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GSTP1 c.341C>T Gene Polymorphism increases the risk of Oral Squamous Cell Carcinoma

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Running title: *GSTP1* polymorphism in oral cancer

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Highlights

- Association of functional SNP in Glutathione S transferase P1 gene with oral cancer was evaluated.
- Frequency of *GSTP1 c.*341 T allele was higher among oral cancer patients than in normal health individuals.
- 341 T allele is associated with the risk of oral squamous cell carcinoma.

Abstract: Glutathione S Transferases (GST) are anti-oxidant enzymes involved in detoxification of cellular and exogenous carcinogens and oxidative products of reactive oxygen species. Genetic polymorphisms can attenuate the detoxification capacity of GST and consequently increase the susceptibility to carcinogenesis. There are eight classes of GST enzymes of which pi subtype is the predominant form expressed in the oral mucosa. c.341C>T single nucleotide polymorphism (rs1138272) in *GSTP1* gene, is a functional variation that reduces the enzymatic activity of GST pi. We carried out a 1:2 case-control study involving 270 individuals to determine the association of c.341C>T variation with the risk of oral squamous cell carcinoma. *GSTP1* c.341C>T variation was genotyped by PCR-RFLP method. GST pi expression in the tumour sample was determined by immunohistochemistry. Tobacco consumption was the major risk factor among cancer patients. The odds ratio for the risk of oral squamous cell carcinoma in individuals with the minor allele was 4.5 (0.95 CI = 2.3 – 8.9; P =0.000004). The genotype was found to follow dominant mode of inheritance (OR 4.4 [0.95 CI = 2.1 – 9.2]; P = 0.00006). Our results support the conclusion that c.341C>T variation in *GSTP1* increases the risk of OSCC in patients habituated to tobacco consumption.

Keywords: Oral Squamous Cell Carcinoma, *GSTP1,* Risk factor.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer worldwide and is the major cause for cancer morbidity and mortality in developing countries. Age standardized incidence rate in India is 12.6 per 100 000 population [1]. The risk factors for OSCC include environmental, high risk strains of human papilloma virus and genetic factors [2]. Genetic risk factors include single nucleotide polymorphisms (SNP) that increase the mutation rate in oral epithelial cells. Most of the OSCC associated SNPs occur in genes involved in pathways of carcinogen metabolism, DNA repair, cell cycle control, extra-cellular matrix integrity, immunity and inflammation [3,4].

Metabolism of carcinogens occurs through bioactivation and detoxification process. Genetic polymorphisms that impair the activity of detoxifying enzymes can contribute to carcinogenesis. Some of the detoxifying enzymes with established role in carcinogenesis are glutathione-S-transferases (GSTs), microsomal epoxide hydrolase (mEH), and uridine 5'-diphosphate (UDP)-glucuronosyltransferase (UGTs) [5].

GSTs are phase II detoxifying enzymes involved in cellular protection from xenobiotics (including carcinogens) and oxidative stress. GSTs are classified into eight classes as α (alpha), μ (mu), κ (kappa), ω (omega), π (pi), σ (sigma), θ (theta), and ζ (zeta) and are expressed in various tissues like liver, kidney, lung, stomach, ovaries, colon, pancreas, prostate and spleen [6,7]. Of the eight groups, GST pi is the predominant form expressed in the oral mucosa which is often found to be overexpressed in oral cancers [8]. c.341C>T SNP in *GSTP1* gene (rs1138272) is a functional polymorphism that affects the

enzymatic activity of GST pi [9]. In light of the importance of GST pi in the protection of oral mucosa against carcinogens, it is possible that functional polymorphism that affects its activity can contribute towards susceptibility to oral carcinogenesis.

We hypothesized that c.341C>T SNP in *GSTP1* gene may increase the susceptibility to oral cancer by lowering the protection of oral mucosa against exogenous carcinogens. To the best of our knowledge there is no information on the association between *GSTP1* c.341C>T polymorphism with OSCC and therefore the present work was undertaken. The objectives of this study were (i) to determine the association of *GSTP1* c.341C>*T* polymorphism with the risk of OSCC and (ii) to determine the influence of *GSTP1* c.341C>T polymorphism on GST pi expression in the tumor.

2. Materials and Methods

2.1. Study design: The study was initiated after obtaining permission from Institutional Ethics Committee of Sri Devaraj Urs Medical College. Patients and control individuals were recruited between 2014 and 2017. Informed consent from each participant was obtained in writing before collecting the sample. In this case-control study a total of 90 participants were recruited from the Department of Otorhinolaryngology, R.L. Jalappa Hospital and Research Centre, Kolar, Karnataka. Tumor specimens were collected from the patients after surgical resection and blood samples were collected from the control individuals. All the tissue samples were taken from patients confirmed histologically as OSCC on biopsy. A questionnaire gathering details on demographics (age, gender), addictions (tobacco use, smoking and alcohol) and family history of cancer were collected systematically using a study proforma before recruitment of the study participants. Clinical and histopathological details were collected from patients' medical records. Staging was done according to7th

edition of American Joint Committee on Cancer TNM (T – Primary tumor staging, N – Nodal status, M – Metastasis) staging system for OSCC. Patient population included 20 males and 70 females. Age group of the patients ranged from 21 to 80 years. Controls were age, gender, and geographically matched individuals who had no history or signs of oral cancer.

The primary objective of the study was to evaluate the association of *GSTP1* c.341C>T SNP with the risk of OSCC. The secondary objective was to evaluate the correlation of this SNP with the expression of *GSTP1* in the tumour tissue. To accomplish this, we firstly determined the genotype of *GSTP1* c.341C>T SNP using genomic DNA purified from the tumor specimens of OSCC patients (n = 90) and peripheral blood of control participants (n = 180). The minor allele frequency of these two groups was compared by using chi-square test. Secondly, we randomly selected 5 tumor samples from each genotype group (CC, CT, TT), determined the expression level of GST pi protein by IHC and analysed the correlation between GST genotype and expression by chi-square test.

2.2. DNA isolation: Resected tumor specimens were stored at −80 °C and blood sampleswere stored at 4 °C until processing. Tissue homogenate of the specimen was used for the genomic DNA isolation by previously published method with the following modification [10]. Tissue sample was minced and placed in a micro-centrifuge tube containing cell lysis buffer to which sodium dodecyl sulphate and proteinase K was added. Contents of the tube were incubated at 37 °C until the complete digestion of the tissue. After the tissue digestion NaCl and isopropyl alcohol was used for the precipitation of the DNA. The resultant DNA was washed with ethanol, dried and dissolved in Tris-EDTA buffer. Peripheral blood was used for preparation of genomic DNA from controls [11]. Quantity and purity of the preparation was estimated by UV spectroscopy.

2.3. Detection of Human Papilloma Virus (HPV):

Presence of HPV in the tumor specimens was analysed by polymerase chain reaction based method using GP5+/6+ primers from L1 consensus region of the viral genome. The procedure is described in detail elsewhere [12].

2.4. *GSTP1* c.341C>T genotyping: Genomic DNA was amplified by polymerase chain reaction on Bio Rad C1000 Touch Thermal Cycler. The primer pairs used were 5'- CAA GGA TGG ACA GGC AGA ATG G -3'(forward) 5'-ATG GCT CAC ACC TGT GTC CAT C- 3' (reverse) [9]. 25 µl reaction mixture included 1x assay buffer, 150 ng genomic DNA, 0.2 mM dNTP, 1 picomole of each primer, 1.5 mM $MgCl_2$ and 1 unit *Taq* DNA polymerase (Bangalore Genei, India). The program comprised of an initial denaturation at 95 ℃ for 3mins followed by 30 cycles at 95 ℃ for 30 sec, 63 ℃ for 30 sec and 72 ℃ for 30 sec; final extension involved 7 mins at 72 ℃. The PCR product was analysed on 1 % agarose gel. The 367 bp amplicon was subjected to restriction digestion with 10 units of *Aci1* (New England Biolabs, USA) at 37 ℃ and analysed on 2 % agarose gel with ethidium bromide staining. The wild-type (341 CC) showed two bands of 195 and 172 bp. Three bands of 367, 195, and 172 bp were seen heterozygous (341 CT). The homozygous (341 TT) had one band of 367 bp. Representative agarose gel picture is shown in Figure 1. 10 % of the samples were randomly selected for confirmation and the results were 100 % concordant. Sanger sequenced samples were used as positive controls.

2.5. GST pi immunohistochemistry (IHC): Resected tumor specimens were collected in formalin for IHC analysis. Formalin fixed paraffin embedded blocks (FFPE) were prepared from formalin fixed tissues. 3 µm thick sections were mounted on poly-L-lysine coated slides

and incubated at 60 °C overnight. Sections were de-paraffinized in xylene and rehydrated in decreasing gradients of alcohol. For antigen retrieval, slides were boiled in microwave oven with Tris-EDTA buffer (pH 9.0) for 20 min and further the sections were treated with 3% H_2O_2 for 30 min to inhibit endogenous peroxidase activity. The tissue sections were washed with Tris-Buffered Saline (TBS, pH 7.6) for 15 min. The sections were further incubated with primary anti-GSTP1 antibody (1:50 dilution; #SC-66000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA,USA) for 2 hours, washed with TBS buffer, followed by incubation with mouse/rabbit HRP secondary antibody (Abcam, Cambridge, UK) for 30 min. The immunostain was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin, dehydrated and mounted for analysis. Negative controls were included with the omission of primary antibody with buffer. The slides were assessed using light microscopy. IHC slides were scored independently by two researchers who were blinded w.r.t., the *GSTP1* genotype of the tumour specimen.

3. Statistical analysis: Sample size and power of the study was calculated using OpenEpi web tool using 95 % confidence interval [13]. Normal approximation with continuity correction was considered for power calculation. Statistical analysis was carried using the web based calculation available at www.OpenEpi.com (updated 2013/04/06). Allele and genotype frequencies of the two groups were compared using relevant contingency tables. Difference between the groups was determined by calculating P - value from chi-square test (Fisher's exact test). The study population was tested for conformity to Hardy-Weinberg equilibrium using the web program by Rodriguez and co-workers [14]. P-values <0.05 were considered as statistically significant. Post-hoc power of the study was calculated using the online calculator 'Online Sample Size Estimator' (www. osse.bii.a-star.edu.sg).

4. Results

The demographic profile of the patients and the histopathological profile of the tumor samples are given in Table 1. Age of patients ranged from 21 to 80 years with a mean age of 54.2 ± 12.03 years. Most of the patients were females (78 %) and were from lower socio-economic strata. All the patients in the study group had addiction to one or more carcinogenic substance like betel nut, chewable tobacco, *gutkha* (consists of betel leaf, tobacco, areca nut slaked lime, spices, and catechu packed in tins or pouches), smoking or alcohol. The mean duration of the addiction was 30 ± 12 years. All the patients were habituated to chewing betel nut and tobacco while 5.6 % of the patients were habituated to tobacco, *gutkha* chewing and alcohol followed by smoking (7.8 %). None of the tumour samples were positive for HPV. Thus, tobacco chewing and not HPV infection was the major risk factor in the patient group. The main complaints during initial presentations were burning sensation in oral cavity (88 %), oral ulcer (54 %) and trismus (14 %). Mean duration of symptoms was 10 ± 4 months. Buccal mucosa was the most commonly involved site (63 %); the other sites of lesion were lower alveolus, anterior 2/3rd of tongue, retromolar trigone (RMT) and floor of mouth. 10 patients in the study had locally advanced disease involving buccal mucosa and lower alveolus and it was difficult to identify the epicenter of the disease. They have been termed as lower gingivobuccal sulcus (GBS) cancers. This type of cancers are common in Indian subcontinent. Majority of the patients showed stage IVa tumor (69 %); 22 % showed stage III and another 9 % showed stage II. Squamous cell carcinoma was found to be well differentiated in 73 %, moderately differentiated in 24 % and poorly differentiated in 2 % of the patients. Type 2 diabetes mellitus was the major co-morbidity seen among the patients (~11 %).

GSTP1 c.341C>T SNP was genotyped in 90 patients tumor and 180 normal healthy individuals. The profile of *GSTP1* c.341C>T alleles and genotypes is shown in Table 2. The profile of the genotypes was in agreement with Hardy-Weinberg equilibrium (χ^2 = 2.11). Minor allele frequency (341T) among OSCC patients was 15.6 %, which is about four times higher than its frequency in the control group (3.9 %). The power of the study was 86.8 %. The distribution of the *GSTP1* c.341C>T alleles among case and control groups was compared by means of contingency table. The difference in the distribution profile of the minor allele was found to be statistically significant (P-value = 0.000004; Odds ratio = 4.5 [0.95 CI = 1.9-10.7]). We also found a statistically significant difference in the distribution of the genotypes (P-value = 0.00006).The distribution of the genotypes of *GSTP1* c.341C>T SNP was also compared by considering dominant, recessive, additive and over-dominant genetic models (Table 3). Statistically significant difference was observed in all the four models. Of the four models, the best P-value was observed in the case of dominant genetic mode.

We carried out multivariate logistic regression of the genotype data in the patient group (Supplementary Table 1). The genotype containing the risk allele (CT + TT) did not show statistically significant association with age, gender, stage and grading. However, we found significant association with reference to duration of tobacco consumption. The median duration of tobacco consumption in our patient group was 28 years. The genotype containing the risk allele (CT + TT) was significantly higher among patients with more than 28 years of tobacco consumption. (P-value = 0.027; Odds ratio = 3.55 [0.95 CI = 1.15-10.95].

The GST pi protein expression profile in the tumor samples was analysed by immunohistochemistry (IHC). Representative photomicrographs of IHC analysis are shown in Figure 2. We noticed immunoreactivity for GST pi protein in the tumor cells and not in

the surrounding normal squamous epithelium. Immunoreactivity of the tumor samples was assessed with respect to intensity of staining and the area of tumor tissue being stained.

We evaluated the relationship between GST pi protein expression and the genotype of *GSTP1* c.341 C>T SNP. 5 tumor specimens from each of the 3 genotype groups *viz*., CC, CT and TT were chosen for IHC analysis. Expression levels in the 3 groups are summarised in Table 2. No statistically significant correlation was observed between the *GSTP1* c.341C>T SNP and GST pi protein expression (P = 0.82).

5. Discussion

Tobacco consumption and infection with high risk strains of human papilloma virus are the major environmental risk factors implicated in the development of oral cancers [15]. Our previous study with hospital based population which is predominantly rural in demography showed that tobacco consumption either in smokable or chewable form was the principal exogenous risk factors for oral carcinogenesis rather than infection with human papilloma virus [12]. This observation prompted us for examining the profile of genetic risk factors with particular emphasis on pathways that affect carcinogen detoxification. The practice of chewing tobacco is common to South and South East Asian region [16, 17]. It is consumed in as a co-ingredient with betel quid which is commonly known as “paan”. Betel quid involves wrapping of areca nut, lime, condiments, sweeteners, and sometimes with or without tobacco with fresh leaf of betel plant (*Piper betle*). The combinations of the ingredients are altered according to individual preferences. Tobacco was a common ingredient in betel quid consumed by the patient group in this study.

The major carcinogenic constitutes of tobacco are nicotine, polycyclic aromatic hydrocarbons, nitrosamines, metals and aldehydes [18]. In addition, stable and unstable free radicals and reactive oxygen species (ROS) with the potential for biological oxidative damage are known to occur in the tobacco smoke [19]. Tobacco-specific nitrosamines, *N*-nitrosodiethylamine, *N*-nitrosoanabasine, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone, and *N*′-nitrosonornicotine have been detected in the saliva of betel quid chewers with tobacco [20].

Genetic polymorphisms associated with enzymes involved in carcinogen detoxification can lead to increased susceptibility to carcinogenesis [21]. The toxicological profile of tobacco guided us towards GST pi as it is an important enzyme involved in the detoxification of environmental carcinogens, xenobiotics and products of ROS induced oxidative damage; It is also the major subtype expressed in the oral mucosa. Toxicological importance of GST pi is also corroborated by animal studies. Epoxides of Polycyclic aromatic hydrocarbons (PAH) such as Benzo(a)pyrene [B(a)P] diolepoxide(BPDE), acrolein and other unsaturated carbonyls generated by oxidation of lipids and DNA can be used as substrates by GST pi [22].

Mice with *GSTP1* knock out show increased risk of skin cancer when exposed to carcinogens like polycyclic aromatic hydrocarbons [23]. Most of the genetic association studies have focused on *GSTP1* c.313G>A variant which results in isoleucine to valine substitution at amino acid residue number 105 in the polypeptide chain. Positive association has been observed between c.313A>G SNP and cancers of lung, breast and prostate [24-26]. There are several studies that have found a positive association between c.313A>G and the risk of OSCC [27-29]. However, these results have failed to reach statistical significance at

the level of meta-analysis [30]. This motivated us to explore the involvement of other functional SNPs in *GSTP1* gene with the risk of oral carcinogenesis. Another less explored functional SNP in the *GSTP1* gene is c.341C>T which results in Alanine to Valine substitution at residue number 114 in the GST pi polypeptide chain. Experimental evidences have shown that c.341C>T SNP results in significant reduction of the enzymatic activity of GST pi [31]. Previous studies have found positive association between c.341C>T SNP and risk of cancers of oesophagus, lung, and thyroid [9,32,33]. In this study, we found significant association between c.341C>T SNP and risk of OSCC both at the allele and genotype level with highest association in the additive genetic model. This indicates that GST pi is probably a limiting factor in the oral mucosa for protection against carcinogenesis. Quantitative reduction in GST pi expression due to gene polymorphism may thus reflect as quantitative increase in the risk of carcinogenesis with homozygosity of the functionally reduced minor allele constituting the highest risk followed by its heterozyogisty and least risk in the case homozygosity of functionally normal major allele.

All the patients recruited for the study were found to be habitual consumers of tobacco or *gutkha*. Tobacco consumption in smokable form is common among men while its consumption in chewable form along with betel quid is common among women particularly in the rural demography. Habit of chewing betel quid and tobacco is common among rural women in South and South East Asia and its link to carcinogenesis has been indicated in previous studies [16, 17]. Human papilloma virus was absent in all the tumor samples included in the study. Thus, tobacco consumption was the major exogenous risk factor among the patients. The positive association observed in this study could be due to the homogeneity of the exogenous risk factor in the patient group.

At the level of oral mucosa, GST pi expression is linked with the carcinogen detoxification capacity and protection against carcinogenesis. However, GST pi has opposite effect in the tumor tissue where it is often found to be overexpressed. Increased expression of GST pi contributes towards tumor survival by neutralising electrophilic chemotherapeutic agents like cisplatin and by minimizing the DNA damaging effect of radiation therapy [34]. We examined if c.341C>T SNP also affect GST pi expression in the tumor. We failed to find any evidence for statistically significant association between immunohistochemical grade of GST pi in tumor samples and c.341C>T SNP. This indicates that the mechanism that results in GST pi overexpression is distinct and not linked to the genotype of c.341C>T SNP.

In conclusion, the results of this study indicate that c.341C>T SNP in *GSTP1* gene increases of risk of OSCC in individuals habituated to consumption of tobacco products but it has no mechanistic link with the magnitude of GST pi over expression in the tumor. The genetic association observed in this study is a reflection of oxidative stress potentially caused by chewable tobacco.

Conflict of interests: The authors declare that there are no conflicts of interest.

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Table 1: Demographic and clinicopathological parameters of study population

Parameters	Cases (n = 90)	Controls (n = 180)
Male	20	43
Female	70	137
Age (Mean ± Standard Deviation; years)	54.2 ± 12.03	53.7 ± 12.5
Habits		
Chewing betel quid + tobacco	90	12
Chewing betel quid + tobacco + alcohol consumption	5	-
Chewing betel quid + tobacco + smoking cigarette	7	3
Site of primary tumor		
Buccal mucosa	57	-
Floor of mouth	1	-
Anterior 2/3rd of tongue	6	-
Lower alveolus	14	-
Lower GBS	10	-
RMT	2	-
Grade		
Grade 1	66	-
Grade 2	22	-
Grade 3	2	-
TNM classification		
T_{1-2}	8	-
T_{3-4}	82	-
Stage		
II	20	-
III	18	-

IV	52	-

Table 2: Profile of allele and genotype frequencies of *GSTP1* c.341C>T SNP in the study population

Allele/ Genotype	Patients (n = 90)	Controls (n =180)	P- value[#]	Odds ratio
C	152	346	0.000004[*]	4.5 (2.3 – 8.9)[$]
T	28	14		
CC	67	167	0.00006[*]	4.4 (2.1-9.2)[$]
CT	18	12		
TT	5	1		

[#]Chi-square test (Fisher's exact)

[*] Significant (P ≤ 0.05)

[$]Parenthesis represent 0.95 confidence interval

Table 3: Evaluation of association between *GSTP1* c.341C>T and OSCC risk under different genetic models.

Model	Genotype	P-value[#]
Dominant	CT + TT vs CC	0.00006[*]
Recessive	TT *vs.* CT + CC	0.01[*]
Additive	TT > CT >CC	0.00003[*]
Over-dominant	CT *vs.* CC+TT	0.001[*]

[#]Chi-square test (Fisher's exact)

* Significant (P ≤ 0.05)

Table 4: Correlation between *GSTP1* c.341C>T SNP and GST pi expression in tumor specimens

GSTP1 c.341 C>T genotype	GST pi expression		P-value[#]
	Low	High	
CC (n = 5)	3	2	0.76[*]
CT (n = 5)	2	3	
TT (n = 5)	3	2	

[#]Chi-square test

* Not significant (P ≥ 0.05)

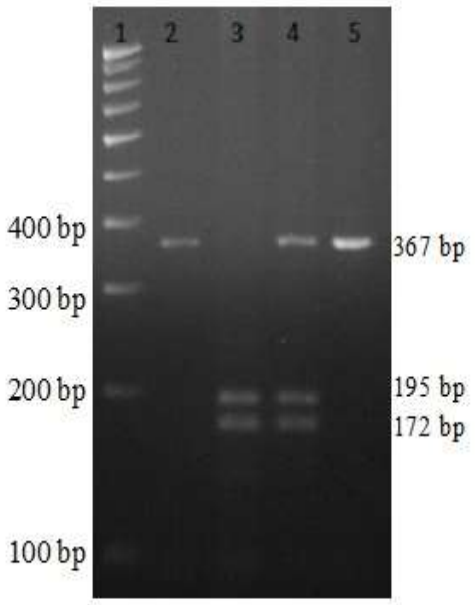

Figure 1: Representative image of agarose gel electrophoresis showing PCR-RFLP pattern for *GSTP1* c.341 C>T SNP. Lane 1: 100 bp DNA ladder; Lane 2: PCR amplicon (367 bp); Lane 3: CC genotype (195+172 bp); Lane 4: CT genotype (367+195+172 bp); Lane 5: TT genotype (367 bp)

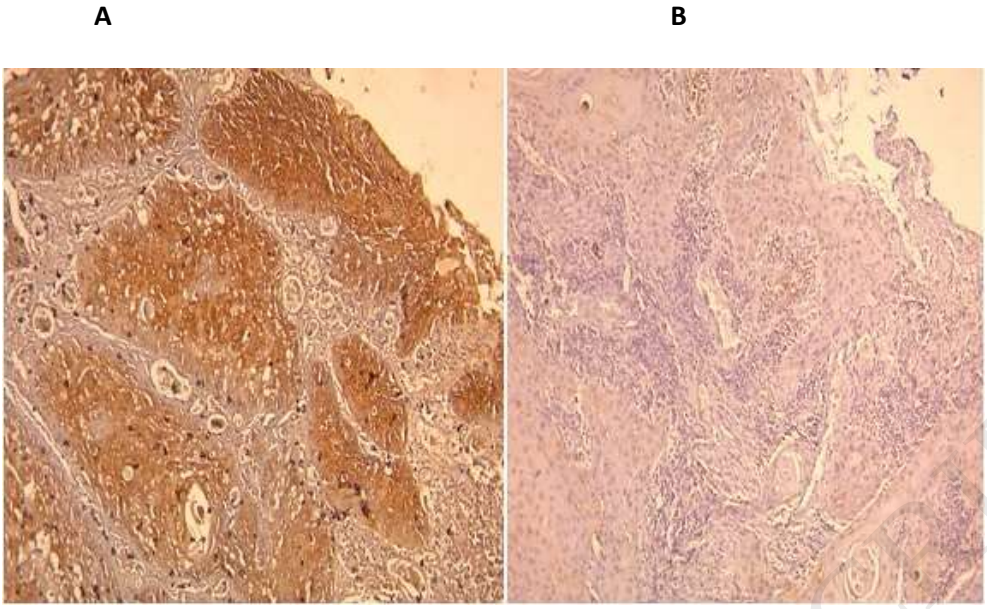

Figure 2: Photomicrograph of OSCC sections showing immunohistochemical staining of GST pi. Panels 'A' shows high expression and 'B' shows low expression (10x magnification).