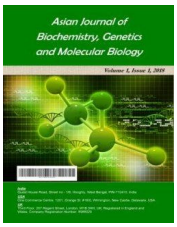

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Expression of *PDCD1* (*PD-1*) Gene among Non-small Cell Lung Cancer (NSCLC) Patients with Real-Time PCR Application

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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

Background: *PD-L1* is the main ligand is expressed on many tumors including lung cancer and is expressed in hematopoietic cells and various leukemia. The aim of this study was to evaluate the expression of *PD-1* gene and the evaluation of cancerous grades of NSCLC and its subclasses from 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 Hospital, Athiyah Hospital, and Masih Hospital). Of the 35 samples collected in 2017, 20% of the patients were women and 80% of them were male. The range of patients' age spectrum was 37 - 80 years. The disease grade of the patients in this study was varied and 22 different grades among them. To investigate the *PDCD-1* gene expression level, after extraction of RNA and cDNA synthesis the Real-Time PCR was done and the expression of the gene was investigated.

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Results: The highest grade was IIIa which contained 6 patients (17.1%). 74% of adenocarcinoma cases were in T-categories of lung cancer and 25% of patients were in grade IIIa. Patients with the grade of T3 were observed in 4 samples, 2 had adenocarcinoma and 2 with SCC with age range of 55 -62 years. The results showed that the expression of *PDCD-1* increased 2.46 Fold more in patients with lung cancer than NSCLC.
Conclusion: The results of this study showed that there is a significant relationship between the *PDCD1* or *PD-1* expression of NSCLC-type lung cancer compared with healthy individuals, and using the RT-PCR for ease and rapidity it can be proved.

Keywords: Lung cancer; NSCLC; PDCD-1 gene; PD-1 gene; Real-Time PCR.

1. INTRODUCTION

Cancer is a chronic and often an inflammatory disease which mechanism of development and dissemination is very complicated. There are several factors, such as environmental factors, lifestyle habits, genetic mutations, and immune system disorders that affect the development of the disease. At present, evidence suggests that tumor development is associated with the formation of a small and specific tumor. Tumor cells can escape stimulation and activity of the immune system and, by using several methods, disrupt the immune system, thus preventing the removal by the host immune system. [1]. Human cancers include a number of genetic and epigenetic changes that can synthesize new antigens that are potentially detectable by the immune system, thereby causing the T cells arm of the immune response in the body. T cells are the first components of the immune system which initially identify the cancer cells as abnormal, and produce a population of cytotoxic T-lymphocytes (CTLs) that can be spread anywhere in the cancer cells and specifically recognize them, and eliminate cancer cells being among take protective immunity against cancer as is dependent on the coordination of CTLs [2].

Lung cancer is currently the leading cause of cancer-related death worldwide. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of lung cancers. Surgery, platinum-based chemotherapy, targeted molecular agents, and radiation therapy is the main treatment choices for NSCLC. Early symptoms of patients with NSCLC especially, in case of lung cancer, are not such obvious [3].

There are two main types of lung cancers: including 80-85% of lung cancers, which is NSCLC and nearly 10-15% of them include small cell lung cancer (SCLC). NSCLC subtypes start with different types of lung cells, but they are classified with each other as NSCLC since the approach to treatment and prognosis are often the same. Approximately 40% of lung cancer cases are adenocarcinoma. This type of lung cancer occurs predominantly in current or former smokers, yet it is the most common type of lung cancer in non-smokers. This type of cancer is more common in women than in men and is likely to occur in young people more than other types of cancers of the lung [4].

Adenocarcinoma is commonly found in the outer parts of the lung. Although it tends to grow slowly against other types of lung cancer, changes in this type of cancers vary from disease to another patient [5]. The second type of NSCLC cancers is called squamous cell carcinoma (epidermoid), which accounts for about 30-25% of all lung cancers. They often have a history of smoking and tend to be found in the central part of the lungs near the main airway (Bronchus) [6]. The third type of cancers of NSCLC cases accounting for ~1-10% of lung cancers and can be observed in any part of the lung. This type of cancers has a rapid growth and spread thus can make the treatment difficult [7].

The planned cell death protein (*PDCD1*) or *PD-1*, whose gene encodes a cell membrane protein from the immunoglobulin family is expressed in pro-B cells and plays an important role in their differentiation. The product of this gene plays an important role in the function of the T cell and contributes to preventing autoimmune diseases [8]. *PD-1* trans-membrane protein of type 1 is a suprammalin immunoglobulin and acts on various types of active immune cells such as T cells, B cells, natural killer cells (NK), NKT cells, DCs, macrophages [9].

PD-1 has two known ligands, including *PD-L1* (*B7-H1*) and *PD-L2* (*B7-DC*) that belong to the family B7 [10,11]. *PD-L1* is the main ligand and is expressed in hematopoietic cells including T cells, B cells, DCs, macrophages and mast cells,

as well as many non-hematopoietic cells such as endothelial cells and epithelial cells [12,13]. *PD-L1* is expressed on many tumors, including cancers in various organs such as head and neck, lung, stomach, colon, pancreas, breast, kidney, bladder, ovary, cervix, as well as melanoma, glioblastoma, multiple myeloma, Lymphoma, and various leukemia. As a result, effective anti-tumor responses are mediated by T-cells expressed by *PD-1* [14-16].

PD-1 is a glycoprotein transmitted from a membrane of type 1, with a weight of 50-55 kD, which is composed of a second extracellular region similar to the antibody variable region with 21-23% sharing sequence with CTLA-4, CD28, and ICOS. The absence of a cysteine proximal membrane *PD-1*, which is required for the homodimerization of other members of the CD28 family, has led to its monomerization in solution and cell surface [17]. The cytoplastic domain of *PD-1* is a two-residue of tyrosine, a proximal membrane that consists of a tyrosine-based inhibitor motif of immune receptor and a tyrosine-based immune receptor [18]. Because ITIM is widely used for inhibitors including 72 CDs, BIIRγFC and KIRs, the remainder of the proximal membrane tyrosine has been proposed, which plays a central role for the *PD-1* inhibitory function. Although intra-laboratory findings, using a mouse-expressing cell lineage of different *PD-1* mutants, revealed that the remaining tyrosine located in the ITSM, not ITIM, was necessary for the inhibition of *PD-1* [19]. Upon stimulation of the antigen, the tyrosine residues located in the ITSM are phosphorylated and utilizes the SHP-2 tyrosine phosphatase protein, which has effective downstream molecules such as syk, PI3K in B cells, and ZAP70, for inhibition of CD3 in T cells. *PD-1* is induced on negative β /α and δ /γ T cells in the thymus and on the T and B cells of the peripheral blood as stimulated upon activation [19-21].

The aim of this study was to evaluate the expression of *PD-1* gene and the investigation of cancerous grades of NSCLC and its subclasses from lung cancer patients in Tehran hospitals using Real-Time PCR.

2. MATERIALS AND METHODS

2.1 Collection of Clinical Samples

Thirty-five clinical samples were collected from patients with NSCLC lung cancer were in three hospitals of Tehran (Khatam Hospital, Atiyah Hospital, and Masih Hospital). Most being 71.4% of them from Masih, 22.8% Atiyah and the lowest being 5.8% were from Khatam Hospitals, respectively. Of the total of 35 specimens taken in 2017, 80% of the patients were men and 20% of these patients were women. The age spectrum of all patients ranged between 37 to 80 years.

2.2 Ethical Considerations

The patients had the consent for this study by filling related form. This study was approved by ethical committee of Islamic Azad University, Tehran, Iran. (ID. 21F/599, 2016).

2.3 Clinical Information of Patients

The disease grades among them in this study extensively varied and 22 various grades were determined. Grades obtained include T2BN1, T2BN0MX, T2N0, IB, IIA, IIB, III, IIIa, T2aN2MX, T1aN0MX, T1bN) MX, T1N1MX, T2aN0, T2N1, T3N0MX-IIB, PT4N0MX, T2, T2AN2MX, T3N1MX, T2aN0M0B1, T3N2MX T3N0MX. However, six patients had an undetermined grade of their disease.

All subtypes of Adenocarcinoma, Adenosquamous, and SCC were observed in the subtypes of the disease. 16 subtypes of adenocarcinoma, 12 subtypes were also identified as SCC and one belonged to adenosquamous subtype. The only sub-type Adenosquamous belonged to the T2BN0MX grade and the Masih Hospital.

2.4 Extraction of RNA from Tumor Tissues

Firstly, 50 to 100 mg of tissue from the sample was cut by a scalpel and placed in a plate. Then after scraping and collection, the samples were homogenized by the scalpel. Subsequently, the tissue was transferred to the DNase and RNase, free and 1000 µl of trizole was added, and by a vortex, the cells were entirely lysed and no particles were observed in the solution and a single solution was obtained. Next, 200 µl of chloroform was added to the microtubes containing the trace elements to separate the phases and by invert for several times. In the next stage, the samples were centrifuged at 13,000 rpm (10 min, 4°C).

Following the centrifugation, three phases were seen. The pink trizole was below, a white precipitate layer containing blood proteins and

the blue supernatant anhydrous phase containing RNA. Next, 400 µl of the supernatant containing RNA was removed gently and accurately without contacting the underlying layer with the RNase free DNase free tip and transferred to a new 1.5 RNase, DNase free microtube, and then to the same amount (400 µl) of isopropanol Add to microtube and incubate for 10 minutes at room temperature.

In the next step, centrifugation was performed for 5 minutes at 13000 rpm at 4°C to the further aim. The supernatant was then completely removed by RNase, DNase free tip, to maintain only RNA precipitate in the microtube. Next, 1000 µl of 75% ethanol was then added to RNA for washing. The centrifuges were then re-centrifuged for 5 minutes at 10,000 rpm at 4°C. After centrifugation, alcohol was completely removed.

In the next step, the microtube 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 30-50 µl and pipetting was performed to completely dissolve the RNA sedimentation. Finally, OD of 3 µl of extracted RNA was read to evaluate its quality using Nanodrop apparatus and the subsequently RNA was transferred immediately to the -70C temperature.

2.5 Synthesis of cDNA

After the RNA was extracted, firstly its concentration was measured to determine the volume required for cDNA synthesis with a spectrophotometer, and an OD of 280nm/260nm ratio was obtained to determine the purity of the RNA, and that higher than 1.6 was adopted. In the next step, using thermal cycler, primers, and RNA from clinical samples, the cDNA was synthesized. For this purpose, the Primescipt (Takara) enzyme and specific primers and oligo-dimetimidine (Oligo-dt) were employed to synthesize cDNA.

2.6 Real-Time PCR Conditions

The reaction of the RT-PCR was performed using the Applied Biosystems StepOnePlus Real-Time PCR Systems. By this technique, the expression of the target gene from mRNA was evaluated by the relative quantitative method and using the SYBR Green dye. The amplification of a gene that was detectable in a cycle called Ct and the resulting Ct compared to the expression of Glyceraldehyde-3 gene -phosphate dehydrogenase (*GAPDH*). The GAPDH enzyme was used as a housekeeping gene and internal control. The primers and materials used in conjunction with the temperature conditions used in Real-Time PCR are shown in Tables 1, 2 and 3.

Table 1. Primers used the gene expression of *PDCD-1* gene

Sequences 5′-3′	Primer name
5- GTG TTG GGA GGG CAG AAG TG -3	*PDCD-1*-F
5- GTG TGG ATG TGA GGA GTG GAT AG -3	*PDCD-1*-R

Table 2. Materials used in the reaction of the RT-PCR for detection of *PDCD-1* 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 RT-PCR for the *PDCD-1* gene

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

3. RESULTS

Patient samples and disease grades: The frequency of 22 grades of the disease was observed in patients in this study. The predominant grade was IIIa grade which was seen in 6 patients (17.1%). After IIIa grade, the T2BN0MX grade was observed with 2 samples. Only one sample was identified from other grades. Six samples were not included in the studied grades. Patient characteristics are shown in Table 4.

Furthermore, 74% of adenocarcinoma cases were in T-category of lung cancer and 25% of patients were in grade IIIa. Those with the T3 grade of the disease included four patients, two of which were adenocarcinoma and two others were SCC and their age ranged from 55 to 62 years old. Three patients were in the T1 category, and all (100%) exhibited adenocarcinoma. In addition, those patients in the IIA and IIB categories belonged to the SCC subclass, and all three patients were from Atiyah Hospital.

Only one IB subclass was seen among the patients, which was also classified under the SCC subclass. Independent t-test revealed 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 no statistically significant difference was observed (p = 0.202) between the two groups.

Linear and logarithmic graphs of real-time PCR amplification and temperature curve for *PDCD-1* gene are shown in Fig. 1. The results showed that the expression of *PDCD-1* gene 2.46 Fold was more common in patients with lung cancer than NSCLC. Comparison of fold change of the *PDCD-1* gene in patients and controls is shown in Fig. 2.

4. DISCUSSION

Quantitative Real-Time PCR (qPCR) quantitative methods is based on the amplification of the gene loci in each PCR cycle or reaction using fluorescent light emitting molecules, and the fluorescence data collected from the PCR timeline should be analyzed by data analysis. Furthermore, the difference between the expression of mRNA in the target gene is evaluated by comparison to the control gene.

The *PD-1* gene investigated in this study was a CD28 family with 23% sequence similarity to another member of this family, CTLA-4 inhibitor, but contrary to the limited expression of CTLA-4 to T cells after activation, *PD-1* can also be induced on T cells, B cells, and myeloid cells. This extensive expression pattern of *PD-1*

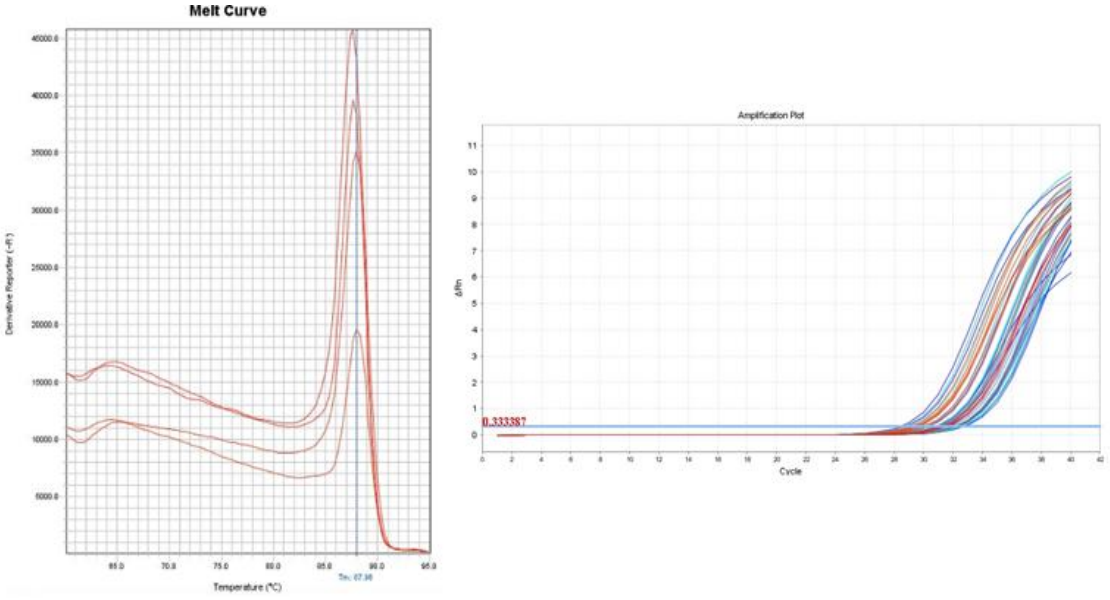

Fig. 1. Linear and logarithmic graphs of Real-Time PCR amplification and temperature curve for *PDCD-1* gene. The x axis contains the number of reaction cycles and a y axis called Delta Rn

Table 4. Patient characteristics with NSCLC

Case no.	Stage	Subtype	Δct Cancer	Gender	Age	Alcohol	Smoking
1	IIIA	Adeno	3.18	M	62	NO	NO
2	IB	SCC	3.83	M	59	NO	NO
3	T2	Adeno	3.74	F	54	NO	NO
4	IIIa	Adeno	4.102	M	53	NO	Yes
5	IIB	unknown	7.56	M	45	NO	Yes
6	unknown	unknown	4.46	M	63	NO	YES
7	unknown	unknown	1.94	M	62	NO	YES
8	T2N1	SCC	2.52	M	61	NO	NO
9	T2b,N1	Adeno	6.06	M	54	NO	NO
10	T3N1MX	Adeno	13.7	F	55	NO	NO
11	III	SCC	2.73	M	50	NO	NO
12	T2a,N0	Adeno	7.63	M	80	NO	NO
13	IIB	SCC	3.18	M	65	NO	Yes
14	T3,N0,Mx-IIB	SCC	4.74	M	56	NO	NO
15	IIIA	Adeno	3.33	M	54	NO	NO
16	unknown	unknown	4.55	F	50	NO	NO
17	IIIA	SCC	1.84	F	62	NO	NO
18	T2a N2 Mx	SCC	8.46	M	60	NO	NO
19	T2aN0M0 IB	SCC	1.99	M	64	NO	Yes
20	unknown	unknown	3.40	M	61	NO	NO
21	T2N0	Adeno	3.82	M	46	NO	NO
22	T2BN0MX	ADENO	2.12	F	37	NO	NO
23	T1N1MX	ADENO	6.37	M	60	NO	NO
24	T2BN0MX	ADENOSQUAMOUS	3.85	M	44	NO	NO
25	T3N2Mx	SCC	3.79	M	62	NO	NO
26	PT4N0MX	SCC	6.41	F	62	NO	NO
27	unknown	unknown	7.46	M	48	NO	NO
28	T3N0MX	Adeno	8.85	F	57	NO	NO
29	unknown	Adeno	2.22	F	58	NO	Yes
30	T1aN0Mx	Adeno	4.11	M	53	NO	Yes
31	T1bN0M0	Adeno	3.81	F	58	NO	NO
32	IIA	SCC	1.21	M	58	NO	Yes
33	IIIA	Adeno	3.27	M	55	NO	Yes
34	IIIA	SCC	4.11	M	67	NO	NO
35	T2AN2MX	ADENO	2.97	M	56	NO	NO

demonstrates its vital and important role in making and maintaining environmental tolerance in a variety of immune cells, thus preventing the development of autoimmune diseases [22,23]. This role of *PD-1* negative inhibitory trait support in establishing and maintaining environmental tolerance has been proven in studies of an animal model deficient in this gene and the self-sustaining disease, such as lupus-like syndrome and arthritis and progressive nephritis. Lymphocyte activation is precisely determined by positive and negative messages. These messages are received via various safety regulatory receivers [24].

Expression of *PDL-1* and *PDL-2* occurs on different tumor cells. Hirano Iwai showed that *PDL-1* on tumor cells suppressed the cytotoxic activity of TCD8 cells using the Mastocytoma 815P cell line with a high expression of *PDL-1*. Until now, using different systems, it has been demonstrated that eradication of the tumor can be accelerated by blocking *PD-1*. In addition, *PD-1* blocking also serves to suppress tumor metastasis using colon cancer cell lines and melanoma [25,26]. In our studies, *PDCD-1* gene expression was found to be higher level in cancer patients than normal conditions and we confirmed this finding using RT-PCR.

Extensive *PD-1* expression, in contrast to the limited expression of other members of the CD28 family in T cells, suggests that *PD-1* regulates a wide range of immune responses compared with

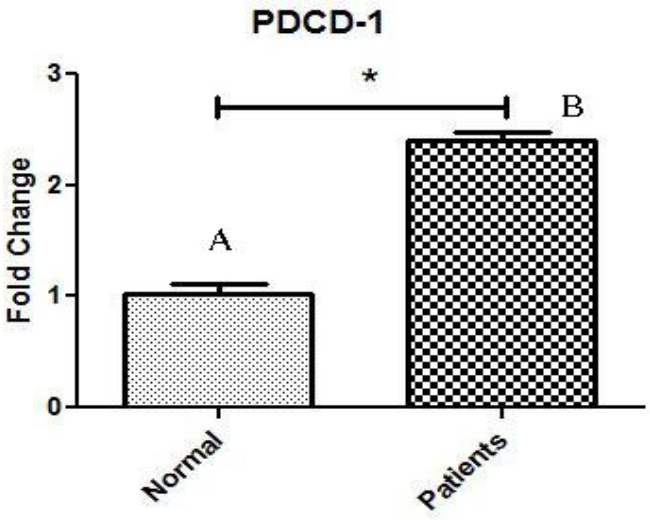

Fig. 2. The fold change expression of PDCD-1 gene. The results showed that the expression of *PDCD-1* gene 2.46 Fold was more common in patients with lung cancer than NSCLC. A: expression of *PDCD-1* gene in control. B: expression of *PDCD-1* gene patients

other members of the CD28 family. Although most analyzes focused on the expression of *PD-1* on the cell surface, Raimondi reported that *PD-1* could also be present in the cytoplasm of T cells with regulatory function [27].

PD-1 plays an essential role in the regulation of autoimmunity, tumor safety, infectious immunity, graft immunization, allergy and specific-compartment immunity. Recently, many groups are trying to produce not only *PD-1* antagonists for the treatment of cancer and infectious diseases, but also their agonists for the treatment of autoimmune diseases, allergies, and retreatment. The damage to many of the autoimmune, allergic, malignant and, in particular, chronic diseases of viral infections is affected by the disruption of the *PD-1* regulatory pathway. Individual differences in expression and, possibly, *PD-1* function appear as polymorphisms in the regulatory sections of the *PD-1* gene. Some studies have examined the association between *PD-1* gene polymorphism and gene expression in vitro and the predisposition and severity of the incidence of these diseases [28].

Genetic analysis of *PD-1* in human diseases has traditionally focused on case-control studies, in which the frequency of allele markers in patient groups and healthy controls have been compared and the differences have been statistically analyzed. Relationships are often expressed as a risk factor or odds ratio; where the disease is more common in a person carrying an individual allele or special marker, in comparison with the person without that allele or marker [29].

5. CONCLUSION

The results of this study highlighted that there was a significant relationship between *PDCD1* or *PD-1* death expression in NSCLC-type lung cancer compared to normal individuals, and its increased level can be easily and rapidly detected using the RT-PCR.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

This study was approved by ethical committee of Islamic Azad University, Tehran, Iran. (ID. 21F/599, 2016).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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