Pak. J. Biochem. Mol. Biol. 2011; 44(2): 51-55

Loss of heterozygosity in p53 gene found in oral squamous cell carcinoma patients of Pakistan

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Abstract: Oral squamous cell carcinoma (OSCC) is the leading cause of death in the developing countries like Pakistan. The major risk factor for developing OSCC is the excessive chewing habit of *paan* (betel quid) *chaliya* (betel nut), tobacco, n*iswar* (type of dipping tobacco, made from fresh tobacco leaves, calcium oxide, and wood ash) *gutka* (a preparation of crushed betel nut, tobacco and sweet or savory flavors) and *manpuri* (the powder of betel nut, tobacco and slaked lime). The p53 gene is the extensively studied tumor suppressor gene involved in the suppression of tumor. The germ line mutation/polymorphism of p53 gene has been reported to be involved in multiple steps of carcinogenesis. It has been reported that exon 4-9 were the hot spots of the mutation in the tumor suppressor gene. Mutagens can damage DNA and generate promutagenic lesions. This study aims to find out the loss of p53 gene functions due to mutation/polymorphism caused by genomic alteration and interaction with tobacco and its related ingredients in Pakistan. A total of 250 OSCC patient's tissue and blood specimens were collected with informed consent from local hospitals of Karachi. The samples were compared with 250 age and sex matched controls. The OSCC was confirmed by histopathology of the tissue samples. Extraction of DNA from blood and tissue samples was carried out and 10 exons of p53 gene were amplified through polymerase chain reaction (PCR) by using forward and reverse primers. The amplified PCR products were checked by agarose gel electrophoresis, and PCR products were screened for mutation(s) by single stranded conformational polymorphism (SSCP). The PCR-SSCP analyses showing mobility shift bands indicated the single nucleotide mutation/polymorphism in tissue and blood samples of the patients. A single nucleotide change on SSCP gels was observed in the coding region of exon 2, 3, 4, 5, 6, 7, 10 and 11. The change was significantly higher in the tissue samples of the OSCC patients but not in their blood. The change of single nucleotide may be responsible for the substitution of amino acid in the p53 protein. This may result in the germ line mutation(s) of the p53 gene due to chewing habits which are involved in different steps of tumorgenesis and increasing the susceptibility of OSCC in Pakistan.

Key words: p53 polymorphism, oral squamous cell carcinoma (OSCC), PCR-SSCP, loss of heterozygosity (LOH), missense mutation.
Received: April 12, 2011 **Accepted**: May 25, 2011
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INTRODUCTION

Oral squamous-cell carcinoma (OSCC) is among the common neoplasm in Pakistan. Excessive use of tobacco, *paan*, *chhaliya*, *gutka*, *niswa*r and *manpuri* are thought to be causing mouth cancer among people of Pakistan. These chewable agents are the sources of specific carcinogens that eventually lead to symptoms of oral cancer including swelling and increased irritability of the tongue attributed to the mutation of p53 gene[1].

The development of tumors has been associated with the multiple genetic changes[2]. Cancer cells exhibit a change in phenotype due to the mutation, which may be responsible for the genomic instability found in the tissues[3].

The oral squamous carcinogenesis is a multistep process in which genetic events lead to the disruption of the normal regulatory pathways that control basic cellular functions including cell division, differentiation, and cell death that alter the normal functions of oncogenes and tumor suppressor genes[4]. The tumor suppressor protein, p53, consists of 393 amino acids, encoded by TP53 gene located at chromosome 17[5]. The p53 gene is a tumor-suppressor gene found mutated in common human cancers. It has an important role of regulating cell cycle in multicellular organisms. It is postulated that DNA repair polymorphisms may also influence the risk of gene mutation[6]. The single nucleotide polymorphisms of the tumor suppressor gene p53 have been shown to be associated with diverse types of human cancer[3].

The p53 protein is stabilized in response to different checkpoints activated by DNA damage, hypoxia, viral infection, or oncogene activation resulting in diverse biological effects, such as cell cycle arrest, apoptosis, senescence, differentiation, and antiangiogenesis[7].

The stable p53 protein is activated by phosphorylation, dephosphorylation and acetylation yielding a potent sequence-specific DNA-binding transcription factor[8]. The wide range of p53's biological effects can in part be explained by its activation of the expression of number of target genes including p21WAFI, GADD45, 14-3-3 sigma, bax, Fas/APO1, KILLER/DR5, PIG3, Tsp1, IGF-BP3 and others[9]. Genetic polymorphism in the genes involved in tumorgenesis may determine individual susceptibility to cancer[3].

MATERIALS AND METHODS

Sample collection

A total of 250 control subjects from the normal population and 250 malignant patients of oral squamous cell carcinoma (OSCC) were included in the study. All the individuals gave informed consent for participation in the study and the procedures were approved by the regulations of institutional ethical committees of concerned institutes for the use of human subjects in research. Detailed information on general demographic data, current and past history of cigarette smoking, chewing habits, medical data, occupations, family diseases and dietary habits of the subjects were recorded. Blood and tissue samples of OSCC patients were collected from, Darul Sehat hospital, Abbasi Shaheed hospital, Civil hospital, OMI hospital and Jinnah Post Graduate Medical center (JPMC), Karachi. Blood, from controls as well as from patients, was collected in 10 ml vacutainers containing ACD (acid citrate dextrose). About 1 gram of the cancerous tissue from the patients was collected in a sterile container. The piece of tissue was surgically dissected from the cancerous lesions of OSCC patient and placed on ice. The diagnoses of OSCC in the patients were confirmed by histopathological examination of tissue.

Total genomic DNA extraction from whole Blood and tissue

The genomic DNA was extracted from the white blood cells of tumors and blood by standard protinase K digestion and phenol-chloroform extraction. The whole blood cells were treated with the red cell lysis buffer (10 mM $KHCO_3$, 150 mM NH_4Cl and 0.5 mM EDTA) and white cells were separated by centrifugation. The white cells were suspended in saline Tris EDTA (STE) buffer (100 mM NaCl, 50 mM Tris and 1 mM EDTA). Frozen tissue was crushed in the liquid nitrogen directly suspended in STE buffer. The cell lysate was digested overnight at 55˚C with 10% SDS and proteinase-K (20 mg/ml)[10, 11].

PCR amplification

Each PCR reaction was carried out in a total volume of 50µl. The reaction contained 50 ng of genomic DNA. The master mix contained 1X concentration of 10X PCR buffer (15mM Tris-HCL pH 8.75 at 25˚C, 500mM $(NH_4)_2SO_4$ and 0.1% Triton X-100), 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 0.6 µM of each forward (F) and reverse (R) primers and 1.5 units of *Taq polymerase.* Amplification was performed in ABI thermo cycler for 35 cycles[7,12].

Single stranded conformational polymorphism (SSCP) analysis

The amplified PCR product, 5 µl of each, was mixed with 5µl of SSCP gel loading dye (95% formamide, 20 mM EDTA, pH 8.0, 0.05% xylene cyanol, and 0.05% bromophenol blue), denatured at 95˚C for 7 minutes and chilled quickly on ice for 5 minutes. Samples were loaded on 8-10% native polyacrylamide gels (37:1, acrylamide: bisacrylamide) depending on the expected product sizes. Gels containing TBE buffer (89mM Tris, 89mM boric acid, 2 mM EDTA) were run with a discontinuous buffer system. Electrophoresis was carried out at 30 watts for 5 hours at 2˚C. Gels were silver stained[13] and dried (using Bio-Rad gel dryer) on Watman paper and saved for record.

RESULTS

The frequencies distribution of OSCC was observed with chewing habits in the patients. The highest percentage of the patients was found in g*utka* chewers than *niswar*, *manpuri*, tobacco, *chaliya* and *paan* (Figure 1).

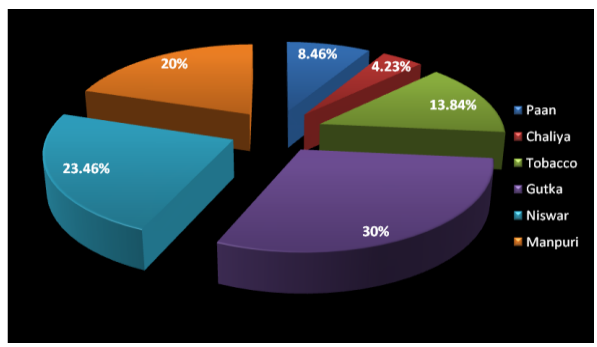


Figure 1: Distribution of OSCC patients according to their chewing habits.

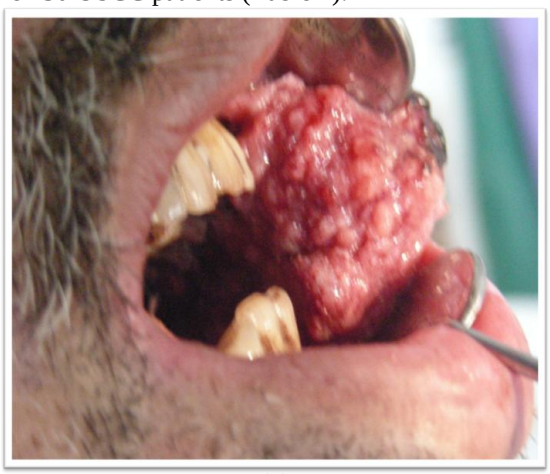
Clinical examination

There was an association between p53 gene mutations with clinical diagnosis. The metastasis was found in the patients with previous history of erythroplakia (red lesions) and leukoplakia (white lesions). These lesions were significantly observed in *gutka*, *niswar* and *manpuri* chewers with continuous bleeding and pus formation. The patients with *paan*, *chaliya* and tobacco chewing were found with the sore and swollen throat with pale or ulcerated oral lesions (Figure 2).

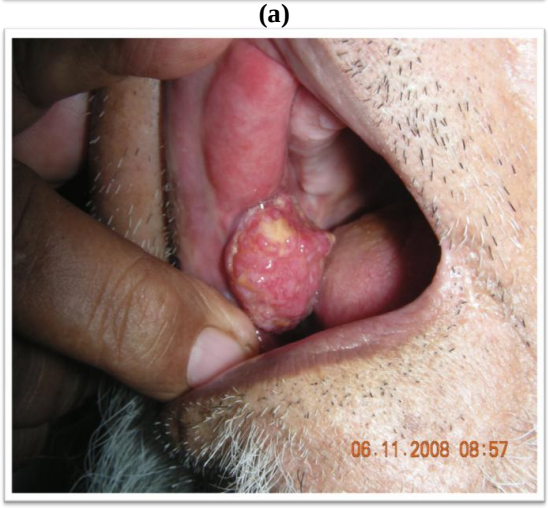
Molecular analyses

Mutational screening of 10 exons was carried through SSCP analysis and data was recorded in the form of percentage altered mobility shift in the bands. A majority of the tumor DNA samples showed abnormal bands in exon 2-3, 4, 5, 6, 7, 10 and 11. No abnormal band was found in exon 8 and

9 when compared with controls (Figure 3). The frequency of altered abnormal band was 155 in exon 2-3, 219 in exon 4, 201 in exon 5, 194 in exon 6, 189 in exon 7, 178 in exon 10 and 157 in exon 11 from the 250 OSCC patients (Table 1).



(a)

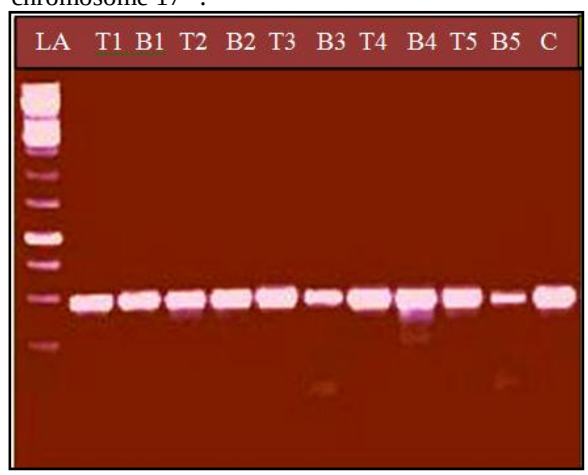


(b)

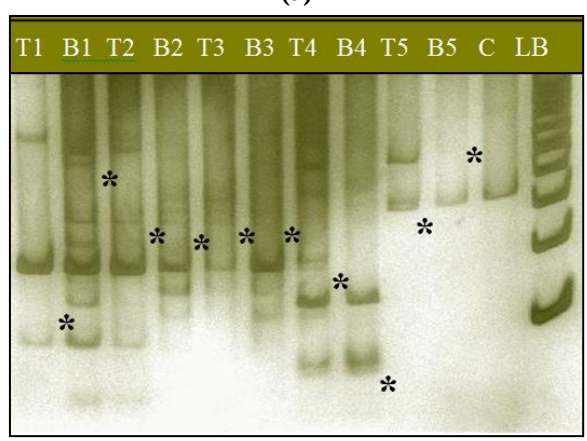
Figure 2: Extensive metastatic lesions in clinically diagnosed patients of OSCC: (a) M*anpuri* addict; (b) G*utka* addict.

DISCUSSION

It has been reported that the mutation in the tumor suppressor gene TP53 can actively participate in cellular transformation[14]. The gene may be inactivated by several types of mutations like frame shift, missense and/or nonsense mutations. This may lead to the improper synthesis of the gene product. Almost 90% of the p53 mutations are missense mutations leading to the synthesis of a stable protein but lacking in its specific DNA binding function and accumulating in the nucleus of the tumor cells[15]. Transfection of various p53 mutations into cells devoid of endogenous p53 leads to an increase in their carcinogenicity, which varies according to the type of mutation[16, 17]. These mutations inactivate the multiple pathways of the gene by insertion and/or deletion of nucleotide(s), which may lead to the formation of mutant protein or absence of protein. It has been observed that cancers have the high frequency of p53 gene mutations and loss of heterozygosity (LOH) in the short arm of the chromosome 17[18].



(a)



(b)

Figure 3: The PCR-SSCP analysis of p53 gene. (a) 1.2% Agarose gel electrophoresis of the exon 5 PCR product of 248 base pair; (b) 8% Polyacrylamide gel electrophoresis for single stranded conformational polymorphism (SSCP) of the samples. Lane T1-T5 (Tumor tissue samples) Lane B1-B5 (Blood of the patients), Lane LA- 1 kilo base pair DNA ladder, Lane LB- 100 base pair DNA ladder; * The bands with mobility shift showing mutation, Lane C (Blood of controls).

The presence of p53 mutation is generally unambiguous. The mutations of the wild-type p53 block cell cycle progression in late G1 phase in the presence of DNA damage or mediates apoptosis and permits DNA repair and cell cycle re-entry. p53

mutant cells lose the ability to inhibit cell growth after DNA-damaging chemotherapy and γ-radiation[19]. Therefore, the p53 was a candidate gene to examine in OSCC with gene amplification because wild-type p53 maintains genomic stability and ploidy, whereas altered cell cycle arrest, gene amplification potential and aneuploidy occur with loss of wild-type p53. Furthermore, p53 SSCP analysis showed both mutation and LOH[20]. It has been reported that the clinical specimens of primary carcinoma showed mutations of p53 exon 7[21].

Table 1: Frequencies distribution of mobility shift bands on SSCP gels indicating the mutation/polymorphism in tumor and blood samples of OSCC patients in Pakistan

	OSCC Tumor	OSCC Blood
Exon 2-3	155 (62%)	78 (31.2%)
Exon 4	219 (87.6%)	71 (28.4%)
Exon 5	201 (80.4%)	29 (11.6%)
Exon 6	194 (77.6%)	88 (35.2%)
Exon 7	189 (75.6%)	10 (04 %)
Exon 10	178 (71.2%)	56 (22.4%)
Exon 11	157 (62.8%)	21 (8.4%)

The PCR amplification and SSCP analyses of p53 gene in exon 2, 3, 4, 5, 6, 7, 10, and 11 showed presence of mutation(s) in the OSCC patients when compared with controls indicative of some point mutations involved in the p53 gene. About 90% of the OSCC patients showed the mutation through altered abnormal band in exons of p53 gene which may lead to the expression of a mutant protein. These mutations may be associated with loss of allele located on the short arm of chromosome 17 and loss of heterozygosity (LOH) in p53 gene. Higher frequency of the mutations was detected in the coding region of the gene in tumor tissues indicating germ line mutation at somatic cell level. These mutations may have clinical significance in the OSCC patients in Pakistan with the chewing habits of *paan*, *chaliya*, tobacco, *niswar*, *gutka*, and *manpuri*.

These findings could be further confirmed through direct sequencing to find out the codon specific point mutation which may prove the detection of amino acid changes in the p53 proteins and to understand the alterations in the p53 pathway due to the mutations in OSCC patients. However, genetic profiling and molecular control of various pathways will allow more accurate diagnosis and assessment of the prognosis of oral cancers and may lead to novel approaches in early diagnosis and therapy of the disease.

ACKNOWLEDGEMENTS

The study was funded by the Higher Education Commission-Research Project No: 20-695.

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