Evaluation of P53 Polymorphism Susceptibility in Oral Cancer in Population From Gujarat, India

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ABSTRACT

PS3 is a most common genetic alterations found in human cancers particularly in tobacco related tumours of the oral cavity. So many polymorphisms have been found in p53 gene. Amongst them polymorphism at codon 72 in exon 4, codon 249 in exon 7, 16bp duplication in intron 3 and G>A transition in intron 6 have been suggested to affect the levels of p53 gene expression as well as its function. Hence the present study evaluated the role of these p53 polymorphisms in oral squamous cell carcinoma from the population of Gujarat, in West India. Method: The current study includes 113 control samples and 102 oral squamous cell carcinoma cases. Genotype frequencies of these four p53 polymorphisms were determined by the PCR-RFLP method. Result: Genotypic distribution of Arg/Pro & Pro/Pro at exon 4 were 28.43% & 22.54% respectively in cases. Prevalence of 16 b1p duplication in intron 3 was 18.62% in cases and 9.73% in controls & that of G>A transition in intron 6 was 31.37% in cases and 62.83% in controls. Conclusion: The results suggested that there is a significant association between all the above polymorphisms and risk of developing oral squamous cell carcinoma in Gujarat, India.

KEYWORDS  
Oral squamous cell carcinoma, P53 polymorphisms, PCR, RFLP

Introduction

Oral cancer is the sixth most common cancer worldwide (Liviu & Johan, 2012). It includes a group of neoplasms affecting any region of the oral cavity, pharyngeal regions and salivary glands. However, this term tends to be used interchangeably with oral squamous cell carcinoma (OSCC), which represents the most frequent of all oral neoplasms. It is estimated that more than 10% of all oral neoplasms are OSCC (Anastasios & Markopoulos, 2012). The most important risk factors for oral squamous cell carcinoma are use of tobacco or betel quid and the regular drinking of alcohol beverages. The highest incidence and prevalence of oral squamous cell carcinoma is found in the Indian subcontinent where the risk of developing oral squamous cell carcinoma is increased by the very prevalent habit of chewing tobacco, betel quid and areca-nut (Feller et al., 2012).

Despite the advances of therapeutic approaches, percentages of morbidity and mortality of OSCC have not improved significantly during last 30 years. Percentages of morbidity and mortality are 6.6/100,000 and 3.1/100,000 in males and 2.9/100,000 and 1.4/100,000 in females respectively (Anastasio & Markopoulos, 2012; Mehrotra & Yadav, 2006). Since, the oral cavity is more accessible to complete examination, it could be used in early detection of precancerous and cancerous lesions. But either due to ignorance or inaccessibility of medical care, the disease gets detected in later stages. Thus there is a need for improvement in early detection of oral carcinomas, because in the initial stages, treatment is more effective and the morbidity is minimal (Mehrotra & Yadav, 2006).

The development of OSCC is a multistep process requiring the accumulation of multiple genetic alterations, influenced by a patient’s genetic predisposition as well as by environmental influences. Such genetic alterations consist of two major types: tumor suppressor genes, which promote tumor development when inactivated; and oncogenes, which promote development when activated (Choi & Myers, 2008).

P53 has pleiotropic functions in the modulation of genomic stability of cells. Disruption of p53 activity is commonly found in human cancers. So far many polymorphisms have been described in this gene. The most commonly studied one is a single nucleotide polymorphism (SNP) at codon 72 in exon 4 of p53 gene, which results in the substitution of arginine (Arg) by proline (Pro) in the transactivating domain. These two polymorphic variants (Pro72 and Arg72) alter the structure and function of the p53 protein. The potential consequence of this amino acid exchange is differences in the susceptibility to malignant transformation, induction of apoptosis, and transcriptional activity. The arginine (Arg72) allele increases the ability of p53 to locate to mitochondria and induce cellular death, whereas proline (Pro72) exhibits a lower apoptotic potential and an increased cellular arrest (Lakshmi et al., 2012).

P53 mutations are distributed in all coding exons, with a strong predominance in exons 4-9, encoding the DNA-binding domain of the protein. About 30% of all mutations fall within six ‘hotspot’ codons and are detectable in almost all type of cancer (codons 175, 245, 248, 249, 273, 282). The results of several studies have shown that codon 249 is one of the most important sites introduced as a hot-spot for p53 gene. Specific base substitutions particularly G->T transversion at third position of codon 249 (AGG to AGT) in exon 7 of the p53 gene results in replacement of arginine with serine. Plenty of literature has been published regarding the mutation at codon 249 in hepatocellular carcinoma and lung cancer but there are no reports in concern to the relation between mutation at codon 249 and oral cancer from Gujarat population (Vijayaraman et al., 2012).

Apart from the mutations in the codons, numerous sequence variations are present in the p53 gene. These variations are intronic and have no cancer related biological consequences. However, two intrinsic polymorphisms in p53 gene; 16 bp duplication in intron 3 and G>A transition in intron 6 have been suggested to affect the levels of p53 gene expression as well as its function. Introns were originally believed to be non-functional because they do not code for proteins, still they have been implicated in regulation of gene expression and DNA-protein interactions. Sequence variations in introns may affect function of proteins and hence cancer risk. Studies of
the association between certain types of cancer and intron 3 and intron 6 polymorphisms have showed conflicting results (Patel et al., 2013).

So present study aimed to analyze whether the polymorphisms in exon 4, exon 7, intron 3, and intron 6 of the p53 gene are associated with differential oral cancer risk in Gujarati population from west India where incidence of oral cancer is dramatically high.

Material and methodology

Study subjects: This study was approved by the Institutional Human Research Ethics Committee, Pramukhswami Medical College, Karamsad. Patients, histopathologically diagnosed and operated for oral squamous cell carcinoma, were included as test subjects for this study. Control subjects were voluntary healthy individuals genetically unrelated to the patients and had no previous history of any type of cancer. Informed consent was taken from all the test and control subjects.

Methodology: Blood samples from all the study subjects were collected in EDTA vacutte and stored at 4°C until analysis. All the collected blood samples were then processed for DNA isolation at CHARUSAT institute, Changa. DNA isolation was done using HiPure Multi-Sample DNA Purification Kit manufactured by HIMEDIA, following manufacturer's instructions and isolated DNA was stored at -20°C until analysis. Genotyping was carried out by polymerase chain reaction followed by Restriction Fragment Length Polymorphism (PCR-RFLP) method. Exon 4, exon 7, intron 3 and intron 6 were amplified within in which the polymorphisms fall, using primers as mentioned previously (Mitra et al., 2003; Vijayaraman et al., 2012). The PCR products were resolved by 2% gel electrophoresis and 15% polyacrylamide gel electrophoresis and then visualized after staining with ethidium bromide.

The result for 16 bp duplication in intron 3 was directly interpreted from 15% PAGE analysis of the PCR products. PCR products of intron 3 were result in either 432 or 448 bp DNA fragments depending on the presence of 16 bp duplication in intron 3 of template genomic DNA. Homozygotes for the absence of duplication (A1/A1) produced band of 432 bp DNA fragment; heterozygotes produced both the bands of 432 & 448 bp (A1/A2); and homozygotes for the presence of 16 bp duplication (A2/A2) produced band of 448 bp DNA fragment.

Intron 6 was amplified separately by using primers as described previously (Mitra et al., 2003). The resulting PCR product was 913 bp DNA fragment which was further digested with enzyme NciI (Thermoscientific, Bangalore), followed by 15% PAGE and visualized after staining with ethidium bromide. Homozygotes for the absence of NciI restriction site produced band of 563 bp DNA fragment (A/A); heterozygotes produced both the bands of 432 & 277 bp DNA fragments (G/A); and homozygotes for the presence of 16 bp duplication (A1/A1) produced band of 448 bp DNA fragment. Amplified product of intron 6 were 913bp DNA fragment which after NciI digestion formed different small DNA fragments of 563 bp, 268 bp and 277 bp DNA fragments (G/A); and homogygotes for the presence of NciI restriction site produced bands of 286 bp and several small fragments from the 286 bp DNA product of the PCR reaction. If there is a polymorphism at codon 249, it results in an uncleaved 158 bp fragment and this feature will be distinguished from that of normal samples on 15% PAGE. Absence of the band at 286 bp (full-length PCR products) provides a control for complete digestion of the PCR product.

Statistical analysis: The statistical analysis was done by using SPSS software version 17. Chi-Square was used to analyze categorical variables and the association between the p53 polymorphism and risk factors of the oral squamous cell carcinoma. Comparisons were made between different genotypes and P-values less than 0.05 were considered statistically significant.

Results

Demographic profile of the study subjects are described in Table-1 (located in appendix A). There were total of 87 (85.3%) males and 15 (14.7%) females included as a test subjects having oral squamous cell carcinoma. In control subjects there were 44 (38.9%) males and 69 (61.1%) females. Age range of 46-65 found more prevalent (71.5%) in oral carcinoma.

Tobacco habits were found more prevalent (95.09%) in oral cancer cases as compared to control cases. Tobacco habituates were found more prone to develop oral cancer as compared to tobacco non-habituates. Drinking alcohol did not found any significant role in oral squamous cell carcinoma. As shown in Table-2 (located in appendix A).

Detection of all the polymorphisms of p53 by PCR-RFLP were conducted successfully in all the cases and controls. The genotypic distribution of 16 bp duplication in intron 3, G>A transition in exon 4, Arg72Pro in codon 72 of exon 4 and Arg/Pro frequency at codon 249 of exon 7 among tests and controls is provided in Table-3 (located in appendix A). All the polymorphisms of p53 shows significant association with oral squamous cell carcinoma over the controls. Among them 16 bp duplication in intron 3 of p53 gene show less significant association comparative to three other polymorphisms of p53 (p value - 0.035). There was strong association of G>A transition in intron 6 of p53 gene with risk of oral squamous cell carcinoma (p value - <0.001). Polymorphism in codon 72 of exon 4 and codon 249 of exon 7 were also found significant role in developing risk of oral squamous cell carcinoma.

As shown in figure-1 (located in appendix B), Lane 1 represent 100 bp DNA ladder. Lane 2,3,5 represent homozygous for absence of 16 bp duplication (A1/A1). Lane 4 represents heterozygous for 16 bp duplication (A1/A2). Lane 6 represents homogygous for the presence of 16 bp duplication (A2/A2) in intron 3 of p53 gene.

Amplified product of intron 6 were 913bp DNA fragment which after NciI digestion formed different small DNA fragments of 563 bp, 286 bp, 277 bp according to the presence or absence of G>A transition in intron 6 of p53. DNA fragment of 350 bp found in all cases which represent the presence of a nonpolymorphic NciI site in the amplicon.

As shown in Figure-2 (located in appendix B), Lane 9 represents the 100 bp DNA ladder; Lane 2 represents homozygous for the absence of NciI restriction site (A/A); Lane 1, 4, 6, 7, 10,11 represent heterozygous for NciI restriction site (A/G); Lane 3, 5, 8 represent homozygous for the presence of NciI restriction site (G/G).

The amplified product of exon 4 was 390 bp DNA fragment. After treatment with enzyme BstU1, 309bp DNA fragment got digested and formed two fragments of 175 bp and 134 bp according to the presence or absence of BstU1 restriction site.

As shown in figure-3 (located in appendix B), Lane 3 represents 50 bp DNA ladder; Lane 1, 4 represent homozygous for the absence of BstU1 restriction site (Pro/Pro); Lane 2, 5 represent...
sent heterozygous for the Arg/Pro polymorphism; Lane 6 represents homozygous for the presence of BstU1 restriction site (Arg/Arg).

Upon the digestion with BstU1 enzyme, Arg was cleaved by enzyme and formed two DNA fragments of 175 bp and 134 bp in 50 cases (49.01%) which represent homozygous for the absence of restriction site. In 23 cases (22.54%) proline allele was not cleaved by enzyme, and produced a single band of 309 bp DNA fragment which represent homozygous for the absence of restriction site (Pro/Pro). 29 cases (28.43%) found heterozygous for Arg/Pro allele of exon 4 of p53 gene yeilding three bands of 309 bp, 175 bp and 134 bp DNA fragments.

The amplified product of codon 249 of exon 7 was 158 bp DNA fragment which was further cleaved by the enzyme HaeIII, and produced four DNA fragments of 92 bp, 66 bp, 42 bp, and 39 bp according to the presence or absence of polymorphism in codon 249 of exon 7 of p53 gene.

As shown in figure-4 (located in appendix B), Lane 1 represents 50 bp DNA ladder; Lane 2,4 represent homozygous for the presence of restriction site (Arg/Arg); Lane 3,5 represent heterozygous for the Arg/Ser allele at exon 7 of p53 gene. Arg allele was cleaved by enzyme HaeIII in 37 cases (36.27%) and produced four fragments of 92 bp, 66 bp, 42 bp, and 39 bp. Homozygous pattern of Ser allele was found in 28 cases (27.45%) produced only a single fragment of 158 bp.

Discussion
The present study evaluated the role of polymorphisms i.e., 16 bp duplication in intron 3, G>A transition in intron 6, Arg/Pro in codon 72 of exon 4, and Arg/Ser in codon 249 of p53 tumor suppressor gene in order to predict the risk of oral squamous cell carcinoma in population from Gujarat, West India. The control and the cases were belong to same ethnicity and were from the same geographic location. An allele frequency of Arg72Pro polymorphism has been reported to vary with respect to ethnicity and latitude (Nagpal et al., 2002).

The allele frequency of proline at codon 72 varies from 0.12-0.69 worldwide (Francisco et al., 2011) whereas for the indian population; it ranges from 0.42-0.72 (Nagpal et al., 2002; Tandle et al., 2001; Mitra et al., 2003; Mittal et al., 2011; Suresh et al., 2011). Risk of oral cancer was also estimated in association with p53 genotypes of all three polymorphic loci. There are evidences that the codon 72 polymorphism had a profound effect on the primary structure of p53 protein and its biochemical and biological activities (Matlashewski et al., 1987; Ozeki et al., 2011). In addition, p53 mutant acts as a more potent inhibitor of p73, which is responsible for apoptosis in tumor cells but not in normal cells (Dumont et al., 1999). The arg-72 form has a much stronger capacity to induce apoptosis than the Pro-72 form of p53 in tumor cells but not in normal cells (Dumont et al., 2003).

We observed the significant association between intron 3 and intron 6 polymorphisms and oral cancer risk. Recent researches suggest that intronic polymorphisms may affect the function of wild type p53 protein and hence cancer risk (Avigad et al., 1997; Lehman et al., 2000; Gemignani et al., 2004). However in cell culture analysis, these two intronic polymorphisms i.e, 16 bp duplication in intron 3 and G>A transition in intron 6, did not seem to be sufficient to impair p53 function during the neoplastic transformation but required an additional coding region mutation. Wu et al. (2002) have observed that proline at exon 4 in conjunction with intron 3 and intron 6 variant alleles exert a protective effect rather than a detrimental effect for lung and colorectal cancers though they found significant risk of lung cancer associated with these variants.

Table-1: Demographic detail

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TEST GROUP</th>
<th>CONTROL GROUP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>26-35</td>
<td>05 (4.9%)</td>
<td>00 (0.0%)</td>
</tr>
<tr>
<td>36-45</td>
<td>18 (11.7%)</td>
<td>04 (2.6%)</td>
</tr>
<tr>
<td>46-55</td>
<td>32 (20.3%)</td>
<td>08 (4.8%)</td>
</tr>
<tr>
<td>56-65</td>
<td>30 (19.2%)</td>
<td>03 (1.7%)</td>
</tr>
<tr>
<td>66-75</td>
<td>02 (1.3%)</td>
<td>00 (0.0%)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>77 (55.3%)</td>
<td>16 (10.7%)</td>
</tr>
</tbody>
</table>

Table-2: Distribution of the risk factors

<table>
<thead>
<tr>
<th>RISK FACTORS</th>
<th>TEST (n=112)</th>
<th>CONTROL (n=113)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco chewer</td>
<td>97 (95.09%)</td>
<td>07 (6.19%)</td>
</tr>
<tr>
<td>Tobacco non-chewer</td>
<td>05 (4.9%)</td>
<td>106 (93.80%)</td>
</tr>
<tr>
<td>Alcohol drinker</td>
<td>60 (58.82%)</td>
<td>00</td>
</tr>
<tr>
<td>Alcohol non-drinker</td>
<td>42 (41.18%)</td>
<td>113 (100%)</td>
</tr>
</tbody>
</table>

Appendix – A
Table-3: Genotypic profile of all polymorphisms of p53

<table>
<thead>
<tr>
<th>POLYMORPHISMS</th>
<th>GROUP</th>
<th>P53 GENOTYPE</th>
<th>TOTAL</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TEST</td>
<td>(1-1)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 bp Duplication (Intron 3)</td>
<td>Test</td>
<td>48 (48.03%)</td>
<td>102</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>73 (64.60%)</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1-2)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G&gt;A Transition (Intron 6)</td>
<td>Test</td>
<td>28 (27.45%)</td>
<td>102</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>11 (9.73%)</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>Codon 72 (Exon 4)</td>
<td>Test</td>
<td>50 (49.01%)</td>
<td>102</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>13 (11.50%)</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>Codon 249 (Exon 7)</td>
<td>Test</td>
<td>37 (36.27%)</td>
<td>102</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>70 (61.94%)</td>
<td>113</td>
<td></td>
</tr>
</tbody>
</table>

Note: * Here in Table-3,

1= A1 at intron 3, A at intron 6, Proline at exon 4 and Arginine at exon 7
2= A2 at intron 3, G at intron 6, Arginine at exon 4 and Serine at exon 7

Appendix – B

Figure-1 : The amplified product of intron 3 of p53 gene. As shown in figure-1, Lane 1 represent 100 bp DNA ladder. Lane 2,3,5 represent homozygous for absence of 16 bp duplication (A1/A1). Lane 4 represents heterozygous for 16 bp duplication (A1/A2). Lane 6 represents homozygous for the presence of 16 bp duplication (A2/A2) in intron 3 of p53 gene.

Figure-2 : The amplified product of intron 6 of p53 gene after NciI digestion. As shown in figure-2, Lane 9 represents the 100 bp DNA ladder; Lane 2 represents homozygous for the absence of NciI restriction site (A/A); Lane 1, 4, 6, 7, 10, 11 represent heterozygous for NciI restriction site (A/G); Lane 3, 5, 8 represent homozygous for the presence of NciI restriction site (G/G).

Figure-3 : The amplified product of exon 4 of p53 gene after BstU1 digestion. As shown in figure-3, Lane 3 represents 50 bp DNA ladder; Lane 1, 4 represent homozygous for the absence of BstU1 restriction site (Pro/Pro); Lane 2, 5 represent heterozygous for the Arg/Pro polymorphism; Lane 6 represents homozygous for the presence of BstU1 restriction site (Arg/Arg).

Figure-4 : The amplified product of exon 7 of p53 gene after HaeIII digestion. As shown in figure-4, Lane 1 represents 50 bp DNA ladder; Lane 2, 4 represent homozygous for the presence of restriction site (Arg/Arg); Lane 3, 5 represent heterozygous for the Arg/Ser allele at exon 7 of p53 gene.

References

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