

Expression of *PDCD1* (*PD-1*) Gene among non-small cell lung cancer (NSCLC) Patients with Real-Time PCR application

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.

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

28 **Introduction**

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

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

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

55 Adenocarcinoma is commonly found in the outer parts of the lung. Although it tends to grow
56 slowly against other types of lung cancer, changes in this type of cancers vary from disease to
57 another patient [5]. The second type of NSCLC cancers is called squamous cell carcinoma

58 (epidermoid), which accounts for about 30-25% of all lung cancers. They often have a history of
59 smoking and tend to be found in the central part of the lungs near the main airway (Bronchus)
60 [6]. The third type of cancers of NSCLC cases accounting for ~1-10% of lung cancers and can be
61 observed in any part of the lung. This type of cancers has a rapid growth and spread thus can
62 make the treatment difficult [7].

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

69 *PD-1* has two known ligands, including *PD-L1* (*B7-H1*) and *PD-L2* (*B7-DC*) that belong to the
70 family B7 [10, 11]. *PD-L1* is the main ligand and is expressed in hematopoietic cells including T
71 cells, B cells, DCs, macrophages and mast cells, as well as many non-hematopoietic cells such as
72 endothelial cells and epithelial cells [12, 13]. *PD-L1* is expressed on many tumors, including
73 cancers in various organs such as head and neck, lung, stomach, colon, pancreas, breast, kidney,
74 bladder, ovary, cervix, as well as melanoma, glioblastoma, multiple myeloma, Lymphoma, and
75 various leukemia. As a result, effective anti-tumor responses are mediated by T-cells expressed
76 by *PD-1* [14-16].

77 *PD-1* is a glycoprotein transmitted from a membrane of type 1, with a weight of 50-55 kD,
78 which is composed of a second extracellular region similar to the antibody variable region with
79 21-23% sharing sequence with CTLA-4, CD28, and ICOS. The absence of a cysteine proximal
80 membrane *PD-1*, which is required for the homodimerization of other members of the CD28
81 family, has led to its monomerization in solution and cell surface [17]. The cytoplasmic domain of
82 *PD-1* is a two-residue of tyrosine, a proximal membrane that consists of a tyrosine-based
83 inhibitor motif of immune receptor and a tyrosine-based immune receptor [18]. Because ITIM is
84 widely used for inhibitors including 72 CDs, BIRγFC and KIRs, the remainder of the proximal
85 membrane tyrosine has been proposed, which plays a central role for the *PD-1* inhibitory
86 function. Although intra-laboratory findings, using a mouse-expressing cell lineage of different
87 *PD-1* mutants, revealed that the remaining tyrosine located in the ITSM, not ITIM, was

88 necessary for the inhibition of *PD-1* [19]. Upon stimulation of the antigen, the tyrosine residues
89 located in the ITSM are phosphorylated and utilizes the SHP-2 tyrosine phosphatase protein,
90 which has effective downstream molecules such as syk, PI3K in B cells, and ZAP70, for
91 inhibition of CD3 in T cells. *PD-1* is induced on negative β/α and δ/γ T cells in the thymus and
92 on the T and B cells of the peripheral blood as stimulated upon activation [19-21].

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

96 **Materials and methods**

97 **Collection of clinical samples**

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

103 **Ethical considerations**

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

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107 **Clinical information of patients**

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

113 All subtypes of Adenocarcinoma, Adenosquamous, and SCC were observed in the subtypes of
114 the disease. 16 subtypes of adenocarcinoma, 12 subtypes were also identified as SCC and one

115 belonged to adenosquamous subtype. The only sub-type Adenosquamous belonged to the
116 T2BN0MX grade and the Masih Hospital.

117 **Extraction of RNA from tumor tissues:**

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

125 Following the centrifugation, three phases were seen. The pink trizole was below, a white
126 precipitate layer containing blood proteins and the blue supernatant anhydrous phase containing
127 RNA. Next, 400 μl of the supernatant containing RNA was removed gently and accurately
128 without contacting the underlying layer with the RNase free DNase free tip and transferred to a
129 new 1.5 RNase, DNase free microtube, and then to the same amount (400 μl) of isopropanol Add
130 to microtube and incubate for 10 minutes at room temperature.

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

136 In the next step, the microtube was placed on ice for 10 minutes to completely dry the RNA
137 sediment. Based on the volume of the obtained coil, the amount of DEPC water was added at the
138 end of 30-50 μl and pipetting was performed to completely dissolve the RNA sedimentation.
139 Finally, OD of 3 μl of extracted RNA was read to evaluate its quality using Nanodrop apparatus
140 and the subsequently RNA was transferred immediately to the -70C temperature.

141 **Synthesis of cDNA**

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

148 **Real-Time PCR conditions**

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

156 **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	<i>PDCD-1</i> -F
5- GTG TGG ATG TGA GGA GTG GAT AG -3	<i>PDCD-1</i> -R

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

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

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 159 **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	-

160 Results

161 Patient samples and disease grades: The frequency of 22 grades of the disease was observed in
 162 patients in this study. The predominant grade was IIIa grade which was seen in 6 patients
 163 (17.1%). After IIIa grade, the T2BN0MX grade was observed with 2 samples. Only one sample
 164 was identified from other grades. Six samples were not included in the studied grades. Patient
 165 characteristics are shown in Table 1.

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

172 Only one IB subclass was seen among the patients, which was also classified under the SCC
 173 subclass. Independent t-test revealed that the average age of the patients with a subtype of
 174 adenoma was 56.7 years and the SCT subtype was 60.7 years, and no statistically significant
 175 difference was observed ($p = 0.202$) between the two groups.

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

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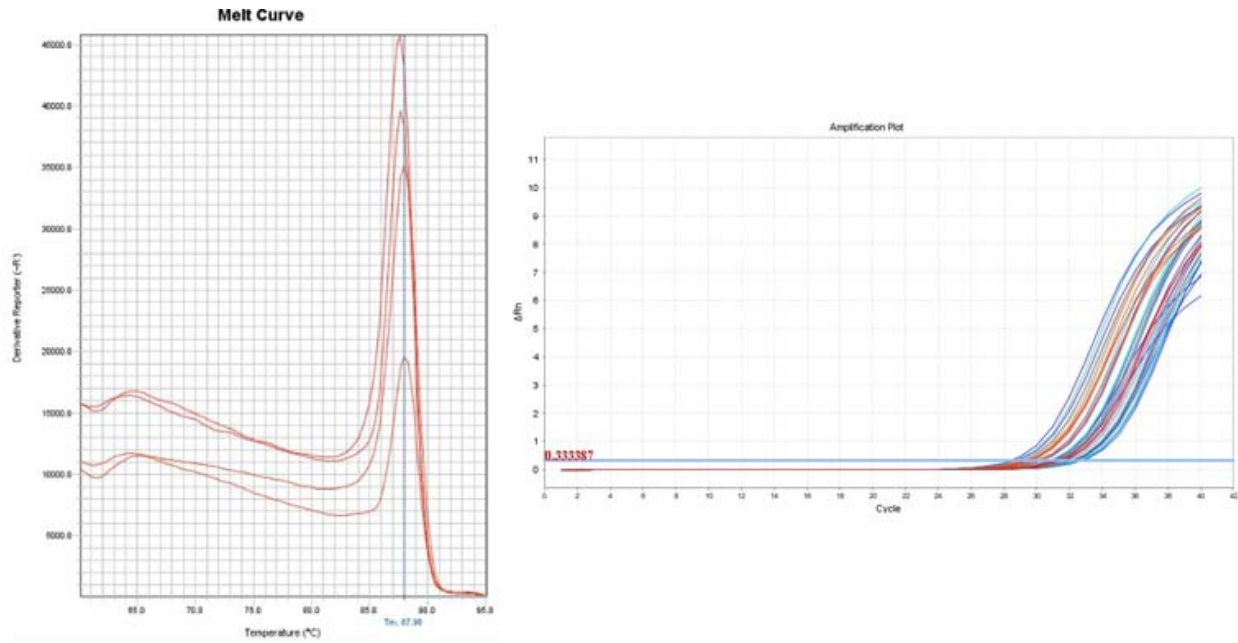
187 Table 1. Patient characteristics with NSCLC

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

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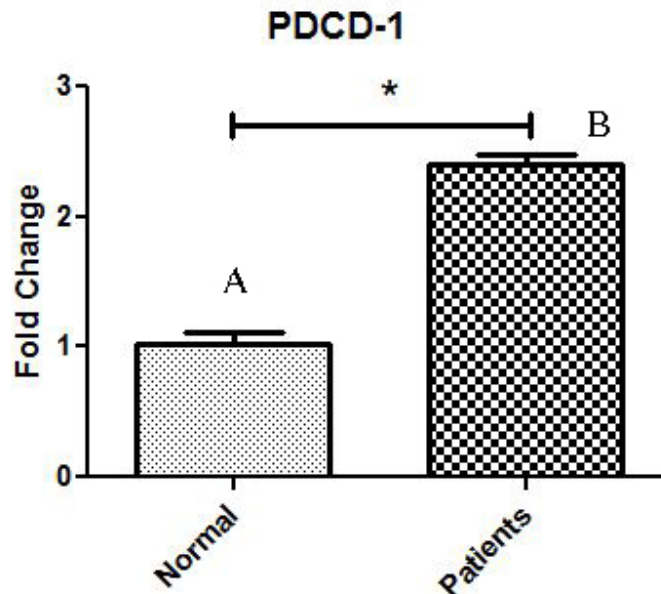
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191 Figure 1: Linear and logarithmic graphs of Real-Time PCR amplification and temperature curve
 192 for *PDCD-1* gene. The x axis contains the number of reaction cycles and a y axis called Delta
 193 Rn.

UNDER REVIEW



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

198

199 **Discussion**

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

205 The *PD-1* gene investigated in this study was a CD28 family with 23% sequence similarity to
 206 another member of this family, CTLA-4 inhibitor, but contrary to the limited expression of
 207 CTLA-4 to T cells after activation, *PD-1* can also be induced on T cells, B cells, and myeloid
 208 cells. This extensive expression pattern of *PD-1* demonstrates its vital and important role in
 209 making and maintaining environmental tolerance in a variety of immune cells, thus preventing
 210 the development of autoimmune diseases [22, 23]. This role of *PD-1* negative inhibitory trait
 211 support in establishing and maintaining environmental tolerance has been proven in studies of an

212 animal model deficient in this gene and the self-sustaining disease, such as lupus-like syndrome
213 and arthritis and progressive nephritis. Lymphocyte activation is precisely determined by
214 positive and negative messages. These messages are received via various safety regulatory
215 receivers [24].

216 Expression of *PDL-1* and *PDL-2* occurs on different tumor cells. Hirano Iwai showed that *PDL-*
217 *1* on tumor cells suppressed the cytotoxic activity of TCD8 cells using the Mastocytoma 815P
218 cell line with a high expression of *PDL-1*. Until now, using different systems, it has been
219 demonstrated that eradication of the tumor can be accelerated by blocking *PD-1*. In addition,
220 *PD-1* blocking also serves to suppress tumor metastasis using colon cancer cell lines and
221 melanoma [25, 26]. In our studies, *PDCD-1* gene expression was found to be higher level in
222 cancer patients than normal conditions and we confirmed this finding using RT-PCR.

223 Extensive *PD-1* expression, in contrast to the limited expression of other members of the CD28
224 family in T cells, suggests that *PD-1* regulates a wide range of immune responses compared with
225 other members of the CD28 family. Although most analyzes focused on the expression of *PD-1*
226 on the cell surface, Raimondi reported that *PD-1* could also be present in the cytoplasm of T
227 cells with regulatory function [27].

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

238 Genetic analysis of *PD-1* in human diseases has traditionally focused on case-control studies, in
239 which the frequency of allele markers in patient groups and healthy controls have been compared
240 and the differences have been statistically analyzed. Relationships are often expressed as a risk

241 factor or odds ratio; where the disease is more common in a person carrying an individual allele
242 or special marker, in comparison with the person without that allele or marker [29].

243

244 **Conclusion**

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

248 **Ethical considerations**

249 This study was approved by ethical committee of Islamic Azad University, Tehran, Iran. (ID.
250 21F/599, 2016).

251 **Consent Disclaimer:**

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

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258 **References:**

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