RESEARCH COMMUNICATION

Genetic Variations in Carcinogen Metabolizing Genes Associated with Oral Cancer in Pakistani Population

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Abstract

Background: Xenobiotics are metabolized by either phase I enzymes like *CYP1A1* or phase II enzymes like GSTs. Polymorphisms in the encoding genes (*CYP1A1, GSTM1, GSTT1* and *GSTP1*) potentially may thererfore contribute towards risk association for oral cancer. Methodology: These genes were investigated via a case control study consisting of 228 oral cancer patients and 150 cancer free normal individuals as controls. DNA was extracted from WBCs for genotyping. Polymerase chain reaction–single stranded conformational polymorphism (SSCP) was used for screening *CYP1A1* and *GSTP1* genes mutations. Deletion of *GSTM1* and *GSTT1* genes were analyzed by multiplex PCR. Results: Two novel mutations were found in this study in relation to oral cancer. A substitution mutation of A_{2842} with C resulting in missense tyrosine to serine formation along with a frameshift mutation due to insertion of thymidine at nucleotide 2842 resulting in 495 nucleotide sequence to alter was found in oral cancer patients. *GSTM1* and *GSTT1* deletion polymorphism was found in significantly higher number of individuals (OR=2.08, CI 1.05-4.2; OR=1.5, CI 0.9-2.4 respectively) compared to controls. 10 patients had deletion of both *GSTM1* and *GSTT1* genes. *GSTP1* gene was also found to have novel substitution mutations of A_{2848} to T and G_{2849} to A in exon 7 resulting in leucine to leucine and alanine to threonine formation respectively. Two intronic deletions of cytosine at positions 1074 and 1466 was found in intron 3 and 4 in patients and no control had these exonic or intronic variants in GSTP1 gene. Conclusion: These results suggest that accumulation of genetic changes in *CYP1A1*, *GSTM1*, *GSTT1* and *GSTP1* genes are associated with increased risk of oral cancer.

Keywords: Oral cancer - *CYP1A1* - *GSTM1* - *GSTT1* - *GSTP1*- polymorphisms - Pakistan

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Introduction

Incidence of oral cancer has increased in the last few years in South East Asia (Buch et al., 2002; Devasena et al., 2007), probably due to increased intake of tobacco. Numerous epidemiological studies indicate that xenobiotic metabolizing genes polymorphisms are associated with increased risk of oral cancer. Most carcinogens are metabolized via complex enzymatic mechanism involving both activation by phase I enzymes and detoxification by phase II enzymes. The phase I enzymes are responsible for either detoxification of xenobiotic or converting them into an intermediate compound that can be recognized by the phase II enzymes. Phase II enzymes make these compounds more electrophilic and easily detoxified.

So far, 4 different sequence polymorphisms have been reported in *CYP1A1* gene, first known as *CYP1A1**2 involves a T_{6235} to C transition in the 3' noncoding region (Kawajiri et al., 1990; Jun et al., 2010), second known as *CYP1A1**3 involve a A_{4889} to G transition in exon 7 (Hayashi et al., 1991; Jun et al., 2010), third known as *CYP1A1**4 involves a T_{5639} to C transition in intron 7 (Crofts et al., 1993), and fourth known as *CYP1A1**5 involves a C_{4887} to A transition in exon 7 (Cascorbi et al., 1996; Jun et al., 2010).

GSTM1 and *GSTT1* are frequently reported to show deletions of entire genes in different population (Stacy and Andrew, 2000; Toru et al., 2008). Deletion of *GSTM1* and *GSTT1* gene are known to lack respective enzyme activity and are said to be null genes (Egan et al., 2004). To date two polymorphic alleles are known for *GSTP1*, *GSTP1**B and *GSTP1**C, in addition to the wild-type allele, GSTP*A has been reported in humans (Ali et al., 1997). Both alleles have an A-to-G transition at nucleotide 313 (codon 104), causing an isoleucine-to-valine change. The *GSTP1**C allele has, in addition to the substitution at nucleotide 313, a C-to-T transition at nucleotide 341 (codon 113) that changes alanine to valine (Zimniak et al., 1994).

The present study was designed to look at the genetic changes of *CYP1A1*, *GSTM1*, *GSTT1* and *GSTP1* genes and their possible association with risk of oral cancer in Pakistani population.

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Materials and Methods

Pathologically confirmed 228 oral cancer patients along with age and sex matched 150 cancer free normal healthy individuals as controls were recruited from National Oncology and Radiotherapy Institute (NORI) and Pakistan Institute of Medical Sciences (PIMS), Islamabad from March 2008 to September 2009 with a prior approval from Ethical Committees of both university and hospitals. All study individuals participated on a volunteer basis. All subjects were personally interviewed according to a structured questionnaire. Blood was collected from individuals with their informed consent. Subject blood was sampled before starting the therapy.

Phenol–chloroform extraction protocol was used for DNA isolation (Baumgartner et al., 2001; Vierhapper et al., 2004). Dilutions of 5ng were made of each DNA isolated for subsequent use.

Primer 3 input software version 0.4.0 was used for primers designing and BLAST using NCBI PRIMER BLAST. All of the photographs of electrophoresis were read by two technicians blind to each other's assessments. PCR product of *CYP1A1* and *GSTP1* genes were analyzed by Single stranded conformational polymorphism (SSCP) using the procedure described by Patrichia et al., (2009) and Amalio et al., (1993) with some minor modifications. SSCP results were analyzed with gel documentation system (BioDocAnalyze Biometra) after ethidium bromide staining and photographed. The samples showing mobility shifts were then sequenced from Macrogen (Korea).

Statistical analysis was performed by using SPSS statistics 17.0 software and GraphPad Prism 5 for calculating odds ratio, 95% confidence interval and standard deviation.

Results

The present case control study consisted of 228 oral cancer patients and 150 cancer free controls. The mean age of cases was 47.4 (±16.3) years and the mean age of controls was 46.0 (±17.7) years. No difference of cancer infectivity was observed in both males and females.

Mutations in CYP1A1 gene

None of the already reported variants for *CYP1A1* were found in our study however novel substitution and frameshift mutations were found in 16 oral cancer patients (Figure 1). The substitution involved A_{2842} to C mutation in exon 2. This A_{2842} to C mutation caused a change in DNA sequence from TAC to TCC and resulted in UCC which codes serine, whereas wild type UAC codes for tyrosine. This tyrosine to serine mutation is in conserved P450 domain and not in the transmembrane domain. Frameshift mutation due to insertion of thymidine at nucleotide 2842 was also found in oral cancer cases having A to C substitution mutation. Due to frameshift mutation the conserved core structure is altered thus proper folding and heme-binding ability of cytochrome P450 molecules is disturbed. Thus the protein structure of *CYP1A1* gene is altered.

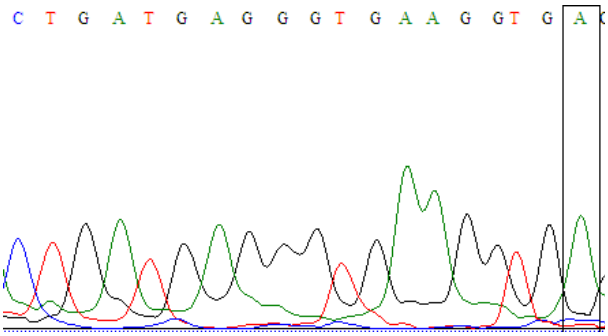

Figure 1. Bioedit Software Figure Showing the Position of T Insertion at Nucleotide 2842 in Exon 2 of CYP1A1 Gene Causing a Frameshift Mutation in HNC Patients. Reverse Primer was Used for Sequencing

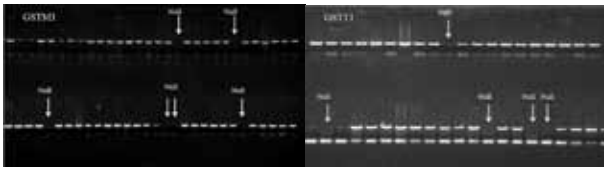

Figure 2. 2% Agarose Gel Electrophoresis showing GSTM1 and GSTT1 Band as Well as Null Genotypes

Table 1. The Genotype Frequencies of the Genotypes at CYP1A1, GSTM1, GSTT1 and GSTP1 Genes in the Oral Cancer Patients (n=228) and Controls (n=150)

Gene	Polymorphism	Cases	Controls	OR (95% CI)
CYP1A1	A to C	16	0	-
	Frameshift (T insertion)	16	0	-
GSTM1	Null	35	12	2.08 (1.05-4.2)
	Positive (Normal)	193	138	-
GSTT1	Null	57	28	1.5 (0.9-2.4)
	Positive (Normal)	171	122	-
GSTP1	A to T and G to A (Exonic)	24	0	-
	Two C deletions (Intronic)	9	0	-

Polymorphisms in GSTM1 and GSTT1 genes

Significantly higher number of oral cancer patients had *GSTM1* deletion genotype as compared with the controls ($P<0.05$). The odds ratio for *GSTM1* null genotype in oral cancer patients compared with the controls was 2.08 with 95% CI of 1.05-4.2 (Figure 2). Also it was found that 57 oral cancer patients had *GSTT1* gene deletion. *GSTT1* deletion genotype was found significantly higher ($P<0.05$) in patients compared to controls. The odds ratio for *GSTT1* gene deletion compared to controls was 1.5 with 95% CI of 0.9-2.4. However, it was found that 10 patients showed deletion of both *GSTM1* and *GSTT1* genes.

Mutations in GSTP1 gene

The current study found that 11% oral cancer patients had exonic substitution mutations. Patients had substitution mutations of A_{2848} to T and G_{2849} to A in *GSTP1* gene in exon 7 (Figer 3). The A_{2848} to T substitution causes a sense mutation changing amino acid coding sequence from CUU to CUA at codon 166. The amino acid sequence CUU codes for leucine and CUA also codes for leucine.

Table 2. Age, Sex Ratio and Tobacco Users with CYP1A1, GSTM1, GSTT1 and GSTP1 Gene Polymorphisms in Cases

Gene	Polymorphism	Age	Male:Female	Tobacco Users
All study group	All study group	47.4 (±16.3)	1:01	55%
CYP1A1	A to C and Frameshift (T insertion)	49.9(±16.5)	1:2	69%
GSTM1	Null Cases	49 (±12.5)	3:2	60%
	Null Controls	46 (±13.4)	2:1	50%
GSTT1	Null Cases	45 (±11.4)	2:1	70%
	null Controls	44 (±13.8)	2:1	53%
GSTP1	A to T and G to A (Exonic)	46.3 (±15.9)	2:1	62.50%
	Two C deletions (Intronic)	47.9(±12.9)	8:1	77.80%

Whereas, at codon 167, G_{2849} to A substitution causes a missense mutation resulting in change of amino acid coding sequence from GCC to ACC. GCC codes for alanine while ACC code threonine. These substitution mutations are in the C terminal region of *GSTP1* gene. No normal control had these mutations.

Results of sequencing of exon 4 and 5 along with intronic exonic junctions showed two deletions of cytosine. These deletions are in intron 3 and 4 and found 4% patients. Intronic deletions of C_{1074} and C_{1466} were found in patients, whereas no control showed these deletions. These variants were in the non coding region therefore they had no effect on protein structure.

The results for *CYP1A1*, *GSTM1*, *GSTT1* and *GSTP1* gene variations suggest increased association of these genes as a risk factor for oral cancer in Pakistani population.

Discussion

The current case control study found an increased risk of oral cancer associated with *CYP1A1* substitution and frameshift mutations, *GSTM1* and *GSTT1* gene deletions and exonic and intronic variants in *GSTP1* gene.

CYP1A1 polymorphism are frequently reported in literature, 12 nucleotide polymorphisms at positions 3229, 3219, 134, 1636, 2414, 2453, 2455, 2461, 2500, 2546, 3205, and 3801 have been reported in addition to a frame-shift mutation due to a single base insertion between 2346 and 2347 have been found so far as cited in article by Duk *et al*. (2004). Nine polymorphisms among them are associated with amino acid substitutions (Spurr et al., 1987; Hayashi et al., 1991; Crofts et al., 1993; Cascorbi et al., 1996; Smart et al., 2000; Chevalier et al., 2001; Saito et al., 2002). Insertional mutation of 33 nucleotide sequence causing frameshift in *CYP1A1* is also reported in earlier studies (Xiang et al., 2001). The population frequencies of various *CYP1A1* polymorphisms follow diverse ethnic and/or geographic specific patterns (Garte et al., 2001). In the present case- control study none of the already reported variants of *CYP1A1* gene were observed in Pakistani population. However, novel mutations in exon 2 of *CYP1A1* gene were observed, a substitution mutation which causes tyrosine to change in serine at amino acid number 110 of *CYP1A1* gene. Tyrosine to serine substitution mutation causes a change in conserved domain of Cytochrome P450. This mutation cause, a change in the protein structure as an aromatic amino acid is changed into a non aromatic amino acid and subsequently gene function is also altered. Due to frameshift mutation all the amino acids after the insertion are altered leading to unstable/ altered protein expression. Therefore these mutations might lead to imbalanced functional activity in detoxification process as *CYP1A1* is a key enzyme that converts PAHs into active carcinogens (Hecht et al., 1993; Bartsch et al., 2000). Mutated *CYP1A1* gene cannot convert carcinogen into a hydrophilized form required for phase II enzyme activation (*GSTM1*, *GSTP1*, *GSTT1*) for the process of detoxification.

Deleted genotype of *GSTM1* gene was observed to be associated with oral cancer in Pakistani population. *GSTM1* gene is deleted in many populations with oral cancer (Cheng et al., 1999; Kim et al., 2000), population-based studies conducted among Chinese and Korean reported a frequency of nearly 50% for the *GSTM1* deletion genotype (Lee et al., 1995 ; Kim et al., 2000; Landi et al., 2000). *GSTM1* deletion genotype varies by ethnic group among African, Asian, Hispanic, European (Mishra et al., 2004), Caucasians (Nazar et al., 1999; Naoe et al., 2000; Palli et al., 2000; Naveen et al., 2004), French (Park et al., 2000) and Asians (Kihara et al., 1997). Our results of deletion of *GSTM1* gene in oral cancer are in accordance with previously published data (Pemble et al., 1994; Rebbeck et al. , 1997; 1999; Naveen et al., 2004; Patrick et al., 2009).

In this study a significant number of oral cancer patients with *GSTT1* deletion genotype have been found when compared with the controls. Similar results, regarding *GSTT1* gene deletions, found in Pakistani population has also been reported in different populations such as, Americans (Mishra et al., 2004; Nazar et al., 1999; Palli et al., 2000), Italians (Schneider et al., 2004), Caucasians, Black Brazilians of South America and Amazonian Brazilians (Setiawan et al., 2000). Asian populations are reported to have the highest *GSTT1* deletion genotype. Presence of null genotypes for *GSTM1* and *GSTT1* has been reported in different populations in both cancer individuals and cancer free controls. In this study, cancer free Pakistani population is also reported to have *GSTM1* and *GSTT1* null genotype, and the percentage of individuals with *GSTM1* and *GSTT1* null genotypes are reported to be 23% and 45% (Stacy and Andrew, 2000) similar to other populations in Singapore (Singh et al., 2009), Turkey (Toefil et al., 2007), Poland (Trizna et al., 1995), China (Setiawan et al., 2000) and in Japan (Mishra et al., 2004). Indians also have higher frequency of *GSTM1* and *GSTT1* deletion genotype (Mishra et al., 2004; Naveen et al., 2004; Singh et al., 2009). Although variant alleles for *GSTM1* and *GSTT1* have been described in literature, but the gene deletion seems to be related

to disease susceptibility. The normal individuals with *GSTM1* or *GSTT1* gene deletions are more susceptible to oral cancer.

No already reported variants of *GSTP1* gene were found. This result is in disagreement with most previously published studies (Joanne et al., 2000; Cho et al., 2006; Peters et al., 2006). One possible reason for this may be due to changing trends of *GSTP1* polymorphisms in different populations. Moyer (2008) found 35 SNPs in four ethnic groups in America, 17 of these SNPs were novel. To the best of our knowledge, this study is the first ever to report 4 novel mutations in *GSTP1* gene in Pakistani population. Two silent mutations with intronic deletions of C and two exonic nonsynonymous substitution mutations altering *GSTP1* mRNA expression are found. The exonic substitutions result in leucine to leucine formation and a nonsynonymous alanine to threonine. These two exonic mutations are present at codon 166 and 167. They are in the GST motif II (α6 helix residues 150–167 and the preceding loop residues 137-149). GST motif II contains the "hydrophobic staple" made up of Ile149 and Tyr154 necessary for GST folding (Cocco et al., 2001); mutations in this motif have been shown to affect folding and refolding pathways of the enzymes (Dragani et al., 1997; Rossjohn et al., 2000; Cocco et al., 2001). It is hypothesized that the GST motif II is involved in the nucleation mechanism of the protein and that the substitution of alanine by threonine may alters this transient substructure. The current mutation causes a change in C terminal protein domain altering the functional activity of *GSTP1*. Mechanistically, two single nucleotide variations in the non-coding region of the *GSTP1* gene may either result in differential binding of putative regulatory proteins, or it may be in linkage disequilibrium with other mutations affecting *GSTP1* inducibility.

In conclusion, The current variations in C*YP1A1, GSTM1, GSTT1* and *GSTP1* genes may be one of the several factors associated with oral cancer risk. Polymorphisms in these genes, and alterations in their expression and function, may increase or decrease carcinogen activation/detoxification followed by a variation of cancer risk. .

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