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 a 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).

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 catalyses 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*1B and GSTP1*1C, in addition to the wild-type allele, GSTP1*A (Ali et al., 1997). Both alleles present an A-to-G transition at nucleotide 313 (codon 104), causing an isoleucine-to-valine change. 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, along with a 100 bp DNA ladder (10 ng/L). 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 DNA ladder (10 ng/L). 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.

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

| Exons | Primer Sequences (5’-3’) | Product size (bp) |
|-------|--------------------------|------------------|
| Exon1F | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon1R | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon2aF | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon2aR | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon2bF | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon2bR | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon3F | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon3R | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon4F | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon4R | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon5F | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon5R | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon6F | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon6R | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon7aF | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon7aR | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon7bF | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon7bR | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon7cF | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon7cR | GGGGCTTGATGCCATCAGTTGC | 459 |

| GSTP1 gene | Exon4F | GGGGCTTGATGCCATCAGTTGC | 459 |
|-------------|-------|--------------------------|------------------|
| Exon4R | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon5F | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon5R | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon6F | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon6R | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon7F | GGGGCTTGATGCCATCAGTTGC | 459 |
| Exon7R | GGGGCTTGATGCCATCAGTTGC | 459 |
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 CUU 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.
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 non-
synonymous 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 (a 6 helix residues 150-167 and the pre-
ceding loop residues 137-149). GST motif II contains the
“hydrophobic staple” made up of Ile149 and Tyr154, nec-
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., 2001 Rossjo

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 in-
volved 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 environmental substances.

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The current mutation causes a change in the C-terminal
protein domain, altering the functional activity of GSTP1.
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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 af-
flecting GSTP1 inducibility.

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