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Research Article

Mutational analysis of xenobiotic metabolizing genes (*CYP1A1* and *GSTP1*) in sporadic head and neck cancer patients

Nosheen Masood and Mahmood Akhtar Kayani

Cancer Genetics Laboratory, Department of Biosciences, COMSATS Institute of Information and Technology, Islamabad, Pakistan.

Abstract

CYP1A1 is the phase I enzyme that detoxifies the carcinogen or converts it into a more electrophilic form, metabolized by phase II enzymes like GSTP1. These detoxifying genes have been extensively studied in association with head and neck cancer (HNC) in different ethnic groups worldwide. The current study was aimed at screening genetic polymorphisms of genes *CYP1A1* and *GSTP1* in 388 Pakistani HNC patients and 150 cancer-free healthy controls, using PCR-SSCP. No already known variants of either gene were found, however a novel frameshift mutation due to insertion of T (g.2842_2843insT) was observed in the *CYP1A1* gene. A statistically significant number (5.4%) of HNC cases, with the mean age of 51.75 (±15.7) years, presented this frameshift mutation in the conserved domain of *CYP1A1*. Another novel substitution mutation in was found in the *GSTP1* gene, presenting TA instead of AG. The g.2848A > T polymorphism causes a leucine-to-leucine formation, whereas g.2849G > A causes alanine-to-threonine formation at amino acid positions 166 and 167, respectively. These exonic mutations were found in 9.5% of the HNC patients and in none of the controls. In addition, two intronic deletions of C (g.1074delC and g.1466delC) were also found in 11 patients with a mean age of 46.2 (±15.6) years. In conclusion, accumulation of mutations in genes *CYP1A1* and *GSTP1* appears to be associated with increased risk of developing HNC, suggesting that mutations in these genes may play a role in the etiology of head and neck cancer.

Key words: GSTP1, CYP1A1, Head and neck cancer, polymorphisms, mutations.

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Introduction

Head and neck cancer (HNC) includes carcinomas of the oral cavity, pharynx, and larynx. It is the sixth most frequent cancer worldwide (Devasena *et al.*, 2007), amounting to half a million diagnosed cases every year (Faheem, 2007). HNC represents 40.1% of all cancers registered (Parkin *et al.*, 1993) and is the second most prevalent in the Pakistani population (Hanif *et al.*, 2009).

Many environmental factors, including smoking and alcohol consumption, as well as genetic factors, are responsible for the development of HNC. Tobacco addiction is an important and strong risk factor associated with HNC (Rajani *et al.*, 2003), however the majority of tobacco-addicted individuals do not develop this type of cancer (Lewin *et al.*, 1998). The reason for this contrast is probably the fact that both exogenous exposure and genetic predisposition are involved in the development of HNC (Peters *et al.*, 2006; Devasena *et al.*, 2007).

Send correspondence to Nosheen Masood. Cancer Genetics Laboratory, Department of Biosciences, COMSATS Institute of Information and Technology, Park Road, Islamabad, Pakistan. E-mail: nosheenmasood@hotmail.com.

Polymorphisms in the carcinogen-detoxifying genes may increase or decrease carcinogen activation or detoxification, with a consequent variation in cancer risk (Curran *et al.*, 2000; Devasena *et al.*, 2007). 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, which involves their elimination. Hence, the coordinated expression and regulation of phase I and II enzymes determine the outcome of carcinogen exposure. Sequence variations or polymorphisms in these genes can alter the expression, function and activity of these enzymes and, consequently, the cancer risks (Duk *et al.*, 2004).

Cytochromes P-450 (phase I enzyme) that are known to exhibit polymorphism include CYP1A1, CYP1B1 (Crofts *et al.*, 1993; Bartsch *et al.*, 2000), CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 (Bartsch *et al.*, 2000). Polymorphism of the *CYP1A1* gene has been studied most extensively in relation to HNC (Toru *et al.*, 2008). It is located on chromosome 15q22-24 and encodes

an aromatic hydrocarbon hydroxylase that converts PAHs to carcinogen and is predominantly expressed in extra-hepatic tissues (Crofts *et al.*, 1993). Its polymorphisms have been shown to increase the microsomal catalytic activity for activating pro-carcinogens (Cascorbi *et al.*, 1996).

GSTP1 is located on chromosome 11q13 and encodes one of the phase II detoxifying enzymes. GSTP1 catalyse the conjugation of glutathione (GSH) to toxic compounds, resulting in more water-soluble and less biologically active products that are 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 (Ali *et al.*, 1997). Both alleles present an A-to-G transition at nucleotide 313 (codon 104), causing an isoleucine-to-valine change. The *GSTP1**C allele presents a C-to-T transition at nucleotide 341 (codon 113), in addition to the substitution at nucleotide 313, that changes alanine to valine. These two GSTP1 proteins differ in specific activity, affinity for electrophilic substrates and heat stability (Ali *et al.*, 1997 Zimniak *et al.*, 1994).

These gene polymorphisms show different trends in different ethnic groups and have been found to be common in South East Asia (Rajani *et al.*, 2003; Devasena *et al.*, 2007). The current study was designed to search for *CYP1A1* and *GSTP1*gene polymorphisms in a Pakistani sample.

Material and Methods

The present case-control study consisted of 388 cases with pathologically confirmed head and neck cancer and 150 cancer-free healthy individuals, matched for age and gender, as controls. They were all recruited from the National Oncology and Radiotherapy Institute (NORI) and the Institute of Medical Sciences (PIMS), Pakistan, from March 2008 to September 2009, with prior approval from the Ethics Committees of both the CIIT and the hospitals. All study subjects participated on a volunteer basis, with informed consent. All subjects were personally interviewed according to a structured questionnaire.

Blood from the subjects was sampled before starting therapy. Blood samples were collected in EDTA-containing tubes and stored at 20 °C until further use. DNA was isolated using an organic protocol with phenol-chloroform extraction, as previously described (Baumgartner-Parzer *et al.*, 2001; Vierhapper *et al.*, 2004). The isolated DNA was electrophoresed on 1% ethidium-bromide-stained agarose gel, and photographed (BioDocAnalyze Biometra). Dilutions of 5 ng were made of each isolated DNA and stored at 4 °C until further use.

Primers for all exons of *CYP1A1* and *GSTP1* were synthesized by using the primer 3 input software version 0.4.0 and BLAST using NCBI PRIMER BLAST (Table 1). 2 µL of DNA (10 ng/µL) were added to a 20 µL PCR reaction mixture composed of 2 µL PCR buffer, 2 µL of each primer (10 mM), 0.24 µL deoxynucleotide triphosphate (25 mM) and 0.2 µL Taq polymerase (5u/µL). The reaction mixture was then placed in a 9700 ABI Systems thermal cycler for 5 min at 94 °C and subjected to 30 cycles at 94 °C for 25 s, annealing temperature for 1 min, and 72 °C for 1 min, followed by a final step at 72 °C for 10 min, and held at 4 °C. In order to avoid any false-positive alteration, a proofreading polymerase reaction was also performed in this regard.

Amplification products were resolved on 2% ethidium bromide-stained agarose gel, along with a 100 bp

Table 1 - Primer sequences used in PCR SSCP for *GSTP1* and *CYP1A1*.

	Exons	Primer Sequences (5'-3')	Product size (bp)
CYP1A1 gene	Exon1F	GGTTGTGATTAGTTCTTTGG	459
	Exon1R	GTGTTGAAAAGGAGAGGAGT	
	Exon2aF	GAATGAAATGGAGTTGGATT	381
	Exon2aR	AGGATCGTATTCTCTGCTGT	
	Exon2bF	AGAACCAAGGCTCCATAAT	476
	Exon2bR	ATTGCATGAATGTGGTTAGA	
	Exon3F	CCTTCTCTCCATTCCCCTGT	150
	Exon3R	GTAAGACAAAGGCTGGTGCTG	
	Exon4F	GCCTGGGTTAAGTATGCAGAT	154
	Exon4R	CTGACAGGGCACCCAATACT	
	Exon5F	TGACACTTTGAATGCTCTTTCC	154
	Exon5R	AAACCAAACCCATGCAAAAG	
	Exon6F	AGGACCCTGGAGTCGATTG	163
	Exon6R	AGCTCCTGGCACTGGTAGAG	
	Exon 7aF	GCATTGATCCTCCTGTCCAT	594
	Exon 7aR	CAGAGGCAAGTCCAGGGTAG	
	Exon 7bF	TGTCTACCTGGTCTGGTTGG	600
	Exon 7bR	CCTCCAGGACAGCAATAAGG	
	Exon 7cF	CTGCCAAGAGTGAAGGGAAG	590
	Exon 7cR	AACACAGAATGGGGTTCAGG	
GSTP1 gene	Exon1F	AGTTCGCTGCGCACACTT	465
	Exon1R	GACGTCCTGGGTCCCCTA	
	Exon2F	GTCCCCAGTGCCGTTAGC	277
	Exon2R	GATAAGGGGGTTCGGATCTC	
	Exon3F	GGAGGAACCTGTTTCCCTGT	277
	Exon3R	GTCCCCCGATCCTAGTCAC	
	Exon4F	GGGGCTGTGACTAGGATCG	237
	Exon4R	GGGCAGCTGATTTAAACAAAA	
	Exon5F	ACAGACAGCCCCCTGGTT	227
	Exon5R	AAGCCACCTGAGGGGTAAG	
	Exon6F	GCAAGCAGAGGAGAATCTGG	278
	Exon6R	GCTAAACAAATGGCTCACACC	
	Exon7F	AGACCTAGGGGATGGGCTTA	451
	Exon7R	GTGCTGGAGGAGCTGTTTTC	

DNA ladder. All gel electrophoresis photographs were analyzed by two technicians blind to each other's assessments.

The PCR product was submitted to single-strand conformational polymorphism (SSCP) analysis, according to the procedure described by Telenti *et al.* (1993) and Sheen *et al.* (2009). After ethidium bromide staining, the SSCP results were analyzed with a gel documentation system (BioDocAnalyze Biometra) and photographed. The samples showing mobility shifts were sequenced.

Forty-eight samples were screened based on SSCP analysis and sequenced by Macrogen (Korea) using forward and reverse primers. The reverse-primer-sequenced results were made forward-complementary and analyzed using BioEdit v 7.0.5 software. The reference sequences for *CYP1A1* (MIM ID-108330 and NG_008431.1) and *GSTP1* (MIM ID-134660 and NG_012075.1) were obtained from NCBI. Statistical analysis was performed by using the SPSS statistics 17.0 software and GraphPad Prism 5 Demo for calculating odd ratios, with a 95% confidence interval.

Results

No previously reported polymorphisms of the *CYP1A1* gene were found in the present study. Instead, a novel frameshift mutation due to thymidine insertion (g.2842_2843insT) was found (Figure 1). A significant number of patients had a mutation in exon 2 of the *CYP1A1* gene, not observed in any of the controls. Due to this mutation, the conserved core structure was altered, which disturbs the proper folding and heme-binding ability of the cytochrome P450 molecules. This frameshift mutation causes a change in the subsequent 495 nucleotide sequence, altering the protein structure of the *CYP1A1* gene. The mean age of patients showing the frameshift mutation was 51.75 (±15.7) years and 62% were males (Table 2).

Thirty-seven SSCP variants for *GSTP1* exon 7 were sequenced (Figure 2). A significant number ($p < 0.001$) of patients had substitution mutations of g.2848A > T and g.2849G > A in exon 7 of the *GSTP1* gene (Figure 3). The g.2848A > T mutation causes a sense mutation, changing the amino acid coding sequence from CUU to CUA at codon 166. Both the amino acid sequences CUU and CUA code for leucine. However, at codon 167, g.2849G > A causes a missense mutation, resulting in the change of the amino-acid-coding sequence from GCC to ACC. GCC

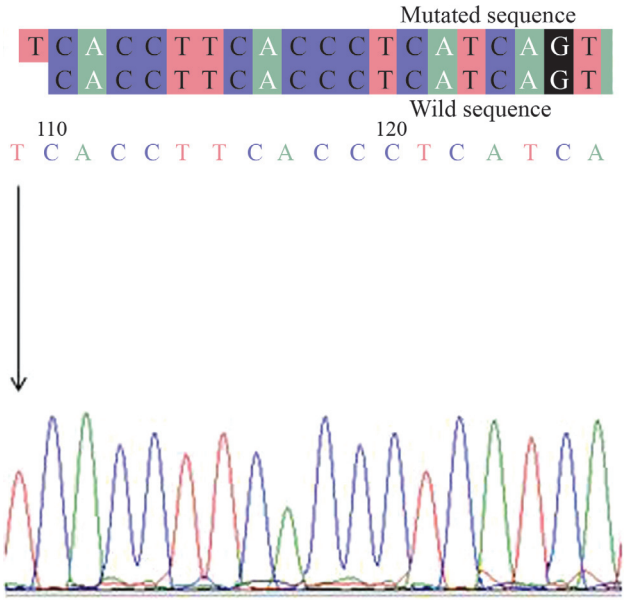

Figure 1 - Position of T insertion at nucleotide 2842 in *CYP1A1* exon 2, causing a frameshift mutation, in HNC patients.

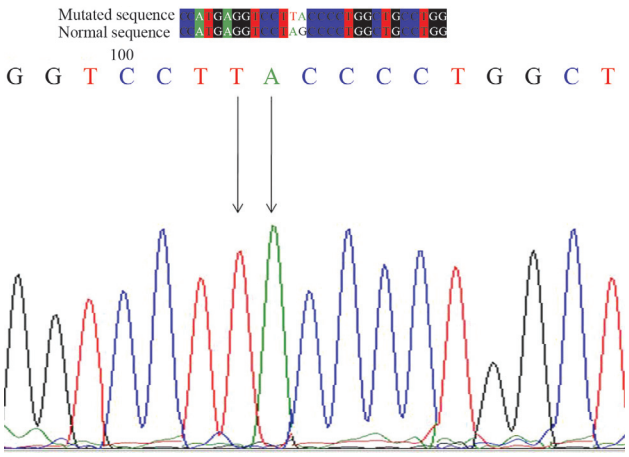

Figure 2 - Substitution mutations of A to T and G to A at positions 2848 and 2849 of the *GSTP1* gene, respectively, in HNC patients.

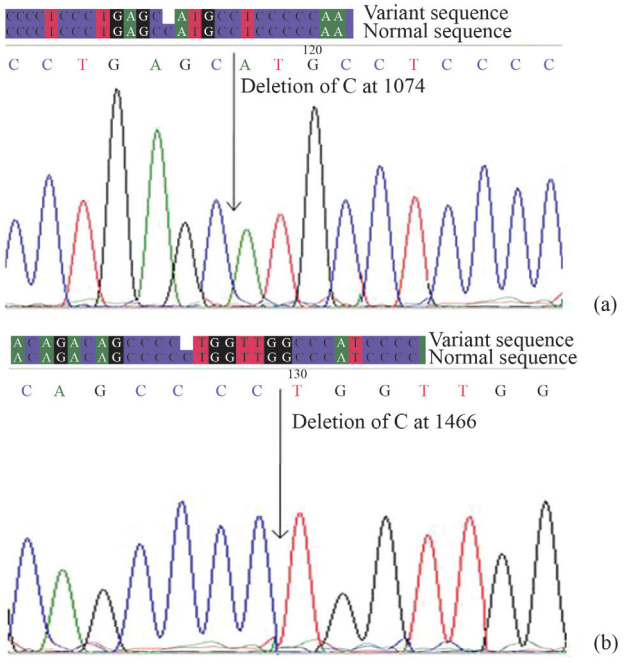

Figure 3 - Sequencing results showing deletion of C between C and A in intron 3 at position 1074 (a) and deletion of C in intron 4 of the *GSTP1* gene (b) in HNC patients.

Table 2 - Statistical evaluation of frameshift mutation in gene *CYP1A1* and exonic and intronic mutations in gene *GSTP1* in HNC patients

	CYP1A1 mutation		*GSTP1* substitution mutation		*GSTP1* intronic variation	
Variables	Frame-shift	OR (CI 95%)	A > T and G > A	OR (CI 95%)	at 1074	OR (CI 95%)
Total of patients	21	9.4 (1.3-70.8)	9.50%	15.7 (2.14-115.5)	2.80%	4.3 (0.56-34)
Gender						
Female	62%	1.6 (0.09-29.8)	32.40%	0.48 (0.22-1.03)	18.20%	0.22 (0.22-1.03)
Male	38%	0.6 (0.03-11.3)	67.60%	2.08 (0.97-4.45)	81.80%	4.5 (0.94-21.53)
			Age (years)			
Mean	51.75 ± 15.7		46.2 ± 15.6		48.18 ± 11.8	
< 48	52%	1.1 (0.06-20.0)	45.90%	0.85 (0.41-1.75)	45.50%	0.83 (0.424-2.85)
> 48	48%	0.9 (0.05-16.5)	54.10%	1.17 (0.57-2.42)	54.50%	1.2 (0.35-4.1)
			Smoking			
Yes	62%	1.6 (0.09-29.8)	64.90%	1.85 (0.87-3.89)	63.60%	1.75 (0.49-6.22)
No	38%	0.6 (0.03-11.3)	35.10%	0.54 (0.26-1.14)	36.40%	0.57 (0.16-2.03)
			Occupation			
Job	9	0.75 (0.04-13.7)	35.10%	0.3 (0.13-0.76)	45.50%	0.69 (0.13-3.72)
Jobless	8	0.62 (0.03-11.3)	35.10%	0.3 (0.13-0.76)	18.20%	0.05 (0.006-0.43)
House- wife	4	0.2 (0.01-4.62)	29.80%	0.18 (0.07-0.49)	36.30%	0.33 (0.06-1.86)
			Location of Cancer			
Oral cavity	76%	3.2 (0.17-61.02)	64.90%	3.4 (1.3-8.8)	81.80%	20.25 (2.32-176.8)
Pharynx	14%	0.17 (0.01-3.4)	18.90%	0.05 (0.02-0.2)	9.10%	0.01 (0.0005-0.18)
Larynx	10%	0.11 (0.005-2.4)	16.20%	0.03 (0.02-0.13)	9.10%	0.01 (0.0005-0.18)

codes for alanine, while ACC codes for threonine. These substitution mutations are located in the C-terminal region of the *GSTP1* gene. These mutations were observed in a statistically significant (OR 2.08, 95% CI 0.97-4.45) number of male patients. Cancer of the oral cavity was found to be the most prevalent ($p < 0.05$, OR 3.4, 95% CI 1.3-8.8) in these patients (Table 2). These mutations were not observed in any of the healthy controls.

The results of exon 4 and 5 sequencing, along with intron-exon junctions, showed cytosine deletions. These deletions were located in introns 3 and 4, and were found in 2.08% of the patients (Figure 3). Intronic deletions (g.1074delC and g.1466delC) were found in a statistically significant ($p < 0.05$) number of patients and in none of the controls. The mean age of patients showing these deletions was 48.18 (± 11.8) years, and a significant ($p < 0.05$, OR 4.5, 95% CI 0.94-21.53) number of patients were male and had cancer of the oral cavity (OR 20.25, 95% CI 2.32-176.8) (Table 2).

Discussion

In an earlier study, 13 nucleotide polymorphisms (at 12 positions: 3229, 3219, 134, 1636, 2414, 2453, 2455, 2461, 2500, 2546, 3205, and 3801) and one frameshift mutation due to a single-base insertion between 2346 and 2347 in *CYP1A1* have been reported in the Korean population (Duk *et al.*, 2004). Among these, nine polymorphisms were associated with amino acid substitutions (Spurr *et al.*, 1987; Hayashi *et al.*, 1991; Crofts *et al.*; 1993; Cascorbi *et al.*, 1996; Smart and Daly, 2000; Chevalier *et al.*, 2001; Saito *et al.*, 2002). Insertion mutations of 33 nucleotide sequences causing frameshift mutations in *CYP1A1* have also been found (Xiang *et al.*, 2001). The population frequencies of the various *CYP1A1* polymorphisms follow diverse ethnic and/or geographic patterns (Garte *et al.*, 2001).

In the present study, none of the so far reported variants of the *CYP1A1* gene were observed in the Pakistani population. Instead, a novel frameshift mutation (g.2842_2843insT) affecting 495 nucleotide sequences was observed in the studied patients. Due to this mutation, all the amino acids subsequent to the insertion were changed and the protein structure was altered, leading to an altered protein expression.

Similar to *CYP1A1*, no previously reported variants were found for the *GSTP1* gene. These results are different from most of the studies in the literature (Curran *et al.*, 2000; Cho *et al.*; 2006; Peters *et al.*, 2006). A possible reason for this may be the variation in *GSTP1* polymorphisms in different populations. Moyer *et al.* (2008) found 35 SNPs in four ethnic groups in America, and 17 of these SNPs were novel mutations.

This study is the first to report four novel mutations in the *GSTP1* gene in the Pakistani population. Two silent mu-

tations with intronic deletions of C, one exonic nonsynonymous and one synonymous substitution mutations altering *GSTP1* mRNA expression were found. The exonic substitutions result in leucine-to-leucine formation and a nonsynonymous alanine-to-threonine. These two exonic mutations are located at codon 166 and 167, and they are in the GST motif II (α 6 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 (Dragani *et al.*, 1997); mutations in this motif have been shown to affect folding and refolding pathways of the enzymes (Dragani *et al.*, 1997; Cocco *et al.*, 2001 Rossjohn *et al.*, 2000; Stenberg *et al.*, 2000). 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 alter this transient substructure. The current mutation causes a change in the C-terminal protein domain, altering the functional activity of GSTP1. Mechanistically, two single nucleotide polymorphisms in the intronic 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, mutations in genes *CYP1A1* and *GSTP1* were found to be significantly higher in Pakistani patients with HNC compared to healthy controls. However, to determine the role of these genetic changes in increasing the cancer risk, an integrated analysis of many genes involved in cancer development is required. The identification of mutations in genes associated with the xenobiotic metabolism may provide a basis for understanding the high degree of individual variability in the susceptibility to the adverse effects of environmentalsubstances.

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