1 *Original Research Article*

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3 Expression of *PDCD1* (*PD-1*) Gene among non-small cell lung 4 cancer (NSCLC) Patients with Real-Time PCR application

5

6 **Abstract**

7 **Background:** *PD-L1* is the main ligand is expressed on many tumors including lung cancer and 8 is expressed in hematopoietic cells and various leukemia. The aim of this study was to evaluate 9 the expression of *PD-1* gene and the evaluation of cancerous grades of NSCLC and its 10 subclasses from lung cancer patients in Tehran hospitals using Real-Time PCR.

11 **Materials and Methods:** A total of 35 clinical samples were collected from patients with 12 NSCLC-derived lung cancer from three hospitals in Tehran (Khatam Hospital, Athiyah Hospital, 13 and Masih Hospital). Of the 35 samples collected in 2017, 20% of the patients were women and 14 80% of them were male. The range of patients' age spectrum was 37 - 80 years. The disease 15 grade of the patients in this study was varied and 22 different grades among them. To investigate 16 the *PDCD-1* gene expression level, after extraction of RNA and cDNA synthesis the Real-Time 17 PCR was done and the expression of the gene was investigated.

18 **Results:** The highest grade was IIIa which contained 6 patients (17.1%). 74% of 19 adenocarcinoma cases were in T-categories of lung cancer and 25% of patients were in grade 20 IIIa. Patients with the grade of T3 were observed in 4 samples, 2 had adenocarcinoma and 2 with 21 SCC with age range of 55 -62 years. The results showed that the expression of *PDCD-1* 22 increased 2.46 Fold more in patients with lung cancer than NSCLC.

23 **Conclusion**: The results of this study showed that there is a significant relationship between the 24 *PDCD1* or *PD-1* expression of NSCLC-type lung cancer compared with healthy individuals, and 25 using the RT-PCR for ease and rapidity it can be proved.

26 **Keywords**: Lung Cancer, NSCLC, *PDCD-1 gene*, *PD-1 gene*, Real-Time PCR

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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

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(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

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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.

Materials and methods

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.

Ethical considerations

The patients had the consent for this study by filling related form.

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.

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

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

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

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

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

139 **Synthesis of cDNA**

140 After the RNA was extracted, firstly its concentration was measured to determine the volume
141 required for cDNA synthesis with a spectrophotometer, and an OD of 280nm/260nm ratio was
142 obtained to determine the purity of the RNA, and that higher than 1.6 was adopted. In the next
143 step, using thermal cycler, primers, and RNA from clinical samples, the cDNA was synthesized.

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144 For this purpose, the Primescipt (Takara) enzyme and specific primers and oligo-dimetimidine
145 (Oligo-dt) were employed to synthesize cDNA.

146 **Real-Time PCR conditions**

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

154 **Table 1: Primers used the expression of the *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

155 **Table 2: Materials used in the reaction of the real-time PCR for the *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

156

157 **Table 3: Temperature and time used for real-time 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	-

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158 **Results**

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

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

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

174 Linear and logarithmic graphs of real-time PCR amplification and temperature curve for *PDCD-*
175 *1* gene are shown in Fig. 1. The results showed that the expression of *PDCD-1* gene 2.46 Fold
176 was more common in patients with lung cancer than NSCLC. Comparison of fold change of the
177 *PDCD-1* gene in patients and controls is shown in Fig. 2.

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185 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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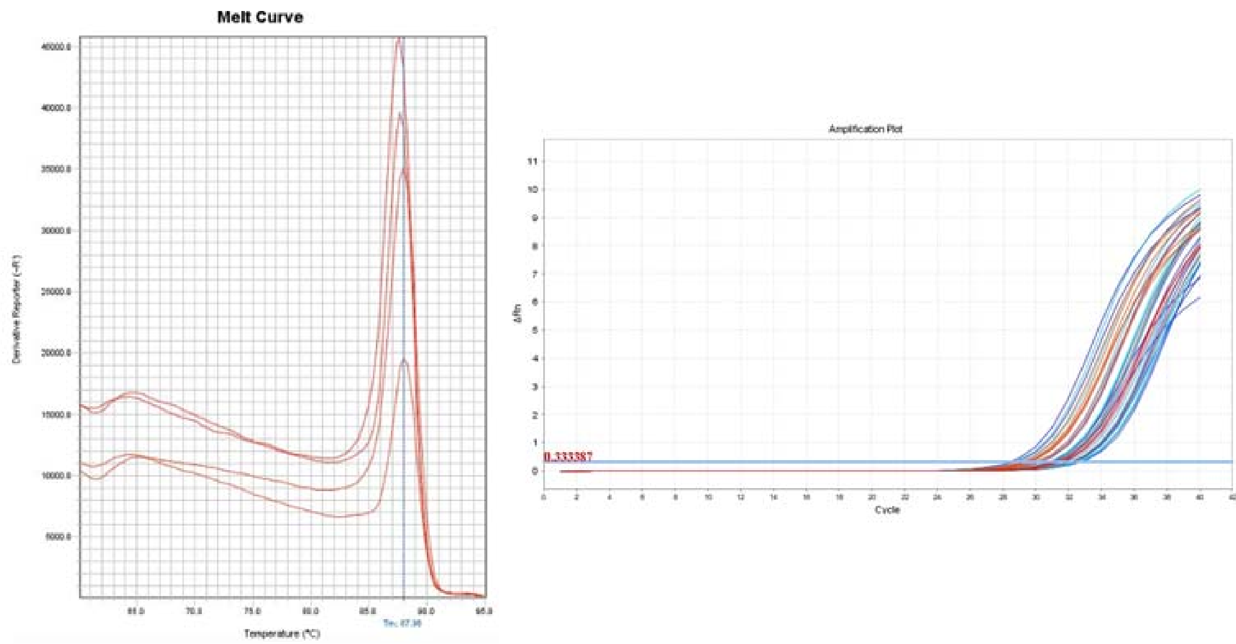
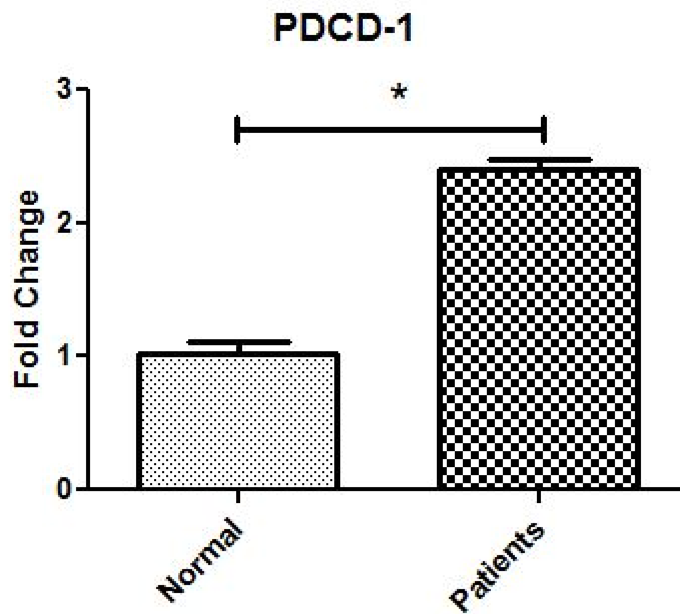


188

189 **Figure 1: Linear and logarithmic graphs of Real-Time PCR amplification and temperature**

190 **curve for *PDCD-1* gene**



191

192 Figure 2: The fold change expression of PDCD-1 gene.

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194

195 **Discussion**

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

201 The *PD-1* gene investigated in this study was a CD28 family with 23% sequence similarity to
202 another member of this family, CTLA-4 inhibitor, but contrary to the limited expression of
203 CTLA-4 to T cells after activation, *PD-1* can also be induced on T cells, B cells, and myeloid
204 cells. This extensive expression pattern of *PD-1* demonstrates its vital and important role in
205 making and maintaining environmental tolerance in a variety of immune cells, thus preventing
206 the development of autoimmune diseases [22, 23]. This role of *PD-1* negative inhibitory trait
207 support in establishing and maintaining environmental tolerance has been proven in studies of an
208 animal model deficient in this gene and the self-sustaining disease, such as lupus-like syndrome
209 and arthritis and progressive nephritis. Lymphocyte activation is precisely determined by
210 positive and negative messages. These messages are received via various safety regulatory
211 receivers [24].

212 Expression of *PDL-1* and *PDL-2* occurs on different tumor cells. Hirano Iwai showed that *PDL-*
213 *1* on tumor cells suppressed the cytotoxic activity of TCD8 cells using the Mastocytoma 815P
214 cell line with a high expression of *PDL-1*. Until now, using different systems, it has been
215 demonstrated that eradication of the tumor can be accelerated by blocking *PD-1*. In addition,
216 *PD-1* blocking also serves to suppress tumor metastasis using colon cancer cell lines and
217 melanoma [25, 26]. In our studies, *PDCD-1* gene expression was found to be higher level in
218 cancer patients than normal conditions and we confirmed this finding using RT-PCR.

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

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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].

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.

Ethical Approval:

As per international standard or university standard ethical approval has been collected and preserved by the authors.

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