Clinical and Prognostic Significance of R282W p53 Gene Mutation in North India Patients with Non Small Cell Lung Cancer

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Abstract

Background: p53 plays a central role in protecting the integrity of the genome. Its activity is ubiquitously lost in cancers, either by inactivation of its protein (p53 pathway) or by mutation in the p53 gene, thereby indicating its importance in understanding cancer and as a therapeutic target. Given the high frequency of the hotspot R282W p53 gene mutation in our NSCLC patients, we have evaluated the association of R282W mutation with the progression of the malignancy.

Methods: Blood DNA was extracted from cases. The R282W hotspot p53 gene mutation was detected by using ASO-PCR. Most of the NSCLC patient's submitted samples for EGFR gene mutation analysis.

Results: The clinical significance of R282W hotspot p53 gene mutation in exon 8, codon 268 (C>T) was studied in hundred clinically confirmed NSCLC patients samples. Sixty two of hundred (62%) cases were reported positive for R282W p53 mutation. The clinically significant difference was reported between early and the advanced stages (72% *vs.* 43%) (p<0.007). Similarly higher frequency of this mutation was reported in adenocarcinoma (76.08%) than squamous cell carcinoma 27 (50%) (p<0.0134). Significantly higher frequency of R282W p53 mutation was reported in distant metastasis 23 (85.18%) than the metastasis (<0.0075), current smokers than the ex smokers (p=0.02).

These findings suggest that stage, smoking, histological type, metastasis is strongly associated with the incidence of R282W mutation. Other variables as gender, age, smoking level, and family history of any cancer does not showing any significant association with p53, R282W mutation. Slightly lower overall survival was reported in NSCLC patients with R282W mutation than wild p53 cases (p<0.049).

Conclusion: Our results suggest that the hotspot R282W p53 mutation may influence the susceptibility, progression of NSCLC patients in Indian population. Large population-based prospective studies are required to validate our findings.

Keywords: NSCLC: Non Small Cell Lung Cancer; ADC: Adeno-Carcinoma; SCC: Squamous Cell Carcinoma; ASO: Allele Specific Oligonucleotide

Introduction

Non Small Cell Lung Cancer (NSCLC) is the major cancer killer worldwide in both sexes, accounting for >1.2 million deaths each year [1]. As of 2002, the one year prevalence of lung cancer in India for males was 11,511, and the 5 year prevalence was 27,477 accounting for approximately 3% of global prevalence, and 55% of total prevalence in South Central Asia [2]. The p53 gene, located on the short arm of human chromosome 17, encodes for a nuclear phosphoprotein involved in the regulation of cell proliferation [3]. The mutant gene product, which tends to accumulate to high levels in cancer cells, is believed to exert a dominant negative effect over coexpressed normal p53. Alterations of either the gene or protein product have turned out to be one of the most common changes identified in human malignancies. In resected lung cancers, point mutations of the p53 gene have been found in all histologic types, including approximately 45% of resected NSCLC [4,5]. In most studies, it has found that the risk factors of getting lung cancer are related to high percentage of passive smoking [6], cooking oil vapours [7,8] and occupational exposures [9].

The genetic mistake of p53 in NSCLC, as a result of either p53 protein over-expression [10] or p53 gene mutation [11,12], is found to be strongly correlated with tumor grade and can predict a poor prognosis [13,14]. The p53 gene is a large gene composed of 11 exons and 10 introns that are made up of 420,000 bp [15,16]. However, 90%

of the known mutations exist in exons 4–9, 70% of research studies focusing on exons 5–8. This focus is because p53 exons 5–8 code for the DNA-binding domain of the p53 protein, an area where the structure can be highly affected by sequence changes [17]. In solid tumours, p53 gene mutations are generally considered to be a late event in carcinogenesis because they pre-dominate in advanced stages of the disease and have been correlated with short survival in carcinoma of the breast, prostate, lung, and stomach [18,19].

The main reason why the p53 gene is so frequently mutated in cancers (overall in over 50% of invasive cancers) is that the p53 protein plays multiple, coordinated anti-proliferative roles in response to many different types of stress stimuli [4,20]. The p53 tumor-suppressor gene is commonly mutated in human cancer [21], and 30%–80% of human

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carcinomas contain sectors with a mutation in this gene, depending on the type and stage of the tumor investigated [22,23]. Mutation in p53 has been found to be a prognostic factor in several cancers, but whether it is an independent prognostic factor, is not yet known [24-26]. The p53 plays a central role in protecting the integrity of the genome. Its activity is ubiquitously lost in cancers, either by inactivation of its protein (p53 pathway) or by mutation in the p53 gene, thereby indicating its importance in understanding cancer and as a therapeutic target. Given the high frequency of the p53 gene mutation in NSCLC patients, we investigated whether the R282W p53 gene mutation influences progression of Non small cell lung cancer in north Indian populations.

Materials and Methods

Study population

Non Small Cell lung cancer patients were assessed on the basis of clinical and pathological examinations. This study is a Hospital-based study conducted on North Indian population. All incidents of NSCLC cases are newly diagnosed during the study period, Ethical committee approved the study. The procedures followed according to the ethical standards of responsible committee of the Institutes/Hospitals, to participate in a face-to-face interview using a structured questionnaire.

Selection criteria

Senior pathologists confirmed all diagnoses. We interviewed and collected the demographic factors data from the patient. We collected the information on age, smoking, chewing, usual alcohol intake, and previous cancer diagnoses. Participant's family history of cancer and the clinical information for these cases are obtained from medical records, tumor size, stage, and chemotherapy drugs. Patients were recruited based on inclusion and exclusion criteria, which were determined before the beginning of the study.

Sample collection

A total of 100 Non Small Cell lung cancer patients are enrolled in the study. Sampling was done from two major hospital, MAMC and Associated hospitals New Delhi, All India Institute of Medical Sciences, New Delhi and Department of Radiation Oncology, SKIMS, Srinagar, India between the periods June 2009 to September 2012. Most of the NSCLC patients were referred to our molecular oncology lab for EGFR gene mutation screening. From each patient 3 to 5 ml peripheral blood sample was collected in EDTA collection vials in the Molecular oncology lab.

DNA extraction from blood

Genomic DNA was extracted from blood samples using DNA sure blood mini kit (Nucleo-pore Genetix brand) according to the manufacturer's protocol.

Mutation Analysis by ASO-PCR

The mutation was detected by allele specific oligonucleotide PCR in which allele specific primers were used, one was specific for C allele and other primer was for T allele. The R282W p53 mutation in exon 8(C>T) was evaluated with reaction mixture of 25 μl containing 2.5 μl of 400 μg/μl DNA, 0.25 μl of 25 pmol of each primer (Table 1) and previously used by Stoehr et al. [27] 2.5 μl of 10X PCR buffer, 2.5 μl of 10 mM dNTP mixture, 0.3 μl of 3U/L Taq polymerase and 15.75 μl of nuclease free water.

The thermal cycler conditions used consists of 35 cycles of denaturation for 40 sec at 94°C, annealing for 40 sec at 58°C, and extension for 40 sec at 70°C.

The amplification was performed under the following conditions: 95°C for 5 minutes for initial denaturation followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 40 seconds, extension at 72°C for 1 minute 30 seconds and completed with a final elongation step at 72ºC for 5 minutes. The PCR products were visualized with ethidium bromide on a 2% agarose gel under a UV transilluminator. The amplimers obtained had a band-size of 207 bp (Figure 1).

Statistical Analysis

The variables measured in the study were investigated for association by using the Hardy Weinberg equilibrium equation. The differences in the incidence of p53 mutations among dependent variable like tumor type, stage, histological type, cytological type, sex, smoking history, smoking level, metastasis and family history with of any cancer were calculated by the Chi square test. The Kaplan-Meier method was used to calculate the overall survival in all 100 patients with p53 R282W mutations or without p53 R282W mutations. Statistical difference was considered significant for P values <0.05. SPSS version17 was used for analysis.
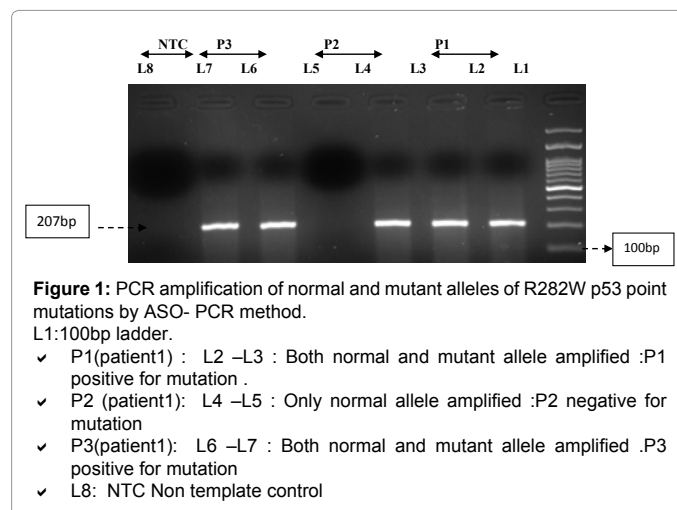
Result

Demographic characteristics of study population

The study population consists of clinically confirmed NSCLC patients. All demographic features of the subjects are depicted in the table 2. A total of hundred Non Small Cell lung cancer patients were analyzed among which 47 were adenocarcinoma and 53 squamous cell carcinoma and 35 patients were in early Stage (I & II) and 65 in advanced Stage (III & IV). Seventy smoked cigarettes, bidi and hukka (pipe) and 30 cases were of non-smokers were defined as subjects who had not smoked ever while in smokers there were 28 cases of current smokers and 42 were ex-smokers. In hundred cohorts of NSCLC patients, 27 were metastasised and 63 were none metastasised as

Primer type	Sequences	AT	Size
Forward	CTTGTCCTGGGAGAGACC	58°C	207
Forward	CTTGTCCTGGGAGAGACT		
Reverse (common)	GAGCTGGTGTTGTTGGGC		

Table 1: Primer sequences, annealing temperature and product size.

Figure 1: PCR amplification of normal and mutant alleles of R282W p53 point mutations by ASO- PCR method.
L1:100bp ladder.
- ✔ P1(patient1) : L2 –L3 : Both normal and mutant allele amplified :P1 positive for mutation .
- ✔ P2 (patient1): L4 –L5 : Only normal allele amplified :P2 negative for mutation
- ✔ P3(patient1): L6 –L7 : Both normal and mutant allele amplified .P3 positive for mutation
- ✔ L8: NTC Non template control

depicted in table 2. Out of hundred NSCLC patients, 62(62%) were positive for p53 R282W mutation and 38 were negative. The difference was statistically significant (p<0.0011). The p53 mutation status in relation to clinicopathological features is shown in the table 3.

Frequency of R282W p53 mutation with respect to histological type, gender and age

The significant association was seen between R282W mutation and histological types of lung cancer patients. The higher frequency was seen in ADC (76%) than SCC (50%). The difference was statistically significant (p<0.0134). There was not much difference in R282W mutation in NSCLC patients with respect to gender however the higher frequency of this mutation was reported in lower age group ≤ 45(84.61%) than higher age group >45(58%).

Frequency of R282W mutation with respect to stage

Among the different stages, the higher frequency of R282W mutation was reported in NSCLC patients in advanced stage (72%) than the early stages (42.85%). The difference was statistically significant (p<0.0007). Also the progression was reported to be faster among the NSCLC patients in advanced stage with R282W mutation.

Frequency of R282W p53 mutation with respect to smoking type

It has been indicated that there is a strong coincidence of G to T transversion hotspots in lung cancers and sites of preferential formation of PAH adducts along the p53 gene. A significant association was seen between the frequency of R282W p53 mutation in current smokers (82.14%) and exsmokers (52.38%), (p<0.02). There was a
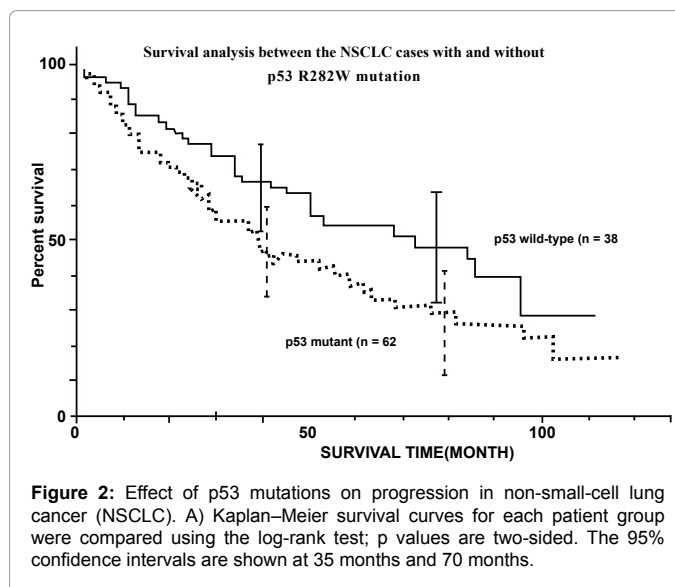

Figure 2: Effect of p53 mutations on progression in non-small-cell lung cancer (NSCLC). A) Kaplan–Meier survival curves for each patient group were compared using the log-rank test; p values are two-sided. The 95% confidence intervals are shown at 35 months and 70 months.

strong coincidence of C to T transversion hotspots in our lung cancers patients.

Frequency of p53 R282W mutation with respect to metastasis

A significant association was seen between R282W p53 gene mutation and metastasis. The significantly higher frequency of R282W p53 mutation was reported in NSCLC patients with metastasis (85%) (p<0.0075).

Frequency of p53 R282W mutation with respect other clinicopathological feature

There was no significant association between level of smoking (mild, moderate and heavy), cytological type, family history and p53 R282W gene mutation.

Survival Analysis

Overall survival of patients with stage I, II, or IIIA NSCLC was statistically significantly lower in those with p53 mutant tumours than in those with p53 wild-type tumors (p<0.049). The survival analysis between the NSCLC cases with and without R282W p53 mutation show some good correlation (Figure 2).

Recent studies have indicated that there is a strong coincidence of G to T transversion hotspots in lung cancers and sites of preferential formation of PAH adducts along the p53 gene. The identification of R282W p53 mutation acting as modifiers of p53 protein may assist in the assessment of individual cancer progression and risk in NSCLC families. It may also play an important role in the delineation of cancer screening and intervention guidelines in these patients.

It may also help in appropriate management protocols, taking into account the risk of developing cancer either earlier or later in life.

Discussion

To the best of our knowledge, the present study is the first report of prevalence of p53 R282W (exon-8, C>T) mutation in NSCLC patients from India. NSCLC is characterized by multiple genetic alterations in proto-oncogenes and in the tumour suppressor genes. p53 is the most common molecular events in the NSCLC, suggesting a key role in lung tumour carcinogenesis. Mutations in codons 175,

Variable	No of patients (%)	P Value
Total No of cases.	100	
Sex		
Males	80	0.95
Females	20	
Age (Years)		
≤45	13	0.134
>45	87	
Smoking Status		
Non Smokers	30	0.6242
Smokers	70	
Current Smokers	28	0.02
Ex. Smokers	42	
Smoking Level (Pack Year)		
Mild (≤10)	07	0.4471
Moderate (≤ 40)	33	
Heavy (> 40)	30	
Histological type		
ADC	46	0.0134
SCC	54	
Stage of the disease		
Early Stage (I & II)	35	0.007
Advanced Stage (III & IV)	65	
Metastasis		
Positive	27	0.0075
Negative	73	
Family History		
Significant	18	0.8553
Non Significant	82	

Table 2: Demographic Characteristics of NSCLC Patients.

P53 R282W p53 gene mutation					
Variables	+ve case	- ve case	Chi-Square	df	P value
Males	50(62.5%)	30(37.5%)	0.0027	1	0.95
Females	12(60%)	8(40%)			
Age group					
≤45	11(84.61%)	2(15.38%)	2.2343	1	0.134
>45	51(58.62%)	36(41.37%)			
Stage of the disease					
Early Stage (I & II)	15(42.85%)	20(57.14%)	7.1718	1	0.007
Advanced Stage (III & IV)	47(72.30%)	18(27.69%)			
Smoking status					
Non Smoker	17(56.66%)	13(43.33%)	0.24	1	0.6242
Smokers	45(64.28%)	25(35.72)			
Current Smoker	23(82.14%)	5(17.85%)	5.25	1	0.02
Ex Smoker	22(52.38%)	20(47.61%)			
Smoking level (pack year)					
Mild (≤10)	6(85.71%)	1(14.28%)	1.61	2	0.4471
Moderate (≤ 40)	20(60.60%)	13(39.39%)			
Heavy (> 40)	19(63.33%)	11(36.66%)			
Histological type					
ADC	35(76.08%)	11(23.91%)	6.1105	1	0.0134
SCC	27(50%)	27(50%)			
Cytological type(ADC)					
Well Differentiated	6(77.77%)	3(22.22%)	0.66	2	0.7189
Moderately Differentiated	9(75%)	3(25%)			
Poorly Differentiated	20(80%)	5(20%)			
Cytological type(SCC)					
Well Differentiated	14(46.66%)	16(53.33%)	2.8	2	0.2466
Moderately Differentiated	10(66.66%)	5(33.33%)			
Poorly Differentiated	3(33.33%)	6(66.66%)			
Metastasis					
Positive	23(85.18%)	4(14.81%)	7.1447	1	0.0075
Negative	39(53.42%)	34(46.57%)			
Family history of any cancer					
Significant (Positive)	11(61.11%)	7(38.88%)	0.0332	1	0.8553
Non Significant (Negative)	51(62.19%)	31(37.80%)			

Table 3: Clinicopathological feature of NSCLC patients.

245, 248, 273, and 282 are the most common in sporadic tumours [28]. In present study the frequency of p53 R282W mutations 62%, patient with adenocarcinoma have high frequency of p53 R282W mutation (76.08%) than the squamous cell carcinoma (50%).

Present study revealed a significant association of p53 R282W (exon-8, C>T) with increased risk of NSCLC if patients in advanced Stage (III & IV) found more risk for the p53 R282W gene mutation 72.30% than the early Stage

(I & II) 42.85%. In addition current smokers have high frequency of p53 R282W (82.14%) mutation than ex smokers (52.38%) and non smokers (56.66%). Patients with distant metastasis also have high frequency for p53 R282W mutation in comparison to case without any distant metastasis, where the metastasis have 85.18% positive cases for mutation while no metastasis cases is with 53.42%. Other group like gender, age, smoking level, cytological type and patients with family history with any type of cancer does not show any significant association. Frequency of p53 R282W mutation in poorly differentiated cytological type in ADC (80%) is high and moderately differentiated cytological type is high in SCC (66.66%). As we analysed the level of smoking those who smoked more than 10 pack year have high frequency of p53 R282W mutation.

Rozenblum et al. [29], who analyzed p53 in exons 2–11 in first passage xenografts from 47 resected pancreatic cancer, recently reported p53 mutation in 76% of cases. Investigators analyzed p53 chiefly in exons 5–8, which is highly conserved through evolution and presumably of functional importance, 95% of the reported mutations have been found in exons 5–8 [30]. However, of 560 mutations in entire coding region of p53 was sequenced, 87% were in exons 5–8, and most of the others were in exons 4(8%) and 10(4%) [4].

Information on p53 database has indicated that 80% are GC to AT transitions occurring predominantly at CpG islands. Mutations in five hotspots codons (175, 245, 248, 273 and 282) accounted for approximately 43% of all p53 mutations in colorectal cancer [31]. All mutations were present in exons 5–8, which encode the DNA binding domain. Based on the updated p53 gene mutation database containing 5961 mutations, codons 175, 245, 248, 249, 273 and 282 were identified as hotspots mutation in human cancers [32].

Conclusion

The incidence of hotspot R282W p53 mutation in our NSCLC patients showed a tendency toward association with progressive disease status. Although the associations appeared to be statistically significant in our population, these initial findings should be independently

verified by other large independent population-base studies to validate our findings.

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