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Cytochrome P450 1A1 and Glutathione S-Transferase Pi 1 Mutations in Pharyngeal and Laryngeal Carcinoma

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Abstract

In the current case control study, 94 pharyngeal, 67 laryngeal cancer cases and 150 cancer free controls were screened via PCR-SSCP assay. Mean ages of pharyngeal, laryngeal cancer patients and control was 48.14 (± 16.7), 48.56 (± 17.4) and 46 (± 17.69) years respectively. Results revealed two novel mutations in CYP1A1 gene, a substitution mutation of $A_{2842}C$ resulting in missense tyrosine to serine formation and frameshift mutation due to insertion of thymidine at nucleotide 2842 resulting in 495 nucleotide sequences to alter. It was found that 3.2% pharyngeal and 2.98% laryngeal cancer patients had these mutations in CYP1A1. In GSTP1 gene exon 7, an $A_{2848}T$ substitution causes a leucine to leucine formation whereas $G_{2849}A$ substitution causes alanine to threonine formation at amino acid 166 and 167 respectively. These exonic mutations were found in 7.4% pharyngeal cancer and 9% laryngeal cancer patients. Two intronic deletions of C at nucleotide 1074 and 1466 were found in 1% pharyngeal and laryngeal cancer patients. Accumulation of mutations in CYP1A1 and GSTP1 genes seem to be associated with increased risk of pharyngeal and laryngeal cancer development.

Keywords: CYP1A1; GSTP1; Pharyngeal cancer; Laryngeal cancer; Pakistani population

Introduction

Polymorphisms in the carcinogen detoxifying gene may increase or decrease carcinogen activation or detoxification followed by variation of cancer risk [1]. Most of the carcinogenic moieties are metabolically processed by xenobiotic-metabolizing enzymes in two broad steps: phase I mediated by Cytochrome p450s (CYPs) and phase II catalyzed by glutathione S-transferases (GSTs). Phase I reactions expose functional groups of the substrates and therefore yield highly reactive intermediates. These intermediates form the substrates for phase II reactions that involve their conjugation with endogenous molecules such as glutathione (GSH) and thus facilitate their elimination. Hence, the coordinated expression and regulation of phase I and II enzymes determines the outcome of carcinogen exposure. Sequence variants or polymorphisms in these genes can alter the expression, function and/or activity of these enzymes and, in turn, cancer risks [2].

The Cytochrome P-450 (phase I enzyme) that are known to exhibit polymorphism include CYP1A1, CYP1B1 [3], CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 [4]. Polymorphism of CYP1A1 gene has been studied with relation to pharyngeal and laryngeal carcinoma but with some conflicting results [5]. Four different sequence variants have been reported in CYP1A1 gene, first known as *CYP1A1*2* involves a T6235 to C transition in the 3' non-coding region, second known as *CYP1A1*3* involve a A4889 to G transition in exon 7, third known as *CYP1A1*4* involves a T_{5639} to C transition in intron 7, and fourth known as *CYP1A1*5* involves a C4887 to A transition in exon 7 [6].

GSTP1 is located on chromosome 11q13 and is one of the phase II detoxifying enzymes. GSTP1 catalyze the conjugation of glutathione (GSH) to toxic compounds, resulting in more water-soluble and less biologically active products that may be easily excreted. To date two polymorphic alleles are known for GSTP1, GSTP1*B and GSTP1*C, in addition to the wild-type allele, GSTP*A [7]. 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 [8].

As polymorphism in these genes are common in studies conducted in South East Asia [9] and show different trends in ethnic groups. The current study is designed to find out germline mutations in CYP1A1 and GSTP1 genes in Pakistani population suffering from pharyngeal and laryngeal carcinoma.

Materials and Methods

The present case-control study consisted of 94 pharyngeal cancer and 67 laryngeal cancer cases along with age and sex matched 150 cancer free normal individuals as controls. They were recruited from National Oncology and Radiotherapy Institute (NORI) and Pakistan Institute of Medical Sciences (PIMS) from March 2008 to September 2009 with a prior approval from Ethical Committees of both CIIT and respective hospitals. All study subjects participated on a volunteer basis with informed consent. All subjects were personally interviewed according to a structured questionnaire. Subjects' blood was sampled before starting the therapy.

Blood samples were collected in EDTA-containing tubes and stored at -20°C until further use. DNA was isolated, using organic protocol with phenol–chloroform extraction as previously described [10,11]. Electrophoresis was performed on isolated DNA in 1% ethidium-bromide stained agarose gel and photographed (BioDocAnalyze Biometra). 5 ng dilutions were made of each DNA isolated and stored at 4°C until use.

Primers for all exons of CYP1A1 and GSTP1 were designed by using primer 3 input software version 0.4.0 and BLAST using NCBI PRIMER BLAST. 10 ng/µl DNA (2 µl) was added to a 20 µl PCR mixture

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composed of PCR buffer (2 µl), 10 mM of each primer (2 µl), 25 mM deoxynucleotide triphosphate (0.24 µl) and 5 u/µl Taq polymerase (0.2 µl). The reaction mixture was placed in 9700 thermal cycler of ABI systems for 5 min at 94°C and subjected to 30 cycles at 94°C for 25 sec, annealing temperature for 1 min and 72°C for 1 min, followed by a final step at 72°C for 10 min and hold at 4°C.

Amplification products were separated on a 2% ethidium bromide–stained agarose gel along with 100 bp DNA ladder. All the patients and control DNA was amplified for all exons of CYP1A1 and GSTP1 gene with exon specific primers. All of the photographs of gel electrophoresis were read by two technicians blind to each other's assessments.

PCR product was analyzed by Single stranded conformational polymorphism (SSCP) through the procedure described by Patricia et al. [12]. SSCP results were analyzed with gel documentation system (BioDocAnalyze Biometra) after ethidium bromide staining and photographed. The samples showing mobility shifts from the controls were then sequenced.

21 samples were screened out from SSCP and were sequenced from Macrogen (Korea). Reverse primer was used for sequencing. The sequenced results were made forward complementary before analysis using BioEdit v 7.0.5 software and analyzed. Statistical analysis was performed by using SPSS statistics 17.0 software and GraphPad Prism 5.

Results

No already reported polymorphisms in CYP1A1 gene were found in the current study. However, two novel mutations in CYP1A1 gene were found in patients but not in controls. One substitution mutation of A_{2842} with C and second a frameshift mutation due to insertion of thymidine at position 2842 (Figures 1 and 3). The A_{2842} to C substitution 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 the conserved P450 domain and not in the transmembrane domain.

Due to frameshift mutation following 495 nucleotides sequence protein structure was altered. These mutations were present in 2nd exon of CYP1A1 gene and 3.2% pharyngeal and 2.98% laryngeal cancer patients had these CYP1A1 mutations.

15 amplified products showing altered mobility patterns for GSTP1 exon 7 were sequenced. The results of sequencing revealed two substitution mutations of A_{2848} to T and G_{2849} to A in GSTP1 gene in exon 7 (Figures 2 and 4). 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. 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. These exonic mutations were found in 7.4% pharyngeal cancer and 9% laryngeal cancer patients. No normal control showed these mutations.

Results of sequencing of exon 4 and 5 along with intronic exonic junctions showed deletions. Two intronic deletions of C_{1074} and C_{1466} in intron 3 and 4 respectively were found in patients whereas no control showed these deletions (Figure 5). 1% pharyngeal and 1% laryngeal cancer patients had these deletions.

Discussion

This case control study is the first research conducted in Pakistani population on CYP1A1 and GSTP1 genes in relation to pharyngeal and laryngeal cancer patient. The current case control study found novel substitution and frameshift mutations in pharyngeal and laryngeal cancer patients whereas no control had these mutations. Thirteen nucleotide polymorphisms in CYP1A1 gene have been reported by different researchers as cited in article by Park et al. [13] among them 12 are at positions 3229, 3219, 134, 1636, 2414, 2453, 2455, 2461, 2500, 2546, 3205, and 3801, and 13th is a frame-shift mutation due to a single base insertion between 2346 and 2347. A study on Korean population of Asian origin found no already reported polymorphisms of CYP1A1 gene [13]. Among these, nine polymorphisms were associated with amino acid substitutions [14]. Insertional mutation of 33 nucleotide sequence causing frameshift in CYP1A1 is also reported in earlier studies [15]. The population frequencies of various *CYP1A1* polymorphisms follow diverse ethnic and/or geographic specific patterns [16]. This is the first case-control study in Pakistani population on head and neck cancer reporting none of the already reported variants of CYP1A1 gene but a novel substitution and a frameshift mutation.

No already reported variants of GSTP1 gene were found. This result was in disagreement with most previously published studies [17]. One possible reason for this may be due to changing trends of

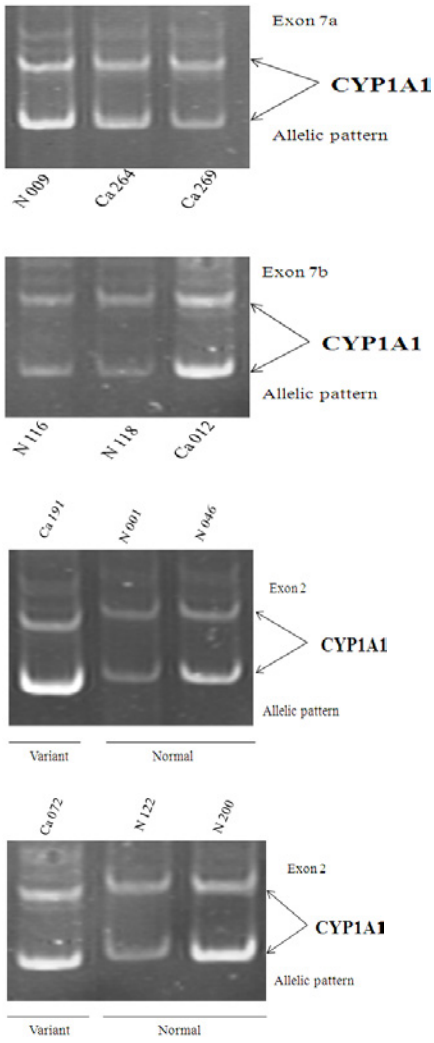

Figure 1: PAGE picture showing SSCP of normal and cancer patients with normal and variant bands for CYP1A1 gene exon 7 and exon 2.

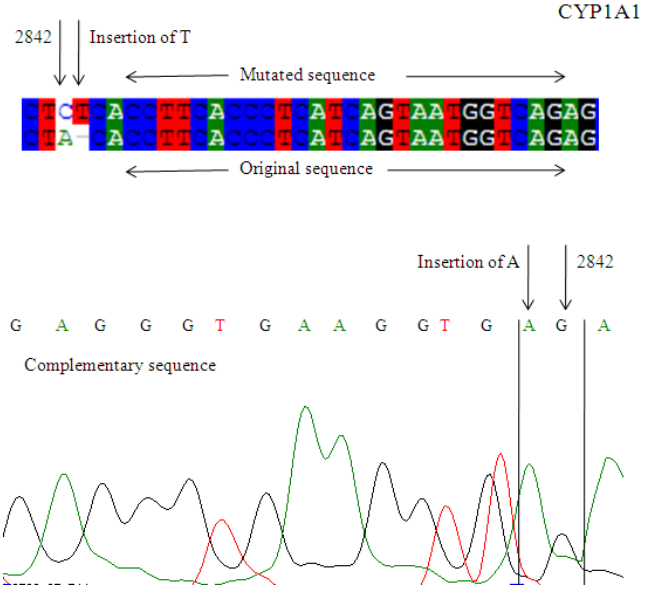

Figure 2: BioEdit software figure showing the position of T insertion at nucleotide 2842 in exon 2 of CYP1A1 gene causing a frameshift mutation and A to C substitution mutation in pharyngeal and laryngeal cancer patients. Reverse primer was used for sequencing.

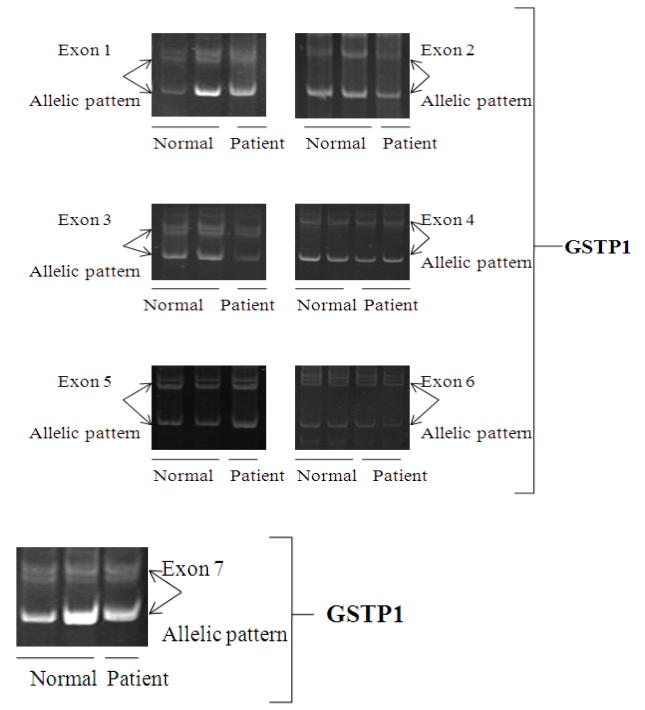

Figure 3: PAGE picture showing SSCP of normal and cancer patients with normal and variant bands for GSTP1 gene exons 1 to 7.

GSTP1 polymorphisms in different populations as this is the first ever case control study in Pakistani population on pharyngeal and laryngeal cancer. Different variations are observed in populations as Moyer et al. [18] found 35 SNPs in four ethnic groups in America, 17 of these SNPs were novel. Pakistan is in the South Asian region that is rich in genetic variations having novel as well as reported polymorphisms which ultimately effects disease progression [19] (Table 1).

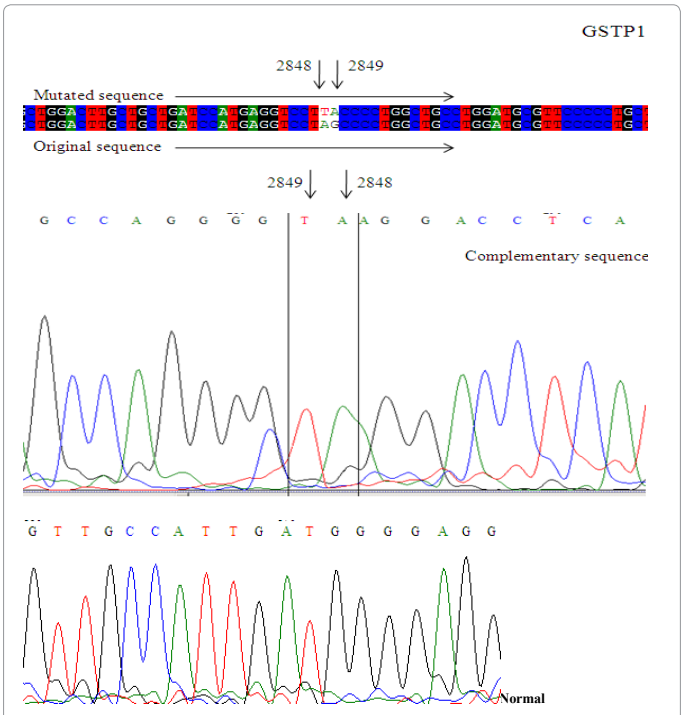

Figure 4: Normal sequencing and substitution mutations of A to T and G to A in pharyngeal and laryngeal cancer patients in GSTP1 gene at position 2848 and 2849. Reverse primer was used for sequencing.

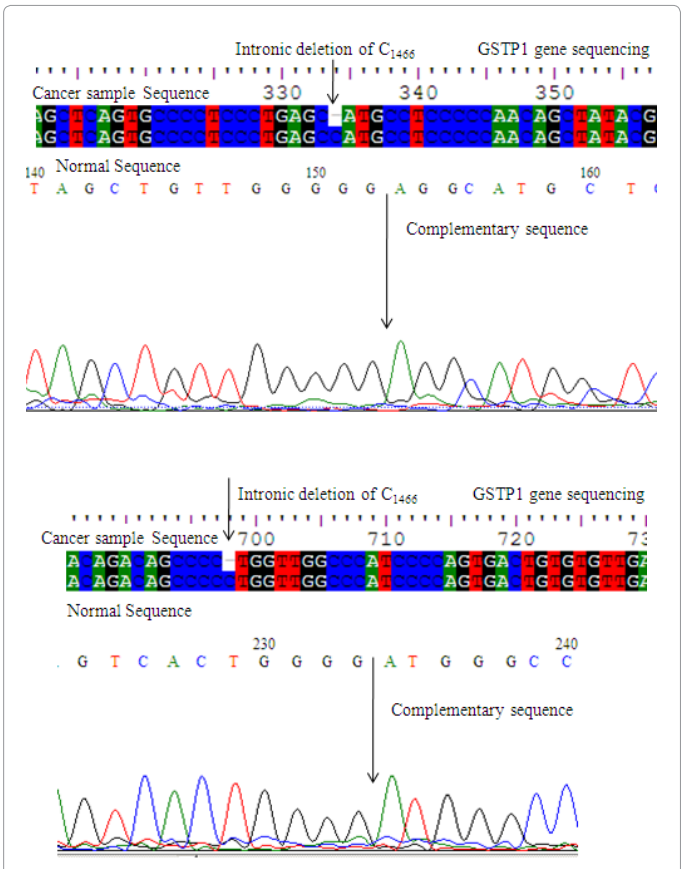

Figure 5: Results of sequencing of *GSTP1* gene showing intronic deletion of C in the intron 3 and 4.

	Cancer of Pharynx	Cancer of Larynx
Male	49	34
Female	45	33
Age	48.1 (± 16.7)	48.57 (± 17.4)
Smoker	54	34
Non smoker	40	33

Table 1: Demographic details of laryngeal and pharyngeal cancer patients.

To the best of our knowledge, this study is the first to report 4 novel mutations in GSTP1 gene in Pakistani population. Two silent variations with intronic deletions of C and two exonic nonsynonymous substitution mutations altering GSTP1 mRNA expression are found. These two exonic mutations are present at codon 166 and 167 and 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 [20]; mutation in this motif have been shown to affect folding and refolding pathways of the enzymes [20]. 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 mutations 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. The A/T and G/A mutations could contribute to inter individual differences in the metabolism of other GSTP1 substrates.

Although SSCP based analysis was accurate as previously novel mutations along with already known mutations were also reported using the same methodology [21] and the same time one of our colleague found an already reported mutation in XRCC1 gene in these patients. But sensitivity and technique limitations were also taken in consideration while conducting this research and further confirmation was done via sequencing.

CYP1A1 and GSTP1 genes mutations are present in pharyngeal and laryngeal cancer patients in Pakistani population. However, for determining the role of genetic mutations as cancer risk requires integrated analysis of many genes involved in cancer development. The identification of mutations in genes associated with xenobiotic metabolism provides a basis for understanding the high degree of individual variation in susceptibility to the adverse effects of environmental chemicals.

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Conflict of Interest

This research has no conflict of interests.

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