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RESEARCH ARTICLE

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Novel association of oral squamous cell carcinoma with *GSTP1* Arg187Trp gene polymorphism

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Abstract

Aims: Glutathione S-transferase subtype pi 1 (GSTP1) is an enzyme that is involved in the detoxification of carcinogenic substances. Arg187Trp is a functional polymorphism in the corresponding *GSTP1* gene that reduces the enzymatic activity by 45%. We evaluated, for the first time, the association of Arg187Trp with the risk of oral squamous cell carcinoma and compared it with other established *GSTP1* polymorphisms viz, Ile105Val and Ala114Val.

Materials and Methods: We carried out a 1:2 case-control study by recruiting 100 patients with oral squamous cell carcinoma and 200 age and gender-matched healthy individuals. Ile105Val, Ala114Val, and Arg187Trp polymorphisms were genotyped by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and their distribution in the study groups was compared by chi-squared test (Fisher's exact).

Results: The minor allele of Ala114Val and Arg187Trp were more common in patients than in controls. In contrast, the distribution of Ile105Val minor allele was similar in the two groups. The differential distribution was also significant at the level of genotypes.

Conclusions: These results indicate that *GSTP1* Arg187Trp is associated with the risk of developing oral squamous cell carcinoma. Our study underlines the importance of detoxification pathway in the risk of carcinogenesis.

KEYWORDS

GSTP1, oral squamous cell carcinoma, recurrence, risk factor

1 | INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the sixth most common cancer in the world.[1] Oral cancers are a major public health burden as they constitute 15% of all the cancers and less than 60% of the patients survive for more than 5 years.[2] Buccal mucosa is the commonly affected site followed by the alveolar surface, hard palate, anterior 2/3rd of tongue, and floor of mouth.[3] The common risk factors for the development of OSCC are tobacco use, infection with high-risk strains of human papillomavirus and genetic factors.[4]

Genetic risk factors linked to oral carcinogenesis are gene polymorphisms that compromise the functional integrity of critical pathways like carcinogen detoxification, DNA repair, cell cycle control, extra-cellular matrix integrity, immunity, and inflammation.[5,6] Glutathione S-transferase pi 1 (GSTP1) is an important enzyme involved in carcinogen detoxification in the oral mucosa.[7]

Abbreviations: GSTP1, Glutathione S-transferase pi 1; OSCC, Oral squamous cell carcinoma.

GSTP1 mediates detoxification by conjugating carcinogenic substances to glutathione, a major cellular antioxidant. The enzyme is often over expressed in OSCC and has been linked to the failure of chemotherapy.[8,9] In contrast, the downregulation of GSTP1 has been shown to increase DNA damage on exposure to carcinogens.[10] Glutathione S-transferase pi 1 (*GSTP1*) gene which codes for the GSTP1 enzyme is located at 11q13.2. Single nucleotide polymorphisms (SNP) in the *GSTP1* gene that reduce its enzymatic activity have been linked to the risk of OSCC.[11] GSTP1 is the major GST enzyme expressed in the oral mucosa.[8]

Functional studies have identified three SNPs in the *GSTP1* gene, which result in the insignificant loss of enzymatic activity.[11] These are Ile105Val (c.313A > G; rs1695), Arg187Trp (c.559C > T; rs45549733), and Ala114Val (c.341C > T; rs1138272). The residual enzyme activities of these variants are 21.8%, 55.2%, and 79.9%, respectively. Both Ile105Val and Ala114Val variants have been associated with cancers of head and neck, thyroid, breast, lung, gastric, hepatocellular, and prostate.[12-20] Arg187Trp is the second most debilitating variant known in the *GSTP1* gene. To the best of our knowledge, there are no studies on the association of this variant with any cancer. We undertook this study to evaluate the association of Arg187Trp with OSCC and compare it with previously established SNPs viz., Ile105Val and Ala114Val.

2 | MATERIALS AND METHODS

2.1 | Study design

We carried out a 1:2 case-control study by including 100 patients with OSCC as cases and 200 healthy individuals as controls. Patients were recruited from the Department of Otorhinolaryngology, R.L. Jalappa Hospital and Research Centre, Kolar, Karnataka, India. Controls were recruited from the local population. Both patients and control individuals were recruited between 2014 and 2017. Informed consents were obtained from the study participants in writing before the recruitment. The study was reviewed and approved by the Institutional Ethics Committee of Sri Devaraj Urs Medical College, Kolar, India. Tumor specimens were collected from the patients after surgical resection and blood samples were collected from the control individuals. A structured questionnaire was used to collect information on demographic parameters (age and gender), habits (tobacco use, smoking, and alcohol), and family history of cancer. Clinical and histopathological details were collected from the medical records of each patient. The staging was done according to the 7th edition of American Joint Committee on Cancer TNM (T – Primary tumor staging, N – Nodal status, and M – Metastasis) staging system for OSCC.

2.2 | Genomic DNA preparation

Genomic DNA was prepared from resected tumor specimens obtained from patients and from peripheral blood obtained from controls. Tumor specimen was homogenized and used for genomic DNA isolation by the previously published method with the following modification.[21] The tissue sample was minced and placed in a microcentrifuge tube containing cell lysis buffer to which sodium dodecyl sulfate (20%) and proteinase K (20 mg) were added. Contents of the tube were incubated at 37°C until the complete digestion of the tissue. After the tissue digestion NaCl (5 M) and 1:1 volume of isopropyl alcohol was used for the precipitation of the DNA. The resultant DNA was washed with 70% ethanol, dried and dissolved in Tris-ethylenediaminetetraacetic acid (EDTA) buffer. Genomic DNA from the peripheral blood of controls prepared by the salting-out method.[22] Quantity and purity of the preparation were estimated by UV spectrophotometry.

2.3 | Detection of HPV

HPV status of the tumor specimens was evaluated by the polymerase chain reaction (PCR) method by using GP5+/6+ primers from L1 consensus region of the viral genome as per the method described by Rajesh et al.[23]

2.4 | Genotyping of *GSTP1* SNPs

GSTP1 SNPs were genotyped by the PCR-restriction fragment length polymorphism (PCR-RFLP) method. PCR and RFLP conditions are summarized in Suppporting Information Supplementary Table 1. The PCR was performed in 25 μl final volume containing 1 pM of each primer, 1 mM deoxyribonucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, 100 ng of genomic DNA, and 1 unit of *Taq* DNA polymerase. An aliquot of the amplicon in each case was incubated at with 10 units of respective restriction enzyme for 16 hours and the digestion pattern was analyzed on 3% agarose gel (Suppporting Information Supplementary Figure 1).

2.5 | Statistical analysis

Statistical analysis was carried out using the web-based calculation available at www.OpenEpi.com(updated

2013/04/06) and SPSS software version 20. The control population was tested for conformity to Hardy-Weinberg Equilibrium by using the web program by Rodriguez et al.[24] Allele and genotype frequencies of the case and control groups were compared by calculating *P*-value from the chi-square test (Fisher's exact test). *P*-values ≤ 0.05 were considered as statistically significant, where *P*-value was significant and odds ratio (OR) was calculated with 0.95 confidence interval (CI). Linkage disequilibrium between SNPs and loci disease linked haplotype was determined using a SHEsis web-tool.[25]

3 | RESULTS

The baseline demographic, clinical, and histopathological parameters of the study participants are summarized in Table 1. Age of the patients ranged from 21 to 80 years with a mean age of 53.8 ± 10.7 years. Majority of the patients were females (79%). Tumors of the buccal mucosa were most common (65%); the other sites of the lesion were lower alveolus, anterior 2/3rd of the tongue, retromolar trigone (RMT), and floor of the mouth. Eleven 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. These tumors are described as lower gingivobuccal sulcus (GBS) cancers which are unique to the Indian subcontinent. Staging of the tumor indicated that the majority of the patients had stage IVa tumor (59%) followed by stage III (21%) and stage II (20%). Squamous cell carcinoma was found to be well differentiated in 71%, moderately differentiated in 27%, and poorly differentiated in 2% of the patients. All the patients included in the study were found to be habituated to one or more carcinogenic substance like betel nut, chewable tobacco, *gutkha,* smoking, or alcohol. Chewing betel nut and tobacco quid was the most common habit seen in all the patients. The median duration of the use of chewable tobacco was 31 years. Only 7% of the patients smoked tobacco along with chewing. Six percent of the patients were habituated to tobacco chewing along with *gutkha* and alcohol consumption. All the tumor samples were found to be negative for HPV by the PCR test. Therefore, tobacco use in chewable form was the major risk factor in the patient group. The patients were followed for a period of 3 years. Ten patients were lost to follow-up. Of the remaining 90 patients, 10 patients died (cause not known), 18 patients presented with recurrence, and 62 patients showed disease-free survival.

TABLE 1 Demographic and clinico-pathological parameters of the study participants

Parameters	Patient (n = 100)	Control (n = 200)
Gender		
Male	21	44
Female	79	156
Age (mean ± SD; y)	54 ± 12	55 ± 13
Habits		
Chewing betel quid + tobacco	100	12
Chewing betel quid + tobacco + alcohol consumption	6	-
Chewing betel quid + tobacco + smoking cigarette	7	3
Site of primary tumor		
Buccal mucosa	65	-
Floor of mouth	1	-
Anterior 2/3rd of tongue	7	-
Lower alveolus	14	-
Lower GBS	11	-
RMT	2	-
Grade		
Grade 1	71	-
Grade 2	27	-
Grade 3	2	-
TNM classification		
T_{1-2}	8	-
T_{3-4}	92	-
Stage		
II	20	-
III	21	-
IV	59	-

GBS, gingivobuccal sulcus.

We first carried out a single locus analysis of the three *GSTP1* SNPs. The distribution of allele and genotype frequencies in the study groups are shown in Table 2. The distribution of the genotypes of all the three SNPs in the control group was in agreement with Hardy-Weinberg equilibrium ($\chi^2 \leq 3.84$). The minor allele frequency of Ile105Val, Ala114Val, and Arg187Trp among patients with OSCC were 34%, 15.5%, and 11%; these frequencies were 1.2, 4.1, and 6.3 times higher than that in the control group. Statistically significant difference between patient and control groups with respect to minor allele frequency was observed with Ala114Val and Arg187Trp but not with Ile105Val. The power of the study based on normal approximation with continuity correction was 86.8% and 98.8% with respect to Ala114Val and Arg187Trp. Patient and control groups were also compared at the level of genotype distribution.

TABLE 2 Profile of allele and genotype frequencies of *GSTP1* SNPs in the study groups.

GSTP1 SNP	Allele/genotype	Patient (n = 100)	Control (n = 200)	*P*- value†	HWE (χ^2)
Ile105Val	Ile	132	272	0.64	
	Val	68	128	OR: 1.09 (0.95 CI: 0.76-1.56)	
	Ile/Ile	46	97	0.71	2.16
	Ile/Val	40	78		
	Val/Val	14	25		
Ala114Val	Ala	169	385	9.16×10^{-7}	
	Val	31	15	OR: 4.7 (0.95 CI: 2.47-8.95)	
	Ala/Ala	74	186	$1 \times 10^{-5*}$	1.98
	Ala/Val	21	13		
	Val/Val	5	1		
Arg187Trp	Arg	178	393	$2 \times 10^{-6*}$	
	Trp	22	7	OR: 6.9 (0.95 CI: 2.9-16.5)	
	Arg/Arg	79	193	$2 \times 10^{-6*}$	0.06
	Arg/Trp	20	7		
	Trp/Trp	1	0		

*Significant ($P \leq 0.05$).

†The Chi-square test - Fisher's exact.

HWE: Hardy-Weinberg equilibrium; SNPs, single nucleotide polymorphisms.

Statistically significant difference was observed only with Ala114Val and Arg187Trp but not with Ile105Val. The distribution of the genotypes was further compared by considering various genetic models (Supporting Information Supplementary Table 2). It also presented a statistically significant difference between patient and control groups was observed with Ala114-Val and Arg187Trp but not with Ile105Valin all the four models. We then carried out a pair-wise linkage disequilibrium test with Arg187Trp, Ala114Val, and Ile105Val. We did not observe any significant linkage between the three SNPs (Supporting Information Supplementary Figure 2).

We carried out univariate and multivariate analysis of the positively associated SNPs with clinicopathological features and risk factors (Table 3). Both Ala114Val and Arg187Trp were not associated with age, gender, or tumor grade. Arg187Trp, but not Ala114Val, was associated with the longer duration (≥ 31 years) of tobacco use in both univariate and multivariate models. We also carried out a univariate and multivariate analysis with respect to tumor recurrence (Table 4). Duration of tobacco use (≥ 31 years) was significantly associated with recurrence in both univariate and multivariate models.

4 | DISCUSSION

In this study, we have explored the association of *GSTP1* SNPs Ile105Val, Ala114Val, and Arg187Trp with OSCC in a cohort of Indian patients. To the best of our knowledge, this is the first study to elucidate the correlation between Arg184Trp and the risk of OSCC. Furthermore, the association was evaluated in conjunction with the previously associated SNPs viz, Ile105Val and Ala114Val. Together, the three SNPs constitute a panel of *GSTP1* variants with biochemically verified functional impact. The findings of the study are: (i) both Arg187Trp and Ala114Val conferred a higher risk of OSCC but not Ile105Val, (ii) Arg187Trp, Ala114Val, and Ile105Val were not in linkage disequilibrium, and (iii) duration of tobacco use and not *GSTP1* SNPs were associated with tumor recurrence. The absence of a strong linkage between Arg187Trp and Ala114Val indicates that their parallel association with OSCC is independent and unlikely due to co-occurrence on the same chromosome. Together, our findings provide evidence to implicate Arg187Trp as an independent risk locus in the pathogenesis of OSCC.

Several studies have explored the association of Ile105Val with the risk of OSCC.[19,26,27] However, the association failed to be reproduced at the level of meta-analysis.[28] This motivated us to evaluate the association of other functional SNPs in the *GSTP1* gene with the risk of OSCC. The lack of association between Ile105Val and OSCC observed in this study agrees with the meta-analysis. The positive association observed between Ala114Val and OSCC is in consonance with the previous studies.[29] SNPs in the *GSTP1* gene have been evaluated for association with various biological phenomena like the risk of carcinogenesis, therapeutic response of pharmacological drugs, autoimmunity, infertility, and radiotoxicity.[30-33] These association

TABLE 3 Univariate and multivariate analysis of association between OSCC and *GSTP1* SNPs (Ala114Val and Arg187Trp)

		Ala114Val		Arg187Trp	
Factor	**n**	**Univariate *P*-value[†]**	**Multivariate *P*-value[#]**	**Univariate *P*-value[†]**	**Multivariate *P*-value[‡]**
Age[§]		0.33	0.29	0.33	0.62
≥50 years	52				
<50 years	48				
Gender					
Male	21	0.72	0.68	0.22	0.25
Female	79				
Tumor grade					
G1	72	0.20	0.14	0.58	0.59
G2+G3	28				
Duration of tobacco use[§]		0.82	0.74	0.01*	0.04*
≤31 years	43			OR: 3.87 (0.95 CI: 1.35 - 11.05)	OR: 3.62 (0.95 CI 1.08- 12.16)
>31 years	57				

*Significant ($P \leq 0.05$).

[†]Chi-squared test – Fisher's exact.

[‡]Logistic regression analysis.

[§]Dichotomised by median.

studies were mostly carried out with Ile105Val and Ala114Val. In contrast, the literature on Arg187Trp is negligible. We found only one reference for Arg187Trp in a patent application dealing with the prediction of susceptibility to skin damage (US Patent number US20160068904A1).

An important covariate in our OSCC cohort was the use of tobacco mostly in the chewable form. Chewing paan (a preparation of areca nut rolled in betel leaf) is a traditional practice in India and other south and south-east Asian countries.[34,35] Though the practice is receding due to modernization, it continues in underdevelopment pockets mostly among older people. Tobacco is a common coingredient in paan preparation thus making its use a risk factor for cancer. Infection with high-risk strains of human papillomavirus is another major risk factor associated with oral carcinogenesis. Human papillomavirus infection was completely absent in our patient group. We have previously shown that tobacco use and not human papillomavirus infection is the major exogenous risk factor for oral cancer in the rural scenario.[29] Higher prevalence of the tobacco use in the patient group may have contributed to the positive association. We could not add another control group comprising of OSCC-free tobacco chewers. The cultural stigma attached to the tobacco use, particularly by women, makes it difficult to elicit unambiguous from volunteers. The predicament was further compounded by the presence of a large fraction of women in the patient group, thereby, requiring an equal number in the control group for gender matching. There is no strong evidence to suggest that habituation to tobacco use is determined by the *GSTP1* gene.

Association of *GSTP1* variants with cancer risk is often influenced by environmental factors. For instance, the association of Ile105Val and gastric cancer risk is higher in patients with exogenous risk factors like *Helicobacter pylori* infection, smoking, or alcohol use,[36] the association of Ile105Val with the risk of lung and bladder cancers is higher in smokers compared with nonsmokers,[37,38] the association of Ile105Val with risk of prostate cancer was higher in patients exposed to carcinogens like polycyclic aromatic hydrocarbons.[15] The involvement of gene-environment interaction was also noticed in this study. Arg187Trp was significantly higher among patients with longer duration of tobacco use. Thus, the positive association observed with Arg187Trp may be due to habituation to tobacco use. Whether the association between Arg187Trp and OSCC replicates in patients not habituated to tobacco use needs to be examined using a different demographic group.

In conclusion, the results of this study show that Arg187Trp in *GSTP1* gene increases the risk of OSCC. This underlines the significance of the detoxification pathway in carcinogenesis.

TABLE 4 Univariate and multivariate analysis of tumor recurrence

Risk factor	Univariate *P*-value[†]	Multivariate *P*-value[‡]
Gender		
Male Female	0.2	0.1
Age[§]		
≤50 years >50 years	0.70	0.61
Stage		
T_{1-2} T_{3-4}	0.93	0.69
Tumor grade		
G1 G2+G3	0.53	0.56
Ile105Val[¶]	0.41	0.40
Ala114Val[¶]	0.71	0.69
Arg187Trp[¶]	0.26	0.26
Duration of tobacco use[§]		
≤31 years >31 years	0.05* OR: 2.92 (0.95 CI:0.85 - 9.74)	0.03* OR: 5.06 (0.95 CI: 1.13 - 22.61)

*Significant ($P \leq 0.05$).
†The Chi-square test (Fisher's exact).
‡Logistic regression.
§Dichotomized by median.
¶*P* value calculated using dominant genetic model.

CONFLICT OF INTERESTS

DR, SB, AMSM, and AVMK are listed as inventors in an Indian patent application concerning this data. RS has no conflict of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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