Carcinogenesis vol.20 no.5 pp.743–748, 1999

ACCELERATED PAPER

Glutathione *S*-transferase M1 and T1 null genotypes as risk factors for oral leukoplakia in ethnic Indian betel quid/tobacco chewers

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Oral cancer is the most common cancer in males and third most common in females in India, the main causative agent being the use of chewing tobacco with or without betel quid (BQ). However, nothing is known about the role of the host metabolic genes in oral cancer in ethnic Indian population. In this study, the prevalence of *GSTM1* and *GSTT1* null genotypes (*GSTM1*2* and *GSTT12) in oral premalignant leukoplakia cases and controls was ascertained in genomic DNA by a multiplex PCR technique. Biopsies taken from 98 oral leukoplakia patients and exfoliated cells from 82 healthy controls both of Indian ethnicity were analysed. *GSTM1*1* (active) was present in 83% and *GSTT1*1* (active) was present in 78% of all control subjects, while prevalence of *GSTM1*2* and *GSTT1*2* null genotypes was significantly higher among oral leukoplakia cases. The prevalence of *GSTM1*2* in leukoplakia cases was 81.6% compared with 17% in controls [odds ratio (OR), 22; 95% confidence interval (CI), 10–47] and *GSTT1**2 was 75.5% in the cases versus 22% in controls (OR, 11; 95% CI, 5–22). Combined null genotypes of *GSTM1* and *GSTT1* prevailed in 60.2% of the cases with none detected in controls. Glutathione *S*-transferase M1 and T1 enzymes are both known to catalyse detoxification of reactive oxygen species, lipid peroxidation products and tobacco-derived carcinogens that have been found in the saliva of BQ/tobacco chewers. Our results, still requiring confirmation by a larger study, demonstrate that the null genotypes of both *GSTM1* and *GSTT1* increase with high penetrance, separately or in combination, the risk for developing leukoplakia in an Indian ethnic population.**

Introduction

Oral cancer incidence ranks fifth in the global cancer burden (1), and a 2- to 3-fold mortality increase has been recorded in eastern and central European countries in the last 3 decades (2). In India, oral cancer, constituting 9.8% of an estimated 644 600 incident cancer cases in 1992 (3), ranks first among all cancer cases in males and is the third most common among females in many regions, with age-standardized incidence rates from 7–17/100 000 persons/year (4); the incidence rate being higher than the western rate of 3–4/100 000/year (5).

Abbreviations: BQ, betel quid; CI, confidence interval; GST, glutathione *S*-transferase; HNSCC, head and neck squamous cell carcinoma; LOH, loss of heterozygosity; OR, odds ratio; PAHs, polycyclic aromatic hydrocarbons; ROS, reactive oxygen species; TSNA, tobacco-specific nitrosamines.

Oral cancer, a malignancy of the lip, mouth or tongue, is predominantly a squamous cell carcinoma. The prognosis is poor, and severe functional and cosmetic defects accompany its treatment. Oral cancer has been causally associated with chewing of tobacco with or without betel quid (BQ) in India and other Asian countries, whereas in western countries, cigarette smoking and heavy alcohol consumption are the main risk factors (6). Unlike tobacco smoke, which contains many carcinogenic pyrolysis products, such as polycyclic aromatic hydrocarbons (PAHs), aldehydes and nitrosamines (7), chewing of tobacco with BQ increases exposure to carcinogenic tobacco-specific nitrosamines (TSNA) [~20 μg/day in smokers (8) versus ~1000 μg/day in chewers; J.Nair, unpublished data] and to nitrosamines derived from areca nut alkaloids**.** Furthermore, reactive oxygen species (ROS), implicated in multistage carcinogenesis, are also generated in substantial amounts in the oral cavity during chewing (9,10). As an early sign of damage to oral mucosa, BQ/tobacco chewers often develop leukoplakia and submucous fibrosis, both well established precancerous lesions, which are accessible to diagnosis and present an important indicator for oral cancer risk. Histopathological examination of clinically diagnosed leukoplakia excludes any other definable lesion and establishes the degree of epithelial dysplasia (11). Some 2–12% of these lesions have been reported to turn malignant over several years (12), although a recent report suggests that a third of presenting patients will develop oral cancer (13).

Molecular epidemiological studies have now provided evidence that individual susceptibility to cancer is mediated by both genetic and environmental factors. The inherited differences in the effectiveness of detoxification/activation of carcinogens play a crucial role in host susceptibility. Thus, there is an urgent need for molecular markers that can predict whether a premalignant lesion will develop into an aggressive or metastasizing tumour. The μ (*GSTM1*) and θ (*GSTT1*) members of the glutathione *S*-transferase (*GST*) multigene family are candidate cancer-predisposing genes; they are mostly involved in the detoxification of a wide range of environmental and tobacco carcinogens, endogenously produced ROS and lipid peroxidation products, yielding excretable hydrophilic metabolites (reviewed in 14–18). The known substrates for GSTM1 include metabolically generated epoxide intermediates of benzo[*a*]pyrene and other PAHs (16), whereas the substrates for GSTT1 include alkyl halides, found in cigarette smoke, and lipid peroxides (16). The null genotypes that are associated with a lack of enzyme function exist at both these loci and their association with smoking-induced cancer has been investigated. Homozygous deletions of *GSTM1* (*GSTM1*2*) have been associated with higher risk of laryngeal, lung, bladder, colon and gastrointestinal cancers (19–23). The null genotype of *GSTT1* (*GSTT1*2*) has been reported to be associated with an increased risk of brain (24) and colorectal cancer (25). Oral premalignant lesions and cancers attributable to tobacco and BQ chewing constitute a significant public

health burden, particularly in India, yet no studies have investigated the impact of genetic determinants on host susceptibility to this oral disease in Indian populations. To address this question, we have established the distribution frequency of *GSTT1* and *GSTM1* genotypes in a cross-section of an asymptomatic Indian population and compared these with the prevalence in oral leukoplakia cases. We report here for the first time a very strong effect of *GSTM1* and *GSTT1* deletion polymorphisms on the risk of developing oral premalignant lesions and by inference on oral cancer in ethnic Indian BQ/tobacco chewers.

Materials and methods

Study subjects

All subjects were ethnic Indians. Information on tobacco chewing, smoking and alcohol habits was obtained. Cancer of any site or any other serious disease excluded the patients from the study.

Control subjects

The allelic background prevalence in the normal asymptomatic Indian population was evaluated by analysing population controls drawn from healthy individuals visiting dental clinics for some dental procedure or healthy volunteers from Bombay, India, without any prior diagnosis of oral cancer or premalignant lesions. They were not matched for age, gender or habits with the leukoplakia cases. Each subject rinsed their mouth three times with sterile water and, thereafter, oral exfoliated cells were collected in sterile saline containing 25 mM EDTA by scraping the buccal mucosa with a fresh tooth brush. Collected samples were centrifuged, washed in saline, re-suspended and stored in saline at –80°C until use.

Cases

Subjects with clinically confirmed oral premalignant lesions, recruited for an intervention study in Trivandrum, India, were randomly selected to enter the present study as cases. The categorization of leukoplakias into homogeneous and non-homogeneous lesions was by clinical features, and the exclusion of malignancy was by histopathology of the lesions. The biopsies of oral mucosa lesions were fixed in formaldehyde, and paraffin blocks were available for our study.

DNA isolation

Genomic DNA from formaldehyde-fixed, paraffin-embedded biopsies or oral exfoliated cells was extracted using a standard method (26) with a modification to include an additional aliquot of proteinase K after 8 h incubation, and a further incubation of 18 h. A similar protocol for DNA extraction from formaldehyde-fixed, paraffin-embedded lung tissue has been reported for PCR-based genotyping of cancer susceptibility genes (27).

Genotyping assays

The homozygous null polymorphisms *GSTM1*2* and *GSTT1*2* were determined using a modified multiplex PCR approach for simultaneous replication of both genes for molecular analysis (28). The co-amplification of an albumin gene fragment served as an internal positive control for a successful amplification reaction. The sequences for the primer pairs were as follows: GSTM1, 5′-GAACTCCCTGAAAAGCTAAAGC-3′ and 5′-GTTGGGCTCAAATATACGGTGG-3′; albumin, 5′-GCCCTCTGCTAACAAGTCCTAC-3′ and 5′-GCCCTAAAAAGAAAATCGCCAATC-3′; GSTT1, 5′-TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCACCGGATCATGGCCAGCC-3′.

PCR was performed in a 50 µl reaction volume containing 50 mM KCl, 2.5 mM $MgCl_2$, 200 mM Tris–HCl (pH 8.3), 200 mM dNTPs, GSTM1 primers at 3 µg each, GSTT1 primers at 1 µg each, albumin primers at 600 ng/ml each, 50–100 ng of genomic DNA and a drop of mineral oil, placed in a preheated PCR machine at 99.9°C for 5 min. Taq DNA-Polymerase (2.5 U; Boehringer Mannheim, Germany) was added to each tube, and the PCR was performed in a Perkin-Elmer GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, USA). After an initial denaturation at 95°C for 5 min, amplification was carried out for 40 cycles at 94°C for 1 min, 64°C for 1 min and 72°C for 1 min, followed by final elongation at 72°C for 7 min. The *GSTM1*2* (homozygous null) was evidenced by the absence of a 215 bp fragment, the *GSTT1*2* (homozygous null) was evidenced by the absence of a 480 bp fragment, and the presence of the 350 bp albumin fragment was indicative of a successful PCR (Figure 1). Informative PCR amplifications were observed for *GSTM1* and *GSTT1* in the 82 controls and 100 out of 114 cases. Two cases were further excluded from the study as complete data on them was not available. Additionally, ~30% of the samples were also screened for *GSTM1* or *GSTT1* using different sets of primers. *GSTM1* polymorphism was determined using a three-primer based PCR assay, enabling us to amplify *GSTM1* (230 bp) and *GSTM4* (157 bp) genes simultaneously (29). The amplification of *GSTM4* provides a positive control for each reaction. *GSTT1* was also ascertained independently by the co-amplification of *GSTT1* (480 bp) and *CYP1A1* (312 bp) as simultaneously positive control (30). The results of these analyses were identical to those obtained by the multiplex PCR.

The nomenclature for the polymorphisms in *GSTM1* and *GSTT1* genes used are as follows (31). *GSTM1*1* active genotype comprises the following functional allele configurations: *GSTM1*1A/*1A*, *GSTM1*1B/*1B*, *GSTM1*1A/*2* and *GSTM1*1B/*2*. The non-functional null or deleted allele is *GSTM1*2* and the corresponding deficient phenotype was termed *GSTM1* null phenotype. Similarly, *GSTT1*1* represents the active genotype, while the non-functional genotype null or deleted allele was denoted as *GSTT1*2*.

Statistical analysis

Chi-square test used for the comparison of proportions, Fisher's exact test for the joint distribution of the genotypes and a multiple logistic regression model was calculated and conditional logistic regression analysis used to obtain odd ratios (ORs) and their 95% confidence intervals (CIs) using a statistical analysis software (SAS, Cary, NC).

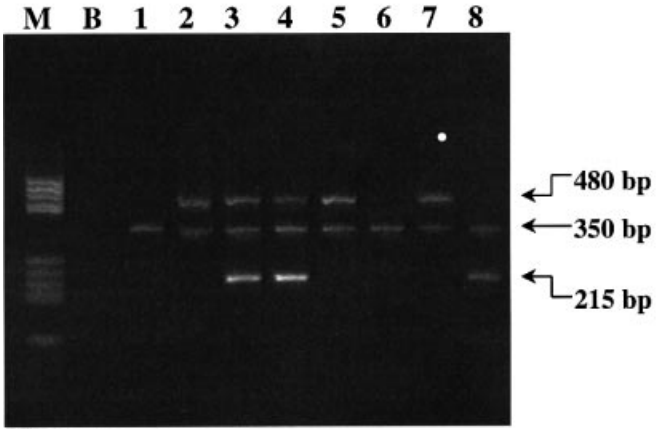

Fig. 1. A representative multiplex PCR analysis of *GST* polymorphisms. *GSTM1* and *GSTT1* gene PCR products resolved by agarose gel electrophoresis. Lane M, DNA molecular weight marker V (Boehringer Mannheim). Lane B, negative control. Lanes 1–8 are samples. A 350 bp DNA fragment corresponding to the albumin gene product provides an internal positive control for each reaction and can be seen in all PCR reactions. A 215 bp product is present only in those samples containing the *GSTM1*1* gene while a 480 bp product is present only in those samples containing the *GSTT1*1* gene.

Table I. Characteristics of the study population

	Healthy controls	All leukoplakia cases
Mean age in years (SEM)	41.8 (1.2)	51.2 (1.4)
No. males, (%)	62 (75.6)	65 (66.3)
No. females, (%)	20 (24.4)	33 (33.7)
No. tobacco chewers with or without BQ, (%)	67 (81.7)	96 (98)
No. current smokers, (%)	16 (19.5)	24 (24.5)
No. alcohol consumers, (%)	23 (28)	52 (53)

Results

The characteristics of the study population are shown in Table I. The age distribution was slightly different between cases and controls, the mean age being ~42 and 51 years for controls and cases, respectively. Approximately 82% of controls and 98% of cases were chewers. In India, tobacco is chewed with lime or in the form of a BQ that consists of the leaf of the betel vine (*Piper betle L*) wrapped around areca or betel nut (nut of *Areca catechu L*), slaked lime, catechu (extract of *Acacia*

Table II. Number of cases and controls and OR (95% CI) of oral leukoplakia in relation to *GSTM1* and *GSTT1* genotypes

Genotypes	Controls (n = 82)	All leukoplakia cases (n = 98)	Homogeneous leukoplakia (n = 59; 60.2%)	Non-homogeneous leukoplakia (n = 39; 39.8%)
GSTM1				
*GSTM1*1*, *n* (%)	68 (82.9)	18 (18.4)	14 (23.7)	4 (10.3)
*GSTM1*2*, *n* (%)	14 (17)	80 (81.6)	45 (76.3)	35 (89.7)
		($P < 0.0001$)*	NS ($P = 0.10$)**	
Unadjusted OR (95% CI)		22 (10–47)	16 (7–36)	43 (13–139)
GSTT1				
*GSTT1*1*, *n* (%)	64 (78)	24 (24.5)	18 (30.5)	6 (15.4)
*GSTT1*2*, *n* (%)	18 (22)	74 (75.5)	41 (69.5)	33 (84.6)
		($P < 0.0001$)*	NS ($P = 0.09$)**	
Unadjusted OR (95% CI)		11 (5–22)	8 (4–17)	20 (7–54)
*GSTM1*1* and *GSTT1*1*, *n* (%)	50 (61.0)	3 (3.1)	3 (5.1)	0 (0)
*GSTM1*2* and *GSTT1*1*, *n* (%)	14 (17.0)	21 (21.4)	15 (25.4)	16 (15.4)
*GSTM1*1* and *GSTT1*2*, *n* (%)	18 (22.0)	15 (15.3)	11 (18.6)	4 (10.3)
*GSTM1*2* and *GSTT1*2*, *n* (%)	0 (0)	59 (60.2)	30 (50.9)	29 (74.3)
P-values***		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
At least 1 active, *n* (%)	82 (100)	39 (39.8)	29 (49.1)	10 (25.6)
Unadjusted OR[a]		∞	∞	∞

All OR were calculated with the control group as reference.
**P*-values of comparison of 'all leukoplakia' versus 'control' using χ^2 test.
***P*-values of comparison of 'non-homogeneous leukoplakia' versus 'homogeneous leukoplakia' using χ^2 test.
****Analysed by Fisher's exact test for the joint distribution of *GSTM1* and *GSTT1* in cases compared with controls.
[a]ORs calculated for *GSTM1*2* and *GSTT1*2* with at least one active as the reference group.

catechu L) and tobacco. The two non-chewer leukoplakia cases were heavy smokers and alcohol users. Smokers constituted 19% of controls and 24.5% of the cases. Fifty-three percent of cases and 28% of controls were alcohol drinkers. Most leukoplakia cases were in the category of heavy habituated chewers (15–20 quids/day), whereas the chewers among controls were regular but not heavy chewers (i.e. 1–2 quids after the two main meals). As the aim and design of the study was not to re-evaluate well established lifestyle risk factors for oral leukoplakia and cancer (32), no attempt was made to approximate the total amount of chewing, smoking or alcohol consumption in terms of amount per day or duration in years.

Table II shows the distribution of *GSTM1*2* and *GSTT1*2* genotype prevalence in cases and controls. We found a highly significantly increased frequency of *GSTM1* null genotype in all leukoplakia cases of 81.6%, compared with 17% in controls. Individuals with *GSTM1*2* had a 22-fold higher risk of leukoplakia. The homozygous null genotype *GSTT1*2* was found in 75.5% of cases and in 22% of controls, conferring an 11-fold higher risk. A total of 60.2% of the cases presented homozygous deletion of both *GSTT1* and *GSTM1*, but none was detected among healthy control chewers, revealing a potentiation of risk in subjects with both null genotypes for oral cancer when they chew tobacco.

Based on clinical features, the leukoplakias were further subclassified into homogeneous and non-homogeneous leukoplakias (Table II) due to the reported higher rate of malignant transformation of non-homogeneous leukoplakias (11,12). When differentiating by the type of leukoplakia, there appeared to be a non-significant increased prevalence of the *GSTM1*2* in non-homogeneous cases 35/39 (89.7%) as compared with homogeneous leukoplakia cases 45/59 (76.3%). The prevalence of *GSTT1*2* was 33/39 (84.6%) and 41/59 (69.5%) in non-homozygous versus homogeneous leukoplakia, respectively. However, the prevalence of both *GSTM1*2* and *GSTT1*2* was 74.4% in non-homozygous and significantly different from 50.8% in homogeneous leukoplakia ($P = 0.0002$).

Table III. Conditional ORs and 95% CI from multiple logistic regression analysis of oral cancer risk factors

Variable	Odds ratio[a] (Wald 95% CI)	*P*-value
*GSTT1*2*	16.1 (6.5–40.3)[b]; 46.9 (10.8–203.8)[a]	0.0001
*GSTM1*2*	22.8 (9.2–57.0)[b]; 60.6 (14.6–251.1)[a]	0.0001
Chewing	31.4 (1.5–676.5)[a]	0.0277
Smoking	0.8 (0.2–3.3)[a]	NS
Alcohol	12.3 (2.9–50.7)[a]	0.0005
Sex	10.9 (2.1–55.5)[a]	0.004
Age	1 (0.9–1)[a]	NS

[a]OR for each variable is adjusted for all other variables listed in the table.
[b]Multiple logistic regression with correction for chewing, smoking, alcohol, sex and age was used to determine the effect of the two genotypes separately.
NS, not significant.

When the data were analysed using logistic regression (Table III) controlling for age as a quantitative factor, gender and the influence of three major lifestyle risk factors for induction of oral cancer (i.e. tobacco chewing, smoking and alcohol consumption), a significant association with leukoplakia for both traits was observed for *GSTM1*2* (OR, 22.8; 95% CI, 9.2–57) and *GSTT1*2* (OR, 16.1; 95% CI, 6.5–40.3). However, when adjusting for the other genotype, higher ORs were obtained, *GSTM1*2* (OR, 60.6; 95% CI, 14.6–251.2) and *GSTT1*2* (OR, 46; 95% CI, 10.8–203.8) due to the fact that in this population a significant association between *GSTM1* and *GSTT1* genes was seen ($P < 0.001$). As expected, the magnitude of risk for oral leukoplakia was highest for chewing (OR, 31; $P = 0.028$), followed by that for alcohol abuse (OR, 12; $P = 0.0005$). Smoking did not emerge as a significant risk factor probably as the number of smokers was too low in our

study. An apparent gender difference in risk for leukoplakias was observed with a female to male OR = 10 ($P = 0.004$), requiring verification, as the proportion of females and males among controls was different from our cases. The distribution of the combined genotypes of *GSTM1* and *GSTT1* was significantly different in cases as compared with controls ($P < 0.0001$). The combined null genotype of both *GSTM1* and *GSTT1* conferred a highly significant risk for oral leukoplakia when compared with the category of at least copy of the genes present. Thus, the *GSTM1* and *GSTT1* deletion genotype alone and even more in combination was demonstrated to highly predispose habitual BQ chewers to the development of oral leukoplakia, a precancerous lesion.

Discussion

Ethnic differences in the prevalence of the *GSTM1* null genotypes have been reported to vary between 22–35% in Africans, 38–67% in Caucasians and 33–63% in East Asian populations (33). The Pacific islanders (Oceania) have the highest reported frequency, *GSTM1*2* ranges from 64% to as high as 100% in Kiribati natives (14). The *GSTT1*2* genotype varies from 10–18% in Caucasians (20,24,34) to 58% in the Chinese (35). To our knowledge, this is the first report on the prevalence of these genotypes in an ethnic Indian population and among premalignant leukoplakia cases from India.

In the ethnic Indians, we observed that the prevalence of 17% ($n = 82$) for *GSTM1*2*, was relatively lower than the figure of ~50% reported in Caucasian populations, while the reported prevalence of *GSTT1*2* at 23% was only marginally higher. Furthermore, we did not find any subject with null genotype for both *GSTM1* and *GSTT1* in 82 controls, suggesting that this combination is rare in the Indian ethnic population. The prevalence of *GSTM1*2* in 'Asian' Indians from Los Angeles or Malaysia was reported to be 36 or 33%, respectively (36,37). The prevalence of *GSTT1*2* in Indians from Malaysia and Singapore was 16%, while the double deletion was seen in only 5% of these subjects (35). These data confirm that there are large ethnic differences in the prevalence of these polymorphic GST enzymes which are known to catalyse the detoxification of tobacco-derived carcinogens and endogenous reactive products derived from lipid peroxidation (38). We demonstrated previously that betel nut and catechu, the two major ingredients of BQ, generate ROS and 8-oxo-guanine in DNA *in vitro* at the alkaline pH provided by lime (39). ROS in part are responsible for the increased frequency of micronuclei observed in hamster cheek pouch upon exposure to BQ ingredients (10) and in exfoliated buccal mucosa cells of chewers (40). We also detected the formation of OH radicals in the oral cavity of subjects during chewing of BQ (9), which may cause direct damage in the oral mucosa and promote growth of initiated oral epithelial cells. The contribution of the *GST* supergene family to oxidative stress resistance is well established (41), and therefore the absence of one or more of the GST enzymes would result in increased ROS-mediated damage. Thus, antioxidants and free radical scavengers such as vitamins A, E and C, and beta-carotene, have widely been used as cancer chemopreventive agents to induce regression of precancerous oral lesions such as leukoplakia and erythroplakia (42).

The major cancers attributable to tobacco are those of the lung, bladder and oral cavity (7,32). Our results indicate that tobacco use to a large extent and alcohol abuse to a lesser extent are significant risk factors for oral premalignancy in concordance with the established associations between oral leukoplakia and carcinogen exposure through chewing tobacco or drinking alcohol (43). In this study, smokers constituted a low percentage of the subjects, and therefore the risk due to smoking could not be seen. Whether the risk difference according to gender seen in this study (Table III) is real, needs to be verified in a larger study, although a higher susceptibility to tobacco-associated cancers has been reported for women (44).

At the beginning of our study, there were only very few reports that related *GST* genotypes and oral cancer risk. The GSTM1 enzyme had been reported to be present in oral tissue (45,46), and a preliminary study (45) demonstrated that there was a relatively low fraction of oral and laryngeal cancer patients exhibiting detectable levels of GSTμ protein (28% of patients with oral/oropharyngeal cancer versus 60% in controls). Trizna *et al.* (47) reported an association between *GSTM1*2* genotype in 42 cases and an increased risk for head and neck cancer. No association was detected between *GSTM1* and/or *GSTT1* genotypes and oral cancer in English Caucasians (25). Among Caucasian oral cancer patients, no association between *GSTM1*2* and risk was found (48), but individuals with mutated *CYP1A1* (exon 7 Ile/Val polymorphism) were at increased risk. In Taiwanese patients (49), null genotypes of *GSTM1* and/or *GSTT1* were associated with increased oral cancer risk, only compared with those that had both *GSTM1* and *GSTT1* active. Among non-chewers, only *CYP2E1* c1/c2 and c2/c2 genotypes were found to confer high risk as compared with c1/c1 genotypes. Thus, from these data the influence of *GSTM1*/*GSTT1* null genotype on oral cancer risk was not clearly demonstrated.

Based on our results, there was a significantly higher prevalence of *GSTM1*2* or *GSTT1*2* in all leukoplakia cases, 59% of the cases were null for both the *GST*s. This figure rose to ~73% in non-homogeneous leukoplakia. The malignant transformation of the non-homogeneous lesions, involving erythroplakia and nodular leukoplakia, is known to be particularly high and reportedly ranges from 15 to 40% over a varying period of time (12). The corresponding figures for patients from Japan and Western countries ranged from 17 to 35%, respectively (50,51). BQ chewing is an ancient and socially accepted practice, the introduction of tobacco reinforced this practice and now almost all habitual chewers use BQ with tobacco. Almost every BQ/tobacco-chewing related oral malignancy is preceded by a clinically distinct premalignant stage at the site of cancer development (52). Our results revealed that genetically predisposed BQ/tobacco chewers are much more susceptible to environmental and life-style risk factors. Heavy chewers with either *GSTT1*2* or *GSTM1*2* genotype had an 11- to 22-fold higher risk of presenting with clinically observable leukoplakia than subjects with active *GSTT1*1* or *GSTM1*1*. The genotype combination of *GSTM1*2* and *GSTT1*2* among ethnic Indians conferred a significantly high risk for oral leukoplakia and subsequently for heavy chewers to oral cancer. Because of the small study size, the absence of double deletion in the control population resulting in an OR = ∞, does not preclude its prevalence. Therefore, assuming the value of double deletion as 1, an OR > 123 (95% CI, 16–918) was obtained. In a recent analysis (53; J.Y.Park, personal communication), the oral cancer risk in African Americans, when stratified by *GSTM1*2* and high tobacco use (>20 pack-years, i.e. no. of packs of cigarettes smoked/day during 1 year),

compared with *GSTM1* positive genotype and <20 pack-year group, was of similarly high magnitude (OR, 112; 95% CI, 6–2001).

A genetic progression model for head and neck squamous cell carcinoma (HNSCC) to explain the field cancerization concept has been proposed, by which an entire epithelial surface is primed for neoplastic changes following prolonged carcinogen exposure leading to focal areas that progress at different rates towards invasive cancer (54,55). Microsatellite analysis in head and neck cancer for allelic loss at 10 major chromosomal loci demonstrated that the spectrum of chromosomal deletion progressively increases at each histopathological step from benign hyperplasia to dysplasia to carcinoma *in situ* to invasive cancer (55). GSTM1 and GSTT1 loci are not reported to be linked as individuals who are GSTT1*2 are not necessarily GSTM1*2. While there is no current evidence suggesting that GSTM1 and/or GSTT1 are lost in the pathologies of HNSCC, the question nevertheless has to be addressed whether the high incidence of these null alleles in these biopsies could be a consequence of allele loss. It has been suggested that, because amplification of chromosome 11q13 is common in the HNSCC and because GSTP1 is in the 11q13 amplicon, it is possible that the behaviour of carcinoma *in situ* is mediated by GSTP1 allelism (56). The GSTM1gene has been mapped to chromosome 1p13.3 and GSTT1 gene to chromosome 22q11.2. As loss of heterozygosity (LOH) has also been reported in oral cancers on chromosomes 1p11–13 (57) and chromosome 22q13 (58); gene deletion due to LOH on the specific amplicons can at present not be excluded as an additional contributory factor. Studies are underway to verify this possibility.

HNSCCs that develop in patients from India and South-East Asia frequently have abnormalities of *ras* oncogenes including mutations, LOH (H-*ras*) and amplification (K- and N-*ras*) in contrast to low prevalence of mutations in these ras genes in the same malignancies from Western countries (59). An increase of somatic mutations, leading to tumour formation, has been linked with GST deficiencies (33). Thus, the deficiency of both GST detoxifying enzymes in tobacco smokers and heavy chewers could lead to higher carcinogenic exposure and to multiple and progressive genetic aberrations. Our results suggest that carcinogenic intermediates derived from BQ/tobacco or generated during habitual chewing (60) are substrates for GSTT1 and GSTM1 enzymes and that oral leukoplakia risk is increased more than additively for individuals who have both *GSTT1* and *GSTM1* null genotypes. Our finding that the double null genotypes were significantly more frequent among the non-homogeneous leukoplakia cases is in keeping with the reported higher malignant transformation of this type of leukoplakia. These latter results need to be confirmed, given the small sample size of our study and a lack of adjustment for amount and duration of tobacco chewed.

In conclusion, our results revealed that null genotypes of *GSTM1* and *GSTT1*, both individually and in combination, are high penetrance genetic risk factors for developing oral leukoplakia that consequently modulate the risk of developing cancerous oral lesions in habituated BQ/tobacco chewers of Indian ethnicity. Based on these data, which still require confirmation by a larger study, identification of high-risk subjects could now be explored to advise them on cessation of the habit before development of clinical symptoms of malignancy.

Acknowledgements

We are grateful to Dr R.Sankaranarayanan (IARC, Lyon, France) for providing oral biopsy blocks and critical suggestions in the manuscript, and to Dr H.-R. Scherf (DKFZ) for his help in processing the oral biopsy blocks. We thank Dr A.Kopp-Schneider (DKFZ) for helping us in the statistical analysis. This work was supported by DKFZ visiting scientist award (U.J.N.).

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Received October 8, 1998; revised February 24, 1999; accepted February 25, 1999