potential biomarkers for detection of different cancer subtypes categorized by origin, histology, aggressiveness or chemosensitivity (4). Because of their significant involvement in many cancer types, miRNAs are among the most attractive targets for therapeutic interventions in cancer. The results of some studies have confirmed various biological functions for miRNA-601, whereas others have shown abnormal expression of miRNA-601 in various tumours. Moreover, this miRNA might play a

different regulatory role in the pathological processes of different tumours (5).

miR-601 represses nuclear factor-kappa B (NFκB) transcription factor-dependent reporter expression, which is considered a main factor of immune-oncogenesis pathway. A limited number of studies on miR-601 indicated that miR-601 could affect a variety of signalling pathways; it has been reported that miR-601 may be involved in cell fate determination (6).

Overexpression of programmed cell death-ligand 1 (*PD-L1*) in many cancers, including NSCLC tumour cells, has been reported. *PD-L1* overexpression appears to permit cancer cells to inhibit the immune response. Antibodies against PD-1/PD-L1 signalling are promising treatments for cancer, including melanomas and NSCLC (3). Because the relation between *PD-L1* expression and miRNA is unclear; therefore, examining the relationship between *PD-L1* expression and miRNA may help to discover a possible mechanism of cancer development.

Recent studies show that tobacco smoking is a main risk factor for lung cancer. The tobacco smoke-induced pulmonary cellular network is an exclusive environment that contributes to the progression of carcinogenesis along with lung inflammatory, structural, and stromal cells (7). Cigarette smoke, by affecting alveolar macrophages, can cause downregulation of macrophage receptor (*MARCO*) gene expression in macrophages and, in turn, attenuate the innate immune system (8).

In addition to smoking, *Mycoplasma* is another significant factor that may be associated with lung cancer. Recent evidences show a potential association between different species of *Mycoplasma* and human cancers (9). *Mycoplasma* are small, cell-free bacteria enclosed by a membrane (10). More than 100 *Mycoplasma* species have been identified in humans, but only a few are reported to produce diseases in humans, among which *Mycoplasma pneumonia* (*M. pneumonia)* is the best known and most studied.

There are few studies on *miRNA-601*, *PD-L1* and *Mycoplasma* and their relationship with lung cancer (11). Therefore, this study aimed to compare the level of miRNA-601 and *PD-L1* expression, as biomarkers, in the sera of patients with NSCLC and healthy individuals in order to diagnose the stages of NSCLC and determine its relationship with smoking and *Mycoplasma* infection in Iran.

Materials and Methods

Patients and specimens

This case-control study enrolled 80 patients with lung cancer and 80 healthy individuals who referred to multiple hospitals in Tehran (Iran) between 2019 and 2020. The Institutional Review Board and Ethical Committee of Zahedan University of Medical Sciences (IR.ZAUMS.REC.1398.298) approved this study. Written informed consents were signed by all patients. A questionnaire that contained the demographic data of all patients was also completed prior to the laboratory examinations. Clinical and pathological findings, and tumour grade of the patients were recorded. Healthy individuals came to the hospitals for check-up and had no abnormalities or pulmonary manifestations present during the physical examinations or in their laboratory results. They did not have any considerable history of cancer or previous medical diseases. Inclusion criteria for patients were: i. Known lung cancer and ii. Complete clinical and pathologic information. Patients with the following criteria were excluded from the study: i. Metastases, ii. Previous treatments such as chemotherapy or radiotherapy, and iii. History of other chronic diseases like diabetes mellitus, liver diseases, etc.

miRNA-601 and *PD-L1* expression analysis

Total RNA extraction

A total of 5 ml blood and respiratory secretions from each participant were decanted into test tubes. Total RNA was extracted from each sample using the RNX-Plus kit (SinaClon, RN7713C, Iran) according to the manufacturer's instructions. Qualification and quantification of RNA were assessed using a NanoDrop machine (Thermo Fisher Scientific, USA) and electrophoresis was performed on a 1% agarose gel.

Real-time polymerase chain reaction assay

RNA from the samples was extracted using a First Strand cDNA Synthesis kit (SinaClon Co., Iran) according to the manufacturer's instructions. Real-time PCR was performed for miRNA-601 and *PD-L1,* as the targets, and *β-actin*, as the reference gene. Table 1 lists the primers used for these genes.

Table 1: Primer sequences used for amplification of *miRNA-601,* programmed cell death-ligand 1 (*PD-L1*) and detection of *Mycoplasma pneumoniae*

Target	Primer sequences (5´-3´)	Reference
miRNA-601	F: GCTCGCTTCGGCAGCACATATAC	(12)
	R: GGTCCGAGGTATTCGCACTGGATA	
PD-L1	F: GTTCTGCGCAGCTTCCCG	(13)
	R: ACCGTGACAGTAAATGCGTTC	
β-actin	F: CGGCCAGGTCATCACCATT	(14)
	R: CACAGGACTCCATGCCCAG	
GSO	F: GGG AGCAAACAGGATTAGATA CCT	(12)
	R: TGCACCATCTGTCACTCTGTTAACCTC	
MGSO	F: AAGGACCTGCAAGGGTTCGT	(12)
	R: CTCTAGCCATTACCTGCTAA	

A total volume of the 20 µl PCR reaction, which included 4 µl distilled water, 10 µl master mix, 4 µl cDNA, and 1 µl primers was used. The amplification program was designed according to the appropriate annealing temperature: 1 cycle at 95°C for 60 seconds, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 40 seconds, and 72°C for 45 seconds. Expression of the target *miRNA-601* was normalized by miRNA REST software. No miRNA was used as the negative control and all tests were performed in triplicate.

Detection of Mycoplasma

Briefly, *Mycoplasma pneumoniae* DNA was extracted from patients' respiratory secretions by using a DNA Extraction kit (DNP, EX6071, SinaClon Co, Iran) and all steps were done according to the manufacturer's instructions. The extracted DNA was stored. Specific primers for GSO and MGSO were used to identify *Mycoplasma* and *M. pneumonia,* respectively (Table 1). The PCR reaction consisted of: 10.7 µl H_2O, 2 µl buffer, 1.5 µl $MgCl_2$, 1.5 µl dNTP, 1 µl forward primer, 1 µl reverse primer, 0.3 µl Taq DNA polymerase, and 2 µl DNA in a total volume of 20 µl. The first denaturation reactions were conducted at 94˚C for 5 minutes, followed by 30 cycles of denaturation at 94˚C for 30 seconds, primer annealing at 56˚C for 60 seconds, and extension at 72˚C for 60 seconds followed by a final extension step at 72˚C for 5 minutes. The expected size for the PCR product for GSO was 163 bp and for MGSO, it was 277 bp. The standard reference strain *M. pneumonia*, NCTC 29342, was used as a positive control strain and *Escherichia coli* (*E. coli*) was used as the negative control strain (15).

Statistical analysis

Statistical analyses were performed using SPSS version 22 (IBM, Armonk, NY, USA) and REST version 2009. Prism version 8.42 (GraphPad Software, Inc., San Diego, CA) was used to create the graphs. $P<0.05$ were considered to be statistically significant. Values are shown as the mean ± standard deviation (SD).

Results

Patients' demographic data

Table 2 lists the basic demographic characteristics of all the patients. There were no significant differences in the distribution of sex and mean age between the control and patient groups. In total, 80 patients (54 males and 26 females) and 80 healthy individuals (48 males and 32 females) enrolled in the study. The mean age in the patient group was 66.57 ± 11.06 years and the control group mean age was 61.96 ± 9.82 years. More than half of the patients (67.5%) were active smokers. About 35% of patients had a family history of gastric cancer. There were 28.8% of patients diagnosed with stage I cancer, 26.3% with stage II, 23.8% with stage III and 21.3% with stage IV (Table 2).

Table 2: Clinicopathological characteristics of patients related to *PD-L1* expression, *miR-601* expression and *M. pneumonia*

Characteristic	*PD-L1* expression (n=80)		*M. pneumoniae* (n=80)		*miR-601* expression (n=80)		Total (n=80)
	Positive (n=66)	Negative	Positive (n=5)	Negative	Positive (n=71)	Negative	
Age (Y)							
<50	10 (12.5)	70 (87.5)	1 (1.25)	79 (98.7)	14 (17.5)	66 (82.5)	66.57 ± 11.06
50-60	22 (27.5)	58 (52.5)	2 (2.5)	78 (97.5)	28 (35)	52 (65)	
>60	34 (42.5)	46 (57.5)	2 (2.5)	78 (97.5)	29 (36.2)	51 (63.8)	
Sex							
Female	34 (42.5)	46 (57.5)	2 (2.5)	78 (97.5)	37 (46.25)	43 (57)	26 (32.5)
Male	32 (40)	48 (60)	3 (3.7)	77 (96.25)	34 (42.5)	46 (57.5)	54 (67.5)
Smoking status							
No	47 (58.7)	33 (41.3)	3 (3.7)	77 (96.25)	40 (50)	40 (50)	54 (67.5)
Yes	19 (23.7)	61 (76.3)	2 (2.5)	78 (97.5)	31 (38.7)	49 (61.3)	26 (32.5)
Stage							
I	12 (15)	68 (85)	0 (0)	80 (100)	13 (16.25)	67 (83.75)	23 (28.8)
II	13 (16.25)	67 (83.75)	0 (0)	80 (100)	14 (17.5)	68 (82.5)	21 (26.3)
III	18 (22.5)	62 (77.5)	5 (45)	75 (55)	20 (25)	60 (75)	19 (23.8)
IV	23 (28.75)	57 (71.25)	3 (3.7)	77 (96.25)	24 (30)	56 (70)	17 (21.3)

Datae presented as n (%).

microRNA expression levels in serum

The expression pattern of *miRNA-601* in patients with different tumour stages is shown in Figure 1. Overall, *miRNA-601* expression was lower in cancer cells compared to normal tissues. A significant trend was found for the decreased expression of *miRNA-601* from stage I to stage IV. *miRNA-601* expression in patients with tumour stages II, III and IV was significantly lower compared to the control group (P<0.01). There was no significant difference in the expression levels of serum *miRNA-601* between patients with tumour stage I and the control group. The expression pattern of *miRNA-601* in patients with tumour stages I and II was significantly higher compared to those with tumour stages III (P<0.01) and IV (P<0.001, Fig.1).

Figure 1 shows the expression pattern of *PD-L1* in patients with different tumour stages. *PD-L1* expression in patients was significantly higher compared to the control group. A significant relationship was found between tumour stage and expression levels of *PD-L1*. There was a significant trend observed in the increased expression of *PD-L1* from tumour stage I to stage IV. Patients with tumour stage IV had significantly higher levels of *PD-L1* expression compared to tumour stages I, II and III (Fig.1).

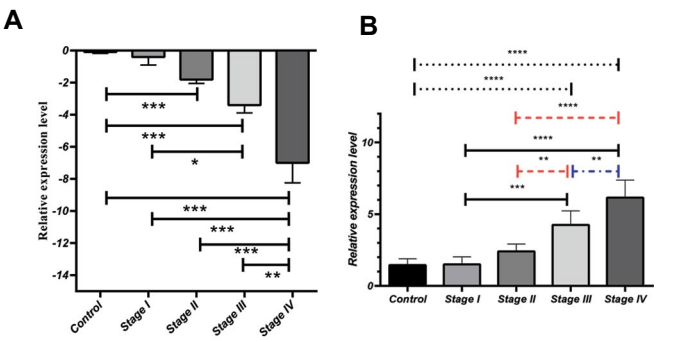

Fig.1: Gene expression analysis of *miRNA-601* and *PDL-1* at different stages of non-small-cell lung cancer (NSCLC) according to real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR). **A.** Gene expression analysis of *miRNA-601* at different stages of non-small-cell lung cancer (NSCLC) according to RT-qPCR. Relative expression is normalized with the *β-actin* gene. Error bars indicate the standard deviation (SD) of three independent replicates. **B.** Gene expression analysis of *PD-L1* at different stages of NSCLC by RT-qPCR. Relative expression is normalized with the *β-actin* gene. Error bars indicate SD of three independent replicates. *; P≤0.05, **; P≤0.01, ***; P≤0.001, ****; P≤0.0001 using one-way ANOVA and Tukey's test.

Detection of Mycoplasma in specimens and its relationship with smoking

Of the 80 cultured samples, 26 (32.5%) were grown in broth medium with evidence of a colour change from red to yellow. In the agar culture, 17 colonies were isolated and *M. pneumonia* was confirmed by PCR in 17 (21.25%) samples (Fig.2).

According to the PCR results, there was no *Mycoplasma* detected in NSCLC patients with tumour stages I and II. However, 61.3% of these patients were smokers. In contrast, *Mycoplasma* was identified in the majority of patients with stages III and IV disease. In this step, 14 individuals were identified as smokers out of 17 patients with stage III NSCLC. From these, 9 (64.3%) were positive for *Mycoplasma* and *M. pneumoniae* was detected in only 5 patients (45%). *Mycoplasma* was also found in patients with stage IV disease. According to the results, out of 20 smokers with stage IV cancer, 8 were positive for *Mycoplasma* and 3 patients had *M. pneumoniae*.

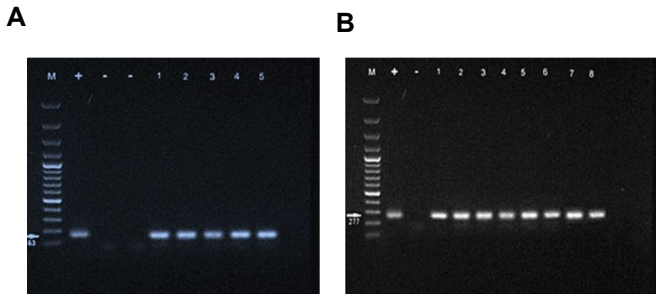

Fig.2: Agarose gel electrophoresis of polymerase chain reactin (PCR) products for detection of *Mycoplasma* and *Mycoplasma Pneumoniae*. **A.** Agarose gel electrophoresis of PCR products for detection of *Mycoplasma*. **B.** Agarose gel electrophoresis of PCR products for detection of *Mycoplasma pneumoniae* (*M. pneumonia*). Lane M; 100 bp DNA ladder; lane +; The positive control, lane – ; The negative control, and lane 1, 2, 3, 4, 5, 6, 7 and 8; The samples.

Correlation of *miR-601* with *PD-L1*, *NFκB* expression and *Mycoplasma*

Figure 3 shows the correlation of *miR-601* with *PD-L1* expression using Pearson's correlation. There was no significant difference between *miR-601* with *PD-L1* expression. A correlation between *miR-601* and *Mycoplasma* was assessed by Pearson's correlation. An inverse correlation was observed between *NFκB* and *miR-601*, but this correlation was not significant. There was no significant correlation between *miR-601* and *Mycoplasma*. The correlation of *miR-601*, *PD-L1* and *Mycoplasma* with age, gender, cancer stage and smoking is shown in Table 2. Kaplan-Meier survival analysis was used to predict patient prognosis with *miR-601*, *PD-L1* and *Mycoplasma* (Fig.4).

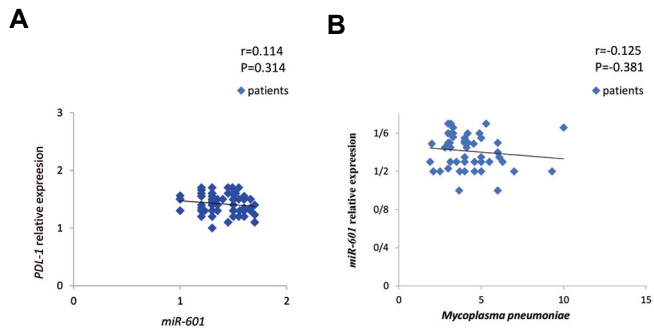

Fig.3: Correlation of *miR-601* expression with *PD-L1*, *Mycoplasma pneumoniae* and *NFκB*. **A.** Correlation of *PD-L1* with *miR-601* expression. **B.** Correlation of *miR-601* with *NFκB*. **C.** Correlation of *miR-601* with *M. pneumonia*.

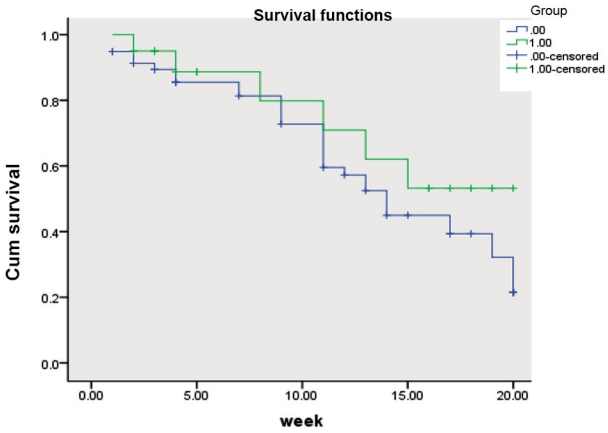

Fig.4: The kaplan-meier survival curve of the patients with lung cancer.

Correlation of nuclear factor-kappa B with *miR-601*

An inverse correlation was observed between *NFκB* and *miR-601*, but this correlation was not significant (Fig.5).

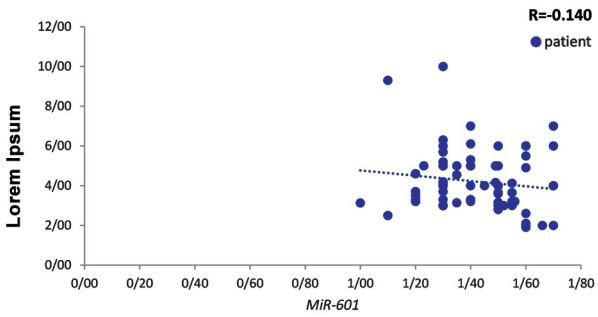

Fig.5: Correlation of *miR-601* with *NFκB* expression.

Discussion

miRNAs are small noncoding RNAs that are negative regulators of gene expression. They bind to the 3' untranslated region of a transcript and inhibit translation. Numerous clinical studies have shown that expression of miRNAs is significantly altered during various types of cancers in humans (16, 17). There is increasing evidence that a group of miRNAs play a role in the development and progression of lung cancer. It has been proposed that deregulation of miRNAs may contribute to altered gene expressions in NSCLC. For example, an inverse relation between *KRT6A* expression and miR-375 levels has been demonstrated in patients with SCC, one of the most common subtypes of NSCLC. In these patients, *KRT6A* expression increased significantly compared to patients with adenocarcinoma. KRT6A belongs to the keratin protein family and is responsible for squamous epithelium epidermalisation (18). Several researchers have attempted to profile serum miRNA expression in order to predict NSCLC. Accordingly, four miRNAs (miR-486, miR-30d, miR-1, miR-499) have been identified using genome analysis as noninvasive serum biomarkers that predict survival in patients with NSCLC. The serum miRNAs are resistant to RNAase hydrolysis; therefore, determination of their fingerprints in patients is a clinically applicable method for lung cancer prognosis (19).

Bioinformatics approaches have predicted that mammalian miRNAs can play regulatory roles for approximately 30% of all protein-coding genes (12). Post-transcriptional regulation of various genes by miRNA may contribute to the emergence of different histological phenotypes in NSCLC, and this relation between miRNA and mRNA may be used for therapeutic purposes miRNAs affect responses to chemotherapy, radiotherapy and targeted therapy (20). The results of several studies have shown that miRNAs have an important role in lung cancer. In the current study, we choose miRNA-601 because of the lack of information for hsa-miR-601 (6).

It has been found that has-miRNA-601 negatively regulates translational initiation. Introduction of has-miRNA-601 to cells causes upregulation of the actin cytoskeleton and downregulation of the Fas-induced apoptosis pathway. Previous studies have explored the expression patterns, clinical value and functional role of miR-601 in different cancers, including gastric cancer, colorectal cancer and hepatocellular carcinoma (6). In this research, we evaluated miRNA-601 expression in sera of patients with lung cancer. Our findings revealed that miRNA-601 expression significantly decreased in cancer cells from patients with stages II, III, and IV cancer, which suggested its potential role in negative regulation of apoptosis pathways.

Consistent with the present results, Ohdaira et al. (6) reported that miR-601 inhibited proliferation, migration and invasion of prostate cancer stem cells.

The role of miR-601 in has also been studied in other cancers. For example, Song et al. (21) observed a significant reduction in miR-601 expression in liver cancer cells, which was associated with tumour spread and metastasis.

In similar studies, the expression of miR-601 in liver cancer cells and in colorectal cancer cells was examined (22). Thus, miR-601 can be introduced as a biomarker for cancer diagnosis.

Interestingly, in contrast to the mentioned studies, Min et al. (23) reported that miR-601 was associated with the spread of gastric cancer and a reduced diagnosis. The ability of miRNAs as diagnostic tools for cancer is undeniable; nevertheless, it is necessary to undertake comprehensive studies to determine guidelines.

In addition to their role in cancer, miRNAs play a regulatory role in controlling the immune system. Therefore, miRNAs can play an important role in preventing cancer through the immune system. NFκB plays a key role in the innate and acquired immune responses in humans and the spread of cancer. According

to our data, the decrease in miR-601 expression might be related to its role in the negative control of apoptosis pathways and suppression of NFκB signalling, which was similar to its role in lung cancer (24). The role of this gene in liver, prostate, breast, colorectal and gastric cancers has also been evaluated and confirmed (6).

With the discovery of the immune checkpoint protein, there has been a deep interest in producing antibodies that block PD-1 and PD-L1 for treatment of certain types of cancers. The PD-1 signalling pathway negatively regulates T cell-mediated immune responses and acts as a mechanism for tumours to evade an antigen-specific T cell immunologic response. It plays a role in promoting cancer development and progression by elevating tumour cell survival. With this background, PD-1 signalling represents a valuable diagnostic and therapeutic target for novel and effective cancer immunotherapies. This new immunotherapy, applied in the treatment of NSCLC, uses monoclonal antibodies directed against *PD-L1* to inhibit its interaction with the PD-1 receptor (12). In our study, we found an overexpression of *PD-L1* in all stages of NSCLC. The *PD-L1* gene expression in stage I was 1.5 fold in comparison to the normal samples and the highest level of *PD-L1* gene expression was found in patients with tumour stage IV. Compatible with our results, overexpression of *PD-L1* by NSCLC cells was reported in several large studies (25).

In addition, a relation between the presence of *M. pneumoniae* and lung cancer has been reported. This study, similar to our study, showed that the frequency of *M. pneumoniae* in patients with lung cancer was higher than the control group. *M. pneumoniae* may possibly play a role in lung cancer progression (26).

The relation between *Mycoplasma* and lung cancer development was examined in the present study. We found that patients with tumour stages III and IV were positive for *Mycoplasma*. However, the bacterium was not detected among patients with stages I and II disease, which indicated the presence of this infection during the late stages of lung cancer. In other words, it could be hypothesized that patients with advanced lung cancer are more susceptible to infection by *Mycoplasma* than those with early stages of the disease. Apparently, the presence of an association between specific infections and cancer remarkably affects both prevention and diagnosis. The results of clinical studies have proven the contribution of *Mycoplasma* in oncogenic transformation (27). Previous studies demonstrated that *Mycoplasma* is one of the strong inducers of bone morphogenetic protein (BMP), which is highly elevated in lung tumours. Pro-oncogenic pathways activated in the presence of BMP2 led to lung tumour development in mice (28). Our findings supported the results of previous studies that implied an association between lung carcinoma and pulmonary infections. Patients with late stages of lung cancer and more than 70 years of age are at increase risk for pulmonary infections (29, 30). As mentioned, miRNA-601 can potentially control the immune response via regulation of NFκB. Low miRNA-601 expression during stages III and IV lung cancer appears to impact the immune response and lead to bacterial colonization, infection, and acute inflammation. However, this is not observed during early stages of the disease. According to the previous studies, NFκB is strongly activated in prostate, breast, and lung cancers. Activation of NFκB has been reported in both SCLC and NSCLC (24, 31).

Smoking is an important agent of lung cancer (7) and these patients usually present with a long history of smoking. We observed that 75% of stages I and II lung cancer patients, 74% of patients with stage III disease, and 71% of patients with stage IV disease were cigarette smokers, which is a high incidence for smoking and cancer. Nicotine and its derivatives have a regulatory role on proliferation and apoptosis of bronchial epithelial cells because they bind to nicotinic acetylcholine receptors (nAChR) and activate the Akt pathway (32). Research has shown that the methylation profile of some important genes, which are frequently methylated in NSCLC, is different in smokers (33). It is well-established that smokers are at higher risk of bacterial infections than non-smokers. Our results showed that 24% of smokers were positive for *Mycoplasma* and 23.5% of these had *M. pneumoniae*. Overall, smokers harbour fewer normal microflora in their nasopharyngeal tract, which leads to colonization of pathogenic bacteria. According to previous studies, tobacco can induce physiological alterations in humans, increase bacterial virulence, and weaken the immune response (34). *M. pneumoniae* plays a role in chronic obstructive pulmonary disease (COPD). On the other hand, smoking strongly promotes COPD by affecting the function of alveolar macrophages, which disrupts the first line of defense against pathogenic microorganisms (8).

The link between smoking and lung cancer has been clearly shown in previous studies. Ozlü et al. (35) reported that 90% of patients with lung cancer had a history of smoking. In another study, Capewell and colleagues found that only 2% of patients with lung cancer were non-smokers (36).

Conclusion

Our results indicated that serum miRNA-601 expression in lung cancer was significantly decreased during the late stages of this disease. miRNA could be a potential noninvasive tool for prediction of lung cancer before disease progression. miRNA expression profiles are extensively used for lung cancer therapy. In this technique, the miRNA is replaced by cancer cells via transfection of the target miRNA gene with a vector; subsequently, miRNA expression can be changed to natural levels in cancer cells. Finally, the expression levels of the target genes can be regulated naturally. The observed overexpression of the *PD-L1* gene in this study indicated that *PD-L1* could be a potential biomarker for anti-PD-1/PD-L1 therapy in smoking-related lung cancer. Generally, the results of the present study can be applied in the context of lung cancer markers for

screening and diagnostic testing procedures for early detection of cancer. More extensive research should be carried out with larger sample sizes. In lung cancer, other complementary biomarkers would be a useful approach to achieve this aim and bring higher precision to cancer screening. In addition to its role in lung cancer prognosis, miRNA-601 can be used as a biomarker for post-bacterial infections that occur during the late stages of lung cancer. Based on the results obtained in our study, we hypothesize that late stages of lung cancer may promote *Mycoplasma* infections in these patients and particularly in those who have a history of smoking.

Acknowledgements

The authors are thankful to the director and staff of the Pasargad Microbiology Research Group for their professional collaboration. There is no financial support and conflict of interest in this study.

Authors' Contributions

M.G.; Contributed to the experimental work, data and statistical analysis, data interpretation, and wrote the manuscript. B.Kh.; Contributed to the experimental work, sample collection, formulation of the main idea, and edited the manuscript. K.A.; Participated in all of the experimental work, and introduced several valid articles and references. All authors read and approved the final manuscript.

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