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Research Article

Evaluation of P53 Core Region Variation among Oropharyngeal Cancer Patients in India

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ABSTRACT:

BACKGROUND: Oropharyngeal cancer is one of the most common forms of cancer in Indian subcontinent affecting especially middle-aged persons. Just like other form of cancer, a change in P53 genomic element is also needed to be investigated for proper prognosis of the disease.

OBJECTIVE: The purpose of this study is to investigate the genetic variation(s) in p53 core region and make an association with oropharyngeal cancer patients in India.

MATERIALS AND METHODS: DNA was isolated from 25 patients (n=25) and age matched healthy individuals (n=50). HPV detection was done with broad ranges of HPV specific primers. P53 core region representing exons (5 to10) were amplified and sequenced. Functional consequences were analyzed with web based bioinformatics tool PredictSNPv1.0 and PROVEAN. Protein secondary structure was predicted by SOPMA and PSIPRED.

RESULTS: Sequencing showed a heterozygous polymorphism in exon5 (g7675181A>G reverse strand) which changes amino acid Q to R in codon 144 among patients. This change is predicted to be “neutral” by PredictSNPv1.0. SOPMA shows shifting of coil forming amino acid affiliation towards beta-sheet motif while PSIPRED depicts the opposite. PSIPRED also predicted a short beta-sheet formation as a consequence of Q144R.

CONCLUSION: Q144R might not be the lone player in disease prognosis via changing secondary structure propensity, although 100% allelic heterozygosity has been observed amongst patients. A large meta-analysis is needed to be performed to get a clearer picture and possible use of Q144R as oropharyngeal biomarker.

Key Words: single nucleotide polymorphism, TP53, oropharyngeal cancer, India, biomarker

[I] INTRODUCTION:

Human p53 gene is located on the short arm of chromosome 17 (17p13.1) and it consists of 10 protein coding exons. TP53 codes a 393 amino acid long protein of approximately 53kDa in size. TP53 is one the most extensively studied gene because of its cell cycle, apoptotic and

transcriptional regulatory function [1]. The central portion of p53 (amino acid 102 to 290 approximately) contain four conserved region where 80 to90% of the mutations occur [2, 3]. Mutations sometime disrupt the structure and function of normal p53 which leads to cancer. Oropharyngeal cancer is a subtype of head and neck cancer and one of the most common forms of cancer in Indian subcontinent [4]. Both smoke and smokeless tobacco products are considered as risk factors in oral and oropharyngeal cancer [5]. Like other head and neck cancer, oropharyngeal cancer originates from the squamous cell in oropharynx. Human papilloma virus (HPV) can be an etiologic agent in oropharyngeal cancer and therefore HPV-positive and HPV-negative got distinct set of molecular features [6]. In current study, we aim to assess genetic variation in p53 core region and make an association study with HPV-negative oropharyngeal squamous cell carcinoma.

This study reports one exonic SNP in TP53 gene among HPV-negative oropharyngeal patients.

[II] MATERIALS AND METHODS:

2.1. Cellular sample: Total 25 oropharyngeal patients have been selected from N.R.S Medical College, Kolkata. A total 50 healthy individual were selected as control. Controls were at the same age range as of patients and no history of cancer in familial background. Informed consents were obtained from each patient under strict privacy. Clinical datasheets were collected as well. Patients only with confirm oropharyngeal cancer has been included in this study. Five millimeter of peripheral blood was collected in EDTA tube from patients as well as controls. Genomic DNA was isolated using DSRGT DNA-MX kit as per manufacturer protocol.

2.2. HPV testing: Polymerase chain reaction were performed for the detection of HPV using broad range primer previously described [7, 8].

2.3. TP53 exon amplification: Polymerase chain reaction (PCR) amplification is done using primers specific to P53 core region i.e. Exon 5 to Exon 10

Exon	Forward Primer (5'>3')	Reverse Primer(5'>3')	PCR condition
E5-6	TTTCTTTGCTG CCGTCTTCC	GGGAGGTCAA ATAAGCAGCA G	94°C/10m;94°C/1m; 53.3°C/1m;72°C/90 s;72°C/5m; 30cycles
E7	TGCTTGCCACA GGTCTCC	GGTCAGAGGC AAGCAGAGG	94°C/10m;94°C/1 m;60°C/1m;72°C/ 90s;72°C/5m; 35cycles
E8	GGGACAGGTA GGACCTGATT	TAACTGCACCC TTGGTCTCC	94°C/10m;94°C/1m; 60°C/1m;72°C/90s; 72°C/5m; 35cycles
E9	GGAGACCAAG GGTGCAGTTA	CCCCAATTGCA GGTAAAACA	94°C/10m;94°C/1m; 60°C/1m;72°C/90s; 72°C/5m; 35cycles
E10	TGCCGTTTTCT TCTTGACTGT	CCAAGGCAGG CAGATCAC	94°C/10m;94°C/1m; 50°C/1m;72°C/40s; 72°C/5m; 30 Cycles

Table1: Primers and PCR conditions that were used to amplify p53 core region exons**.** E represents exon number, m=minutes and s=seconds. Primers are in 5' to 3' direction**.**

PCR products were checked in 1.5% agarose gel with ethedium bromide stain. Amplified products then directly sequenced with ABI3500 sequencer, Applied Biosytem™. Pearson's χ2-test and Fisher's exact test were performed after forming a genotype based contingency table. $P<0.05$ is considered as statistically significant data. Functional consequence of SNP is carried out by online web tool PredictSNP v1.0 [9] and PROVEAN [10, 11]. Secondary structure of protein is predicted by SOPMA [12] and PSIPRED [13, 14].

RESULTS: Simple patient chart indicates a significant p-value which is associated with tobacco consumption (Table 2).Sequencing of TP53 revealed one SNP in exon5. No alterations in genomic sequence have been found in exons 6,7,8,9 and 10.

Attributes	Case	Control	p-value
Gender			
Male	17	30	0.499
Female	8	20	
Age			
30-39	3	13	0.287

40-49	8	17	
Above 50	14	20	
Tobacco consumption			
No	6	19	0.100
Occasional	3	12	
Regular	16	19	

Table 2: Basic information about patients' sex, gender and tobacco habits. Non adjusted two tailed P-value was calculated by chi-square test. P<0.05 was considered to be significant**.**

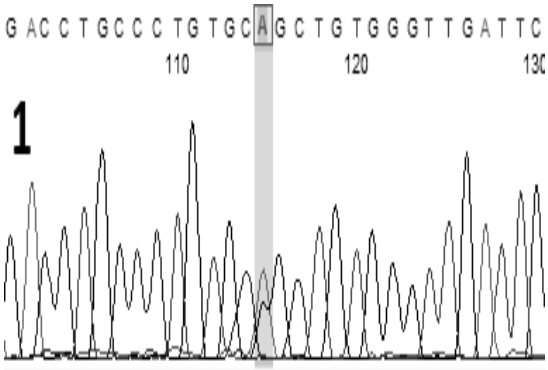
One Intronic SNP rs1625895 have been checked because of its role in several cancers. We found rs1625895 is homozygous for G allele. Interestingly all patients as well as control sample bears GG allelic form. SNP that has been found in exon5 is missense in nature and only heterozygous form (AG) has been found in patients with respect to GG in case of controls. This exonic SNP (g7675181A>G reverse strand, cDNA 431A>G, codon 144) changes a codon and replace amino acid glutamine (Q) with arginine (R) (Table3). Chromatogram for wild type and mutant TP53 has been shown in figure1.

[table3]

Table3: Summary of Intronic (rs1625895) and exonic SNP (Q144R)

Position	Codon/ identifier	WT	MT	Effect
Exon 5	144	CAG	CGG	Missense
Intron 6	rs1625895	A	G	Intronic

WT=Wild type and MT=mutant type. Ancestral allele was considered as wild type allele**.**



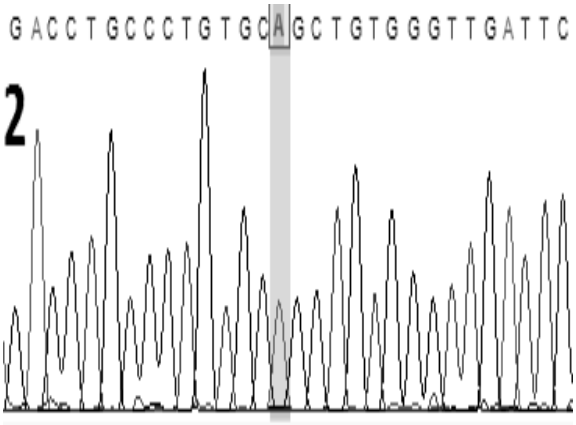


Figure1: Screen shot from TP53 exon 5 sequencing chromatogram shows (1) heterozygous (AG) and (2) homozygous (AA) variation. Variation AG has been observed only in patients (n=25) and AA only in control (n=50) samples. FinchTV software was used to view chromatogram. FinchTV by geospiza® is available at: http://www.geospiza.com/Products/finchtv.shtml.

PredictSNPv1.0 produced mix results about the consequence of Q144R SNP. MAPP and Polyphen2 predicts the SNP to be deleterious (confidence level 41%) but PredictSNP, pHDSNP, Polyphen, SIFT and SNAP predicted it to be neutral (Table4). PROVEAN score for this SNP-2.704 and prediction is "probably deleterious".

Variation	Predict SNP	MAPP	pHDSNP	Polyphen	Polyphen-2	SIFT	SNAP	nsSNP Analyzer	Panther
Q144R	Neu	Del	Neu	Neu	Del	Neu	Neu	Neutral	Neul

Table4. Prediction of SNP effect by different tools using PredictSNP. PredictSNP uses different online tools to assess functional consequence of a variation. Neu='Neutral consequence', Del= 'deleterious consequence'.

Secondary structure prediction by SOPMA yielded a possible change secondary structure (figure2) for mutated p53 where one amino acid in random coil (in wild type) is shifted its affiliation to extended sheet (in mutant) (Table5).

Table5: Comparison between wild type and variation type amino acid configuration using SOPMA

Wild type	Mutant type
Alpha helix (Hh) : 73 is 18.58%	Alpha helix(Hh) : 73 is 18.58%
310 helix (Gg) : 0 is 0.00%	310 helix (Gg) : 0 is 0.00%
Pi helix (Ii) : 0 is 0.00%	Pi helix (Ii) : 0 is 0.00%
Beta bridge (Bb) : 0 is 0.00%	Beta bridge(Bb) : 0 is 0.00%
Extended strand (Ee): 73 is 18.58%	**Extended strand (Ee) : 74 is 18.83%**
Beta turn (Tt) : 31 is 7.89%	Beta turn (Tt) : 31 is 7.89%
Bend region (Ss) : 0 is 0.00%	Bend region(Ss) : 0 is 0.00%
Random coil (Cc) : 216 is 54.96%	**Random coil (Cc):215 is 54.71%**
Ambigous states (?) : 0 is 0.00%	Ambigous states (?):0 is 0.00%
Other states : 0 is 0.00%	Other states : 0 is 0.00%

PSIPRED also reports changes in amino acids' affiliation towards a particular secondary structure motif, albeit contradictory to SOPMA. In PSIPRED prediction method, a beta-sheet transits its affiliation toward coil. In a drastic change a short extended strand is being formed by two leucine residue at position 264 and 265 (figure 3).

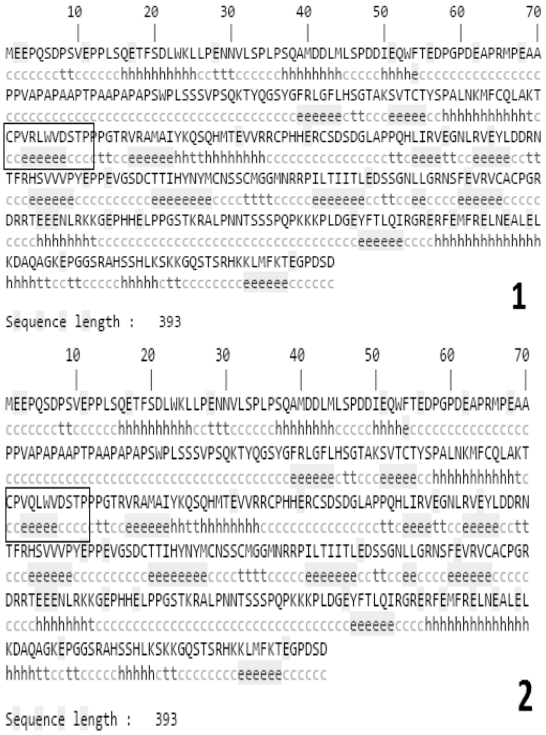


Figure2: SOPMA predicted possible secondary structure of TP53. Wild type (2) has amino acids conferring extended sheets compared to 6 amino acids in mutated form (1). C=coil, e= extended sheets, t= turn and h=helices. Wild type reference protein sequence is taken from transcript ID ENST00000269305.

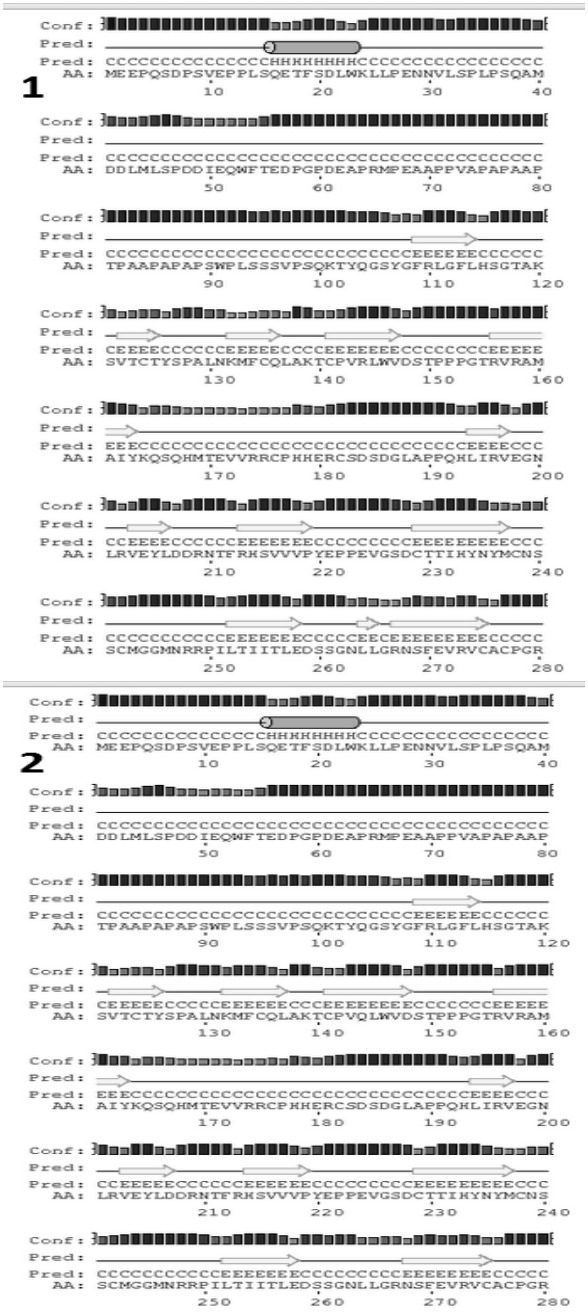


Figure 3: Structure prediction by PSIPRED shown changes in several amino acid affiliations especially around 264-265 position where a small beta-sheet is introduced. However, PSIPRED also predicts sheet to coil transition near 144 residue (1) mutant type (2) wild type. C= coil, E=extended sheets, H=helix. ENST00000269305 was used as reference transcript ID.

DISCUSSION:

TP53 alteration has been found in almost every cancer. Oropharyngeal cancer is not uncommon in this regard. Usually HPV positive oropharyngeal squamous cell carcicoma shows less TP53 alteration than HPV negative oropharyngeal squamous carcicoma, because the origin of cancer depends mainly on HPV oncoprotein, therefore cisplatin and radiation therapy gives good results against HPV-positive oropharyngeal cancer [15]. In India, cancer of tongue and oropharynx constitutes highest malignancies among middle age group (30-50) [4].TP53 mutations especially at codon 72 have been studied extensively in various form of cancer including oropharyngeal squamous carcinoma [16]. MDM2-309 along with p53 codon 72 CC is reported to increase the risk of non-oropharyngeal carcinoma [16]. Codon 72 along with G4C14 to A4T14 is a risk factor in HPV16 type oral carcinoma in never smoker [17]. On contrary, Zang J et al. reported that Codon 72 might not be the risk factor in HPV associated oropharyngeal carcinoma [18]. Other studies came to same conclusion about codon 72 not being the risk factor [19, 20, and 21].

However majority of these works were done in HPV positive oral carcinomas. Hsieh LL reported various form of p53 mutations in Taiwanese oropharyngeal carcinoma population although HPV infection step has not been mentioned. An amino acid change at codon 144 (CAG to CCG, glutamine to proline) is reported in a mouth floor cancer patient with strong history of addiction [22]. Our finding of codon 144 changes amino acid glutamine to arginine. Although PredictSNP (using all 8 SNP pathogenisity identification tool) predicts this SNP as neutral one, secondary structure prediction confirms a conformational change in TP53 structure. In 2005, Kakudo et al. constructed 179 mutant p53s including Q144R. Six novel super mutants including Q144R showed significant increases in apoptosis and did lack the correlation between increased apoptotic ability and transcription activation ability [23]. All of our cancer patients contain Q144R variation, so we assume that increased apoptotic ability does not explain the finding of Q144R in *in vivo*.

Intron6 IVS6+62A>G (rs1625895) another one of the most studied Intronic variation. However this variation affects risk factor in combination with other SNPs [24]. An increased risk in lung cancer [24], breast cancer [25] etc. have already been reported. Study on squamous cell carcicoma of head and neck by paola galli (2009) revealed that rs1625895 can increase the risk of HNC if it works with p73 exon2 G4A. On contrary, reduction of head and neck cancer risk was observed in combinatorial effect of rs1625895 and p53 codon 72 [26]. We found GG homozygosity of rs1625895 in all of the patients as well as controls samples, therefore no further conclusion can be drawn in this regard. Obviously our study is confined with only 25 patients, therefore a large meta-analysis had to be performed to further asses the role of Q144R and rs1625895 in oropharyngeal carcinoma.

CONCLUSION:

SOPMA and PSIPRED revealed a change in p53 secondary structure with no functional consequences. Considering previous studies on Q144R, mutated p53 supposed to check cancer, instead we found all our malignancies contain Q144R variation. As our samples are HPV negative, no viral oncoprotein is present to degrade TP53. Hence there must be some other molecular mechanism or gene(s) is playing a part in oropharyngeal cancer along with p53 Q144R. A thorough analysis with large number samples needed to be done specifically for HPV negative oropharyngeal cancer. A point of interest would be whether we could use Q144R variation as oropharyngeal cancer biomarker even after apparent null effect.

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CONFLICT OF INTEREST:

Authors state no conflict of interest.

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