

Association between GSTT1 and GSTM1 Null Polymorphisms with Pre-Malignant Oral Lesions in North Indian Population

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ABSTRACT

The objective of this investigation is to identify the relationship of GSTT1 and GSTM1 gene polymorphisms with risk of cancer in distinct population in North India with histopathological confirmed oral pre-malignant lesions. A range of parameters such as gender, age, tobacco habit, alcohol consumption and stages of pre-malignant were taken into consideration. Blood samples of 34 histopathological confirmed oral pre-malignant cases, were collected and analyzed for GSTT1 and GSTM1 polymorphisms by PCR method. Thus, the polymorphism of GSTM1 and GSTT1 gene involved may results in the differences in enzymatic activity possibly favoring mechanisms that increase the susceptibility to cancer. Hence, out of 100% pre- malignant population, 72.73% is more susceptible to cancer in case of GSTM1. While in the GSTT1 out of 100% pre- malignant population, 54.55% population is more susceptible to cancer. This study conclude that since the polymorphic genotypes were more in tests samples than the null genotypes, it may be associated with an increased risk of cancer from pre- malignant lesions of oral cancer.

Key words: Null Polymorphism, pre-malignant lesions, GSTT1 (Glutathione S-transferases theta 1), GSTM1 (Glutathione S-transferases Mu 1)

1. Introduction

Oral cancer is the 6th most common cancer in the world which accounts for 350,000 new cases and 128,000 deaths annually. Two-third cases are contributed by the developing countries among the overall cases in the world (Uplap et al. 2011). India alone accounts for one third of the world's oral cancer and has a high rate of pre-malignant lesions (Neufled et al. 2005).

India has one of the highest incidences of oral cancer (age-standardized rate of 9.8 per 10 000) making it the most common cancer among men (men:women ratio 2:1) and accounts for about 30% of all new cases annually. A recent survey of cancer mortality in India shows cancer of the oral cavity as the leading cause of mortality in men and responsible for 22.9% of cancer-related deaths. (Yogesh et al. 2013).

It is the sixth most common cancer reported globally with an annual incidence of over 300,000 cases, of which 62% arise in developing countries. There is a significant difference in the incidence of oral cancer in different regions of the world. The age-adjusted rates of oral cancer vary from over 20 per 100,000 populations in India, 10 per 100,000 in the U.S., and less than 2 per 100,000 in the Middle East (Shankarnarayan *et al.* 1998). Oral cancer is a heterogeneous group of cancers arising from different parts of the oral cavity, with different predisposing factors, prevalence, and treatment outcomes. It has been estimated that around 43% of cancer deaths are due to tobacco use, alcohol consumption, and unhealthy intake of food, inactive lifestyles and infection (Dhami et al.). The use of tobacco is an established etiological factor in the development of cancers of the oral cavity. Tobacco is consumed in both its smoking and smokeless form. The consumption of smokeless tobacco occurs with the concomitant use of several additives that can alter cancer risk (Lyon. 2013).

Tobacco smoke comprises nearly 60 carcinogenic compounds whereas its unburned form contains 16 identified carcinogens. Polycyclic aromatic hydrocarbons (PAHs), nitrosamines, aldehydes and ketones form the major carcinogens present in tobacco (Hecht. 2003)

The use of terms 'oral pre-cancer' and 'oral pre-malignancy' in itself poses problems, since this terminology signifies an invariable development of cancer from such diseases. The use of terms like 'potentially malignant' (Jhonson et al. 1993) signifies more precisely what is actually meant. Notwithstanding the

relevance of this discussion, the designations 'pre-cancer', 'pre-cancerous', 'pre-malignant', and 'precursors' will be used synonymously throughout this review for diseases with a malignant potential. Pre-cancerous lesions include leukoplakia and erythroplakia. There is general agreement that leukoplakia and erythroplakia are clinical diagnoses bearing no connotations as to their histopathology. Classification schemes for lesions of the oral cavity typically have used the clinical appearance of lesions to determine which are premalignant. (Axelle et al. 1996) Leukoplakia and erythroplakia are two clinical lesions widely considered to be premalignant. However, using clinical features to classify lesions is difficult because they vary in appearance and are likely to be interpreted subjectively by the clinician. A histopathologic diagnosis is generally more indicative of premalignant change than clinically apparent alterations. A pre-malignant lesion is a lesion that, while not cancerous, has strong potential for becoming cancerous. It is an apparently benign, morphologically altered tissue which has a greater than normal risk of containing a microscopic focus of cancer at biopsy or of transforming into a malignancy after diagnosis. Precancerous condition can be defined as a disease or patient habit which does not necessarily alter the clinical appearance of local tissue but is known to have a greater than normal risk of pre-cancer or cancer development. A wide spectrum of lesions ranging from dysplasia to in situ carcinoma has to be considered when dealing with oral precancerous conditions. Leukoplakia, exophytic keratosis, and polypoid thickening are early signs which may arouse the suspicion of malignancy (Silverman et al. 1984). Potential etiologic factors may be related to a genetically controlled sensitivity to environmental carcinogens. (Gallo et al. 1997).

Genetic polymorphism is prevalent and plays a vital role in human diseases. Recently, the relationship of genetic polymorphisms and the risk of cancers have been researched widely. (Haitao et al. 2015).

Studies show it takes an average of 3.9 years between the first oral biopsy and the diagnosis of invasive carcinoma. About 5-18% of epithelial dysplasias become malignant (Axelle et al. 1996, Silverman et al. 1984, Gallo et al. 1997, Van der Wall I 2009). With the completion of the first draft of the human genome and the availability of cheaper and quicker technique genotyping technologies, there is a rapidly increasing interest in identifying genes and genetic polymorphisms that predispose people to increased risk of cancer (Zondervan et al. 2002, Kennedy et al. 2003). Polycyclic aromatic hydrocarbons (PAHs), present in tobacco smoke or metabolites of alcohol, are precursors of chemicals that increase the risk of

cancer. Most carcinogens are not biologically active when they enter the body. They need to be converted into biologically active forms before they can interact with host DNA to cause mutations. Most PAHs first require activation by Phase I enzymes, such as cytochrome P4501A1 (CYP1A1), to become an ultimate carcinogen. These activated forms may be subjected to detoxification by Phase II enzymes, especially glutathione S transferases (GSTs) (Lazarus et al. 2000)

The glutathione *S*-transferases (GSTs) are a family of enzymes known to play an important role in the detoxification of several carcinogens found in tobacco smoke. GSTs are dimeric proteins that catalyze conjugation reactions between glutathione and tobacco smoke substrates, such as aromatic heterocyclic radicals and epoxides. Conjugation facilitates excretion and thus constitutes a detoxification step. In addition to their role in phase II detoxification, GSTs also modulate the induction of other enzymes and proteins important for cellular functions, such as DNA repair (Hayes, Pulford. 1995). This class of enzymes is therefore important for maintaining cellular genomic integrity and, as a result, may play an important role in cancer susceptibility. In humans, based on their primary structures, GSTs are divided into seven families/classes: α (alpha), μ (mu), π (pi), τ (theta), θ (sigma), ω (omega), and ξ (zeta) (Mannervik et al. 1985, Mannervik et al. 1992, Meyer et al. 1991). Deletion of these genes results in a lack of enzyme activity and a reduction in the elimination of carcinogenic substances (Rahmi et al. 2009). Two loci in particular, GSTM1 and GSTT1, may be of relevance for susceptibility to squamous cell carcinoma of the head and neck (SCCHN) (Stacy et al. 2001).

The role of the GST enzymes in detoxification mechanisms of the carcinogenic compounds has led to the hypothesis that if the individual's genotype at GST locus encodes a deficient GST enzyme it may result in increased risk of cancer (Meyer et al. 1991). GSTM1 and GSTT1 can both detoxify carcinogenic polycyclic aromatic hydrocarbons, such as benzo[a] pyrene (Pemble et al. 1994) and the absence of the GSTM1 and GSTT1 genes has been reported to increase the risk of several common cancers, particularly those caused by cigarettes smoking including cancers of the mouth, lung, bladder and breast (Hayes, Pulford. 1995, Rebbeck 1997). Although GSTM and GSTT are considered as low penetrance genes they may still contribute significantly to the number of cancer cases in the general population because of their high prevalence (Rebbeck 1997).

The relationship between genetic polymorphism of the above genes and pre-malignant oral lesions was not previously investigated in North India. Therefore, the aim of this study was to investigate the genetic polymorphisms in *GSTM1* and *GSTT1* and its association with pre-malignant oral cancer risk in a specific North Indian population.

2. MATERIALS AND METHODS

2.1 Sample Collection

In present investigation, the required blood samples of 34 cases of clinically confirmed pre-malignant lesions of oral cavity were taken, after obtaining an informed consent from all participants in the study. Personal details were recorded in a questionnaire upon interview. Information regarding age, gender, occupation and details about duration, frequency, nature of tobacco habit (smoking or smokeless), and alcohol consumption were recorded. Patients with pre-malignant lesions were enrolled from I.T.S- Centre for Dental Studies and Research. These were histopathologically confirmed. Genetically unrelated healthy individuals who reported the absence of personal history of cancer of any organ were recruited from the pathology of Surya Hospital- Muradnagar, Ghaziabad, formed the control subjects. Controls enrolled in this study were also matched for age, gender and tobacco habits.

2.2 DNA Extraction

A total 5 milliliter blood samples were collected from 34 patients clinically diagnosed with pre-malignant oral lesions and from 34 healthy individuals. Blood samples were collected into EDTA tubes and stored at -20° C temperature until DNA extraction was performed using standard protocols. DNA was extracted from anti-coagulated (EDTA) samples by proteinase K digestion and phenol-Chloroform extraction.

2.3 Polymorphism in *GSTM1*

For analysis of *GSTM1* m2 polymorphism, an allele-specific PCR method was used as described by Kumar et al. (2010). Briefly, 5 µl reaction mixtures consisted of 50 ng of genomic DNA, 0.2µl of each prime *GSTM1* primers (MF- 5' GAACT CCTGAAAAGCTAAAGC 3' and MR - 5' GTTGGGCTCAAATATACGGTGG 3') . of 0.1µl dNTPs mixture (Bangalore Genei, Bangalore, India), 0.4 µl of MgCl₂, 0.1 µl of taq polymerase with 0.5µl 10×PCR reaction buffer (Bangalore Genei, Bangalore, India). Producing a 205 bp fragment. PCR amplifications were performed in

Eppendorf Mastercycler Gradient-5331 thermal cycler with following PCR temperature profile, as presented in table below:

Amplification steps	Temperature (°C)	Time	Cycles
Initial denaturation	94	3min.	
Denaturation	94	30 sec	} 40
Annealing	55	30 sec	
Extension	72	45 sec	
Final extension	72	5 min	

Table 2.1. Showing temperature profile for PCR amplification for analysis of GSTM1 m2 polymorphism

S.No.	Reagent	Volume
1	10X buffer	0.5µl
2	25 mM MgCl ₂	0.4 µl
3	dNTP mix	0.1 µl
4	Taq Polymerase	0.125 µl
5	Forward Primer	0.2 µl
6	Reverse Primer	0.2 µl
7	MilliQ water	2.475 µl
Total		4.0 µl
8	Genomic DNA (template)- 50ng/ µl	1µl
	Total reaction volume	5 µl

Table 2.2. The reaction mixture for PCR amplification in GSTM1 and GSTT1 polymorphism

2.3 Polymorphism in GSTT1

For analysis of *GSTT1* m2 polymorphism, an allele-specific PCR method was used as described by Kumar et al. (2010). Briefly, 5 µl reaction mixtures consisted of 50 ng of genomic DNA, 0.2µl of each prime *GSTT1* primers (TF - 5' TTCCTTACTGGTCCTCACATCTC 3' and TR- 5' TCACCGGATCAT GGCCAGCA 3'). 0.1µl dNTPs mixture (Bangalore Genei, Bangalore, India), 0.4 µl of MgCl₂, 0.1 µl of taq polymerase with 0.5µl 10×PCR reaction buffer (Bangalore Genei, Bangalore, India). Producing a 205 bp fragment. PCR amplifications were performed in Eppendorf Mastercycler Gradient-5331 thermal cycler with following PCR temperature profile, as presented in table below.

Amplification steps	Temperature (°C)	Time	Cycles
Initial denaturation	94	5min.	
Denaturation	94	30 sec	} 40
Annealing	60	45 sec	
Extension	72	1 min	
Final extension	72	5 min	

Table 2.3. Showing temperature profile for PCR amplification for analysis of GSTT1 polymorphism

3. RESULTS AND DISCUSSION

A. Analysis of PCR products

3.1 GSTM1 Genotyping

After PCR amplification, the PCR products were then separated on 2% agarose gel at 150V for 1.5 h and stained with 1µg/ml ethidium bromide at 25°C for 10 minutes. The gel was carefully taken out after electrophoresis and placed in UV gel doc for analysis of the amplified products. The bands of final PCR products were observed on gel.

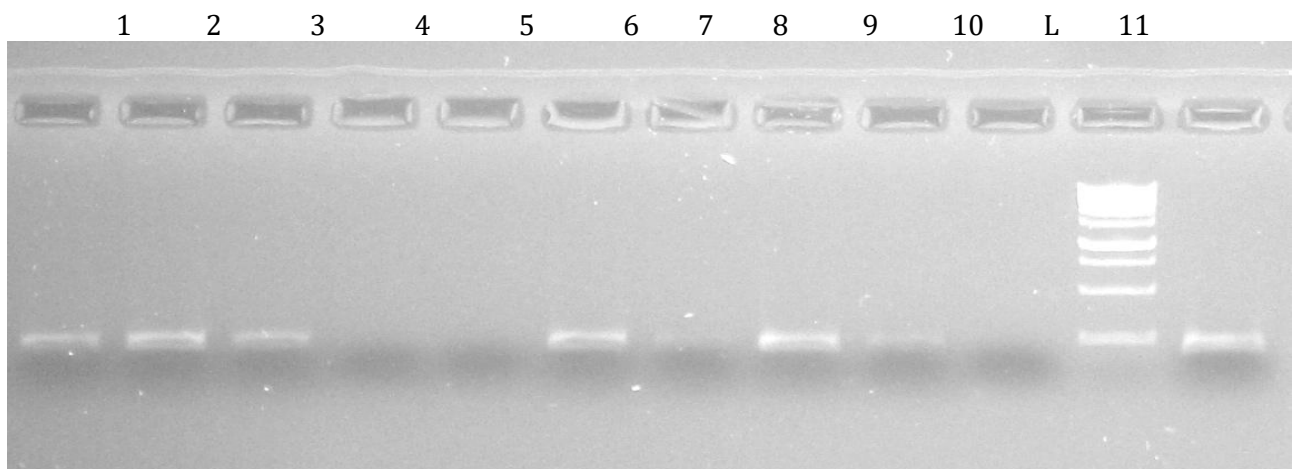


Figure 3.1: PCR products from amplification of GSTM1 gene (315 bp). Lane 4,5,10 did not have bp band, representing GSTM1 null genotype. L= 1Kb ladder.

Lane 4, lane 5 and lane 10 did not have bp representing GSTM1 null genotype. The polymorphism of GSTM1 gene involved may results in the differences in enzymatic activity possibly favoring mechanisms that increase the susceptibility to cancer. Hence, out of 100% pre- malignant population, 72.7% population is more

susceptible to cancer.

3.2 GSTT1 Genotyping

After PCR amplification, the PCR products were then separated on 2% agarose gel at 150V for 1.5 h and stained with 1µg/ml ethidium bromide at 25°C for 10 minutes. The gel was carefully taken out after electrophoresis and placed in UV gel doc for analysis of the amplified products. The bands of final PCR products were observed on ge

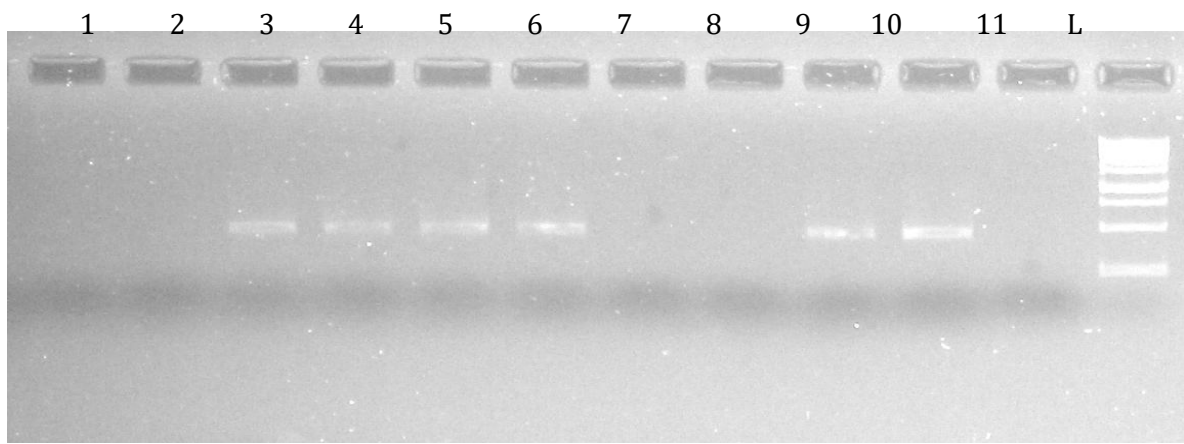


Figure 3.2: PCR products from amplification of GSTT1 gene (480 bp). Lane 1, 2 7, 8 and 11 did not have bp band, representing GSTT1 null genotype. L= 1Kb ladder

Lane 1, lane 2 and lane 7, lane 8 and lane 11 did not have 480 bp representing GSTT1 null genotype. The polymorphism of GSTT1 gene involved may results in the differences in enzymatic activity possibly favoring mechanisms that increase the susceptibility to cancer. Hence, out of 100% pre-malignant population, 54.5% population is more susceptible to cancer.

4. Conclusion

The polymorphism of GSTT1 gene involved may results in the differences in enzymatic activity possibly favoring mechanisms that increase the susceptibility to cancer. Hence, out of 100% pre- malignant population, 54.55% population is more susceptible to cancer.

The polymorphism of GSTM1 gene involved may results in the differences in enzymatic activity possibly favoring mechanisms that increase the susceptibility to cancer. Hence, out of 100% pre- malignant population, 72.73% population is more

susceptible to cancer. Our result conclude that since the polymorphic genotypes were more in tests samples than the null genotypes were more in tests samples than the null genotype, it may be associated with an increased risk of cancer from pre- malignant lesions of oral cancer.

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