Original Article

The synergy of tobacco and alcohol and glutathione S-transferase θ 1 gene deletion and oral squamous cell carcinoma

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the leading cancer among males in India. It is related to tobacco habits and alcohol consumption as well as the individual susceptibility for xenobiotic metabolizing enzyme polymorphisms. Glutathione S-transferase θ 1 (GSTT1) is a Phase II metabolic enzyme which is directly involved in catalyzing chemicals to mutagenic intermediates. This gene is characterized by genetic polymorphism resulting in complete gene deletion and subsequent absence of the enzyme, which ultimately dictates the risk of cancer development. Scraping buccal mucosa to obtain DNA from the cells is a simple, readily acceptable and rapid method to detect and assess the gene.

Aim: To assess GSTT1 gene deletion in individuals giving a history of tobacco smoking and/or chewing and alcohol consumption and absence of clinically detectable lesions; and in OSCC cases to gauge if GSTT1 gene deletion confers protection to an individual and whether it can be used as a "single" marker to arrive at this conclusion. To validate the use of buccal scrape for determining the genotype of an individual by assessing the polymorphism at GSTT1 gene locus (22q11.2).

Materials and Methods: Fifty-two cases were evaluated using buccal mucosal scrapes of tobacco habituates for 8 or more years, without clinically evident lesion (Group I) and from mucosa of tobacco habituates with clinically evident and histopathologically confirmed OSCC (Group II). DNA extraction and genotype at GSTT1 gene locus was determined by polymerase chain reaction assay.

Statistical Analysis: The results were statistically analyzed using Chi-square test.

Results: 90.66% of subjects had GSTT1 null genotype in Group I subjects. In Group II, subjects with both clinically and histopathologically diagnosed oral cancer, about 76.96% had GSTT1 null genotype.

Conclusion: GSTT1 null genotype confers protection to individuals with tobacco habits and alcohol consumption, predominantly to those who used chewable form of tobacco and especially among female population. However, the influence of many other environmental, genetic and epigenetic factors should be considered for the genesis/occurrence of cancer.

Key Words: Alcohol, buccal scrapes, glutathione S-transferase θ 1 gene, oral squamous cell carcinoma, tobacco habits

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the 6th common cancer and is a major health problem in India forming about 10% of the new cancers that occur in all parts of the body.[1] Despite early diagnosis and treatment strategies improving, 5-year survival statistics is approximately 50%. Due to its related high mortality and low cure rate, OSCC represents a major public health problem with a great individual and socioeconomic impact.[2]

Oral carcinogenesis is a complex and multifactorial process modulated by both endogenous and environmental factors.[3] According to the World Health Organization, about 90% of oral cancer in Southeast Asia is attributable to tobacco use (smoking and chewing). Tobacco smoke contains pyrolysis products due to high temperature whereas, smokeless tobacco is rich in nitrosamines-metabolites of nitrosonornicotine: NNN, NNK.[4] Betel quid is chewed with the concomitant use of additives such as betel nut, piper betel leaf, catcheu and slaked lime. The use of betel quid leads to a 50-fold increase in reactive oxygen species generated.[5] Alcohol consumption is also linked to an increased risk of developing cancer.[6] Factors that influence tobacco-exposed individuals to develop a malignancy includes a combination of total tobacco exposure and genetic susceptibility and co-existence of other factors.[7]

All organisms are constantly exposed to foreign chemicals (xenobiotics) which include manufactured and natural chemicals such as drugs, industrial chemicals, pesticides and pollutants.[8] The biotransformation for xenobiotic metabolism involves several enzyme systems that are divided into two phases; Phase I and Phase II. The outcome of biotransformation in most cases is detoxification. Glutathione S-transferases is an important family of Phase II enzymes that catalyze the detoxification of active metabolites of tobacco carcinogens.[9]

GST gene family comprises 16 genes in six subfamilies. Glutathione S-transferase θ 1 (GSTT1) is an enzyme that catalyzes the conjugation of glutathione to a broad variety of carcinogenic compounds, resulting in increased water solubility thus allowing excretion of carcinogenic metabolites formed during Phase I reaction. This class has also been associated with bioactivation and transformation of halogenated compounds to mutagenic intermediates-dichloromethane (DCM). Thus, GSTT1 has both detoxification and activation roles.[6]

GSTT1 gene is characterized by a genetic polymorphism resulting in complete gene deletion with consequent absence of the enzymatic functional activity which is known as null gene. The null genotype has a decreased capacity to detoxify carcinogens present in tobacco smoke, leading to the formation of DNA adducts and DNA damage. The frequency of the null genotype has been determined in different human populations. Despite the strong biological plausibility for the role of gene polymorphism at GSTT1 gene locus in altering individual susceptibility to oral cancer, very little is clear about its possible mechanisms.[10]

The buccal scrape samples for genotyping highlights its clinical usefulness since it is a painless procedure and is easily accessible. Genetic counseling can be delivered to the subjects with increased propensity to cancer development even before the disease has manifested at both molecular level and clinical detectable level. These patients can be followed up at regular intervals and can be monitored to prevent the occurrence of OSCC.

MATERIALS AND METHODS

The study was conducted on buccal mucosa scrapes of patients from Karnataka diagnosed as OSCC and patients with no clinically detectable lesion but giving a positive history of tobacco smoking and/or chewing and alcohol consumption. A total of 55 samples were considered. The study group was divided into:

- Group I/control group (*n* = 25) with a history of tobacco habits (both smokable and smokeless variety) and alcohol consumption for 8–10 years and without clinically detectable lesion
- Group II/OSCC group (*n* = 30 cases) with a history of tobacco habit and alcohol consumption for 8–10 years associated with OSCC.

GSTT1 primer sequences used: Forward: 5'TTCCTTACTGGTCCTCACATCTC3' reverse: 5'TCACCGGATCATGGCCAGCC3'. G3PDH was used as an internal control. The conventional polymerase chain reaction (PCR) technique of amplification was done employing TaqDNA polymerase and a set of 2 (GSTT1, G3PDH) specially designed primers that recognize distinct sequences on target DNA. The purified genomic DNA was amplified by PCR. Amplification products were resolved on 2% ethidium bromide-stained agarose gel along with 100 bp DNA ladder. Electrophoresis results were analyzed in gel documentation system [Figures 1 and 2]. DNA samples showing amplification for G3PDH but not for any exons of GSTT1 were considered as deleted (null) genotype of GSTT1. A 480bp fragment was amplified from chromosome 22q11.2 which is the GSTT1 locus.

Statistical analysis

The results were statistically analyzed using Chi-square test.

Figure 1: Gel Electrophoretic image of squamous cell carcinoma 15, 16, 17 and 18 showing positive band for glutathione S-transferase θ 1 in squamous cell carcinoma 17 only and G3PDH showing positive band in all samples

OBSERVATIONS AND RESULTS

Distribution of subjects in each study group by gender and age

Group I consisted of 58.33% male patients and 41.66% female patients, whereas Group II consisted of 45.16% males and 54.83% female patients. Maximum male subjects in the control group were in the age range of 31–40 years and females were maximum in 51–60 years. As against, maximum male subjects were in the age range of 51–60 years and maximum female subjects were in 41–50 years in OSCC group [Table 1].

Distribution of study subjects by age and habit type

It was observed that tobacco habits both in smoking and chewable form were practiced by the subjects. Along with tobacco-related habits, some subjects also had alcohol consumption habit [Table 2].

Distribution of age with habit duration and gender in both groups

In control group, male subjects were predominantly clustered in 31–40 and 41–50 years age group and the duration of habit ranged from 5 to 15 years, with a peak of 10 to 12 years. The female population had maximum distribution in 41–70 years age groups and with habit duration ranging from 10 to 40 years.

In OSCC group, male subjects were predominantly in the age range of 31–40 years, 51–70 years age groups and habit duration ranged from 7 to 40 years with maximum subjects having 15–25 years of habit. Female subjects had a wide age distribution ranging from 41 to 50, 51 to 60 and 61 to 70 years; with habit duration ranging from 10 to 42 years. No peak was observed.

Comparison of glutathione S-transferase θ 1 results between Group I and Group II

In control group, out of 25 subjects, 23 subjects had GSTT1 null genotype, and 2 subjects had GSTT1 wild genotype.
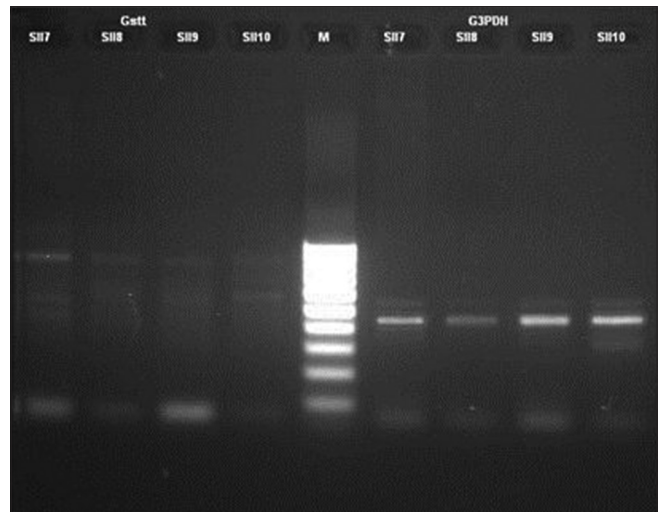

Figure 2: Gel Electrophoretic picture of squamous cell carcinoma 7, 8, 9 and 10 showing positive band for glutathione S-transferase θ 1 as well as G3PDH

Table 1: Distribution of subjects in study groups by gender and age

Age range (years)	Group I/control group (%)		Group II/OSCC group (%)	
	Male	**Female**	**Male**	**Female**
20-30	4.1	4.1	3.22	0
31-40	37.5	4.1	12.5	3.22
41-50	12.5	8.33	6.45	22.5
51-60	4.1	20.8	19.35	12.9
61-70 and above	0	4.1	12.5	16.12

OSCC: Oral squamous cell carcinoma

Table 2: Distribution of study subjects by age and habit type

Age range (years)	Group I/control group (%)		Group II/OSCC group (%)	
	Tobacco	**Tobacco + alcohol**	**Tobacco**	**Tobacco + alcohol**
20-30	8.33	0	0	0
31-40	16.66	25	3.22	9.67
41-50	16.66	4.16	19.35	3.22
51-60	20.83	4.16	33.33	6.45
61-70 and above	4.16	0	19.35	0

OSCC: Oral squamous cell carcinoma

Further, it was seen that out of 23 subjects with null genotype 13 were female and 10 were male subjects.

In OSCC group, out of 30 subjects, 22 subjects had GSTT1 null genotype and 8 subjects had GSTT1 wild genotype. Out of 22 null genotypes, 12 belonged to female subjects and 10 belonged to male subjects. We also observed that out of 8 subjects with GSTT1 wild genotype, 6 subjects used tobacco in chewable form and remaining 2 subjects had no habit history, out of which 1 was female and the other was a male patient [Tables 3 and 4].

Housekeeping gene (G3PDH) was amplified and was present in all the sample lanes; however, the GSTT1 band was seen

Table 3: Expression of glutathione S-transferase 1 in control and study group

	Results		Total
	GSTT 1 wild	GSTT 1 null	
Group I/control group	2	23	25
Group II/OSCC group	8	22	30

GSTT 1: Glutathione S-transferase θ 1, OSCC: Oral squamous cell carcinoma

Table 4: Distribution of glutathione S-transferase θ 1 results with gender in both groups

	GSTT 1 null (%)		GSTT 1 wild (%)	
	Male	Female	Male	Female
Group I/control group	50	41.66	8.33	0
Group II/OSCC group	32.25	38.70	12.9	16.12

GSTT 1: Glutathione S-transferase θ 1, OSCC: Oral squamous cell carcinoma

in 29, squamous cell carcinoma 1 (SCC1) and SCC3 lanes. Hence, except for 29, SCC1 and SCC3, all had GSTT1 gene deletion (NULL genotype).

On comparing the results of both groups, we observed that subjects with GSTT1 null genotype in the control group were 91.66% as against 70.95% in OSCC group. The difference between them was found to be statistically insignificant ($P > 0.001$).

DISCUSSION

Oral cancer is 6th most common cancer that encompasses at least 90% of all malignancies in the world. It ranks 1st among all cancers in males and 3rd among females in India. Despite advances in surgery, radiotherapy and chemotherapy, the 5-year survival rate of OSCC has remained approximately 50%.[1,2]

Oral cancer is a multifactorial process. The main factors implicated in its etiopathogenesis are tobacco, alcohol, microbial infections such as candida, human papillomavirus, Epstein–Barr virus and sunlight. A major role is played by tobacco-related habits and alcohol consumption. Tobacco both in its chewable and smokable form has many carcinogens such as PAH, nitrosamines, aldehydes and ketones. Betel quid chewing is also a popular habit in India and many other South-East Asian countries. Although the composition of betel quid varies in different geographic locations, it generally consists of betelnut (areca nut), piper betel leaf and slaked lime with or without tobacco. At least 6 alkaloids are present in the betel nut itself, of which arecoline and arecadine have been suggested as possible carcinogens.[11]

Xenobiotic metabolism of these procarcinogens to endogenously produced reactive substances is brought by both Phase I and II enzyme systems. The Phase I enzyme leads to the formation of reactive intermediates. These intermediates get acted upon by Phase II enzymes which further results in their metabolic activation or detoxification. Glutathione-S-transferases are a very important family of Phase II enzymes that catalyses the detoxification of a wide variety of active metabolites. GSTT1 is an enzyme that has been associated with both increased water solubility allowing excretion as well as bioactivation and transformation of halogenated intermediates like DCM. Thus, GSTT has both detoxification and activation roles.[6]

The balance between metabolic activation and detoxification pathways differ between individuals and are thought to affect disease susceptibility. This balance may be modified by genetic variations in the xenobiotic metabolizing enzymes. Hence, assessment of an individual's disease risk, by the identification of heritable variations in the expression of these enzymes which could predispose subjects to the development of oral cancer, can prove beneficial in predicting the outcome of such habits in these individuals.[4]

The GSTT1 gene is polymorphic in humans and expression of these genes directly relates to the activity of the enzymes in individuals. The null genotype of GSTT1 has a protective effect as it has decreased capacity to convert the procarcinogens into metabolically active intermediates. Thus, screening of population for GSTT1 genetic expression can be useful in delivering genetic counseling to subjects with susceptible genetic makeup, even before cancer can manifest itself at a clinically detectable level.[4]

The use of buccal scrapes for genotyping is another innovative method used for assessing the genetic make-up of an individual. The main advantage of using buccal cells as samples is that it is a painless, noninvasive procedure and hence very well accepted by patients as it causes little discomfort. Furthermore, acquiring buccal cells is not a very technique sensitive procedure, and a wider population screening can be carried out.

We analyzed the habit frequency in both the genders. We observed that majority of females in their fifth, sixth and seventh decade of life had tobacco chewing history with duration being almost 20–35 years. This explains that majority of female population get into the habit of chewing tobacco which is readily available while doing household chores. In addition, we observed that very negligible population of females had both tobacco and alcohol habits concomitantly as compared to male population, as alcohol consumption by females is considered as a "taboo" in the Indian society.

The frequency of null genotype in the different human population varies from 0.197 in Caucasians to 0.47 in Asians. Roy *et al*. conducted a study to estimate the GSTT1 null genotype in Eastern and Northern India which ranged 6% for

the Lodhas, 9% for Sandals, 39% for Jamatias. There is more variation observed among caste groups in Eastern India than in northern India.[12] Also, intra-ethnic differences were observed at GSTT1 locus in a study conducted by Mishra *et al*.[13] In this study, the frequency of GSTT1 null genotype was 80%, and GSTT1 wild genotype was 20% among the subjects. These variations could be attributed to different geographic locations, intraethnic variation, etc.

In this study, GSTT1 null genotype in the control group was 91.66% in individuals with tobacco and alcohol habit of 8 to 40 years ranging from second to seventh decade of life. After gender stratification, we observed that deletion is seen more commonly in females (100%) than in males. We observed that subjects in their fourth, fifth and sixth decades of life and with habit frequency ranging from 12 to 40 years, commonly culminate to oral cancer. Hence, it proves that GSTT1 null genotype in the control group could be exerting a protective effect. Similar findings were encountered by Anantharaman *et al*. (2007,[4] Peters *et al*.,[14] Evans *et al*.[15] Contradictory results were found in studies by Olshan *et al*.[16] and Sreelekha *et al*.[7]

A study conducted by Nosheen *et al*. states that presence of GSTT1 null genotype is different in the different populations in both cancer individuals and cancer-free individuals.[17]

In this study, the frequency of null genotype in OSCC group was 70.96%. The frequency of GSTT1 null genotype observed in both groups was not statistically different. The results of Group I and Group II contradict each other. Similar findings were observed in a study conducted by Gattás *et al*.[11] The results obtained in group II of our study might mean that — GSTT1 deletions are common in the South Indian population as also stated by Nosheen *et al*. stating that Indians have a higher frequency of GSTT1 deletion genotype. It can also be reasoned out that in our subjects,[17] epigenetic changes can precipitate cancer at a later stage in the patients who were initially protected by GSTT1 null genotype. Epigenetic changes have led to the realization that the packaging of the genome is potentially as important as the genome itself in regulating the essential cellular processes required for preserving the cellular identity and also in giving rise to disease states like cancer.[18] Levels of reduced GSH and activity of GSTs are known to be up-regulated in cancer cells, and certain studies have shown it to be correlated with tumor stage and differentiation. GSTTs are known to regulate the antioxidant status of the cell. Deregulation of the same in the stage of initiation can lead to tumorigenesis. However, deregulation postinitiation might render a survival advantage by blocking apoptosis. Hence, GSTs exhibit a dual role depending on the stage and differentiation of the tumor.[4] The other possibility being that GSTT might not be a single/sole risk predictor in OSCC. The risk factor might be associated with polymorphism of similar genes like GSTM1, GSTP1, CYP1A1, etc., or other genetic mutations. Peters *et al*. showed the ability of polymorphism in the GSTM1, GSTT1 and GSTP1 genes in altering the head and neck cancer risk singly or in combination by examining the gene-environment interaction in detoxifying the tobacco-related carcinogens.[14]

Evans *et al*. demonstrated the association between the presence of GSTT1 gene and head and neck SCC was strongest among heavy smokers with an odds ratio of 2. Stratification by gender demonstrated that deletion of the GSTT1 gene was more pronounced in smoker female population.[15]

However, this study showed that the subjects expressing GSTT1 wild genotype had tobacco chewing as a prime habit. Thus we can infer that components of chewable tobacco, forms the basic substrate for GSTT1 enzyme. This enzyme then converts these components into mutagenic intermediates. GSTT1 phenotype determines the metabolism of methyl chloride and GSTT1 null individuals lack the capacity to metabolize methyl chloride.[14]

Landi *et al*. have shown that for particular chemical substrates, GSTT1 mediated glutathione conjugation can result in activation of a compound to an electrophile that is capable of mutagenesis.[19]

A study conducted by Mahimkar *et al*. showed that individuals carrying GSTT1 null genotype showed a trend toward protection. The protective effect of GSTT1 null genotype was pronounced and specific to tobacco chewers. Also, this protective effect increases with lifetime exposure to tobacco chewing. Similar findings were seen in this study, which can explain the protection offered to all female subjects (100%) in the control group, who lacked GSTT1 gene expression and were exclusively tobacco chewers (8–40 years).[4]

However, a study conducted by Evans *et al*. showed pronounced protection of GSTT1 null genotype in female population but the association was strongest among smokers.[15] It remains possible that the participating controls differ with respect to their frequency of tobacco and alcohol use relative to the included OSCC cases. The conflicting results regarding GSTT1 expression may be due to several confounding factors such as age–sex matched subjects, ethnic variation, different socioeconomic status and technical discrepancies in genotyping assays.

A larger population study is warranted to clarify the protective role of GSTT1 expression against oral cancer. Furthermore, elimination of sampling error and other confounding factors is necessary. Future epidemiological studies focusing on complex

genotypes within the same gene family or related gene families may be helpful in identifying individuals at risk for oral cancer and in elucidating gene-gene interactions.

This study was conducted on DNA extracted from buccal scrapes of the subjects as it received a better patient acceptance and multiple sampling was possible when a large population was targeted for screening. We observed that bands obtained from buccal cells were ill-defined as compared to blood cells during standardization. This could be due to a variety of protein impurities encountered in buccal scrapes from the oral environment (salivary proteins, commensal microbes, food particles, etc.) which interfere with DNA extraction from the buccal cells. With few modifications in the technique like column purification before running PCR, bands within acceptable standards were obtained.

CONCLUSION

Our study, therefore, elucidates the importance of early recognition of risk factors and the possibility of oral cancer occurrence in individuals with specific genetic makeup. This can thus be a preventive measure implemented through genetic counseling to motivate individuals to restrict and withdraw the use of tobacco and alcohol consumption. Thus, the patient can be made aware of his susceptibility toward cancer development well before it manifests itself at both cellular and molecular level.

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Conflicts of interest
There are no conflicts of interest.

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