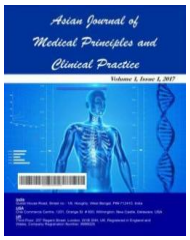

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Study of *PD-L2* Gene Expression in Patients with NSCLC-type Subclasses of Lung Cancer Using Real-Time PCR

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Lung cancer is one of the biggest health problems in the world. Non-small cell lung cancers (NSCLCs), which account for approximately 85% of lung cancer cases, are the leading cause of deaths due to cancer worldwide. The type 1planned death molecule (PD-I, CD279) and its *PD-L1* ligands (B7-H1, CD274) and PD-L2 (B7-DC, CD273) are the key molecules inhibiting the immune regulation. The aim of this study was to investigate the expression of *PD-L2* gene and the evaluation of cancerous stages of NSCLC and its subclasses in lung cancer patients in Tehran hospitals using Real-Time PCR.

Materials and Methods: A total of 35 clinical samples were collected from patients with NSCLC-derived lung cancer from three hospitals in Tehran (Khatam, Athiyah and Masih Hospitals). Of the 35 samples obtained in 2017, 20% of them were women and 80% were men. The age range of patients varied from 37 to 80 years. The disease grade gradients among the patients in this investigation varied and 22 different grades were observed. To investigate the *PDL-2* gene

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expression, after extraction of total RNA and cDNA synthesis using Real-Time PCR, the expression of the gene was investigated.
Results: 74% of adenocarcinoma cases were in T-categories of lung cancer and 25% of patients were in grade IIIa. Patients with T3 stage had four samples, 2 of which were adenocarcinoma and other 2 samples being SCC and their age ranged from 55 to 62 years old. Three patients were in the T1 category, of which 100% had adenocarcinoma. The results showed that expression of *PD-L2* gene was increased 5.45 fold among patients with NSCLC-type lung cancer compared to those with normal condition.
Conclusion: *PD-L2* expression detection methods, including the detection of mRNA expression by using Real-Time PCR and the use of multiple systems for detecting *PD-L2* expression with cell type and in association with other immune markers, can make a deeper understanding of *PD-L2* as a biomarker and help to diagnose lung tumors of the NSCLC type. In our studies, it was found that expression of *PD-L2* gene in cancer patients is higher than normal conditions.

Keywords: Lung cancer; NSCLC; PD-L2; Real-Time PCR.

1. INTRODUCTION

Lung cancer is one of the biggest global health problems accounting for more than six deaths from cancer. Lung cancer is the cause of 12.8% of cases of cancer and 17.8% of deaths due to cancer worldwide [1]. The two main types of lung cancer, including small cell lung cancers (SCLC, formerly called "barley cell carcinoma"), invasive tumors often associated with general symptoms and distant metastases, and non-small cell lung cancers (NSCLCs), which accounts for approximately 85% of lung cancers as the leading cause of deaths from cancer worldwide [1,2]. Among NSCLCs, adenocarcinoma (ADC) and squamous cell carcinoma (SqCC) are two major histological subgroups which have distinct epidemiological, biological and genetic characteristics [3]. SqCC is often observed in men in the lung airway and is related to catarrhal etiology [4]. Adenocarcinoma is the most common type that accounts for 50% of cases and is the most common type of cancer in non-smokers. The third type of NSCLC cancers, large cell carcinoma, sometimes called undesirable carcinoma, is a common type of NSCLC that forms 10 to 15 percent of the lung cancer cases. This kind of cancer has a tendency to spread to lymph nodes and distant places [5].

Stages of Lung Cancer Non-small Cells:

NSCLC: Cancer cells are found in the sputum, but the tumor is not found in imaging or bronchoscope tests in the lungs, or the tumor is very small to check.

Phase 0: Cancer at this stage is also known as carcinoma in the area. The cancer is small and does not spread to the lung tissue or outside the lungs.

NSCLC Stage I: Cancer may be present in the lung tissue, but lymph nodes are not affected.

NSCLC Stage II: Cancer may spread to adjacent lymph nodes or to the wall of the chest.

NSCLC Stage III: Cancer continues to extend from lungs to lymph nodes or adjacent structures and organs, such as the heart, trachea, and esophagus.

NSCLC Stage IV: Cancer metastasis throughout the body and may already affect the liver, bones or brain.

The stages of NSCLC are described using the TNM system standing for T or a tumor that describes the size of the tumor, N or the lymph node shows whether or not there is cancer in the lymph nodes, and the third stage being M or metastasis, which indicates that cancer has spread to other parts of the body, such as the liver, bone, or brain [6,7].

The *PD-L1* and *PD-L2* molecules: The planned type 1 death molecule (PD-I, CD279) and its ligands *PD-L1* (B7-H1, CD274) and *PD-L2* (B7-DC, CD273) are the key molecules inhibiting the immune regulation. This route provides specific goals for cancer immunotherapy. There is considerable evidence that *PD-L2* inhibits immunity by binding to the PD-1 receptor [8]. However, numerous studies have shown that *PD-L2* can act as a stimulant for T-cell proliferation and cytokine production, even in PD-1 T-cell defects or with *PD-L2* mutations not connected to PD-1 [9-11]. These findings

indicate that the *PD-L2* may act via a receptor other than that for PD-1. Most studies using mAb inhibitors highlight that it plays an important role in inhibiting PD-L1 immune responses. However, *PD-L2* plays an important role in responses such as increased airway sensitivity, experimental allergic conjunctiva, and nematode infections [12].

The aim of this study was to investigate the expression of *PD-L2* gene and the evaluation of cancerous stages of NSCLC and its subclasses in patients with lung cancer in Tehran hospitals using Real-Time PCR.

2. MATERIALS AND METHODS

2.1 Collection of Clinical Samples

A total of 35 clinical samples from patients with NSCLC lung cancer were collected from three hospitals in Tehran (Khatam Hospital, Attih Hospital, and Christian Hospital). 71.4% of the patients were from Hospital of Masih, 22.8% of the patients were from Atiyah Hospital, and 5.7% of them were from Khatam Hospital. Of the 35 samples collected in 2017, 20% of the patients were women and 80% of the patients were men. The range of patients varied from 37 to 80 years.

2.2 Clinical Information of Patients

The disease grade gradients in the patients in this study were varied and 22 different grades were observed. Grades obtained include IB, IIB, III, IIA, IIIa, PT4N0MX, T1bN) MX, T1aN0MX, T1N1MX, T2, T2BN1, T2BN0MX, T2N0, T2N1, T2aN2MX, T3N0MX, T2aN0, T2AN2MX, T2aN0M0B1, T3N0MX-IIB, T3N1MX, and T3N2MX. Six patients had an unknown stage of the disease.

All subtypes of Adenocarcinoma, Adenos-quamous, and SCC were observed in the subtypes of the disease. Sixteen subtypes of adenocarcinoma, 1 adenosquamous subtype, and 12 subtypes were also identified as SCC. The only sub-type Adenosquamous belonged to the T2BN0MX Grade which was detected in the Masih Hospital.

2.3 Real-Time PCR

2.3.1 Extraction of RNA from tumor tissues

First, we separated 50 to 100 milligrams of tissue cut with a scalpel and placed it on a plate. Then we scraped and homogenized the slice using a scalpel. Then the tissue was transferred to the RNase, DNase free Cryotubes, and 1000µl trizole was added and the vertex was done lysed and no particles are observed in solution and a homogeneous solution was obtained. Then, 200 µl of chloroform was added to the microtubes containing the trizole to separate the phases and then inverted for several times. In the next step, the sample was centrifuged for 10 minutes at 13,000 rpm at 4°C.

After centrifugation, three phases were formed. The pink trizole was below, a white precipitate layer containing blood proteins and supernatant phase containing RNA. We slowly and accurately extracted the supernatant containing RNA without contacting the underlying layer with the head of the RNase, DNase free sampler taking 400µl of it, and transferred to a new RNase, DNase free 1.5 ml tube, and then the same amount (400 µl) of isopropanol was added to tube and incubated for 10 minutes at room temperature.

In the next step, centrifugation was performed for 5 minutes at 13000 rpm at 4°C for this purpose. The supernatant was then completely discharged by RNase, DNase free, to maintain only RNA pellet. RNA was then added to 1000 µl of 75% ethanol for washing. The centrifuges were then centrifuged again for 5 minutes at about 10,000 rpm at 4°C. After centrifugation, alcohol was completely removed.

In the next step, the microtubule was placed on ice for 10 minutes to completely dry the RNA sediment. Based on the volume of the obtained coil, the amount of DEPC water was added at the end of 50 µl and pipetting was done to completely dissolve the RNA. Finally, for OD, 3 µl of the extracted RNA was taken to evaluate its quality using NanoDrop apparatus and the total RNA was transferred immediately to the -70°C.

2.3.2 Synthesis of cDNA

After extraction of RNA samples, the quantity and quality of RNA were evaluated using spectrophotometry and agarose gel. For this purpose, the enzyme Primerscript (Takara) and specific primers and oligomer dioxymidine (Oligo-dt) were used to synthesize cDNA using thermal conditions and master mix prepared.

2.3.3 Real-Time PCR conditions

The reaction time of the PCR was performed using the Applied Biosystems Step One Plus

Table 1. Primers used the expression of the *PD-L2* gene

Primer TM	Sequences 5′-3′	Primer name
61.1	5-AGT GAT TTC CAA AAG CAG AGG TG-3	*PDL2-F-meh*
60.9	5-ATT ATG ATT TGA GTT TGT GCG AAG C-3	*PDL2-R-meh*

Table 2. Materials used in the reaction of the Real-Time PCR for the *PD-L2* gene

Materials	Volume (µL) in the reaction
RealQ Plus 2x Master Mix Green - Amplicon	7.5
Primer Mix Forward and Reverse (3µM each)	1
cDNA (10 ng/µL)	1
Distilled Deionized Water	5.5
Final volume	15

Table 3. Temperature and time used for Real-Time PCR for the *PD-L2* gene

-	Temperature°C	Time	Repeat
Hot Start Activation	95	15 min	-
Denaturation	95	15 S	40
Annealing and Extension	60	1 min	-

Real-Time PCR Systems. The cDNAs made from specimens with genetic primers and the target designed for RT-PCR was amplified using qualitative PCR. The GADPH enzyme-encoding gene was used as a housekeeping and internal control. Primers and materials used in regard to performing the Real-Time PCR temperature conditions are shown in Tables 1, 2 and 3.

2.4 Ethical Process

The study was approved by the Institutional Review Board of Central Tehran Branch, Islamic Azad University, Tehran, Iran and performed in accordance with the principles of the Declaration of Helsinki and each subject signed an informed consent before participating to the study. All procedures were approved by the relevant ethics committees, and written informed consent was obtained from all participants.

3. RESULTS

3.1 Patient Specimens and Disease Gradients

The frequency of 22 grades of the disease observed in patients in this study was seen as shown in Fig. 1. The highest grade as IIIa grade included 6 patients (17.1%).

An interesting point was that 74% of adenocarcinoma cases were in T-categories of lung cancer and 25% of patients were in grade IIIa.

Patients with T3 grade, 4 samples were obtained, 2 of which were adenocarcinoma and 2 were SCC and their age ranged from 55 to 62 years old. Three patients were in the T1 category, and all of them had adenocarcinoma. Patients in the IIA and IIB categories belonged to the SCC subclass, and all three patients were from Atiyah Hospital.

Only one IB case was seen in the patients, which was also classified under the SCC subclass. The frequency of different grades in patients studied is shown in Fig. 1.

Independent t-test showed that the average age of the patients with a subtype of adenoma was 56.7 years and the SCT subtype was 60.7 years, and this difference was not statistically significant between the two groups (p_{value} = 0.202).

Based on the volume of the obtained coil, the amount of DEPC water was added at the end of 30-50µl and the RNA was completely dissolved by pipetting.

4. DISCUSSION

In this study, we focused on one of the PD-1, *PD-L2* ligands to determine whether or not this might be a new molecular target for lung cancers of the NSCLC type. The results showed that

there was a correlation between expression of *PD-L2* mRNA and lung cancer using RT-PCR. Human cancers occur following many of genetic and epigenetic changes which produce new antigens potentially detectable by the immune system [13]. Tumors develop multiple-stage resistance systems, including local immunosuppression, induction of tolerance, and systemic dysfunction in signaling of T cells [14-16]. In addition, tumors use several paths to escape destruction by the immune system [17]. PD-1 is a key protease immune receptor expressed by activated T cells, which can prevent immunosuppression. PD-1 ligands include *PD-L1* (B7-H1) and *PD-L2* (B7-DC) expressed by tumor and stromal cells [18,19]. Therefore, *PD-L1* and *PD-L2* may act as a molecular target for tumor progression in various types of cancer. Under laboratory conditions, inhibition of PD-1 metabolism and *PD-L1* and *PD-L2* ligands may increase the response of T cells and increase the pre-clinical anti-tumor activity. Research on the role of anti-PD-1 and PD-L2 antibodies in tumors is under advancement [20,21].

Recent studies by Brahmer et al. [22] and Topalian et al. [23] reported the immune system and anti-PD1 or PD-L1 immunotherapy activity in tumors such as NSCLC. In NSCLC, 10% of patients exhibited an anti-PD-L1 antibody response [22], while 18% of NSCLC patients responded to the anti-PD1 antibody. Therefore, the expression of PD-L1 with the response has been reported in recent studies [23]. In our studies, it was also proved that the expression of this gene in our patients was increased.

In our survey, there was a correlation between *PD-L2* expression and tumor formation. *PD-L2* expressing tumor cells are likely to exhibit a high potential for progression of NSCLC conditions. Therefore, it can be concluded that *PD-L2* plays an important role in promoting tumorigenic conditions of NSCLC in patient populations.

PD-L2 is the second known receptor for the T cells from PD-1 [24]. It is a membrane protein encoded by the programmed death of ligand 1 ligand 2 (PDCD1LG2) and is structurally similar to the PD-L1. Although PD-L1 is the dominant ligand for PD-1, PD-L2 can compete with PD-L1 by 2-6 times more than PD-1 with PD-1 [25].

PD-L2 is expressed in relatively few numbers of cells and tissues but is activated in antigen presenting cells (APCs) such as monocytes, macrophages and dendritic cells [26]. However, the role of PD-L2 in inhibiting immunosuppression in the microscopic environment of the tumor and as a marker for clinical features has not yet been determined. Several research groups have recently revealed a possible correlation between expression of PD-L2 tumor and clinical outcome in retrospective cohort studies using IHC staining and various antibodies.

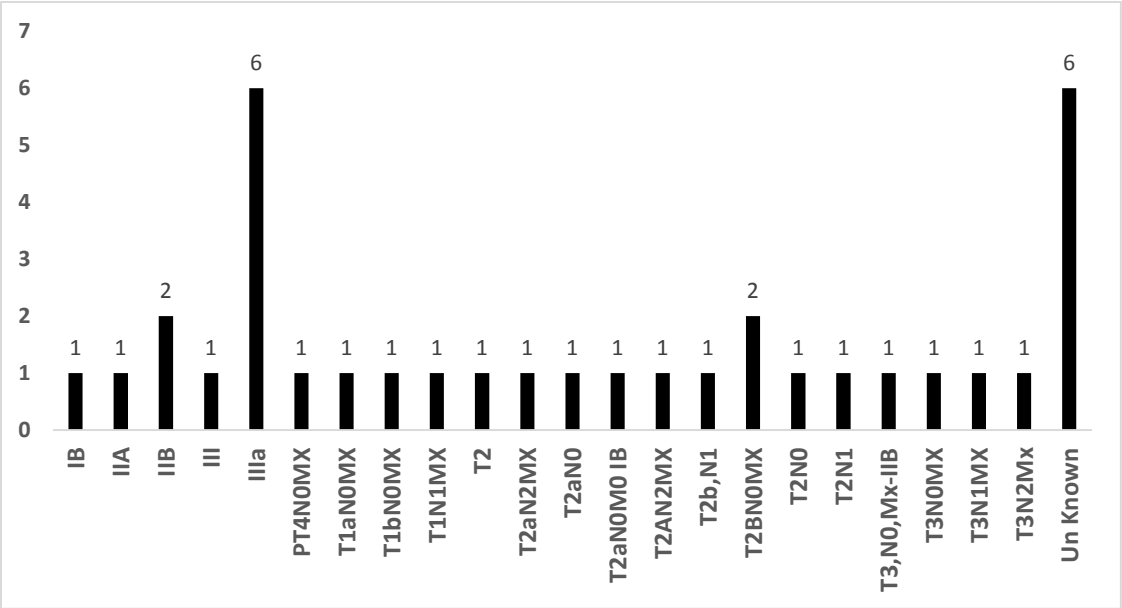

Fig. 1. Frequency of observed gradients in patients studied

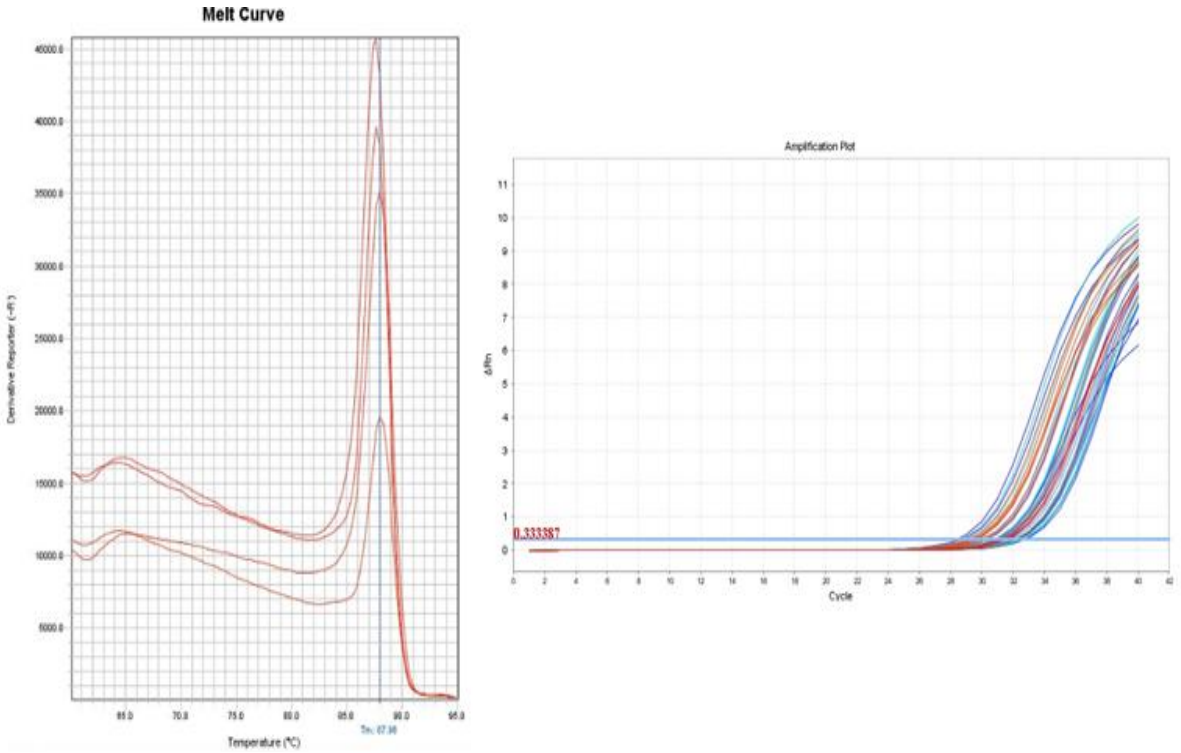

Fig. 2. Linear and logarithmic diagrams of real-time PCR amplification and temperature curve for the *PD-L2* gene

In a study by Shin, they analyzed the expression of *PD-L2* in renal cell carcinoma using IHC and anti-PD-L2 monoclonal antibody in mice and demonstrated an increase in this regard, but found that expression of *PD-L2* had a poor prognostic value in the diagnosis of renal cell carcinoma. The same antibody used in the other study revealed the expression of *PD-L2* in lung polymorphic carcinomas and showed that expression of *PD-L2* had no prognostic effect [27].

It should be noted that anti-PD-1 therapies can stop interactions between PD-L1 and PD-L2 or PD-1 while releasing anti-PD1 antibodies to inhibit PD -1 will release PD-L2 to communicate it [28].

A better understanding of the relationship between the expression of the PD-L1 protein and the expression of other proteins involved in the immune response, especially in patients who do not respond to PD-L1/PD-1 inhibitors, may lead to better treatment for non-respondents to PD-L1/PD-1.

5. CONCLUSION

PD-L2 expression detection methods, including the detection of mRNA expression by using Real-Time PCR and the use of multiple systems for detecting *PD-L2* expression with the cell type, and in association with other immune markers, can contribute to a deeper understanding of *PD-L2* as a biomarker in the diagnosis of NSCLC-type lung tumors. In our studies, it was realized that expression of *PD-L2* gene in cancer patients is higher than those in normal conditions. In the coming years, expression of the *PD-L2* protein may be evaluated along with other information such as immuno-suppression markers or mutations to provide more precise guidance on the clinical use of immunotherapy.

CONSENT AND ETHICAL APPROVAL

The study was approved by the Institutional Review Board of Central Tehran Branch, Islamic Azad University, Tehran, Iran and performed in accordance with the principles of the Declaration of Helsinki and each subject signed an informed consent before participating to the study. All procedures were approved by the relevant ethics committees, and written informed consent was obtained from all participants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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