Increased Risk of Oral Leukoplakia and Cancer Among Mixed Tobacco Users Carrying *XRCC1* Variant Haplotypes and Cancer Among Smokers Carrying Two Risk Genotypes: One on Each of Two Loci, *GSTM3* and *XRCC1* (Codon 280)

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Abstract

An individual's susceptibility to oral precancer and cancer depends not only on tobacco exposure but also on the genotypes/haplotypes at susceptible loci. In this hospital-based case-control study, 310 cancer patients, 197 leukoplakia patients, and 348 controls were studied to determine risk of the disease due to polymorphisms at three sites on *XRCC1* and one site on *XRCC3*. Independently, variant genotypes on these loci did not modulate risk of leukoplakia and cancer except for the *XRCC1* (codon 280) risk genotype in exclusive smokeless tobacco users with leukoplakia [odds ratios (OR), 2.4; 95% confidence intervals (CI), 1.0-5.7]. But variant haplotypes, containing one variant allele, on *XRCC1* increased the risk of leukoplakia (OR, 1.3; 95% CI, 1.0-1.7). Among stratified samples, mixed tobacco users, carrying variant haplotypes, also had increased risk of both leukoplakia (OR, 2.2; 95% CI, 1.3-3.9) and cancer (OR, 1.9; 95% CI, 1.2-3.1). In a previous study on this population, it was shown that the *GSTM3* (*A*/*A*) genotype increased the risk of oral leukoplakia and cancer among smokers, which has also been substantiated in this study with expanded sample sizes. The simultaneous presence of two risk genotypes in smokers, one on each of two loci, *GSTM3* and *XRCC1* (codon 280), increased the risk of cancer (OR, 2.4; 95% CI, 1.0-5.8). Again, smokers carrying two risk genotypes, one on each of two loci, *GSTM3* and *XRCC1* (codon 399), were also overrepresented in both leukoplakia and cancer populations (P_{trend} = 0.02 and 0.04, respectively) but enhancement of risks were not observed; probably due to small sample sizes. Therefore, the presence of variant haplotypes on *XRCC1* and two risk genotypes, one on each of two loci, *GSTM3* and *XRCC1*, could be useful to determine the leukoplakias that might progress to cancer in a group of patients. (Cancer Epidemiol Biomarkers Prev 2005;14(9):2106–12)

Introduction

Tobacco chewing and smoking has been identified as the major risk factors for oral cavity precancer and cancer in India. Polycyclic aromatic hydrocarbons, aldehydes, aromatic amines, nitrosamines, etc., are thought to be carcinogenic components present in tobacco. But chewing of tobacco with betel quid increases the concentrations of carcinogenic tobacco-specific nitrosamines and reactive oxygen species in the mouth (1). Oral leukoplakia, a common precancer lesion among smokers and chewers, is defined as ''a chronic white mucosal maculae, which cannot be characterized clinically or pathologically as any other disease and is not associated with any physical or chemical causative agent except the use of tobacco'' (2). The annual incidence of oral leukoplakia among subjects >15 years of age was reported as 0.2% to 11.7% in different populations of India (3, 4). As an early sign of damage to oral mucosa, tobacco smokers and chewers often develop precancerous lesions such as leukoplakia. This lesion is easily accessible to diagnosis and can be considered as an indicator of oral cancer risk. Studies have shown that although 2% to 12% of precancers are transformed into cancer in different populations, ~80% of oral cancers progress from precancerous lesions (5). Globally, ~500,000 new oral and pharyngeal cancers are diagnosed annually, and three-quarters of these are from developing countries, with ~65,000 cases from India (6, 7). Oral cancer ranked first among all cancer cases in males and was the third most common cancer among females in many regions of India. The age-standardized incidence rate of oral cancer is 7 to 17/100,000 persons/y in India, which is higher than the western rate of 3 to 4/100,000 persons/y (8).

Molecular epidemiologic studies have now provided evidence that an individual's susceptibility to oral leukoplakia and cancer is modulated by both genetic and environmental factors (1, 9). The exposure to environmental agents and by-products of cellular metabolism results in damage to DNA, which if left unrepaired, could lead to the process of carcinogenesis. The entire process leading to DNA damage, and subsequent repair of the damage involves a host of enzymes. DNA damage itself is a consequence of a balance between activation and detoxification of carcinogens that involves phase I and phase II enzymes. Glutathione *S*-transferases (GST), one group of phase II enzymes, detoxify many electrophilic substrates by conjugation with reduced glutathione (10). Different classes of GST isozymes have a common and broad range of substrate specificities and detoxify the reactive metabolites such as benzo-α-pyrene, tobacco-specific nitrosamines, and alkyl halides, etc. (11). Four members of the GST genes such as *GSTM1, GSTT1, GSTP1*, and *GSTM3* display polymorphisms that have been associated with increased risk of cancers (12-14).

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More than 130 genes are known to be involved in the repair of different types of DNA damage involving distinct pathways (15). The major pathways include nucleotide excision repair for the removal of UV damage and bulky DNA adducts, base excision repair for repairing minor base alterations such as oxidative damage and methylated bases, homologous recombination, and nonhomologous end joining for the repair of double-strand DNA breaks. Therefore, repair of damaged DNA is an important biological phenomenon for the integrity of cellular DNA and polymorphisms in DNA repair genes may alter the activity of the enzymes and, thus, modulate cancer susceptibility (16). *XRCC1* plays an important role in the base excision repair pathway and interacts with DNA polymerase-β, poly(ADP)ribose polymerase, and DNA ligase III. It also contains a BRCA1 COOH terminus domain, which is characteristic of proteins involved in cell cycle checkpoint functions; this domain can be responsive to DNA damage. Three nonsynonymous polymorphisms on *XRCC1* were detected at codons 194 (Arg>Trp, *C>T*), 280 (Arg>His, *G>A*), 399 (Arg>Gln, *G>A*) and have been associated with the presence or absence of risk of cancers in breast, stomach, head and neck, and lung in different studies (17). *XRCC3* is one of the DNA repair genes that are involved in the repair of DNA double-strand breaks and polymorphism for a *C>T* substitution at position 18067 in exon 7 results in an amino acid change at codon 241 (Thr>Met). Although the functional relevance of this polymorphism is unknown, few studies have reported that the 241Met allele is associated with increased risk of cancer (18).

Numerous reports have shown that polymorphisms in GSTs could increase the risk of oral precancer and cancer in different populations (1, 9, 19). In this study, we examined polymorphisms at three sites on *XRCC1* and one site on *XRCC3* in patients with oral squamous cell carcinomas and leukoplakia, and in controls to determine risk of diseases due to the presence of variant genotypes on *XRCC1/XRCC3* and haplotypes on *XRCC1*. It is expected that the presence of more than one risk genotype on two low-risk genes (such as *GSTM3* and *XRCC1*) in an individual might augment the risk of precancer and cancer that may not be observed due to the presence of single-risk genotypes. Therefore, patients and controls carrying risk genotypes on two loci (such as *XRCC1* and *GSTM3*) were also compared to understand the risk of disease.

Materials and Methods

Patients, Controls, and Tobacco Habit. Unrelated patients diagnosed with leukoplakia or primary squamous cell carcinoma in the oral cavity were recruited between 1999 and 2004 from the R. Ahmed Dental College and Hospital (Kolkata, India). For all patients, the department of pathology from the same hospital did histopathologic confirmation of the lesions. Unrelated controls who came for treatment of dental ailments, but without any previous and present lesions in the oral cavity, were recruited from the outpatient department of the same hospital. Obtaining informed written consent, all individuals were personally interviewed using a questionnaire. Information on age, sex, occupation, alcohol consumption, type of tobacco habits, daily tobacco use frequency, duration of habits, economic status, place of job, and food habit data were recorded. Findings pertaining to histopathologic diagnosis and clinical staging were obtained from the pathological reports of the biopsy materials.

All subjects in this study were current tobacco users and some reported tobacco habits such as smoking of cigarette and/or bidi, a native cigarette-like stick of coarse tobacco hand-rolled in a dry tembuhurni leaf. Individuals having only smoking habit are termed as ''exclusive smokers'' in this study. The tobacco content of one cigarette is nearly equal to that present in two bidis. Some of the patients and controls had the habit of tobacco chewing or dipping in different forms. Individuals having only tobacco chewing/dipping habit are termed as ''exclusive smokeless tobacco users'' in this study. In India, the prevalent tobacco chewing habits involve use of betel quid (betel leaf with tobacco, areca nut, and lime), ''gutkha'' (dried mixture of betel quid and tobacco sold in attractive pouches), ''mawa'' and ''zarda'' (flavored tobacco) or ''khaini'' (crude form of dried and ground tobacco with lime). The remaining patients and controls had both smoking and chewing/dipping habit simultaneously and are termed as ''mixed'' habituals in this study. Lifetime smokeless tobacco exposure was measured in terms of the frequency of chewing/dipping per day multiplied by the duration of habit. This is termed as chewing-year (CY; taking smokeless tobacco once in a day for 1 year = 1 CY). Similarly, the dose of tobacco smoking was measured in pack-years (PY): one packet per day for 1 year = 1 PY (one pack = 20 cigarettes or 40 bidis). For dose-response calculations, all subjects were subclassified into light and heavy tobacco users. The cut-off point of tobacco dose was defined as the median in the control group and the same cut-off was used in the other two patient groups, i.e., leukoplakia and cancer. Smokeless tobacco users were classified as light (<120 CY) and heavy (≥120 CY), where the median dose of all smokeless tobacco users in control group was 120 CY. Smokers were classified as light (<12 PY) and heavy smokers (≥12 PY), where the median dose of controls was 12 PY. In some of the analyses, exclusive smokers and mixed habituals were pooled in a group and this group was termed as ''smokers''.

Sample Collection and Processing. About 5 mL of blood was collected by vein puncture from patients (cancer = 310, leukoplakia = 197) and healthy controls (n = 348) and stored at −20°C until DNA isolation. Genomic DNA was isolated from whole blood by the salt precipitation method (20). Biopsy materials collected from leukoplakia and cancer lesions were processed for histopathology.

Genotyping

XRCC1 Gene. To determine polymorphisms at codons 194 and 399 of *XRCC1*, PCR products (487 and 615 bp, respectively) were digested with *Msp*I at 37°C for 4 hours and electrophoresed in 1.5% agarose (21). Banding patterns for genotypes at codon 194 were: *Arg/Arg* = 292,174,21 bp; *Arg/Trp* = 313,292,174,21 bp; *Trp/Trp* = 313,174 bp. PCR products for codon 194 polymorphism contain an invariant cutting site for *Msp*I and it serves as an internal control for complete *Msp*I digestion. PCR product for the codon 399 polymorphism showed three kinds of banding patterns for genotypes (*Arg/Arg* = 375,240 bp; *Arg/Gln* = 615,375,240 bp; *Gln/Gln* = 615 bp). PCR product (861 bp) for codon 280 polymorphism was digested with *Rsa*I (16) and electrophoresed in 2.5% agarose to determine genotypes (*Arg/Arg* = 597,202,63 bp; *Arg/His* = 660,597,202,63 bp; *His/His* = 660,202 bp).

XRCC3 Gene. Polymorphism at codon 241 of *XRCC3* was determined generating a 552 bp PCR product, digesting with *Nla*III and electrophoresing in 2.5% agarose gel (22). In addition to the polymorphism at codon 241, one additional monomorphic *Nla*III site, producing a 239 bp DNA fragment, served as an internal control for restriction enzyme digestion. Genotypes were determined by banding patterns such as: *Thr/Thr* (313,239 bp), *Thr/Met* (313,239,208,105 bp), and *Met/Met* (239,208,105 bp).

GSTM1, GSTT1, GSTM3, and *GSTP1 (codon 105)* Genes. Previously, 256 cancer, 109 leukoplakia, and 259 control individuals from the present sample pools were genotyped at these loci (9). In this study, 54 cancer, 88 leukoplakia patients, and 89 controls were additionally recruited and genotyped using the same methods.

Risk Genotypes. Homozygous deletion (or null) at *GSTM1* and *GSTT1* loci, *GSTM3* (*A/A*) and *GSTP1Ile/Ile* genotypes were considered as risk genotypes because these genotypes increased the risk of leukoplakia and cancer (1, 9, 23). At *XRCC1* or *XRCC3* loci, if frequencies of homozygous variant genotypes are low, then combination of heterozygous and homozygous variant genotypes, such as *XRCC3 Met/Met + Met/Thr*, were considered as risk genotypes because studies have shown that variant alleles at these loci increase risk of cancer (15). Patients and controls were also categorized on the basis of presence of single- or double-risk genotype/s or double non–risk genotypes on two different loci in the same individual. For example, individuals carrying [*GSTM3 (A/A) + XRCC3 Met/Met* or *Met/Thr*] were categorized as a group carrying double-risk genotypes; individuals carrying [*GSTM3 (A/A) + XRCC3 Thr/Thr*] or [*GSTM3 (A/B)* or *(B/B) + XRCC3 Met/Met* or *Met/Thr*] were categorized as a group carrying single-risk genotypes; individuals carrying [*GSTM3 (B/B) + XRCC3 Thr/Thr*] were categorized as a group carrying double non–risk genotypes.

Sequencing of PCR Products. Few PCR products (8% of total samples) from all loci were sequenced (ABI prism 3100; Applied Biosystem, Foster City, CA) to confirm the genotypes determined by PCR and PCR-restriction fragment length polymorphism methods.

Statistical Analysis. Age-, sex-, and tobacco dose–adjusted risk of oral cancer and leukoplakia was calculated as odds ratios (OR) with 95% confidence intervals (CI) for all genotypes in all patient samples and in stratified patient samples by multiple logistic regression analysis using SPSS statistical package. χ^2 test with Yates' correction was used for comparison of proportions. Risks of the diseases were also evaluated for heavy and light smoking and chewing doses. Trend tests (P_{trend}) were conducted to determine whether individuals carrying single- or double-risk genotypes are overrepresented in patients compared with controls (24). Frequencies of the haplotypes, resulting from three polymorphisms on *XRCC1* gene, were estimated by the maximum-likelihood method using the expectation maximization algorithm (25).

Results

All patients and controls were ethnically similar and living in and around the city of Kolkata, located on the eastern region of India. Most of the patients and controls belonged to a low-income group (family income <U.S. $100/month), and this is probably one of the reasons they visited government hospitals for treatment. Therefore, it could be assumed that they were similar nutritionally. Both patients and controls had occupations in diverse areas such as agriculture, small industry, car driving, private sector office, small business, etc., but were not exposed to specific toxic chemicals in the workplace. Most of the females were housewives and doing only household jobs. Hence, environmental effects other than tobacco use were negligible. The distribution of demographic characteristics and the tobacco habits of patient and control populations are summarized in Table 1. About 85% of smokers had both cigarette- and bidi-smoking habits, therefore, bidi and cigarette smokers were not analyzed separately (data not shown). In both patient groups, only few (<5%) had occasional alcohol-drinking habits (data not shown); hence, alcohol consumption was not considered in statistical analyses.

The sites of oral cavity affected by leukoplakia were buccal mucosa and the commissure area (76%), buccal mucosa and alveolar sulcus (19%), and tongue (5%). Most of the patients suffered from ulcerative (62%) followed by homogeneous (35%) and nodular (3%) types of leukoplakia. Fifty-two percent of the cancer sites were from buccal mucosa and alveolar sulcus and the remaining sites were distributed equally among lip, tongue, retromolar area, and buccal sulcus. Histopathologically, all malignancies were diagnosed as squamous cell carcinomas of the oral cavity. These were classified as well-differentiated (65%), moderately differentiated (17%), and poorly differentiated (18%) squamous cell carcinomas.

A few genotypes (8% of total samples) at all loci, determined by sequencing method, were identical to those determined by PCR or PCR-restriction fragment length polymorphism methods. Therefore, the remaining genotypes were determined by PCR or PCR-restriction fragment length

Table 1. Characteristics of patients and controls

Subjects and tobacco habits	Cancer (n = 310)	Controls (n = 348)	Leukoplakia (n = 197)
Sex			
Male	197	265	170
Female	113	83	27
Age (years)			
Mean ± SD	55 ± 12.0	50.4 ± 11.5	47 ± 10.8
Exclusive smoking habit			
Exclusive smokers	53	114	111
Lifetime smoking range (PY)	5-60.5	2.5-60	2.5-45
Mean smoking dose ± SD (PY)	14 ± 8	14.5 ± 10.5	12 ± 9.5
Individuals with light (<12 PY) tobacco smoking dose	77	91	94
Individuals with heavy (≥12 PY) tobacco smoking dose	57	95	72
Exclusive smokeless tobacco habit			
Exclusive smokeless tobacco users	176	162	31
Lifetime smokeless tobacco using range (CY)	10-1,250	10-675	10-420
Mean smokeless tobacco dose ± SD (CY)	207 ± 161.7	198 ± 163	160 ± 125
Individuals with light (<120 CY) smokeless tobacco dose	120	113	57
Individuals with heavy (≥120 CY) smokeless tobacco dose	137	121	29
Mixed habituals			
Smoking as well as smokeless tobacco using habit	81	72	55
Lifetime smoking range (PY)	2.5-35	5-45	5-40
Mean smoking dose ± SD (PY)	11 ± 8.1	13.5 ± 8	14.5 ± 9.5
Lifetime smokeless tobacco using range (CY)	10-375	10-925	10-525
Mean smokeless tobacco dose ± SD (CY)	123 ± 88.3	116 ± 116.2	134 ± 125.2

NOTE: Sex distribution: cancer versus control (P = 0.001) and leukoplakia versus control (P = 0.007). Mean age: cancer versus control (P < 0.0001) and leukoplakia versus control (P = 0.0008). Mean smoking doses: exclusive smoker versus mixed habituals in cancer group (P = 0.03). Mean smokeless tobacco doses: exclusive smokeless tobacco users versus mixed habituals in cancer and control groups (P < 0.0001 and 0.0002, respectively). Exclusive smokers and smokeless tobacco users have only smoking and tobacco chewing/dipping habits, respectively. Mixed habituals have smoking as well as smokeless tobacco habits. In a few calculations, mixed habituals and exclusive smokers were pooled to increase the sample size as ''smokers.'' Light and heavy tobacco doses are defined in Materials and Methods.

polymorphism methods. Because the frequencies of variant genotypes on *XRCC1* and *XRCC3* were <2% (except at *XRCC1* codon 399, which is <8%), variant and heterozygous genotypes were pooled as risk genotypes to compare between patients and healthy controls (Table 2). The age-, sex-, and tobacco dose–adjusted distribution of *XRCC1* (at codons 194, 280, and 399), *XRCC3* (at codon 241), *GSTM1, GSTT1, GSTM3*, and *GSTP1* (codon 105) risk genotypes were not significantly different among patients and controls when total sample sizes were taken for analysis. But in the stratified samples, although the sample size was low, the frequency of *XRCC1* risk genotypes at codon 280 was significantly increased in exclusive smokeless tobacco users with leukoplakia versus respective controls (OR, 2.4; 95% CI, 1.0-5.7; data not shown). Like the previous observation (9), the frequency of the *GSTM3* (*A/A*) genotype was significantly increased in smokers with cancer versus respective controls (OR, 1.8; 95% CI, 1.0-3.4; data not shown) and the frequencies of *GSTM3* (*A/A*) and *GSTM1* homozygous deletion genotypes were significantly increased in exclusive smokers with leukoplakia versus respective controls (OR, 2.3; 95% CI, 1.1-4.7; OR, 1.6; 95% CI, 1.0-2.8, respectively, data not shown) even after the addition of some patients (54 cancer and 88 leukoplakia) and 89 controls in the previous sample pools.

Patients and controls carrying single-risk genotypes on either of two different loci or double-risk genotypes or double non–risk genotypes on both loci (such as *GSTM3* and *XRCC1* at codon 399) were categorized into four groups (Table 3). Comparison of frequencies of these individuals by trend test, which included data on single- and double-risk and double non–risk genotypes on a pair of loci, revealed that individuals carrying single- and double-risk genotypes on three pairs of loci such as *GSTM1* and *XRCC3, GSTM3* and *XRCC1* (codon 399), and *GSTM3* and *XRCC3* were overrepresented in exclusive smokers with leukoplakia compared with controls (P_{trend}, 0.04, 0.02, and 0.04, respectively). Similarly, individuals carrying single- and double-risk genotypes on a pair of loci such as *GSTT1* and *XRCC1* (codon 280) were also overrepresented in exclusive smokeless tobacco users with leukoplakia compared with controls (P_{trend}, 0.02). In the cancer population, mixed tobacco users carrying single- and double-risk genotypes on the pair of *GSTP1* and *XRCC1* (codon 194), and smokers carrying single- and double-risk genotypes on the pairs of *GSTM3* and *XRCC1* (codon 280) and *GSTM3* and *XRCC1* (codon 399), were overrepresented in patients compared with controls (P_{trend}, 0.04, 0.03, and 0.04, respectively). But comparison of only double-risk and non–risk genotypes on *GSTM3* and *XRCC1* (codon 280) exhibited enhanced risk of cancer in smokers (OR, 2.4; 95% CI, 1.0-5.8) and none of the double-risk genotypes on other pairs of loci exhibited increased risk of diseases (data not shown).

Haplotype frequencies at three polymorphic sites on *XRCC1* in all patients and controls, as well as stratified samples, were estimated and compared (Table 4). Alleles in the *XRCC1* haplotypes have been arranged in the order: codon 194 (*C/T*)–codon 280 (*G/A*)–codon 399 (*G/A*). The *C-G-G* haplotype, which contains only wild-type alleles, was most common in control and patient samples. Frequencies of variant haplotypes, which contain only one variant allele, i.e., *C-G-A, C-A-G,* and *T-G-G,* were compared in patients and controls either as a pooled group or individually. Distribution of variant haplotypes was significantly different (OR, 1.3; 95% CI, 1.0-1.7) in leukoplakia and controls but not in cancer patients when all samples were considered for analysis. Although the frequencies of pooled variant haplotypes were significantly increased in mixed habituals (i.e., having both smoking and chewing/dipping habits) of leukoplakia and cancer patients when compared with respective controls (OR, 2.2; 95% CI, 1.3-3.9; OR, 1.9; 95% CI, 1.2-3.1, respectively). Individually variant haplotypes, such as *C-G-A* and *T-G-G*, could also increase the risk of leukoplakia and cancer among mixed habituals (Table 4, legend). Distributions of pooled variant haplotypes were similar in exclusive smokers and smokeless tobacco users with cancer, leukoplakia, and controls. The haplotypes that contain more than one variant allele were either absent or less frequent (<2%) in these patient and control groups (data not shown) and, therefore, were not considered for further analysis.

Discussion

In the present study, healthy controls as well as patients with leukoplakia and cancer were recruited from the same ethnic population living in the same geographic location. In India, males use both smoking and smokeless tobacco, whereas females use mostly smokeless tobacco. Although smokers and smokeless tobacco users are equally affected by leukoplakia, it was observed that comparatively more male patients and smokers are present in the leukoplakia population than in the cancer population (Table 1). The reason might be due to the fact that leukoplakia is not initially life-threatening, therefore females (mostly housewives from low-income families and

Table 2. Distribution of risk genotypes at polymorphic sites on *XRCC1, XRCC3, GSTM1, GSTT1, GSTM3*, and *GSTP1* in 197 leukoplakia, 310 cancer, and 348 control individuals

Individuals (risk genotypes)	Control, *n* (%)	Leukoplakia, *n* (%)	Cancer, *n* (%)	OR (95% CI)
XRCC1 codon 194	6 + 57 (18)	3 + 37 (20)		1.1 (0.7-1.8)
(*Trp/Trp+Arg/Trp*)			3 + 58 (20)	1.1 (0.7-1.6)
XRCC1 codon 280	3 + 81 (24)	2 + 53 (28)		1.2 (0.8-1.8)
(*His/His+Arg/His*)			3 + 79 (27)	1.1 (0.7-1.6)
XRCC1 codon 399	27 + 163 (55)	25 + 82 (54)		1.0 (0.6-1.4)
(*Gln/Gln+Arg/Gln*)			32 + 143 (57)	1.0 (0.7-1.4)
XRCC3	8 + 120 (38)	8 + 64 (37)		1.0 (0.6-1.4)
(*Met/Met+Met/Thr*)			12 + 97 (35)	1.0 (0.6-1.2)
GSTM1	117 (34)	75 (38)		1.2 (0.8-1.7)
(homozygous deletion)			104 (34)	1.0 (0.7-1.4)
GSTT1	54 (17)	34 (17)		1.4 (0.8-2.2)
(homozygous deletion)			54 (17)	1.2 (0.7-1.8)
GSTM3	270 (78)	160 (81)		1.1 (0.7-1.7)
(*A/A*)			257 (83)	1.4 (0.9-2.0)
GSTP1 codon 105	184 (53)	104 (53)		1.0 (0.7-1.4)
(*Ile/Ile*)			181 (59)	1.3 (0.9-1.7)

NOTE: To calculate age-, sex-, and tobacco dose–adjusted OR and 95% CI, genotypes of patients and controls were compared. All *GST* genotype data were obtained by pooling the previous published data (from 256 cancer, 109 leukoplakia patients, and 259 controls; ref. 9) with the genotype data of the remaining newly collected samples. *Trp/Trp, His/His, Gln/Gln*, and *Met/Met* are variant genotypes and allele frequencies were: *Trp*, 0.1; *His*, 0.13; *Gln*, 0.32; *Met*, 0.21.

Table 3. Distributions of single/double-risk or double non–risk genotypes on a pair of loci in stratified samples of leukoplakia, cancer, and control populations

	Habit	Gene combination	Patients with				Controls with				P_{trend}
			Non–risk genotypes on both loci, *n* (%)	Risk genotypes on *GST*, *n* (%)	Risk genotypes on *XRCC*, *n* (%)	Risk genotypes on both loci, *n* (%)	Non–risk genotypes on both loci, *n* (%)	Risk genotypes on *GST*, *n* (%)	Risk genotypes on *XRCC*, *n* (%)	Risk genotypes on both loci, *n* (%)	
Leukoplakia	exclusive smokers	*GSTM1-XRCC3*	39 (35)	26 (24)	24 (22)	21 (19)	49 (45)	21 (19)	28 (25)	12 (11)	0.04 0.08
	exclusive smokers	*GSTM3-XRCC1* codon 399	5 (4)	42 (38)	10 (9)	54 (49)	13 (11)	40 (35)	15 (13)	46 (40)	0.09 0.02
	exclusive smokers	*GSTM3-XRCC3*	12 (11)	53 (48)	3 (3)	42 (38)	19 (17)	51 (46)	9 (8)	31 (28)	0.08 0.04
	exclusive smokeless	*GSTT1-XRCC1* codon 280	14 (45)	5 (16)	9 (29)	3 (10)	104 (64)	18 (11)	35 (22)	5 (3)	0.02 0.02
Cancer	mixed habituals	*GSTP1-XRCC1* codon 194	27 (34)	36 (45)	7 (9)	10 (13)	33 (46)	27 (38)	7 (10)	4 (6)	0.04 0.08
	smokers	*GSTM3-XRCC1* codon 280	13 (10)*	79 (60)	6 (5)	33 (25)*	31 (17)*	109 (59)	11 (6)	33 (18)*	0.03 0.03
	smokers	*GSTM3-XRCC1* codon 399	8 (6)	46 (35)	11 (8)	68 (51)	19 (10)	67 (36)	23 (12)	76 (42)	0.07 0.04

NOTE: *n* = number of patients or controls with that habit and genotypes. Exclusive smokers, exclusive smokeless tobacco users and ''smokers'' have been defined in Table 1. Risk genotype on a single locus has been shown in Table 2. In a few individuals, genotypes on *XRCC1* or *XRCC3* could not be determined after repeated attempts. Patients and controls carrying either two non–risk genotypes or single-risk genotype or two-risk genotypes on a pair of loci were compared by P_{trend} test. *OR, 2.4; 95%CI, 1.0-5.8, comparing only double risk and non–risk genotypes in cancer patients and controls.

smokeless tobacco users) would probably avoid lengthy procedures of reporting to the hospital. As a result, females and smokeless tobacco users are underrepresented in the leukoplakia population. All three groups of individuals exhibited good fit with Hardy-Weinberg equilibrium for all loci (data not shown). Adjustments for age, sex, and tobacco doses were done whenever required because there were some significant differences in the collected data in relation to age, sex, PY, and CY among the three studied groups (Table 1).

The risk genotypes (i.e., combined heterozygous and variant genotypes) on codons 194, 280, and 399 of *XRCC1* and *XRCC3* did not increase the risk of leukoplakia and cancer in the study population, either individually or in combination (Table 2 and data not shown, respectively) but genotypes containing variant alleles at *XRCC1* codon 280 increased the risk of leukoplakia in a small number of exclusive smokeless tobacco users (OR, 2.4; 95% CI, 1.0-5.7, data not shown). Although the exact functional roles of these nonsynonymous polymorphisms at *XRCC1* and *XRCC3* are not fully understood, it was argued that these polymorphisms would alter function of the proteins and, hence, risk of the cancer (23). The *G* to *A* transition in *XRCC1* codon 399 results in the change from *Arg* to *Gln* in the *XRCC1* BRCA1 COOH terminus domain that interacts with poly (ADP)ribose polymerase. Hence, the variant amino acid (i.e., *Gln* instead of *Arg*) at 399 of XRCC1 protein may be less efficient in DNA repair. The variant genotype at codon 399 of *XRCC1* increased the risk of oral squamous cell carcinomas in different populations (26, 27). Studies also reported both the presence and absence of association between polymorphism at codon 194 of *XRCC1* and risk of cancer (17, 28). On the contrary, reports have also shown that wild-type allele at codon 194 of *XRCC1* increased bleomycin and benzo-α-pyrene diol epoxide–induced chromosomal breaks in lymphocytes (29). Few studies have assessed the association between the less common polymorphism at codon 280 of *XRCC1* and cancer, but one study reported positive association between variant alleles at codon 280 of *XRCC1* and increased risk of lung cancer (30). A large lung cancer study did not observe positive association between polymorphism at codon 241 of *XRCC3* and increased risk of disease (22). But increased risk of bladder cancer was observed with variant allele at codon 241 of *XRCC3* (31). However, the potential of a polymorphism to impair DNA repair activity may not always be reflected on the increased risk of cancer as other modifiers can interfere in the process. One of the possibilities for not observing the presence of association between variant genotypes at *XRCC1/XRCC3* and increased risk of precancer and cancer in the oral cavity could be the small sample size with variant genotypes in these patient and control populations.

Earlier, it was reported that *GSTM1* homozygous deletion and *GSTM3* (*A/A*) genotype increased the risk of leukoplakia (1, 9) and *GSTM3* (*A/A*) genotype also increased the risk of oral cancer among tobacco smokers (9). After adding some newly recruited subjects (54 cancer, 88 leukoplakia, and 89 control individuals) in the previously collected sample pools, similar results were observed in the present study. *GSTM1* homozygous deletion and *GSTM3* (*A/A*) genotype increased the risk of leukoplakia (OR, 1.6; 95% CI, 1.0-2.8 and OR, 2.3; 95% CI, 1.1-4.7, respectively) in exclusive smokers (data not shown), and *GSTM3* (*A/A*) genotype increased the risk of cancer (OR, 1.8; 95% CI, 1.0-3.4) in smokers (data not shown). Thus, our previous results (9) are also substantiated in this study.

GSTs, such as *GSTM1*, *GSTP1*, and *GSTM3*, have overlapping substrate specificities, so it is expected that tobacco carcinogens, such as polycyclic aromatic hydrocarbons, will also be detoxified by these enzymes (23). But individuals carrying variant genotypes on these loci might have higher levels of DNA adducts in the exposed tissues (32). Consequently, there is a possibility that smokers carrying the *GSTM1* or *GSTP1* or *GSTM3* risk genotype will be susceptible to leukoplakia and cancer if the DNA adducts remain unrepaired. Although nucleotide excision repair is mainly involved in the removal of polycyclic aromatic hydrocarbon–DNA adducts,

these adducts are also repaired by base excision repair mechanisms, thus, supporting a possible involvement of *XRCC1* (33). Polycyclic aromatic hydrocarbons can also be metabolized to electrophiles that bind to DNA and destabilize the *N*-glycosyl bonds, inducing rapid depurination or depyrimidation of adducted bases thus reflecting a base excision repair mechanism. Consequently, an individual carrying two risk genotypes, one on each of two loci (such as *GSTM3* and *XRCC1*), may become more vulnerable to tobacco-related precancer and cancer than those carrying only one risk genotype or two non–risk genotypes on these loci. Significantly more bulky DNA adducts were also detected in WBC of cancer patients and normal individuals carrying variant *XRCC3* genotypes. Although such adducts undergo nucleotide excision repair rather than recombinational repair, concurrent exposure-induced oxidation reactions may lead to interstrand cross-links. Such DNA interstrand cross-links could be repaired by *XRCC3* enzyme. Therefore, individuals carrying variant genotype on *XRCC3* will also be susceptible to smoking-related leukoplakia and cancer. In this study, we had genotype data at GSTs and *XRCC1/XRCC3* loci in patients and controls, so individuals carrying single-risk genotypes at any one of the two loci or double-risk genotypes at both loci or two non–risk genotypes at both loci in patient and control populations were counted and compared to check whether individuals with increasing numbers of risk genotypes are overrepresented in patients compared with controls (Table 3). In leukoplakia patients, exclusive smokers with the increasing number of risk genotypes on three pairs of loci *GSTM1* and *XRCC3*, *GSTM3* and *XRCC1* (codon 399), and *GSTM3* and *XRCC3*, and exclusive smokeless tobacco users with the increasing number of risk genotypes on *GSTT1* and *XRCC1* (codon 280), were overrepresented compared with controls (P_{trend} = 0.04, 0.02, 0.04, and 0.02, respectively). Tobacco-specific nitrosamines and reactive oxygen species generated in smokeless tobacco could increase the levels of 8-hydroxyguanosine in DNA that could be removed by the base excision repair pathway (1, 34). Therefore, the presence of risk genotypes on *GSTT1* and *XRCC1* in an individual might increase the risk of disease among smokeless tobacco smokers, which has been reflected as overrepresentation in leukoplakia patients (Table 3). In cancer patients, mixed habituals with the increasing number of risk genotypes on a pair of loci *GSTP1* and *XRCC1* (codon 194), and smokers with an increasing number of risk genotypes on two pairs of loci, *GSTM3* and *XRCC1* (codon 280) and *GSTM3* and *XRCC1* (codon 399), were overrepresented compared with controls (P_{trend} = 0.04, 0.03, and 0.04, respectively). But the presence of two risk genotypes, one on each of two loci, *GSTM3* and *XRCC1* (codon 280), increased the susceptibility to cancer in smokers (OR, 2.4; 95% CI, 1.0-5.8; Table 3 legend) and none of the other combinations of two risk genotypes, on other pairs of loci, could show increased susceptibility to leukoplakia and cancer, probably due to small sample sizes. This observation indicates that reduced carcinogen detoxification and DNA repair activity by polymorphic *GSTM3* and *XRCC1*, respectively, in an individual, might have enhanced the risk of cancer among tobacco smokers. One interesting feature of this study is that individuals with an increasing number of risk genotypes on the *GSTM3* and *XRCC1* pair (codon 399) were overrepresented in smokers from both leukoplakia and cancer populations. Therefore, there is a possibility that the genotypes at *GSTM3* and *XRCC1* (codon 399) could be useful to identify leukoplakias, which might progress to cancer, but it remains to be tested with more sample sizes.

In this study population, frequencies of pooled variant haplotypes on *XRCC1* are 51% and 56%, respectively, in controls and cancer patients, and these values were 43.9% and 50.5%, respectively, in a Korean population (26). Our present study shows that pooled variant haplotypes (*C-G-A* + *C-A-G* + *T-G-G*) are overrepresented in the leukoplakia population compared with controls (OR, 1.3; 95% CI, 1.0-1.7; Table 4). The mixed habituals of both leukoplakia and cancer patients also had increased frequencies of pooled variant haplotypes, which led to increased risk of diseases (OR, 2.2; 95% CI, 1.3-3.9 and OR, 1.9; 95% CI, 1.2-3.1, respectively). It is interesting to note that the variant haplotypes at these three polymorphic sites consist of at least one variant allele at codons 194 or 280 or 399, which in their homozygous and heterozygous state, exhibited no association with the risk of cancer or leukoplakia even in mixed habituals (data not shown). This could be due to the small sample size and/or less prevalence of variant genotypes in this study population. Another reason could be that specific haplotypes, rather than genotype, are better predictors of gene expression and function (35). One of the variant haplotypes (*T-G-G*) was also observed to be significantly more frequent in head and neck cancer patients from a Korean population (26), and has also been corroborated in this population (OR, 3.5; 95% CI, 1.1-11.7 and OR, 3.1; 95% CI, 1.0-9.4 in mixed habituals of leukoplakia and cancer patients, respectively; Table 4, legend). It was reported that lymphocytes isolated from individuals having homozygous wild genotype at codon 194 (C/C) and homozygous variant genotype at 399 (*A/A*) of *XRCC1* are susceptible to bleomycin

Table 4. Estimated frequencies of *XRCC1* haplotypes in patients and controls

Category of patients	Subjects (chromosome number)	Variants			Wild	OR (95% CI)
		C-G-A, *n* (%)	*C-A-G*, *n* (%)	*T-G-G*, *n* (%)	*C-G-G*, *n* (%)	
All	control (674)	205 (30)	85 (13)	55 (8)	329 (49)	
	leukoplakia (392)	130 (33)	56 (14)	42 (13)	164 (41)	1.3 (1.0-1.7)[a]
	cancer (606)	200 (33)	83 (14)	56 (9)	267 (44)	1.2 (0.96-1.5)
Exclusive smokers	control (219)	64 (29)	28 (13)	19 (9)	108 (49)	
	leukoplakia (220)	74 (34)	24 (11)	25 (11)	97 (44)	1.2 (0.8-1.8)
	cancer (104)	34 (33)	16 (15)	11 (11)	43 (41)	1.4 (0.8-2.3)
Mixed habituals	control (137)	37 (28)	17 (12)	6 (4)	77 (56)	
	leukoplakia (110)	40 (36)[d]	19 (18)	11 (10)[f]	40 (36)	2.2 (1.3-3.9)[b]
	cancer (156)	56 (36)[e]	22 (14)	15 (9)[g]	63 (41)	1.9 (1.2-3.1)[c]
Exclusive smokeless tobacco users	control (318)	104 (33)	40 (13)	30 (9)	144 (45)	
	leukoplakia (62)	16 (26)	13 (21)	6 (10)	27 (43)	1.1 (0.6-1.9)
	cancer (346)	110 (32)	45 (13)	30 (8)	161 (47)	1.0 (0.7-1.3)

NOTE: Genotype data from polymorphisms at codon C194T (*Arg/Trp*), G280A (*Arg/His*), and G399A (*Arg/Gln*) of *XRCC1* were run in the software ''HAPLOFREQ'' to estimate haplotype frequencies. Wild (*C-G-G*) and other haplotypes (containing one variant allele) were compared either individually or pooling the variant haplotypes into a group. Haplotypes containing more than one variant allele are either absent or less frequent (<2%) and, therefore, were not taken for comparison. For calculation of OR in a, b and c: wild and pooled variant haplotypes were compared. For calculation of OR in d, e, f, and g: wild and single variant haplotypes were compared; d, OR, 2.1 (1.1-3.9); e, OR, 1.9 (1.1-3.3); f, OR, 3.5 (1.1-11.7); and g, OR, 3.1 (1.0-9.4).

and benzo-α-pyrene diol epoxide–induced chromosomal breaks (29). These lymphocytes could be assigned to the *194C-399A* haplotype. This haplotype is also present in the variant haplotypes as *194C-280G-399A* (i.e., *C-G-A*), shown in this study (Table 4) and also had increased risk of leukoplakia and cancer in mixed tobacco users (OR, 2.1; 95% CI, 1.1-3.9 and OR, 1.9; 95% CI, 1.1-3.3, respectively; Table 4, legend). Mixed tobacco users had the habits of both smoking and smokeless tobacco (Table 1). Mean smoking doses of mixed habituals in control and leukoplakia populations were similar to those of exclusive smokers in control and leukoplakia populations, whereas mean smoking dose of mixed habituals is significantly less ($P = 0.03$) than that of exclusive smokers in the cancer population (Table 1). Additionally, mixed habituals had smokeless tobacco exposure. Nitrosamines present in smokeless tobacco could also increase the levels of 8-hydroxyguanosine in DNA that could be removed by the base excision repair pathway (34). Although it has not been ascertained but it could be assumed that mixed habituals were exposed to more tobacco carcinogens than exclusive smokers and smokeless tobacco users, and so, became susceptible to cancer and leukoplakia. Therefore, it is possible that carcinogens, such as polycyclic aromatic hydrocarbons in tobacco smoke and nitrosamines in smokeless tobacco, have increased the risk of both leukoplakia and cancer in mixed habituals carrying variant haplotypes because variant alleles on *XRCC1* are less effective in DNA repair (23, 31). The haplotypes were estimated from the genotypes by a statistical method, so it was not possible to identify haplotype(s) from each individual. Hence, it was not possible to compare the effects of variant haplotypes on the risk of leukoplakia and cancer in the background of different *GST* genotypes.

In conclusion, variant haplotypes on *XRCC1* increased the risk of both leukoplakia and cancer among mixed habituals of this population and smokers carrying two risk genotypes, one on each of two loci, *GSTM3* and *XRCC1* (codon 280), were susceptible to cancer. Therefore, a group of variant/susceptible alleles on a group of low risk gene(s), such as *GSTM3* and *XRCC1* might become good markers to know which of the leukoplakias could progress to cancer. However, expression profiles and biological activities of these variant haplotypes and genotypes *in vivo* will provide a better understanding of risk of disease at the molecular level.

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Increased Risk of Oral Leukoplakia and Cancer Among Mixed Tobacco Users Carrying *XRCC1* Variant Haplotypes and Cancer Among Smokers Carrying Two Risk Genotypes: One on Each of Two Loci, *GSTM3* and *XRCC1* (Codon 280)

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