RESEARCH COMMUNICATION

Association of GSTM1 and GSTT1 Gene Deletions with Risk of Head and Neck Cancer in Pakistan : A Case Control Study

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Abstract

Polymorphic deletions of GSTM1 and GSTT1 genes involved in the detoxification of potentially carcinogenic agents may be risk factors for various cancers, including head and neck cancer (HNC). In the present case-control study we aimed to access possible associations of HNC with GSTM1 and GSTT1 null genotypes in a Pakistani population. DNA was extracted from leukocytes of 388 cancer patients and 150 healthy controls by phenol-chloroform procedure. GSTM1 and GSTT1 deletion variants were genotyped by multiplex PCR assay with CYP1A1 as an internal control and further analyzed by primer specific PCR assay and sequencing. Mean age of cases and controls was 48 (+16.6) years with a male to female ratio of 1:1. Cancer of the oral cavity (57%) was most prevalent in the sampled population followed by pharynx and larynx (30% and 13% respectively). A statistically significant (P<0.05) association was observed for both null genotypes in contribution to HNC as compared with the controls. The odds ratio (OR) for the GSTM1 null genotype was 2.3 with a 95% CI of 1.5-5.5 and for GSTT1 OR was 2.04 with 95% CI of 1.3-3.1. These results suggest that the GSTM1 and GSTT1 null genotypes are risk factors for HNC development among the Pakistani population.

Keywords: Head and neck cancer - GSTM1 - GSTT1 - gene deletions - Pakistani population - risk factor

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Introduction

Incidence of HNC has increased significantly worldwide in the last 20 years (Toefil et al., 2007) and is now the fifth most common cancer worldwide (Jun et al., 2010). In Pakistan, rising incidence of HNC is observed in last few years and is ranked as second most prevalent cancer. Head and neck cancer includes the cancers of the oral cavity, pharynx and larynx. Cancer of oral cavity is common, followed by larynx and pharynx (Bhurgri et al., 2006). Its multifactorial etiology includes genetic susceptibility as well as environmental risk factors. Recent evidence indicates that carcinogen metabolizing genes play critical role in determining individual susceptibility to cancer (Patrick et al., 2009).

Carcinogens are detoxified by phase II enzymes, such as glutathione S-transferases (GSTs) which are involved in detoxification of polycyclic aromatic hydrocarbons (PAHs) and benzo (a) pyrene (Schneider et al., 2004). GSTs are family of dimeric protein enzymes known to play an important role in the Phase II detoxification of several carcinogens (Hayes and Pulford, 1995). GSTs catalyze the conjugation reactions between glutathione and carcinogen substrates and facilitate its excretion. Evidence suggests that genetic polymorphisms of these genes might increase individual susceptibility to HNC. Number of published studies have focused on GSTM1 and GSTT1 genetic variation with respect to HNC and have yielded conflicting results (Toru et al., 2008). Whether GSTM1 or GSTT1 polymorphism is a risk factor for HNC remains largely uncertain.

GSTM1 and GSTT1 are known to exhibit deletion polymorphisms (Egan et al., 2004). Persons with homozygous deletions of either the GSTM1 or the GSTT1 gene have no enzymatic functional activity of the respective enzyme and are known as null gene (Egan et al., 2004). This has been confirmed by phenotype assays that have demonstrated 94 percent or greater concordance between phenotype and genotype (Zhong et al., 1991; Warholm et al., 1995; Bruhn et al., 1998). The GSTM1 gene is located on chromosome 1p13.3 (Rossini et al., 2007) and is involved in detoxification of 7, 8-diol-9, 10-epoxide. Three variant alleles have been identified at the GSTM1 gene, one deletion allele and two others (GSTM1a and GSTM1b) that differ by C→G substitution at base position 534 (Rebbeck et al., 1997; Hengstler et al., 1998). Normal and malignant squamous cells of the larynx have been shown to express the GSTM isoform in the highest concentration compared with GSTT (Bongers et al., 1995).

GSTT1 gene is located on chromosome 22q11.23 (Rossini et al., 2007) and is involved in epoxybutanes and ethylene oxide detoxification. Two alleles have been identified at the GSTT1 locus - one functional and

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the other nonfunctional (Pemble et al., 1994). GSTT1 has both detoxification and activation roles (Hayes and Pulford, 1995, Landi, 2000). GSTT1 null genotype has been reported in many studies however, fewer studies have reported any significant GSTT1 null genotype association with HNC (Geisler and Olshan, 2001; Toru et al., 2008).

The present study is designed to find out the GSTM1 and GSTT1 deletions in Pakistani population having head and neck cancer as well as normal healthy controls.

Materials and Methods

Identification of patients and normal controls

The present case-control study consisted of 388 cases with pathologically confirmed head and neck cancer 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), Islamabad from March 2008 to September 2009 with a prior approval from Ethical Committees of both university and hospitals.

All patients and normal individuals participated on a volunteer basis. All subjects were personally interviewed according to a structured questionnaire. Blood was collected from subjects with their informed consent. 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 method with phenol-chloroform extraction as previously described (Vierhapper, 2004). The isolated DNA was electrophoresised on 1% ethidium-bromide stained agarose gel. Electrophoresis results were analyzed in gel documentation system (BioDocAnalyze Biometra) and photographed. 5ng dilutions were made of each DNA isolated and stored at 4°C until use.

Primer designing and multiplex PCR

Primers for all exons of GSTM1, GSTT1 and CYP1A1 (7th exon as internal control) were synthesized by using primer 3 input software version 0.4.0 (Table 1) and BLAST using NCBI PRIMER BLAST. A multiplex PCR was performed. 2μl DNA (10 ng/μl) was added to a 20μl PCR mixture composed of 2μl PCR buffer, 2μl of each primer (10mM), 0.24μl deoxynucleotide triphosphate (25mM) and 0.2μl Taq polymerase (5u/μl). The reaction mixture was placed on a 9700 thermal of ABI systems for 5min at 94°C and then subjected to 30 cycles at 94°C for 25sec, annealing temperature for 1min and 72°C for 1 min, followed by a final step at 72°C for 10min and hold at 4°C.

Amplification products were resolved on a 2% ethidium bromide–stained agarose gel along with 100bp DNA ladder (Figure 2). The photographs of gel electrophoresis were read by two technicians blind to each other's assessments. DNA samples showing amplification for CYP1A1 but not for any exon of GSTM1 and GSTT1 were considered as deleted GSTM1 and GSTT1 genotype respectively.

Technique for deletion detection

For deletion confirmation, forward and reverse primers were designed from intronic sequence before GSTM1 gene exon 1 (M1F and M1R respectively) and after last exon (M2F and M2R). For GSTT1, intronic sequence before exon 1 and after last exon was used for forward and reverse primer designing (T1F, T1R, T2F and T2R respectively). Primers for deletion confirmation were designed by same method as described above. Primer set of CYP1A1 gene was used as internal control.

The study subjects not showing any amplification with GSTM1 and GSTT1 exon specific primers were amplified with specific deletion confirmation primers (Table 1). Deleted genotype samples for GSTM1 were first amplified with M1F, M1R and also with M2F, M2R primers. They all amplified confirming the presence of intronic regions before and after the GSTM1 gene. Then the M1F and M2R primers were used to check GSTM1 deletion. Similarly for deleted GSTT1 gene T1F, T1R and T2F, T2R primers were used for deleted samples amplification. Then T1F and T2R primers were used to obtain a PCR product. All the amplified PCR products were sequenced from Macrogen

Table 1. Primers Sequences Used for GSTM1, GSTT1 and CYP1A1

Gene	Primer Sequence
GSTM1	
1 F	GCGGGAGGAAGTCTTACTGA
1 R	ACACCCCCAACACACACAC
2 F	GCTTCCCTGGTGCAGACA
2 R	GCAGAGGCAGCCACAGGT
3 F	TCCACCTGTCTCAGGGATCT
3 R	TAAGCTGGGGAGAGGAGATG
4 R	CATGTGACAGTATTCTTATTTCAGTCC
4 R	ACTCAATCTCAGCATCACAGC
5 F	GCAAGCACAACCTGTGTGAG
5 R	TGTGCAGGAATGCAAGAGTC
6 F	AGTTCCAGCTTGGGGAAGAT
6 R	CCAAGAATATGTGGGCTGGA
7 F	ATGGTTTGCAGGAAACAAGG
7 R	TCCAGGACTGGGAAAACATC
8 F	GTGTCTGCAGTGGGGTTGT
8 R	AGTCCCTTGGAAGAGGCAGT
GSTT1	
1 F	CCCGCAATTGGACTAAAGAG
1 R	CTCCAAACCAGACCAGCAAT
2 F	GCAGACTGGTGGGAAGAAGA
2 R	TGCCTCTGAAGACTTTAGTTTCCT
3 F	CAGAGCGAGACTCCGTATCA
3 R	CAATTTGGCACAACAGAGGA
4 F	GGCGAGAGAGCAAGACTCAG
4 R	GGCAGCATAAGCAGGACTTC
5 F	ATCTGTGGTCCCCAAATCAG
5 R	GGGGGTTGTCTTTTGCATAG
CYP1A1	
7 F	TGTCTACCTGGTCTGGTTGG
7 R	CCTCCAGGACAGCAATAAGG
Deletion	
M1F	CGTTAGGATCTGGCTGGTGT
M1R	GGGGCTGCACTCAGTAAGAC
M2F	CCTGGATGTCCCATTCATTC
M2R	AGATTGGGTCCTGGAGACCT
T1F	GGCTGACACACTTTCAGTGG
T1R	AGTGCCATCTATCGCATTCC
T2F	GGGGGTTGTCTTTTGCATAG
T2R	CCCAGGCTGGAGTGCAGTGG

Numbers are exons; F, forward; R, reverse

(Korea). Forward primer was used for sequencing. The sequenced results were analyzed using BioEdit v 7.0.5 software. Statistical analysis, for calculating OR, CI and standard deviation, was done by using SPSS statistics 17.0 software and GraphPad Prism 5.

Results

The study comprised of a total of 538 subjects with 388 confirmed HNC cases and 150 normal healthy controls. Male to female ratio was 1:1, this means that both the sexes were equally exposed to HNC. The mean age of head and neck cancer patients was 48 (+16.59) years and normal control was 46 (+17.69). Most prevalent area of cancer in HNC patients was oral cavity (57%), followed by Pharynx (30%) and larynx (13%).

It was found after PCR analysis that 70 patients out of 388 had GSTM1 deletion genotype (Figure 1) which is significantly higher as compared with the controls ($P<0.05$). The odds ratio for GSTM1 null genotype in HNC patients compared with the controls was 2.3 with 95% CI of 1.5-5.5. Patients showing GSTM1 deletion had mean age of 49 (+12.5) years. 50% patients showing GSTM1 deletion genotype have cancer of oral cavity, 45% have cancer of pharynx and 5% have cancer of larynx.

Similarly 110 HNC patients had GSTT1 gene deletion (Figure 1). 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 2.04 with 95% CI of 1.3-3.1. The frequency of GSTT1 deleted genotype was 57 among oral cancer, 40 among pharyngeal cancer and 13 among larygeal cancer patients. Sixteen patients showed deletion

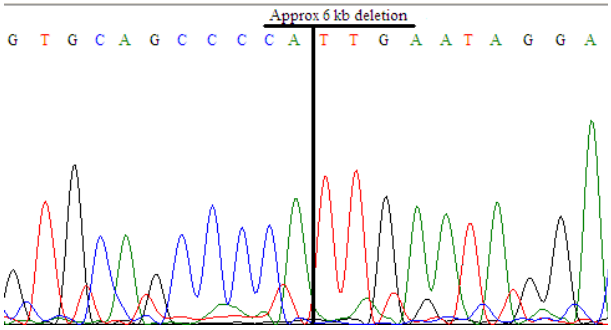

Figure 1. Sequencing Results for the GSTM1 Gene. Forward and Reverse Primers From intronic Portions Before and After the Gene. Shows Nearly 6kb Deletion has the Whole GSTM1 Gene

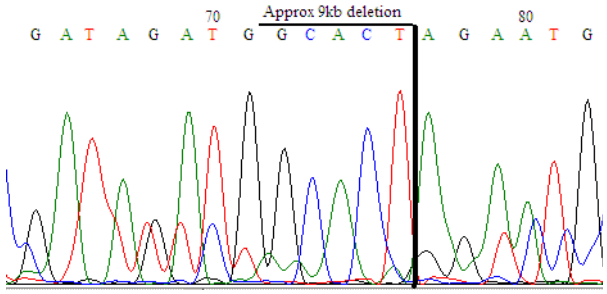

Figure 2. Sequencing Results for the GSTT1 Gene. Forward and Reverse Primers from Intronic Portions Before and After the Gene. Shows Nearly 9kb Deletion has the Whole GSTT1 Gene

of both genes and their mean age was 47 (+9.7) years. The mean age of patients with GSTT1 deletion was 45 (+11.4) years.

GSTM1 deletion was observed in 12 controls with a mean age of 46 (+13.4) years, whereas 28 controls showed GSTT1 deletion genotype with a mean age of 44 (+13.8) years.

To further confirm GSTM1 and GSTT1 gene deletions, PCR product of gene deletion primers were sequenced. The sequencing results of GSTM1 gene revealed an amplified region 98 bp before GSTM1 gene and a region 293 bp after the gene. This indicates that the middle portion containing GSTM1 gene is deleted and the intronic regions are present. The portion of nearly 6 Kbp containing the entire GSTM1 gene is deleted (Figure 1).

Similarly for GSTT1 gene the intronic regions are present but the gene is deleted. The amplified sequence for GSTT1 deleted gene, using intronic sequences, revealed the location of deleted region as 537 bp before the GSTT1 gene and 333 bp after the gene. The total size of GSTT1 deleted gene, along with some intronic portion, is approx. 9 Kbp. These results are presented in Figure 2.

Discussion

The association of GSTM1 and GSTT1 null genotypes with HNC risk was studied in this case- control study. Null genotypes were observed for both GSTM1 and GSTT1 gene. The frequency of these genotypes was significantly higher ($P<0.05$) as compared to the controls.

GSTM1 gene is deleted in many populations with head and neck cancer (Stacy and Andrew, 2001; Toru et al., 2008). A population-based study conducted among the Chinese and Korean reported a frequency of nearly 50% for the GSTM1 deletion genotype (Kim et al., 2000; Park et al., 2000; Setiwan et al., 2000). GSTM1 deletion genotype varies by ethnic group among African, Asian, Hispanic, European (Rebbeck et al., 1999; Cotton et al., 2000), Caucasians (Gertig et al., 1998; Chen et al., 1999; Crump et al., 2000), French (Stucker et al., 1999) and Asians (Stacy and Andrew, 2001). The present case control study found that GSTM1 null genotype is associated with increased risk of HNC (Trizna et al., 1995; Kihara et al., 1997; Jourenkova et al., 1998; Cheng et al., 1999; Nazar et al., 1999). Resultant loss of enzymatic activity due to GSTM1 gene deletion has also been reported in different populations (Zheng et al., 1993; Hayes et al., 1995; Rebbeck, 1997).

In this study a significant number of HNC patients with GSTT1 deletion genotype have been found when compared with the controls. Similar results, regarding GSTM1 and GSTT1 gene deletions, found in Pakistani population has also been reported in different populations such as, Americans (Gertig et al., 1998; Rebbeck et al., 1999; Cotton et al., 2000; Crump et al., 2000), Italians (Salagovic et al., 1999; Palli et al., 2000), Caucasians, Black Brazilians of South America and Amazonian Brazilians (Arodha et al., 1998). Asian populations are reported to have the highest GSTT1 deletion genotype. In one study 58 percent of Chinese and 38 percent of Malaysians had this GSTT1 null genotype (Lee et

al., 1995). These GSTT1 deletions are reported to be associated with laryngeal cancer risk in addition to HNC (Trizna et al., 1997; Jourenkova et al., 1998; Cheng et al., 1999).

Although variant alleles for GSTM1 and GSTT1 have been described in literature, but the gene deletion seems to be related to disease susceptibility. Presence of null genotypes for GSTM1 and GSTT1 has been reported in different populations in both cancer individuals and cancer free controls. Statistically significant difference in gene deletions may contribute to disease susceptibility. 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% (Rehan et al., 2010) similar to other populations in Singapore (Stacy and Andrew, 2000; Toefil et al., 2007) (Lee et al., 1995; Zhao et al., 1995), Turkey (Ada et al., 2004), Italy (D'Alo et al., 2004), Poland (Kargas et al. 2003), China (Setiawan et al. 2000) and in Japan (Naoe et al. 2000). Indians also have higher frequency of GSTM1 and GSTT1 deletion genotype (Mishra et al., 2004; Naveen et al., 2004; Singh et al., 2009). In conclusion the GSTM1 and GSTT1 genes have different deletion frequencies with respect to populations. The current study focuses on two genes involved in head and neck cancer incidence, and shows that individuals with GSTM1 and GSTT1 gene deletions are at an increased risk of developing head and neck cancer in Pakistani population.

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