TP53 is a Mutational Target in Non Small Cell Lung Cancer Patients and its Pro/Pro Variant is Potentially Contributing to Cancer Susceptibility

Saxena Alpana1, Javid J1, Mir R1, Masroor M1, Ahamad I1, Farooq S1, Yadav P1, Zuberi M1, Ajaz Ah Bhat2, Ahmad I, Khalanin T1, Julka PK1, Mohan A1, Lone M1, Banday MA1 and Ray PC1

1Molecular Oncology Lab, Department of Biochemistry, Maulana Azad Medical College and Associated hospitals, New Delhi, India
2Division of Surgical Oncology, Vanderbilt University, Nashville, TN, USA
3King Fahd Medical Research Center, King Abdulaziz University, Kingdom of Saudi Arabia
4Department of Medicine, BCM, Texas, USA
5Department of Radiotherapy, All India Institute of Medical Sciences, New Delhi, India
6Department of Medicine, All India Institute of Medical Sciences, New Delhi, India
7Department of Radiation Oncology, SKIMS, Srinagar, India
8Department of Medical Oncology, SKIMS, Srinagar, India

Abstract

Background: TP53 is one of the most important tumor suppressor genes, regulating various cellular processes and playing a pivotal role in preventing a cell to become malignant. P53 mutant protein containing arginine or proline at codon 72 shows different biological and biochemical activity. So, the aim of the present study was to find out the role of mutated TP53 gene with different codon 72 variants on the clinical outcome of patients suffering from NSCLC.

Materials and methods: A case control study of 100 NSCLC patients and 100 cancer free healthy controls was performed. TP53 codon 72 polymorphism and mutations at exon 5 and 8 were analyzed in NSCLC patients using AS–PCR and survival curves were plotted using Kaplan–Meier analysis.

Results: A statistically significant difference was observed in the frequencies of TP53 codon 72 variants between cases and healthy controls (p<0.003) with a strong association of risk of developing NSCLC with homozygous Pro/Pro genotype, OR 5.3 (95% CI 1.8-15.3, p<0.001). TP53 mutations at exon 5/8 occurred in 78% of the cases and Pro/Pro genotype of codon 72 was associated with increased number of P53 mutations, OR 4.7 (95% CI 0.5-44.8); Pro/Pro homozygotes, 16 of 17 (94.1%); Arg/Pro heterozygotes, 45 of 61 (73.8%); and Arg/Arg homozygotes, 17 of 22 (77.3%). Codon 72 Pro/Pro homozygotes were associated with poor overall survival and the Pro/Pro genotypes with P53 mutations also predicted decreased overall survival. The median survival time for patients with wild type Pro and mutated P53 with Arg/Arg and Pro/Pro codon 72 genotypes were 14.5, 11.5 and 4.0 months respectively (p=0.003).

Conclusion: Pro/Pro variant of P53 codon 72 was associated with increased number of P53 mutations, and was associated with adverse clinical outcome of NSCLC patients of north India.

Keywords: p53 codon 72 polymorphism; p53 mutations; NSCLC patients

Abbreviations: SCC: Squamous Cell Carcinoma; ADC: Adenocarcinoma; NSCLC: Non Small Cell Lung Cancer; AS–PCR: Allele Specific Polymerase Chain Reaction

Introduction

Lung cancer is the most commonly diagnosed cancer worldwide, 1.61 million, 12.7% of the total and is also the leading causes of cancer deaths, 1.38 million, and 18.2% of the total [1]. NSCLC is the major type of lung cancer, accounts for 85% of lung cancer cases with 5-year survival rate of 11 to 15 percent overall, and less than 5 percent at stage III/IV [2]. India has one of the highest cancer rates in world estimated more than 5.5 lakh deaths in 2010 and are projected to increase to 7 lakhs by 2015 [3]. Cancer mortality rates varied greatly in different states, including lung cancer the most common cancer in men in cities like Calcutta, Mumbai and New Delhi, accounting more than 11% in men, aged 30–69 years [3].

Human TP53 gene located in chromosome 17p13.1, encoded by 53 kDa protein of 393 amino acids, plays its anti-cancer role in several mechanisms such as DNA repair, cell cycle arrest and apoptosis etc [4]. Mutations within the p53 gene itself or mutations of downstream mediators of p53 lead to inactivation of its function [4]. More than 18,000 acquired mutations in the p53 gene have been identified in all major types of human cancers. Inactivation of the TP53 tumor suppressor gene is a key and thought to be an early event in lung carcinogenesis [5]. Mutations in TP53 are present in >90% of small cell lung cancers and >50% of non-small cell lung cancers [6-8]. The majority of these mutations are missense mutations in exons 5-8, the DNA-binding domain of p53 [9].

A common polymorphism of p53 at codon 72 (rs1042522) results in either variant of the protein with an arginine (CCG) or a proline (CCC) residues with different tumor suppression abilities [10,11]. The association of p53 codon 72 polymorphism has been studied in
lung carcinoma, but the results are in contradictory [12-20]. Presence of either of the codon 72 variant, arginine or proline had shown a dominant influence on biological and biochemical activities of mutated p53 gene [21-26] So, the aim of the present study was to find the association of mutated p53 gene with different codon 72 variants on the clinical outcome of North Indian NSCLC patients.

Materials and Methods

Sample collection and DNA extraction

Blood samples from newly diagnosed 100 NSCLC patients and 100 non-cancer individuals as controls were selected from an ongoing molecular study of lung cancer in the department of biochemistry, MAMC and associated hospitals New Delhi. Patients with a history of previous cancer or metastasized cancer from other organs except lung were excluded. All controls, like the cases, were the residents of North India. Written informed consent was obtained from all participants and patient follow up was obtained through hospital records as well as by direct patient contact. The study was approved by the institutional ethics committee, MAMC, New Delhi. Blood samples anti-coagulated with EDTA were stored at -70°C until use and genomic DNA was extracted using DNA sure blood mini kit (Nucleo-pore Genetix brand).

Study population

Clinical characteristics among NSCLC patients and healthy controls are summarized in Table 1. Briefly, NSCLC patients constitute equal number of two major subtypes, Squamous cell carcinoma and Adenocarcinoma (50% vs 50%) in early 35% as well as advanced 65% TNM stage including 28% of patients with metastasis to distant organs. Well differentiated Squamous cell carcinoma (56.0%) and poorly differentiated Adenocarcinoma (54.0%) represented a major portion in cytological grade of NSCLC cases. In addition family history of lung cancer or any other cancer was analyzed in just 18.0% cases. Both cases and controls include 80 males and 20 females of age in ≤ 45 group (range, 30-85 years) and controls of 56.19 ± 10.9(range, 32-80 years).

PCR amplification

PCR was performed in 25 µl reaction volume containing 5 µl of 50 ng template DNA, 0.25 µl 25 pmol each Primers, 2.5 µl 10 mM dNTPs, 1.5 µl of 20 mM MgCl₂, 0.3 µl of 5 U/µl Taq polymerase (Fermantas) with 2.5 µl of 10 × Taq Buffer (Fermantas) and 12.7 µl of nuclease free ddH₂O. As negative control, a sample without DNA template was also included in the PCR reaction to ensure that no contamination was introduced. Specific primers and PCR conditions are illustrated in Table 2. PCR products were analyzed on 2.0% agarose gel, stained with ethidium bromide and photographed on a UV Gel doc system (Figure 1).

Statistical analysis

The statistical analysis was performed using the Graph Pad Prism 6.0 and SPSS software version 17. Kaplan–Meier plots and log–rank (Mantel–Cox) test were used to evaluate the relationship between p53 and overall survival of NSCLC patients. The associations between p53 polymorphism/mutations and NSCLC were determined using the logistic regression method to assess odds ratio (ORs) and confidence intervals (95% CI). Allele frequencies between the cases and controls were evaluated by using the Chi–square Hardy-Weinberg equilibrium test and values below 5 were analyzed by Fisher Exact Test. Mean ± SD were calculated through Kolmogorov-Smirnov test (KS-test). Differences were regarded as significant with P<0.05.

Results

p53 polymorphism and NSCLC

The Arg/Pro genotype distributions of p53 codon 72 among cases and controls are summarized in Tables 3 and 4. We observed a statistically significant difference in the frequencies of p53 Arg/Arg, Arg/Pro and Pro/Pro genotypes among patients and healthy controls (Chi square=1.155 and p=0.003). The frequency of Pro/Pro (0.47) was found to be higher among cases whereas controls presented with Arg/Arg genotype frequency in higher rate (0.68). A multivariate analysis based on logistic regression like odds ratio with 95% confidence intervals, revealed higher risk of developing NSCLC in association with homoyzogous Pro/Pro genotype, OR 5.3 (95% CI 1.8-15.3, p<0.001).

Upon further stratification of p53 codon 72 genotypes with various clinical and pathological characteristics of NSCLC patients we observed statistically significant association with TNM stage (p<0.0001), distant metastasis (p<0.0001) and cytological differentiation among...

TP53 mutations at exon 5 or exon 8 occurred in 78% of the non small cell lung cancer patients. The relationships among p53 mutations and p53 codon 72 polymorphism with various clinical and pathological variables are shown in Table 5. Males and old age group patients showed Pro allele dominance than Squamous cell carcinoma but in both the cell types Pro allele was associated with poor cytological differentiation (0.70 and 0.50) respectively. In addition no significant difference was observed in association with smoking status. However, the interaction between p53 codon 72 genotypes with other parameters like age, gender, smoking status or family history of any cancer was not statistically significant.

Table 2: Primers used for p53 polymorphism and mutations.

<table>
<thead>
<tr>
<th>Exon 4 (Codon 72) Polymorphism</th>
<th>Primer Sequence</th>
<th>Tm</th>
<th>PCR Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg/Arg</td>
<td>A1: 5'-TCCCCCTTGCCGTCGCAA-3'</td>
<td>60°C</td>
<td>141bp</td>
</tr>
<tr>
<td></td>
<td>A2: 5'-CTGGTGACGGGCCAGCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg/Pro</td>
<td>P1: 5'-GCGAGAGGTGCTTCGCCC-3'</td>
<td>55°C</td>
<td>177bp</td>
</tr>
<tr>
<td></td>
<td>P2: 5'-CGTGGAAGCTCAGAGCTT-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Over all frequency of p53 exon 4 (72 codon) polymorphism in NSCLC patients and controls.

<table>
<thead>
<tr>
<th>Exon 4 (Codon 72) Polymorphism</th>
<th>Genotype (%)</th>
<th>Pro Allele Frequency</th>
<th>Controls (n=100)</th>
<th>Genotype (%)</th>
<th>Pro Allele Frequency</th>
<th>OR(95%CI)</th>
<th>Chi Square</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg/Arg</td>
<td>22/100 (22.0)</td>
<td>0.47</td>
<td>41/100 (41.0)</td>
<td>0.32</td>
<td>1</td>
<td>11.55</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Arg/Pro</td>
<td>61/100 (61.0)</td>
<td>0.75</td>
<td>53/100 (53.0)</td>
<td>2.2</td>
<td>(1.1-4.1)</td>
<td>5.6</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>17/100 (17.0)</td>
<td>0.15</td>
<td>6/100 (6.0)</td>
<td>5.3</td>
<td>(1.8-15.3)</td>
<td>10.3</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Agarose gel electrophoresis of TP53 gene: a) Codon 72 Polymorphism: P1-homozygous arginine, P2-heterozygous Arg/Pro, P3-homozygous proline and L8-negative control, L1:100bp molecular weight marker. b) Codon 168 mutation: P1 and P3-codon 168 mutant, P2-codon 168 normal, C-negative control c) Codon 282 mutation: P1 and P3-codon 282 mutant, P2-codon 282 normal, C-negative control, L:100bp molecular weight marker.

Table 4:

<table>
<thead>
<tr>
<th>Exon 4 (Codon 72) Polymorphism</th>
<th>Cases (n=100)</th>
<th>Pro Allele Frequency</th>
<th>Controls (n=100)</th>
<th>OR(95%CI)</th>
<th>Chi Square</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg/Arg</td>
<td>22/100 (22.0)</td>
<td>0.47</td>
<td>41/100 (41.0)</td>
<td>0.32</td>
<td>1</td>
<td>11.55</td>
</tr>
<tr>
<td>Arg/Pro</td>
<td>61/100 (61.0)</td>
<td>0.75</td>
<td>53/100 (53.0)</td>
<td>2.2</td>
<td>(1.1-4.1)</td>
<td>5.6</td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>17/100 (17.0)</td>
<td>0.15</td>
<td>6/100 (6.0)</td>
<td>5.3</td>
<td>(1.8-15.3)</td>
<td>10.3</td>
</tr>
</tbody>
</table>
were found to be associated with an increased risk of p53 mutations (OR, 1.24 and 4.2) respectively. Also patients at advanced stage with metastasis to distant organs were at higher risk of p53 mutations (OR, 5.0: CI 1.08-23.0). In addition higher p53 mutation positivity was observed in adenocarcinoma patients (OR, 2.6: CI 0.97-7.2), whereas in both the cell types poor cytological grade represented increased risk of p53 mutations.

Pro/Pro genotype of codon 72 was associated with increased number of p53 mutations, OR (4.7 CI 0.5-44.8); Pro/Pro homozygotes. 16 of 17 (94.1%); Arg/Pro heterozygotes, 45 of 61 (73.8%); and Arg/Arg homozygotes, 17 of 22 (77.3%). However, among Arg/Arg genotypes increased number of p53 Exon 8 mutation was observed than p53 Exon 5 mutation (59.1% vs 40.9%) whereas there was equal prevalence of both the mutations among Pro/Pro genotypes (Figure 2).

Irrespective of p53 codon 72 polymorphism, adenocarcinoma cell type was found to be associated with higher percentage of p53 mutations whereas taking the codon 72 in consideration, all of the variants carried almost same number of mutations in adenocarcinoma, but in case of squamous cell type prevalence of mutations was observed to be more among Arg/Arg codon 72 genotypes. Whereas, among Pro/Pro codon 72 homozygotes p53 mutations were more common in both the histological types.

**Survival outcome NSCLC patients with p53 polymorphism and mutations**

Kaplan-Meier survival analysis was performed to analyze the relationship of p53 codon 72 polymorphism and mutations with overall survival of 100 NSCLC patients with mean follow-up time of 12.0 months (median 10.0; range, 0.5-127.5 months). At the end of the follow up period number of deaths were 64 (64.0%) with mean follow up time of 10.4 months (median 10.0; range, 0.5-26.0 months) and for the patients who survived the follow up period (censored patients), the follow up time was 14.9 months (median 9.8; range, 0.5-127.5 months).

Patients with Arg/Arg codon 72 genotype had the longer survival time and Pro/Pro genotype carriers the lowest with intermediate overall survival among Arg/Pro codon 72 heterozygotes (Figure 3). Median survival time for patients with p53 Arg/Arg, Arg/Pro and Pro/Pro genotypes were 14.5, 11.5, and 4.0 months respectively (p=0.003). Presence of Pro/Pro genotypes in adenocarcinoma and advanced TNM stage patients with metastasis to distant organs also showed lower median survival time than SCC and Early stage NSCLC patients (Median 3.0 and 5.0 vs 8.0 and 9.0 months respectively). Studies have demonstrated the differential mutant p53 activity carrying arginine compared to proline at codon 72. So, we also analyzed the association of codon 72 variants and p53 mutations on overall survival of NSCLC.

**Table 4:** Frequency of P53 exon 4 (72 codon) in NSCLC patients.
Discussion

TP53 tumor suppressor gene is the most commonly mutated gene in almost every types of human cancer and TP53 alterations are the early events in lung carcinogenesis [27-31]. Present study focused on the association between the TP53 mutations on exon 5/8 with common codon 72 polymorphic, Arg/Pro variants of p53 gene and their clinical significance in NSCLC patients of North India.

Several studies have reported a significant association between p53 codon 72 Arg/Pro variations and cancer susceptibility including lung cancer; however, the results have been controversial [12,14,17,20,32-35]. Wild type p53 with Arg72 demonstrated to be more efficient in inducing apoptosis and a potent tumor transformation suppressor than the Pro72 variant [21,36-38]. Arginine form of p53 also showed high binding affinity to MDM2, leads to an enhanced transport to mitochondria and the form is more potent at inducing expression of cell cycle inhibitor p21. [36,39,40]. In vitro study on cell lines predicted decreased DNA repair capacity and apoptosis ability of p53 codon 72 Pro variant following radiations and benzopyrene exposure [33]. TP53 codon 72 polymorphism has been found to be associated with an increased risk of lung cancer [41]. Our results suggest that the p53 codon 72 Pro/Pro is associated with increased risk of developing NSCLC with approximately more than 5 fold increase than homozygous Arg/Arg variants and was an independent prognostic marker of unfavorable clinical outcome of NSCLC patients. Our results were concordant with the results of recent studies based on large lung cancer case control series [32,33]. p53 codon 72 polymorphism shows a strong relation with adenocarcinoma than Squamous cell carcinoma, which have been also suggested by various other studies [17,23,42]. Increase in cancer risk among Pro72 carriers was observed as the number of pack-years smoked increased, the same was observed by Fan et al. [17].

In vitro studies have indicated that the mutated p53 with Arg 72 is more effective in inhibiting P73-dependent apoptosis [22,24,26]. Consistent with our observation, p53 codon 72 Pro variant was a strong predictor of unfavorable clinical outcome of NSCLC patients. Our results were concordant with the results of recent studies based on large lung cancer case control series [32,33]. p53 codon 72 polymorphism shows a strong relation with adenocarcinoma than Squamous cell carcinoma, which have been also suggested by various other studies [17,23,42]. Increase in cancer risk among Pro72 carriers was observed as the number of pack-years smoked increased, the same was observed by Fan et al. [17].

In vitro studies have indicated that the mutated p53 with Arg 72 is more effective in inhibiting P73-dependent apoptosis [22,24,26]. Consistent with our observation, p53 codon 72 Pro/Pro variants were
found to be associated with increased frequencies of p53 mutations with poor prognostic predictors in lung cancer [9,43]. Study on ovarian cancer also reported association of frequent p53 mutations with Pro72 genotype [44]. In contrast, few studies have reported, Arg/72 variants with more number of p53 mutations but the poor prognosis were associated with mutated Pro72 genotypes [45-47]. Adenocarcinoma was associated with frequent p53 mutations but squamous cell carcinoma presented with more prevalence of mutations among Arginine codon 72 genotypes. A strong correlation of p53 mutations was found to be in patients with adenocarcinoma; however, the complete dependence on histology was not statistically significant [43]. TP53 mutations usually precede metastasis [48], our results showed higher frequency of Pro72 homozygotes and a 5 fold risk of developing metastasis with the increased incidence of TP53 mutations in patients of distant organ metastasis. p53 mutation spectra of smokers were entirely distinct with certain mutations at high rates, but were uncommon among non-smoker lung cancer cases [49-51]. Present study shows higher incidence of p53 codon 168 and 282 mutations among nonsmoker patients. Reports from several in vitro and in vivo studies have indicated resistance to chemotherapies in association with mutated p53 gene [52-54], and the presence of p53 codon 72 Arg/Pro variants also plays an important role in the biology of mutated p53 [21-26], confirming the hypothesis that the p53 gene may serve as a marker of tumor response to treatment in NSCLC.

Conclusion

In conclusion, present study demonstrates that the p53 codon 72 polymorphism may contribute to risk of cancer susceptibility and adverse clinical outcome, which is further added by increase in other p53 mutations especially on Pro/Pro variant of p53 codon 72 polymorphism among NSCLC patients of North India. In addition, p53 genotyping with mutation analysis can be useful to select specific candidate of NSCLC for p53 targeted strategies as well as in the management of treatment with conventional chemotherapies. The findings of the current study are limited due to small sample size in the strata, our results need to validate by large size studies, designed to compare characteristics of p53 mutations harboring either Arg 72 or Pro 72 variants of p53 gene.

Acknowledgment

The authors thank the oncologists and patients from Regional Cancer Center SKIMS, Srinagar and All India Institute of Medical Sciences, New Delhi, for their cooperation and participation.

References
