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Mutations in Tumor suppressor *TP53* Gene in Formalin- Fixed, Paraffin Embedded Tissues of Squamous Cell Carcinoma (SCC) of Lung Cancer

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Abstract: The *TP53* gene mutations have been extensively studied in lung cancer and have been used as a means to understand the origin(s) and mechanisms of these mutations in lung cancer development. *TP53* gene mutations occur frequently in many human cancers. Analysis of *TP53* gene mutations can also provide clues to the etiology of tumor formation. Formalin-fixed, paraffin embedded (FFPE) tissues from patients undergone surgery between 1997 to 2005 were provided the pathology department of Afzalipour Hospital, Kerman, Iran. The mutational status of the exons 5 & 8 of the *TP53* gene was screened by PCR followed by sequencing. Of the 25 patient samples sent for sequencing, 22 produced usable results for exon 5 and of these, 18 cases (81.8%) showed mutations. 18 samples also produced usable results for exon 8, and of these, 15 (83.3%) cases contained mutations. We identified a total of 113 mutations in tumor suppressor *TP53* gene of squamous cell carcinoma of lung tumors. At least 69 mutations were found in Exon 5 (in 18 cases) and 44 mutations in Exon 8 (in 15 cases). The most frequent mutations in exon 5 were deletion, G to T transvertion, G to A transition, G to C transversion and in exon 8 were A to T and G to T tansversion and deletion. Mutations were spread throughout the exons 5 & 8 but showed a strong preference for some mutational hotspots. These findings showe that *TP53* gene mutations contribute to the pathogenesis of non-small cell lung cancer significantly and the frequency of the mutations was higher in Iran compared to other areas described in the literature.

Keywords: *TP53* gene; lung cancer; squamous cell carcinoma; mutation.

INTRODUCTION

Despite improvements in diagnosis and therapy of lung cancer in the past two decades, lung cancer still remains the leading cause of death from cancer in both men and women and is suggested to be closely associated with the mutations of the *TP53* gene[1-5].
Lung cancers are classified into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC)[6].

About 80% of primary lung cancers are NSCLC which include two major histological subtypes: squamous cell carcinoma (SCC) and adenocarcinoma (AC) [7].

Cancer biology suggests that the transformation of a normal cell into a malignant cell needs a limited number of crucial genetic alterations. These changes lead to the activation of proto-oncogenes or inactivation of tumor suppressor genes (TSGs) and mutator genes[7,8]. Of major genetic alterations detected in lung cancer are point mutations in the *TP53* tumor suppressor gene[7,9].

The *TP53* gene is located on chromosome 17p13.1 and encodes a multifunctional DNA sequence-specific nuclear phosphoprotein important for keeping the integrity of the genome[10,11]. Following a non-repairable damage to DNA, this protein inhibits progress of the cell cycle from G1 to S phase and promotes apoptosis [12-14].
The *TP53* gene is frequently mutated in a wide variety of human cancers [7,15,16] and studies on lung tumor samples indicate that essentially 100% of SCLC and 50-70% of NSCLC show evidence for inactivating *TP53* mutations [17].

Lung cancer, like many other cancers is the result of a multi-step process [7]. An important issue in understanding the role of *TP53* inactivation in human

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lung cancer was to define the timing of these alterations in the course of tumor progression.

Several different investigators have demonstrated that TP_{53} inactivation is observed in early (preneoplastic dysplasia) stage of NSCLC and have suggested that this might serve as a useful molecular marker for early diagnosis or prognosis of this disease [17,18-20].

In addition to its association with high incidence of cancer, inactivation of TP_{53} also largely determines the properties of the tumor. Tumors with mutated TP_{53} for example can be more anaplastic, have a higher rate of proliferation and have a more aggressive phenotype than similar tumors with normal TP_{53}, thereby giving rise to a worse prognosis. Wild type TP_{53} is considered as a positive prognostic marker in colorectal, breast, bladder, prostate, and hematological malignancies and is usually associated with less aggressive tumors [15].

Levels and activity of p53 increase in response to DNA damage caused by oxidative stress, irradiation, cell adhesion, altered ribonucleotide pools and other stress factors [21]. It is generally assumed that the tumor suppressor properties of the p53 protein are associated with committing cells with severely damaged DNA to apoptosis [22,23]. Cells in which the TP_{53} is inactivated or modified by mutation, are not eliminated by apoptosis and accumulate DNA damage, which can lead to tumor development [23].

Several studies have also shown that the mutated TP_{53} gene correlated with a relapse of malignancy and increased resistance to chemotherapy and/or radiotherapy [24].

These data together suggest pivotal role(s) of p53 in the pathogenesis, extension and clinical outcome of malignancy. It has been shown that TP_{53} gene mutations in lung cancers are different from those in other cancers and that an excess of G to T transversion is a characteristic of lung tumors [7].

Recent studies from Europe and Asia have suggested geographic, ethnic and histologic differences in TP_{53} mutation frequencies and types in lung cancer [7].

The present study was conducted to investigate the TP_{53} mutations in patients with squamous cell carcinoma (SCC) of lung cancer hospitalized in Afzalipour Hospital in Kerman, Iran. Formalin-fixed, paraffin embedded (FFPE) tissues from patients undergone surgery between 1997 to 2005 for lung cancer were evaluated. The mutational status of the TP_{53} gene (exons 5 & 8) was screened by PCR followed by sequencing.

Samples that are routinely and widely used for molecular biology research and pathology examinations are formalin-fixed, paraffin embedded (FFPE) tissue blocks. Fixation with formalin is essential for archiving purposes and maintaining the cell morphology. On the other hand, the solubilization of DNA from formalin-fixed specimens is negatively correlated with the duration of formalin treatment and the yield of DNA extractions may be seriously reduced when compared to a fresh specimen[25,26]

MATERIAL AND METHODS

Tissue preparation: FFPE tissue blocks of squamous cell carcinoma which were fixed between 1997 to 2005 were kindly provided by professor Tabrizchi and professor Dabiri from pathology department of Afzalipour Hospital, Kerman, Iran

After removing the surrounding paraffin the tissues were minced with a surgical blade into 1mm pieces and between 25mg to 75mg of the tissues were transferred into a 1.5 ml microcentrifuge tube. The tissues were deparaffinized as follows [7,26,27].

Deparaffinization: 500 microliters of xylene (Merck Co., Germany) was added to the contents of the tube under a fume hood. The tubes were vortexed for 1 minute and left in a 65 ºC water-bath for 15 minutes. The xylene was decanted and the procedure was repeated two more times.

Xylene removal: To remove the residual xylene, samples were washed five times with ethanol (Merck Co., Germany) at room temperature as follows**.** [7,25,27].

1ml of absolute ethanol was added and mixed by vortex for ten seconds and removed after 10 minutes.1ml of absolute ethanol was added and mixed by vortex for ten seconds and removed after 10 minutes.1ml of 90% ethanol was added and mixed by vortex for ten seconds and after 20 minutes the tube was centrifuged at 8000 rpm (to pack tissues) and ethanol was removed. The procedure was repeated two more times once with 70% and once with 50% ethanol solutions.

The tubes were then left in a 40 ºC oven for 20 minutes to dry the tissues.

Tissue lysis: After the tissues were dried, 500 microliter of lysis solution was added to each tube. The lysis solution contained: 40 mM Tris (Merck Co., Germany), 1mM EDTA (CinnaGen Co., Iran), 0.5% Tween-20 (Merck Co., Germany) and 0.5 µg/µl proteinase K (Bioneer Co, Korea), pH, 8 [7]. The tubes were left in a 60 ºC water bath and were inverted a few times every 30 minutes until the tissue was completely

digestged (temperatures over 60 will inactivate the enzyme rapidly).
The resulting cell lysate was then heated at 95 ºC for 8 minutes to inactivate the proteinase K [7].

Phenol-Chloroform removal of lipids and proteins: An equal volume of Tris-saturated phenol [28] (Merck Co., Germany: pH,8) was added and the tubes were left on a rotating wheel for 10 minutes, then centrifuged at 12000 rpm for 2 minutes.
300 microliters of the upper phase was transferred to a new tube and after adding an equal volume of phenol-chloroform mixture (1:1) (Merck Co., Germany) the tube contents were mixed on the rotating wheel for 10 minutes and were centrifuged at 12000 rpm for 2 minutes.

The upper phase was transferred to a new tube and 2.5 volume of cold, absolute ethanol was added and the tube was left overnight at -20 ºC for DNA to precipitate.

The tubes were then centrifuged for 30 minutes at 4 ºC and ethanol was decanted.
The DNA pellet was washed gently two times with cold 70% ethanol and the pellet was dried completely at room temperature.
The pellet was dissolved in 30 to 70 microliters of sterile distilled water, depending on the size of the pellet.

To complete the solubilization, the tubes were left in a 40 ºC water bath for one hour.
To check the extracted DNA, 5 microliters of the solution was electrophoresed on a 1% agarose gel (Merck Co., Germany) [3].

PCR: The following primers were designed for the PCR amplification of exons 5 and 8. Primers were ordered to and synthesized by Isogen Co., Notherland.

Exon 5- Forward: 5`-TCT-GTT-CAC-TTG-TGC-CCT-GAC-TTT-CAA-C-3`
Exon 5- Reverse: 5`-GCA-ACC-AGC-CCT-GTC-TCT-CCA-3`
Exon 8- Forward: 5`-GTA-GGA-CCT-GAT-TTC-CTT-ACT-GCC-3`
Exon 8- Reverse: 5`-TGA-GGC-ATA-ACT-GCA-CCC-TTG-GTC-T-3

PCR was carried out in a final volume of 25 µl reaction mixture containing:
1x PCR Buffer (CinnaGen Co., Iran), 0.2 mM dNTP mix (CinnaGen Co., Iran), 1.5 mM MgCl2 (CinnaGen Co., Iran), 1 µM forward primer, 1 µM reverse primer, 3 U of Taq DNA polymerase (CinnaGen Co., Iran), 0.1 µg patient's DNA and autoclaved double distilled water. The mixture was heated at 95 °C for 3 min and then subjected to 50 cycles of PCR as follows:
94 °C for 1 min, 68 to 61 °C for 1 min (Touch down PCR), 72 °C for 2 min and at last 72 °C for 5 min.
5 microliters of the PCR products were then electrophoresed on a 2.5 % agarose gel.
The PCR products were sent to Hong Kong (Techdragon Co.) for sequencing. The sequencing was performed by DNA Analyzer ABI PRISM® 3700.

RESULTS AND DISCUSSION

Fig. 1, shows the agarose gel electrophoresis of the PCR products. About 10 µg of DNA was loaded on a 2.5% gel.

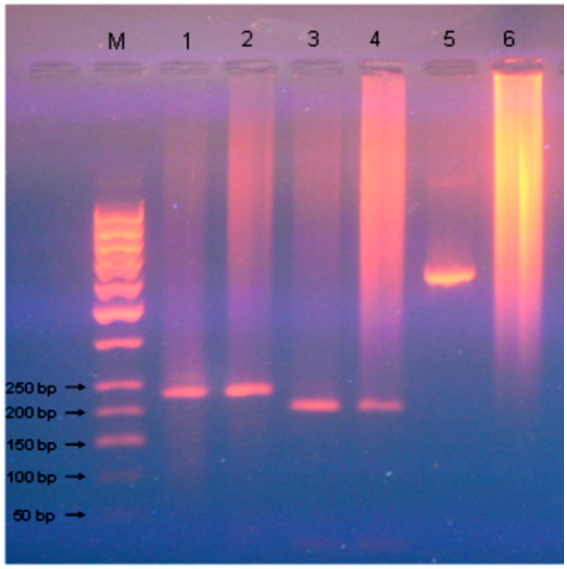


Fig 1: Agarose gel electrophoresis of PCR products. (M) size marker, (1 & 2) PCR products of exon 5 of the TP_{53} gene, (3 & 4) PCR products of the exon 8 of TP_{53} gene, (5) positive control (CinnaGen Co., Iran), (6) genomic DNA extracted from a FFPE sample.

Out of the 25 patient samples sent for sequencing, 22 produced usable results for exon 5 and of these, 18 cases (81.8%) showed mutations. 18 samples also produced usable results for exon 8, and of these, 15 (83.3%) cases contained mutations.

At least 69 mutations were found in Exon 5 (in 18 cases together) and 44 mutations in Exon 8 (in 15 cases together).
In the 18 samples of exon 5, 4 cases showed 1 mutation, 7 cases 2 mutations, 2 cases 3 mutations, 2

cases 4 mutations, 1 case 8 mutations, 1 case 11 mutations and 1 case 21 mutations.
In the 14 samples of exon 8, 6 cases showed 1mutation, 3 cases 2 mutations, 1 case 3 mutations, 2 cases 4 mutations, 1 case 5 mutations, 1 case 7 mutations and 1 case 10 mutations.

As shown in figures 2 and 3, the 69 mutations in exon 5 included 15 (23%) deletions, 12 (17%) G to T transversions, 12 (17%) G to A transitions, 11 (16%) G to C transversions, 7 (10%) A to T tranzversions, 4 (6%) A to C transversions, 3 (4%) addition, 2 (3%) C to T transitions, 2 (3%) T to A tranzversions and 1 (1%) C to A transversions.

The 44 mutations in exon 8 included 8 (18%) deletions, 16 (36%) G to T transversions, 17 (39%) A to T transversions, 2 (5%) additions, 1 (2%) C to T transitions.

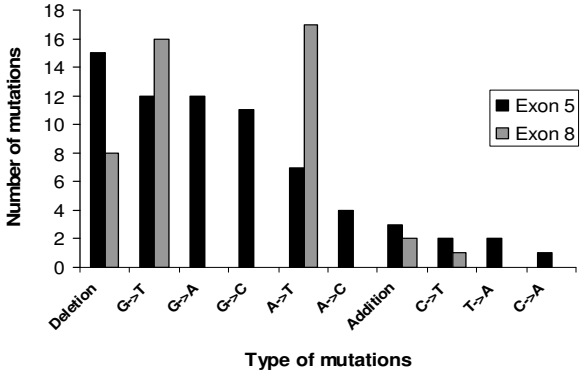
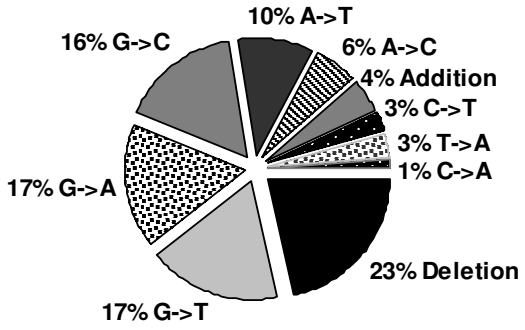


Fig. 2: Type and number of mutations in exons 5 and 8 of the *TP53* gene in squamous cell carcinoma (SCC) of lung cancer

As Fig. 3 shows, in exon 5 the most frequent mutations were deletions, G to T transvertions, G to A transitions and G to C transversions.

In exon 8, A to T and G to T tansversions and deletion mutations were the most frequent mutations.

A:



B:

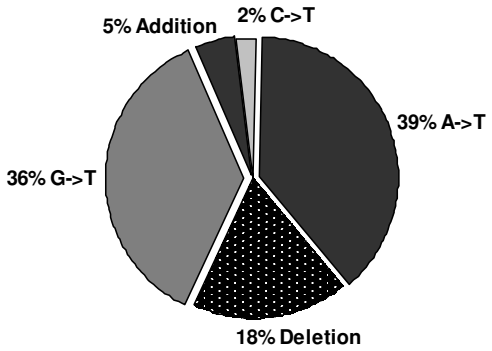
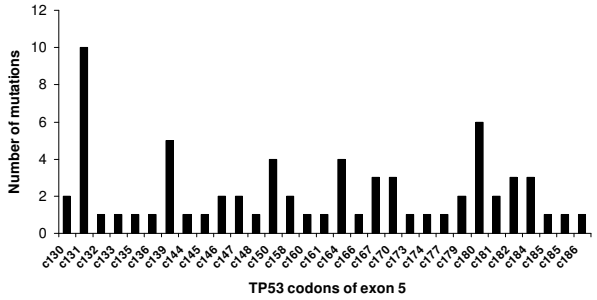


Fig. 3: Percentage of different mutations in exons 5 (A) & 8 (B) in squamous cell carcinoma (SCC) of lung cancer

As shown in figure 4, although mutations were spread throughout the exons 5 & 8, they showed strong preferences for some mutational hotspots. In exon 5 more mutations were observed at codons 131 (10/69, 14%), 180 (6/69, 8.6 %), 139 (5/69, 7.2%), 150 (4/69, 5.7%) and 164 (4/69, 5.7%) and in exon 8 at codons 263, 287, 297, 305 (5/44, 11.3%, each), 296 (4/44, 9%), 290 and 301 (3/44, 6.8 %, each).



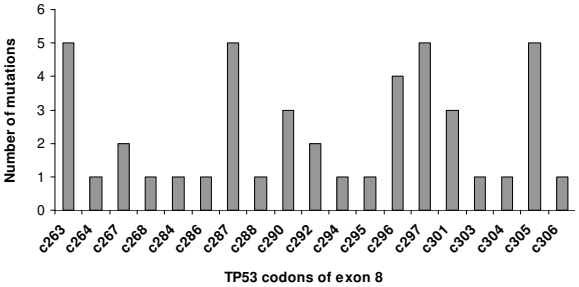


Fig. 4: Distribution of *TP53* gene mutations in Exons 5 & 8 of lung tumors in squamous cell carcinoma (SCC) of lung cancer

CONCLUSION

In this study, we identified a total of 113 mutations in TP_{53} gene of squamous cell carcinoma of lung tumors. 18 (81.1%) out of 22 samples sequenced for exon 5, showed mutations in this exon and 15 (83.3%) out of 18 samples sequenced for exon 8 showed mutation in this exon. These findings suggest that TP_{53} gene mutations contribute significantly to the pathogenesis of non-small cell lung cancer and the frequency of mutaions in Kerman/Iran is significantly higher compared to other parts of the world.
Tagawa et al. found genetic alteration of the TP_{53} gene (in exons 5 to 8) in 34% of non-small cell lung cancer patients [24].

Gao *et al*. examined the exons 5 to 8 of TP_{53} and found mutations in 35% of cases of SCC of lung cancer [7]. Matsuzoe et al. found mutations in 38% of exons 5 & 8 of TP_{53} gene ini non-small cell lung cancer patients [29]. Gesner et al. investigated mutations of the TP_{53} gene in NSCLC patients. TP_{53} exons 5 to 8 were amplified using nested PCR and subsequently sequenced. Mutations were found in 36.4% of patients [30]. The higher rate of mutations in lungs cancer patients in Iran is most probable related to the higher prevalence of smoking and air pollutants.

Smoking is implicated as the prominent cause of lung cancer. Among the cigarette smoke components, PAHs (polycyclic aromatic hydrocarbons) are strongly implicated as the causative agents in the development of these cancer.

TP_{53} mutation in both exons may arise by PAHs. Many investigations have focused on the role of PAHs in causing G to T transversions in hotspots in the TP_{53} gene sequence [31-33].

The high incidence of G to A transitions in exon 5 of our samples suggests the role and importance of nitrosamines such as NNK, found in tobacco smoke, in lung carcinogenesis [34-36].

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