

# Clinical implications of cytosine deletion of exon 5 of P53 gene in non small cell lung cancer patients

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## Abstract

**Aim:** Lung cancer is considered to be the most common cancer in the world. In humans, about 50% or more cancers have a mutated tumor suppressor p53 gene thereby resulting in accumulation of p53 protein and losing its function to activate the target genes that regulate the cell cycle and apoptosis. Extensive research conducted in murine cancer models with activated p53, loss of p53, or p53 missense mutations have facilitated researchers to understand the role of this key protein. Our study was aimed to evaluate the frequency of cytosine deletion in non-small cell lung cancer (NSCLC) patients. **Methods:** One hundred NSCLC patients were genotyped for P53 (exon5, codon 168) cytosine deletion leading to loss of its function and activate the target genes by allele-specific polymerase chain reaction. The P53 cytosine deletion was correlated with all the clinicopathological parameters of the patients. **Results and Analysis:** 59% cases were carrying P53 cytosine deletion. Similarly, the significantly higher incidence of cytosine deletion was reported in current smokers (75%) in comparison to ex-smoker and non-smoker. Significantly higher frequency of cytosine deletion was reported in adenocarcinoma (68.08%) than squamous cell carcinoma (52.83%). Also, a significant difference was reported between p53 cytosine deletion and metastasis (64.28%). Further, the majority of the cases assessed for response carrying P53 cytosine deletion were found to show faster disease progression. **Conclusion:** The data suggests that there is a significant association of the P53 exon 5 deletion of cytosine in codon 168 with metastasis and staging of the disease.

**Key words:** Non-small cell lung cancer, adeno-carcinoma, squamous cell carcinoma, allele specific oligonucleotide, P53 (exon5, codon 168) cytosine deletion

## Introduction

The p53 gene, located on the short arm of human chromosome 17, encodes for a nuclear phosphoprotein involved in the regulation of cell proliferation.<sup>[1]</sup> Non-small cell lung cancer (NSCLC) is the major cancer killer worldwide in both sexes, accounting for >1.2 million deaths each year.<sup>[2]</sup> Wild-type p53 gene protein is an important component of the pathway leading from DNA damage to apoptosis because p53 protein is implicated in multiple functions that include control of cell cycle, DNA repair, cell senescence, genomic stability, and stress responses.<sup>[3,4]</sup> P-53 gene abnormalities are the most frequent genetic events illustrated to date.<sup>[5,6]</sup>

P53 mutation and aberrant p53 gene product expression in over half of adult cancers, including lung, breast, colon, oesophagus, and skin cancers, are now considered to be one of the most common genetic features in a wide range of human cancers.<sup>[7,8]</sup> It has been shown that angiogenesis is required for the growth and metastasis of human solid tumours,<sup>[9]</sup> and several studies have demonstrated that the p53 tumour suppressor gene plays an important role in controlling tumour angiogenesis.<sup>[10,11]</sup> These alterations can be explained by the presence of regionally distinct carcinogens in both smoke and air, interacting with local environmental cofactors, in the development of lung cancer. Besides, the genetic mistake of p53 in NSCLC, as a result of either p53 protein over expression<sup>[12]</sup> or p53 gene mutation,<sup>[13,14]</sup> is found to be strongly correlated with tumour grade and can predict a poor prognosis.<sup>[15,16]</sup> Steels *et al.* showed that the mutated p53 gene leads to poor survival in adenocarcinoma as well as in squamous cell carcinoma (SCC) in all stages of the disease.<sup>[17]</sup>

The p53 protein has been aptly referred to as the “guardian of the genome” because the p53 gene is induced by DNA damaging agents and subsequently either delays

cell-cycle progression, or steers the damaged cell headlong into programmed cell death. The p53 protein is a nuclear transcription factor that binds to the p21 promoter inducing its expression and inhibiting cell-cycle progression at the G1/S cell-cycle checkpoint.<sup>[18]</sup> Inactivation of the p53 gene appears to be the most common genetic alteration in human cancers and contributes to the development of over 50% of all human cancers.<sup>[19]</sup> The genetic mistake of p53 in NSCLC, as a result of either p53 protein over expression<sup>[20]</sup> or p53 gene mutation,<sup>[21]</sup> is found to be strongly correlated with tumour grade and can predict a poor prognosis.<sup>[22]</sup> Genetic abnormality of the TP53 in lung cancers has been shown to be associated with a poorer survival prognosis and increased cellular resistance to therapy.

## Materials and Methods

### Study subjects

The study included 100 clinically confirmed NSCLC patients. All demographic characteristics of NSCLC patients has been depicted in Table 1. Among 100 cases, 47 adenocarcinoma and 53 SCC, 80 were males and 20 were females, 86 cases of NSCLC are <45 age group and 14 of >45 age group. 35 Patients was in early Stage (I and II) and 65 in advanced Stage (III and IV). 70 cases of smokers had smoked cigarettes, bidi and hukka (pipe), 30 cases 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. According to pack year those 7 patients who have smoked <10 pack year are mild smokers, 33 patients are <40 pack year is moderate smokers, and 30 are Heavy smokers has smoked >40 pack year. Cytological investigations of adenocarcinoma's patients 9 were well differentiated, 13 were moderately differentiated and 25 were poorly differentiated. Cytological investigations of SCCs patients 31 were well differentiated, 12 were moderately differentiated and 10 were poorly differentiated. In hundred cohorts of NSCLC patients, 28 were metastasised, and 72 were none metastasised. Among 100 NSCLC patients, only 18 patients has the familial history of cancer, and 82 patients do not have any familial history of cancer. This study has been approved by the institutional ethics committee, Maulana Azad Medical College, New Delhi.

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### Sample collections and DNA isolation

A blood sample collected in EDTA vials and stored in  $-20^{\circ}\text{C}$ . Genomic DNA was extracted from using DNA sure blood mini kit (Nucleo-pore Genetix) according to the manufacturer's instructions.

### Genotyping

To search the deletion of cytosine at codon 168 (exon5) of p53 gene, allele-specific oligonucleotide (ASO) polymerase chain reaction (PCR) was used. Genotyping was done in 100 NSCLC subjects. The PCR amplification was done in 25  $\mu\text{l}$  reaction mixture containing 2.5  $\mu\text{l}$  of 400  $\mu\text{g}/\mu\text{l}$  DNA,

Primer type	Sequences	AT	Size
Forward (wild type)	CTACAAGCAGTCACAGCAC	55°C	150
Forward (mutant type)	CTACAAGCAGTCACAGCAA		
Reverse (common)	ATCAGTGAGGAATCAGAGG		

A 2.5  $\mu\text{l}$  of  $\times 10$  PCR buffer, 2.5  $\mu\text{l}$  of 10 mM dNTP mixture, 0.3  $\mu\text{l}$  of 3U/L Taq polymerase, 15.75  $\mu\text{l}$  of nuclease-free

**Table 1: Clinicopathological feature of NSCLC patients. P53, Exon 5 cytosine deletion of codon 168**

Variables	Positive case (%)	Negative case (%)	$\chi^2$	df	P
Gene					
P53	59 (59)	41 (41)	12.72	1	0.00036
Gender					
Males	48 (60)	32 (40)	0.06	1	0.7
Females	12 (60)	8 (40)			
Age group					
$\leq 45$	8 (57.14)	6 (42.85)	0.03	1	0.9
$> 45$	52 (60.46)	34 (39.53)			
Stage					
Early stage (I and II)	23 (65.71)	12 (34.28)	0.49	1	0.5
Advanced stage (III and IV)	37 (53.84)	28 (43.07)			
Smoking status					
Non smokers	17 (56.66)	13 (43.33)	3	2	0.1
Current smokers	21 (75)	7 (25)			
Ex. smokers	22 (52.38)	20 (47.61)			
Smoking level (pack year)					
Mild ( $\leq 10$ )	06 (85.71)	01 (14.28)	2.03	2	0.38
Moderate ( $\leq 40$ )	20 (60.60)	13 (39.39)			
Heavy ( $> 40$ )	17 (56.66)	13 (43.33)			
Histological type					
ADC	32 (68.08)	15 (31.91)	1.8	1	0.1
SCC	28 (52.83)	25 (47.16)			
Cytological type					
Well differentiated	6 (66.66)	3 (33.33)	0.09	2	0.9
Moderately differentiated	9 (69.23)	4 (30.76)			
Poorly differentiated	18 (72)	7 (28)			
Cytological type					
Well differentiated	12 (38.70)	19 (61.29)	4.7	2	0.09
Moderately differentiated	3 (25)	9 (75)			
Poorly differentiated	7 (70)	3 (30)			
Metastasis					
Positive	18 (64.28)	10 (35.71)	0.4	1	0.6
Negative	41 (56.94)	31 (43.05)			
Family history of any cancer					
Significant (positive)	11 (61.11)	07 (38.88)	0.004	1	0.9
Nonsignificant (negative)	48 (58.53)	34 (41.46)			

NSCLC=Non-small cell lung cancer, ADC=Adeno-carcinoma, SCC=Squamous cell carcinoma

water and 0.25  $\mu\text{l}$  of 25 pmol of each primer<sup>[23]</sup>. The primer properties and sequence is depicted in Table 2. A programmable thermal cycler was used to perform 40 cycles of denaturation for 40 s at  $94^{\circ}\text{C}$ , annealing for 40 s at  $55^{\circ}\text{C}$ , and extension for 40 s at  $70^{\circ}\text{C}$  to obtain the 150 bp amplified product. PCR products were visualized on 2% agarose gel containing ethidium bromide as depicted in the figure No 1.

### Statistics

Several variables included in this history to investigate the association with NSCLC using the Hardy-Weinberg equilibrium equation. The differences in the incidence of p53 deletion of cytosine nucleotide among dependent variable like tumour 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 positive p53 deletion of cytosine and negative for p53 deletion of cytosine. A  $P < 0.05$  were considered statistically significant.

### Results

Clinically diagnosed 100 NSCLC patients were used to analyse the cytosine deletion of P53 in exon-5. Out of 100 NSCLC patients, 59 (59%) were positive and was found statistically significant ( $P = 0.00036$ ). The clinicopathological information of NSCLC patients is shown in Table 1.

**Table 2: Demographic characteristics of NSCLC patients**

Variable	Number of patients (%)	$\chi^2$	df	P
Total number	100			
Gender				
Males	80	0.06	1	0.7
Females	20			
Age (years)				
$\leq 45$	14	0.03	1	0.9
$> 45$	86			
Smoking status				
Non smoker	30	3	2	0.1
Current smoker	28			
Ex. smoker	42			
Smoking level, pack year				
Mild ( $\leq 10$ )	07	2.03	2	0.38
Moderate ( $\leq 40$ )	33			
Heavy ( $> 40$ )	30			
Histological type				
ADC	47	1.8	1	0.1
SCC	53			
Stage				
Early stage (I and II)	35	0.49	1	0.5
Advanced stage (III and IV)	65			
Metastasis				
Positive	28	0.4	1	0.6
Negative	72			
Family history				
Significant	18	0.004	1	0.9
Nonsignificant	82			

NSCLC=Non-small cell lung cancer, ADC=Adeno-carcinoma, SCC=Squamous cell carcinoma

### Association and frequency of cytosine deletion of p53 with respect to gender and age

The present study indicates that deletion of cytosine in exon 5 of the p53 gene is equally contributed in males (60%) as well as in female (60%). However, >45 age group patients have 60.46% cases of cytosine deletion as compared to <45 age group.

### Association and frequency of cytosine deletion of p53 with respect to stage, smoking status and level

Non-small cell lung cancer cases diagnosed in early stage (I and II) have high frequency of cytosine deletion (65.71%) and have significant association ( $P = 0.016$ ) in contrast to advanced stage (53.84%). We examined the smoking status of NSCLC cases, where current smokers have a high frequency of cytosine deletion (75%) when compared with nonsmoker and ex-smokers. Cases analysed on the basis of smoking level; only mild smoker (<10 pack year) have high (85.71%) frequency of p53 cytosine deletion.

### Association and frequency of cytosine deletion of p53 with respect to histological type, cytological type, metastasis and family history of any cancer

In this study two types of NSCLC cases were selected (i) adenocarcinoma and (ii) SCC, Adenocarcinoma patients have high frequency (68.08%) of cytosine deletion and was significantly associated as compared to SCC (52.83%). Deletion of cytosine in exon5 of p53 in relation to cytological type of adenocarcinoma patients with poorly differentiated cell type have high frequency (72%) of cytosine deletion when compared with moderate and well differentiated cell type of cases. On the other hand, poorly differentiated cell type cases of SCC have high frequency (70%) with cytosine deletion in exon5 of the p53 gene in comparison to others. NSCLC cases with metastasis positive have high frequency (64.28%) of cytosine deletion in comparison to cases with metastasis negative.

### Point mutation in p53 (Exon-5, cytosine deletion at codon 168)

The amplified PCR product cytosine deletion of cytosine in exon 5 of the p53 gene is 150 bp as shown in the Figure 1. The deletion of cytosine in exon 5 of the p53 gene identified by ASO PCR.

### Point mutation in p53 (Exon-5, cytosine deletion at codon 168)

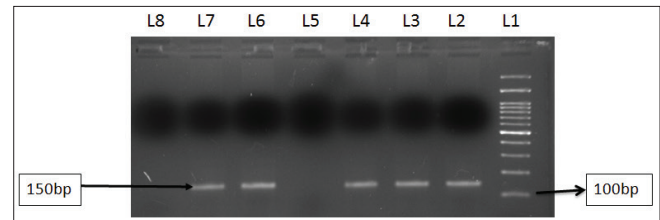
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### Survival analysis

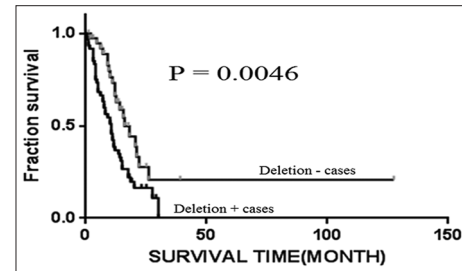
The Kaplan–Meier survival analysis between the NSCLC cases with p53 cytosine deletion in exon5 have less survival and significantly associated ( $P = 0.0046$ ). This study of p53 cytosine deletion in exon5 represents the poor survival of NSCLC patients [Figure 2].

### Discussion

The present study is the first report of the prevalence of p53, deletion of cytosine of exon5 in codon 168 in NSCLC patients from India in best of our knowledge. The P53 tumour-suppressor gene is commonly mutated in human cancer,<sup>[5]</sup> and 30–80% of human carcinomas contain sectors with a mutation in this gene, depending on the type and stage of the tumour investigated.<sup>[15,24]</sup> Mutations are present in exons 5–8, which encode the DNA binding domain.<sup>[25]</sup>



**Figure 1: Detection of p53, Cytosine deletion in exon 8 at codon 168 point mutations by ASO-PCR method, L1 indicates 100bp ladder, L2 lane is mutant, L3 lane is normal, L4 lane mutant, L5 lane is normal, L6-L7 lane in mutant and L8 lane is negative control**



**Figure 2: The Kaplan–Meier survival analysis**

Alterations in the p53 gene play an important role in the development of human lung cancer, occurring at an early stage in tumour development.<sup>[26,27]</sup> P53, deletion of cytosine in exon5 at codon 168 present in 59% of NSCLC cases while adenocarcinoma 68.08% and SCC 52.83%. P53 cytosine deletion is equally prevalent in male and female while in >45 age group of NSCLC patients have a high percentage of cytosine deletion. We found p53 cytosine deletion was associated with the smoking type.

In NSCLC p53, cytosine deletion have been linked to current smokers (75%) smoked cigarette, bidi and chukka may be a risk factor for NSCLC, and we also analysed the level of smoking only mild smokers have high frequency of deletion (85.71%) those who has smoked <10 pack year. Patients in early stage have a high risk of cytosine deletion (65.71%) and poor prognosis of NSCLC patients. Patients diagnosed with adenocarcinoma and poorly differentiated cell types have high (72%) frequency of cytosine deletion while patients diagnosed in SCC have poorly differentiated cell types also have high (70%) frequency of cytosine deletion and poor prognosis of patients. We examined the patients positive for metastasis had high frequency (64.28%) cytosine deletion may be a risk factor for the tumour metastasis in disease progression. Our study demonstrates that deletion in p53 of cytosine is a statistically significant predictor of poor survival in patients with NSCLC and significantly associated (0.0046).

### Conclusion

The data suggests that there is a significant association of the P53 exon 5 deletion of cytosine in codon 168 with metastasis and staging of the disease. P53 cytosine deletion may be the risk factor for the disease progression and can be used as prognostic marker to detect the disease in an early stage.

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