RESEARCH COMMUNICATION

Unusual Intronic Variant in GSTP1 in Head and Neck Cancer in Pakistan

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Abstract

In the present case control study mRNA expression of the GSTP1 gene, encoding a phase II enzyme that detoxifies via glutathione conjugation, was investigated using semiquantitative PCR followed by SSCP for 49 confirmed head and neck (HN) cancer and 49 control samples. It was found that GSTP1 was upregulated in significantly higher number of cancers (OR 4.2, 95% CI 1.2- 15.3). Grade wise correlation was also observed with more up regulation in patients with more advanced grades of HN carcinomas. We also found that 5 patients showed variation in mRNA with a larger product size than expected. Sequencing revealed insertion of an intronic segment between the 6th and 7th exon of the GSTP1 gene. Germline screening was performed showing mobility shifts which suggested mutation at the DNA level resulting in intronic portion retention. This study is of prime importance for drug design and treatment selection to overcome increased resistance of HN cancers to drugs due to alteration in the GSTP1 gene.

Keywords: GSTP1 - head and neck cancer - transcript - mutation - variant

Asian Pacific J Cancer Prev, **13**, 1683-1686

Introduction

Head and neck cancers include cancers of oral cavity, larynx and pharynx. It is sixth most frequent cancer worldwide and is particularly high in south East Asian countries (Johnson, 1991; Parkin et al., 2002) including Pakistan where it is second most prevalent cancer (Faheen, 2007).

In humans, glutathione S transferases (GSTs), supergene family has four distinct isoforms (Mu, Theta, Pi and Alpha). GSTs are responsible for converting a variety of electrophilic and hydrophobic compounds into more soluble forms that are more easily excreted by conjugating them with glutathione (Hayes et al., 2005). Glutathione S transferase pi (*GSTP1*) is the predominant isoform in various malignant tumors of urinary, digestive and respiratory tracts (Nelson et al., 2001; Kim et al., 2009; Scharmach et al., 2009; Arun et al., 2010; Yu et al., 2010). No conclusive results had been made yet regarding association of *GSTP1* expression and malignant transformation. Nevertheless, few studies have suggested increased expression of *GSTP1* as an indicator of premalignant and malignant changes (Aliya et al., 2003; Usarek et al., 2004) while others have suggested expression of *GSTP1* as a sign of carcinogen exposure in the endothelial and oral tissues (Gajewska et al., 2007; Conklin et al., 2009). Few studies have also reported that loss of *GSTP1* expression is suggestive of carcinogenesis (Okino et al., 2007; Ritchie et al., 2009). *GSTP1* is genetically polymorphic, and two *GSTP1* non-synonymous SNPs have been studied extensively in the epidemiological literature (Yang et al., 2006).

Over expression of *GSTP1* due to transcriptional activation or stabilization of the protein or mRNA could potentially be a factor contributing to antitumor drug resistance (Hayes and Pulford, 1995). Variation in the expression of GSTP1 among individuals and ethnic groups has also been reported in literature showing *GSTP1* to be associated with risk of head and neck cancer (Anita et al., 2002). Similar findings regarding the upregulation of these molecules have also been reported in Pakistani head and neck cancer population. A significant correlation of molecules over expression with staging was observed (Nosheen et al., 2011). However, in the present study, identification of variations in *GSTP1* mRNA expression is correlated with head and neck cancer tissue grades, followed by variant studies designed to define the initial stage of mutation of that variation. Specifically, semiquantitative PCR for mRNA expressional variation analysis and PCR-SSCP studies, for germline screening of variants, were carried out.

Materials and Methods

Head and neck cancer patients undergoing surgery at Pakistan Institute of Medical Sciences (PIMS) Islamabad, Allied Hospital Faisalabad and Military Hospital Rawalpindi (Pakistan) were recruited between 2009 and 2010. The study was prior approved from ethical committees of hospitals and university. All 49

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patients were diagnosed with carcinoma of head and neck by oncologists through cytological imaging and histopathological examinations. Informed consent was obtained from patients prior to surgery and interviewed. Clinical data were available for all the specimens tested. Tumor tissue specimen paired with their corresponding adjacent normal tissue were surgically obtained, collected in RNA later (Ambion) and stored at -20˚C until further use. Grading system was used according to guidelines of AJCC.

RNA isolation

RNA was extracted from tissue with TRIZOL (Invitrogen, USA) according to the manufacturer's protocol as described previously (Zhong et al., 2006) and stored at -86˚C until further use. RNA was quantified by spectrophotometry and equal quantity of RNA, for all the samples, was used in subsequent studies.

cDNA synthesis and semi-quantitative reverse transcriptase polymerase chain reaction analysis

Total RNA isolated from controls and tumor tissue was analyzed for *GSTP1* mRNA by semi-quantitative reverse transcriptase-PCR. cDNA was prepared from total RNA as described in manufacturer's protocol (Invitrogen, U.S.A.) and 2 µl of RT product was used for subsequent PCR reactions. Primers were synthesized from Molecular Biology Products with sequences described previously (Ioanna et al., 2001; Fusako et al., 2004). Prior to amplification of *GSTP1* normalization was carried out with β-actin, the housekeeping gene. Aliquots of the PCR reaction were subjected to electrophoresis on 2% agarose gels and PCR fragments were visualized by ethidium bromide staining and photographed on Bio-Video gel documentation system (Biometra). mRNA expression of the housekeeping gene was used as a quality control for the samples showing equal cDNA in all samples.

PCR-SSCP

Single stranded conformational polymorphism was used for DNA mutational screening by the procedure already described (Nosheen & Kayani, 2011).

Statistical analysis

Odds ratio and 95% confidence interval was used to evaluate the significance of results. Statistical analysis was performed by SPSS (8.0) software.

Results

The mean age of head and neck cancer patients was 51.6 (±16.1) years. Male to female ratio was 2:1 with most frequent area of cancer being larynx followed by oral cavity and pharynx in a ratio of 3:2:1 respectively. In later stages of head and neck cancer mean ages were higher as compared to earlier stages.

GSTP1 expression and grade correlation

Semiquantitative PCR results demonstrated that *GSTP1* expression was upregulated in head and neck cancer patients. β actin was used as a housekeeping gene for uniform expression of mRNA representing equal quantity of cDNA. *GSTP1* mRNA expression was significantly upregulated (Figure 1) in head and neck tumor tissue compared to control tissue (OR 4.2, CI 1.2-15.3). *GSTP1* over expression also appeared related to grades of head and neck cancer. *GSTP1* upregulation was less in earlier grades compared to later grades of head and neck cancer tissues.

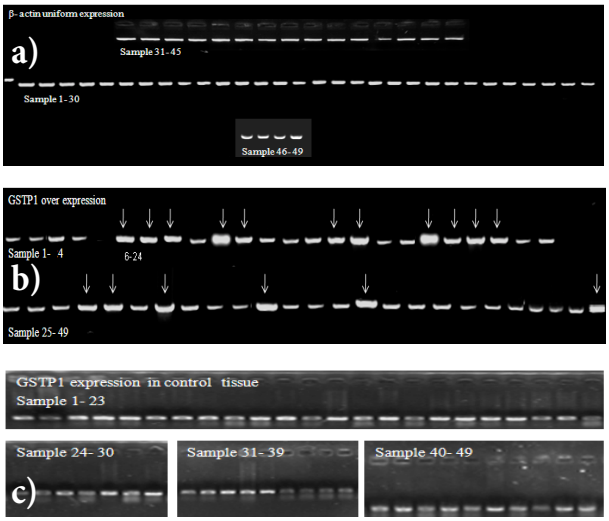

Figure 1. Photograph of 2% Agarose Gel Electrophoresis Showing Amplification in the Samples. (a) β- Actin House Keeping gene, (b) GSTP1 Expression in Control Tissue and (c) GSTP1 Expression in Tumor Tissue

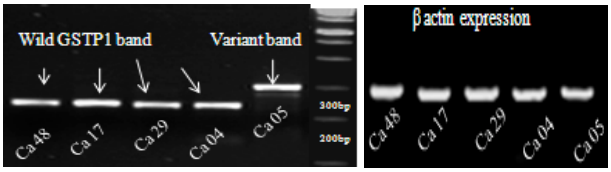

Figure 2. Photograph of 2% Agarose Gel electrophoresis Showing Variant Bands As Well As Normal Bands

Intronic insertion

After semiquantitative PCR a 250bp band size was observed on 2% agarose gel electrophoresis for *GSTP1*. A 300bp band size of *GSTP1* was also observed in 5 tissue samples (Figure 2). The samples were, therefore, checked again for sample contaminations with housekeeping gene. No contaminations were observed and these samples again showed variant bands. These samples were sequenced in order to observe the variation. The results of sequencing of variant bands, as well as control, showed that the variants have intronic insertion (Figure 3 and Figure 4). Insertion of intronic segment between the successive exons 6 and 7 of 177 nucleotides was also transcribed. This mutation leads to stable mRNA synthesis but leading to impaired protein due to stop codons in the intronic segment.

Germline screening

The results of SSCP also showed altered mobility pattern of variants (Figure 5). Therefore it was found that few patients of head and neck cancer had germline mutation leading to transcriptional variants. The intronic portion was also transcribed which contains many stop codons in its sequence. These results further corroborate our notion that the germline of these patients was effected

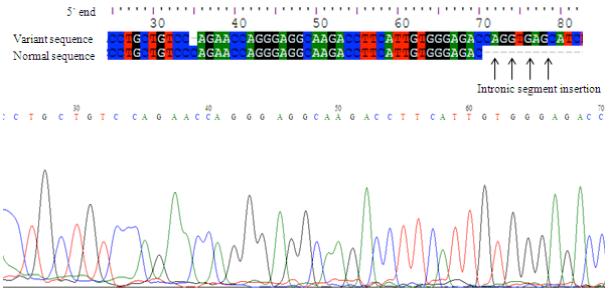

Figure 3. Bioedit Software and Sequencing Pictures Showing Presence of Exonic Region and Intronic Insertion at 5` end. Expression of House Keeping Gene is Uniform in all the Samples

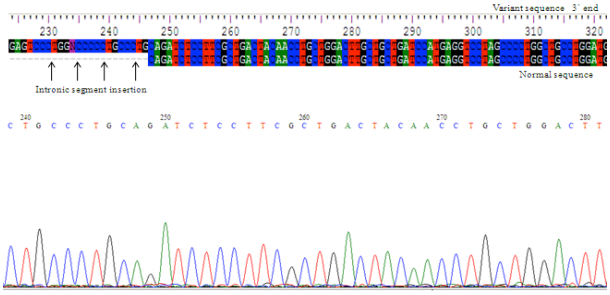

Figure 4. Bioedit Software and Sequencing Pictures Showing Presence of Exonic Region and Intronic Insertion at 3` end

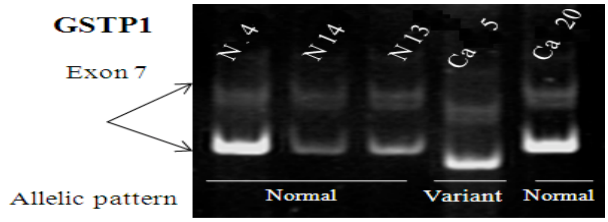

Figure 5. SSCP Picture after Ethidium Bromide Staining Showing Normal and Variant Band with Mobility Shift in Sample with mRNA Expressional Variation

leading to altered mRNA transcription. These results showed that germline mutation in DNA caused faulty transcription containing the intronic segments with stop codon and ultimately the protein is not formed resulting in cancer risk. Because there is significant correlation between DNA mutation and mRNA expression therefore these variants should be considered while designing of drugs or treatment therapy because of the fact that the last two exon of GSTP1 gene are hotspots for mutations.

Discussion

As a complementary approach to confirm the above results, we sought whether the variants of *GSTP1* were at sporadic level or germline level. In order to find the first point of mutation, DNA of these patients and normal controls was screened at germline level. The use of SSCP technique in reporting novel mutations, prompted us to examine these variants for any novel SNPs. We have already successfully reported novel mutations in *GSTP1* gene at DNA levels (Nosheen & Kayani, 2011).

In the present study it was found that *GSTP1* mRNA expression was significantly highly upregulated in head and neck cancer patients and it was directly related to different grades of cancer. As the cancer progresses the expression of *GSTP1* is increased. A variant was also found showing germline mutation leading to altered expression containing intronic portion between exon.

One reason for upregulation of *GSTP1* in head and neck cancer may be correlated to the fact that GSTP1 inhibits apoptosis by reacting with cJUN (Wang et al., 2001; Holley et al., 2007). *GSTP1* over-expression may be due to a number of different mechanisms including gene amplification, transcriptional activation, protein stabilization, and genetic abnormalities (Matthias et al., 1998). Also *GSTP1* expression is regulated by several mechanisms like Sp1 (Moffat et al., 1996), AP1 (Xia et al., 1996), retinoic acid response element (Lo & Ali, 1997) and PKA/CREB1 (Lo & Usman, 2002). Increased levels of *GSTP1* may be occasionally involved in the intrinsic drug resistance of head and neck cancers. However, silencing of this gene has shown to increase tumor sensitivity to same drug. Lower expression of *GSTP1* may be associated with better response to chemotherapy and improved prognosis (Isabel et al., 2010).

We found intronic variant in 5 samples for *GSTP1*, retaining the segment. Germline screening for these samples showed altered mobility shifts suggesting mutation at DNA levels. SNP in germline resulting in intronic segment insertion is not a new phenomenon although novel in relation to head and neck cancer. SNP in intronic sequences like rs1871042 and IVS-16 (Moyer et al., 2008) had already been reported in Caucasian American population with frequency of 0.3 (Moyer et al., 2008). In addition to these, other SNPs in intronic portion 6-7 have also been reported such as rs77993400, rs8191451 and rs45483800 (ensembl). Moyer although reported 35 SNPs, intronic as well as exonic SNPs, but functional consequences of just exonic SNPs was reported. We have already published a detailed germline screening of GSTP1 gene in 437 head and neck cancer patients and have not found any of these SNPs (Nosheen & Kayani, 2011). In this study we found that germline mutation in splice site resulted in a novel retention of whole intronic segment. This insertion caused an increased size of mRNA sequence. In silico functional study related to the mRNA sequence showed that protein function is altered as the intronic insertion has a number of stop codons. Therefore we concluded that mutation at DNA level in splice site resulted in intronic insertion in mRNA. The variant RNA is stable as we observed expression in semiquantitative PCR. Protein was unstable due to many stop codons in the intronic portion.

Functional studies related to the current mutation needs to be explored in detail in order to find the association of these variations with cancer risk. The present intronic insertion should be considered while drug designing or treatment therapy selection due to the role of the *GSTP1* gene in resistance to drugs.

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