

Risk assessment of *p53* genotypes and haplotypes in tobacco-associated leukoplakia and oral cancer patients from eastern India

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The role of 3 *p53* polymorphisms (16 bp duplication at intron 3, codon 72 Arg/Pro and intron 6 *NciI* RFLP at np 13494) as potential markers for indicating cancer risk remains inconclusive. In our case-control study consisting of 197 leukoplakia and 310 oral squamous cell carcinoma (SCC) patients and 348 controls, genotype frequencies at these 3 *p53* loci were determined by PCR-RFLP method and analyzed by multiple logistic regression to determine the risks of the diseases. The 2/2 genotype at codon 72 of *p53* was at risk for developing leukoplakia (OR = 1.6, 95% CI 1.1–2.3), whereas the combination of 1/2 and 2/2 genotypes at intron 3 and 1/1 and 1/2 genotypes at intron 6 conferred a protective effect against leukoplakia and oral SCC development, respectively (OR = 0.5, 95% CI 0.4–0.8 and OR = 0.6, 95% CI 0.5–0.9, respectively). When subjects were stratified according to specific tobacco habit, the risk/protection estimates improved significantly in some cases. Specifically, the exclusive smokers with *p53* codon 72 2/2 genotype showed a higher risk of developing leukoplakia (OR = 2.7, 95% CI 1.2–6.3). Furthermore, a particular *p53* haplotype 1-2-2 was at risk for both tobacco-associated leukoplakia and oral SCC (OR = 1.5, 95% CI 1.1–1.9 and OR = 1.3, 95% CI 1.1–1.7, respectively). Our results show that both specific *p53* genotype and haplotype can indicate risk of tobacco-associated leukoplakia, but risk of development of tobacco-associated oral SCC can be predicted by specific *p53* haplotype only.

Key words: *p53*; polymorphism; leukoplakia; oral SCC; tobacco

Oral cancer constitutes 5.5% of all malignancies globally and is the sixth most common cancer worldwide.¹ Globally about 500,000 new oral and pharyngeal cancers are diagnosed annually, and three-quarters of these are from the developing world with about 65,000 cases from India.^{1,2} Oral squamous cell carcinomas (SCC) are generally associated with tobacco habits (mainly chewing with/without smoking or alcohol consumption) and usually preceded by premalignant lesions, most often a persistent leukoplakia.³ Oral leukoplakia, a common premalignant lesion among smokers, 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.⁴ This lesion is easily accessible to diagnosis and can be considered as an indicator of oral cancer risk. In a 10-year prospective study, carried out in several geographic areas of India with various kinds of tobacco habits, annual age-adjusted incidence rates of leukoplakia per 1,000 individuals per year varied from 1.1–2.4 among men and 0.2–1.3 among women.⁵ Oral leukoplakia has been reported to show a significant tendency to malignant transformation from 0.13–6% and rising to 14% or higher when dysplasia is present.⁶ In Europe, USA, Australia, China and Japan, cigarette, cigar and pipe smoking are the main forms of tobacco use and the effect of tobacco is known, not only to be dose and time dependent but also to act synergistically with the intake of alcohol (spirits) to multiply disease risk. By contrast, in India, Sri Lanka, Papua New Guinea and South East Asia, tobacco chewing is prevalent, usually in the form of a betel quid that consists of the leaf of the betel vine (*Piper betle*) wrapped around areca nut, lime and tobacco; any combination u.

these constituents may be used. Other forms of tobacco use that are also potent causes of oral cancer include the use of nass (an aqueous or oily mixture of tobacco, ash and lime), smoking of *bidi* (cheap cigarettes in which tobacco is rolled in a temburni leaf) and reverse smoking in which the lighted end of a cigarette or cheroot is held within the mouth.⁷ Differences in the clinicopathologic and molecular pathologic profile in the tobacco smoking and alcohol-associated oral cancers in the USA, UK, France, Japan, etc., and the chewing tobacco-associated oral cancers, particularly in India, have been recorded.^{7,8}

Molecular epidemiologic studies have now provided evidence that an individual's susceptibility to cancer is modulated by both genetic and environmental factors.⁹ Possible contribution of inherited polymorphisms in cancer-associated genes, especially *p53*, to the development of cancer risk has been the subject of interest after Storey *et al.* in 1998 reported that the women with *p53* codon 72 2/2 (Arg homozygous) genotype were at higher risk compared to 1/1 (Pro homozygous) genotype in developing cervical cancer upon HPV infection.¹⁰ Since then, a number of studies on different cancer types followed, but no definite conclusion could be reached. Meta analysis reports on the *p53* codon 72 polymorphism in cervical and lung cancer were also inconclusive.^{11–14} The 2 other *p53* polymorphisms viz. 16 bp duplication at intron 3 and intron 6 *NciI* RFLP (at nucleotide position 13494) have also been studied for any association in different cancer types. Homozygous genotypes for the absence of the *NciI* restriction site at intron 6 and presence of 16 bp duplication at intron 3 were reported to increase the risk of lung cancer¹⁵ and ovarian cancer.¹⁶ Also, breast cancer risk by the age of 50 years has been reported to be associated with 16 bp duplication polymorphism at intron 3 both under homozygous and heterozygous conditions,¹⁷ whereas another study with lung cancer patients did not support the observation.¹⁸

It has been proposed that inheritance of specific germline haplotypes based on 3 biallelic polymorphisms of *p53* (16 bp duplication at intron 3, codon 72 Arg/Pro and intron 6 *NciI* RFLP at np 13494) is a better indicator of breast cancer,^{19–22} colorectal cancer²³ and lung cancer risks.¹⁵ The specific haplotype 'p53 1-2-1' exhibited positive association with breast cancer risk,^{20,21} 2-2-1 with increased lung cancer risk,¹⁵ whereas 1-1-1 was found to be

Abbreviations: CI, confidence interval; CY, chewing year; OR, odds ratio; PCR, polymerase chain reaction; PY, pack-year; RFLP, restriction fragment length polymorphism; SCC, squamous cell carcinoma.

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associated with breast risk in a study from Pakistan.²² The absence of the duplication at intron 3, Pro at codon 72 and absence of *NciI* restriction site at intron 6 is defined as allele 1 at each of the 3 loci, respectively.^{24,25} We have adopted a similar allelic nomenclature in this article.

So far, only a few studies have reported a lack of association of the *p53* codon 72 2/2 genotype with susceptibility to head and neck squamous cell carcinoma,^{26–34} but significant difference in haplotype frequency between head and neck cancer patients and controls has been observed.³² To date, there is no report on association of a particular *p53* genotype and/or haplotype in tobacco-associated leukoplakia. In the present case-control study, composed of 197 leukoplakia, 310 oral SCC patients and 348 controls of the eastern Indian population, we explored the potential relationships between these 3 *p53* polymorphisms and risk of oral leukoplakia and cancer. We also examined the relationship between tobacco habit and dose and these polymorphisms for the risk of the diseases.

Material and methods

Patients, controls and tobacco habit

Unrelated patients diagnosed with leukoplakia or primary SCC in the oral cavity during 1999–2003 from the R. Ahmed Dental College and Hospital (Kolkata, India) were recruited in our study. For all patients, the Department of Pathology from the same hospital performed histopathologic confirmation of the lesions. Unrelated controls—those requiring treatment for dental ailments but without any previous and present lesion in oral cavity—were recruited from the outpatient department of the same hospital. No other selection parameters were used in sampling the cases and control subjects to avoid any selection bias. All blood samples were collected from participants with informed consent, and the Institutional Review Board for ethical use of human samples approved the study. Participants were personally interviewed using a questionnaire. Information on age, sex, occupation, alcohol consumption, type of tobacco habit, frequency of daily tobacco use, duration of the habit and economic status were recorded. Data pertaining to histopathologic diagnosis and clinical staging were obtained from the pathologic reports of the biopsy materials. All patients and controls were an ethnically similar caste population, and living in and around the city of Kolkata, located in the eastern region of India. Most of the patients and controls belonged to the low-income group (family income <\$100 USD per month), and this is one of the reasons for which they visited a government hospital for treatment. Both patients and controls reported tobacco habits such as smoking of *bidi* and/or cigarettes and chewing of tobacco in different forms. Some patients and controls reported dual habits comprising both smoking and chewing/dipping of tobacco, whereas the majority had a single habit. All the subjects in the study were regular tobacco users at the time of sample collection. Information provided by the smokeless tobacco users regarding the amount of tobacco used per chew was not reliable. Hence, lifetime tobacco exposure was measured in terms of the frequency of chewing per day multiplied by the duration of the habit. This was termed as chewing-year (CY; consumption of smokeless form of tobacco once in a day for 1 year = 1 CY). Similarly, dose of tobacco smoking was measured as pack-years (PY): 1 packet per day for 1 year = 1 PY (1 pack = 10 cigarettes or 20 *bidis*). For dose-response calculations, all subjects were subclassified into light and heavy tobacco users (both smoking and smokeless form of tobacco separately). The cutoff was defined as the median in the control group and then used in the other 2 subject groups, *i.e.*, leukoplakia and cancer for statistical analysis. Smokeless tobacco users were classified as light (<120 CY) and heavy (≥ 120 CY) where the median dose of all smokeless tobacco users in the control group was 120 CY. Smokers were classified as light (<24 PY) and heavy smokers (≥ 24 PY), where the median dose of controls was 24 PY.

Sample collection and processing

About 5 ml blood was collected by vein puncture from all patient and control individuals and stored at -20°C until DNA isolation. Genomic DNA was isolated from whole blood by salt precipitation method.³⁵ Biopsy materials collected from the patients were used to study histology and stage of differentiation of cells.

Genotyping of p53

Three biallelic *p53* gene polymorphisms (16 bp duplication at intron 3, codon 72 Arg/Pro and intron 6 *NciI* at nt 13494) for leukoplakia, cancer patients and controls were analyzed by PCR followed by variant specific restriction enzyme digestion. PCR was performed with primers that flanked the 2 polymorphic sites 16 bp duplication at intron 3 and codon 72 Arg/Pro as described elsewhere.³² Resulting PCR products were either 432 or 448 bp DNA fragments depending on the absence or presence of 16 bp duplication at intron 3 in template genomic DNA. Length polymorphism was directly evident from 6% polyacrylamide gel analysis of the PCR product. An aliquot of the same PCR product was subjected to restriction digestion with *BstUI* (New England Biolabs, Beverly, MA), which generated DNA fragments of 4 different sizes: 448 bp, *BstUI* digestion resistant (Pro at codon 72) with the intron 3 duplication; 432 bp, *BstUI* digestion resistant (Pro at codon 72) without the intron 3 duplication; 246 bp, *BstUI* digested (Arg at codon 72) with the intron 3 duplication; 230 bp, *BstUI* digested (Arg at codon 72) without the intron 3 duplication.

The third polymorphic site (*NciI* restriction site at intron 6) was amplified separately by PCR with flanking primers as described elsewhere.³² Five microliters of reaction mixture containing 913 bp amplicon were subjected to restriction digestion with *NciI*. The digest contained either 563 bp DNA fragment (in absence of the polymorphic *NciI* site) or a combination of 286 bp and 277 bp DNA fragments (in presence of the polymorphic *NciI* restriction site) together with an invariant 350 bp DNA fragment due to presence of a nonpolymorphic *NciI* site in the amplicon.

Statistical analysis

Pearson χ^2 test or Fischer's exact test (when sample number was <5 in any cell) was used for comparison of *p53* genotype and haplotype distributions between the 3 study groups and also for any difference in sex distribution among groups. Student *t*-test was used to calculate any statistically significant difference of continuous independent variables like age, PY and CY within the control and patient groups. Trend test was conducted to determine any increase in risk with the increase in number of risk alleles.^{36,37} Risk of oral SCC and leukoplakia was calculated as age-, sex-, PY- and CY-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for genotypes in all patient samples by multiple logistic regression analysis using SPSS statistical package.³⁸ Frequencies of the pairwise and extended haplotypes resulting from 3 polymorphisms in the *p53* gene were estimated using HAPLOPOP software.³⁹ Linkage disequilibrium value (D) was calculated through ARLEQUIN software package.⁴⁰

Results

A total of 197 leukoplakia, 310 oral cancer patients and 348 controls were included in our study. The distribution of demographic characteristics of the patients and the control subjects are summarized in Table I. The age and sex distribution between the control and 2 patient groups were found to be statistically significant. The difference in mean smoking exposure (PY) between leukoplakia and controls was not significant, whereas it differed significantly when the cancer group was compared to controls ($p = 0.005$). On the contrary, mean smokeless tobacco exposure (CY) was significantly different in leukoplakia patients ($p = 0.004$) but not in cancer patients (Table I). About 85% of smokers had habits of both cigarettes and *bidis*, so *bidi* and cigarette smokers were

TABLE I - CHARACTERISTICS OF PATIENTS AND CONTROLS

Subjects and tobacco habits		Controls (348) n (%)	Leukoplakia (197) n (%)	Oral cancer (310) n (%)
Sex ¹	Male	265 (76)	170 (86)	197 (64)
	Female	81 (24)	24 (14)	113 (36)
Age (years) ²	Mean \pm SD	50.4 \pm 11.5	47 \pm 10.8	55 \pm 12.0
Habituals	Exclusive smokers ³	114 (33)	111 (56)	53 (17)
	Exclusive smokeless tobacco users ⁴	162 (46)	31 (15)	176 (57)
	Mixed habituals ⁵	72 (21)	53 (27)	81 (26)
Smokers	Lifetime smoking range (PY)	5-125	5-90	5-120
	Mean smoking dose \pm SD (PY) ⁶	27 \pm 19	25 \pm 18	23 \pm 18
	No. of individuals with light (< 24 PY) tobacco smoking dose	91 (49)	94 (57)	77 (57)
	No. of individuals with heavy (\geq 24 PY) tobacco smoking dose	95 (51)	72 (43)	57 (43)
Smokeless tobacco users	Total smokers	186	164	134
	Lifetime smokeless tobacco using range (CY)	10-925	10-525	10-1,250
	Mean smokeless tobacco dose \pm SD (CY) ⁷	166 \pm 161.3	118 \pm 127	174 \pm 192.4
	No. of individuals with light (< 120 CY) smokeless tobacco dose	113 (84)	57 (66)	120 (47)
	No. of individuals with heavy (\geq 120 CY) smokeless tobacco dose	121 (16)	29 (34)	137 (53)
Total smokeless tobacco users	134	86	257	

All subjects are part of an ethnically similar caste population from eastern India.⁻¹ Difference in sex distribution: oral cancer vs. control, $p < 0.05$, and leukoplakia vs. control, $p < 0.05$.⁻² Mean age difference: oral cancer vs. control, $p < 0.05$, and leukoplakia vs. control, $p < 0.05$.⁻³ Current smokers with either cigarette and/or bidi smoking habit.⁻⁴ Current smokeless tobacco users using different forms of smokeless tobacco, i.e. khaini, betel quid, etc.⁻⁵ Mixed habituals have both types of habits simultaneously (smoking as well as smokeless tobacco). Light and heavy tobacco users are defined as the '< median' and ' \geq median', respectively, for PY (i.e., 24) and CY (i.e., 120) in the control group, thereafter used in the other 2 subject groups (both cancer and leukoplakia).⁻⁶ Mean smoking dose: oral cancer vs. control, $p = 0.005$.⁻⁷ Mean smokeless tobacco dose: leukoplakia vs. control, $p = 0.004$.

TABLE II - ASSIGNMENT OF RISK GENOTYPES FOR 3 p53 POLYMORPHISMS IN LEUKOPLAKIA AND ORAL CANCER PATIENTS

Genotypes	Control subjects n (%)	Leukoplakia patients		Oral cancer patients		
		n (%)	OR (95% CI)	n (%)	OR (95% CI)	
Intron 3	1/1	226 (66.1)	150 (78.5)	1.0 (Referent)	218 (71.0)	1.0 (Referent)
	1/2	102 (29.8)	38 (19.9)	0.6 (0.4-0.9)	81 (26.4)	0.8 (0.6-1.2)
	2/2	14 (4.1)	3 (1.6)	0.3 (0.1-1.1)	8 (2.6)	0.6 (0.3-1.4)
	$\chi^2_{HW} = 0.33$		$\chi^2_{HW} = 0.11$	$p_{trend} = 0.002$	$\chi^2_{HW} = 0.02$	$p_{trend} = 0.13$
Codon 72	1/1	98 (28.7)	32 (16.7)	1.0 (Referent)	66 (21.4)	1.0 (Referent)
	1/2	159 (46.5)	92 (48.2)	1.8 (1.1-2.8)	155 (50.3)	1.4 (1.0-2.1)
	2/2	85 (24.8)	67 (35.1)	2.4 (1.5-4.0)	87 (28.3)	1.5 (1.0-2.3)
	$\chi^2_{HW} = 1.62$		$\chi^2_{HW} = 0$	$p_{trend} = 0.0007$	$\chi^2_{HW} = 0.04$	$p_{trend} = 0.06$
Intron 6	2/2	212 (61.6)	132 (69.8)	1.0 (Referent)	217 (70.5)	1.0 (Referent)
	2/1	116 (33.7)	49 (25.9)	0.7 (0.5-1.0)	79 (25.6)	0.7 (0.5-0.9)
	1/1	16 (4.7)	8 (4.3)	0.8 (0.3-1.9)	12 (3.9)	0.7 (0.3-1.6)
	$\chi^2_{HW} = 0$		$\chi^2_{HW} = 0.2$	$p_{trend} = 0.09$	$\chi^2_{HW} = 0.2$	$p_{trend} = 0.03$

The number of samples genotyped are less than the total number of samples in Table 1 due to failure in PCR for one or more locus in 2 few samples even after repeated attempts.-OR, odds ratio; CI, confidence interval. Age-, sex-, PY- and CY- adjusted ORs were calculated.

not analyzed separately. In both patient groups, only a few (<5%) had occasional alcohol drinking history and none of the controls had an alcohol-drinking habit. So, alcohol consumption was not considered in statistical analysis.

The sites of oral cavity affected by leukoplakia were buccal mucosa and 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 buccal mucosa and alveolar sulcus, and the remaining sites were distributed equally between lip, tongue, retromolar area and buccal sulcus. Histopathologically, all malignancies were diagnosed SCC of oral cavity. These were classified as well differentiated (65%), moderately differentiated (17%) and poorly differentiated (18%) SCC.

Table II shows the germline genotype distribution for all 3 different biallelic DNA polymorphisms of the p53 gene in leukoplakia, oral cancer and control subjects. Genotyping of the samples were done by PCR followed by variant specific restriction enzyme diges-

tion. A few PCR products (5%) were sequenced (ABI prism 377; Applied Biosystems, Foster City, CA) to confirm the genotypes at all loci. The sequencing results matched exactly to the genotype data by PCR-RFLP analysis. All 3 groups of individuals exhibited good fit to Hardy Weinberg equilibrium for all 3 loci (Table II).

Homozygous genotypes of allele 1 at intron 3 (absence of 16 bp duplication allele), allele 2 at codon 72 Arg/Pro locus (Arginine at codon 72) and allele 2 at NciI locus (absence of NciI restriction site in intron 6) were overrepresented in cases compared to control subjects (Table II). Before estimating the risk of leukoplakia and oral cancer associated with p53 genotypes, a trend test was done to check whether any specific pattern of risk was exhibited with the increase in a particular allele. The referent genotypes were considered on the basis of the highest proportion of a homozygous genotype at any particular p53 locus in the control group. An increased risk was indeed observed with increase in Arginine allele at codon 72 Arg/Pro locus (allele 2) in leukoplakia patients ($P_{trend} = 0.0007$), but the same was not significant for oral cancer

TABLE III – RISK/PROTECTION ESTIMATES OF p53 INTRON 3, CODON 72 AND INTRON 6 GENOTYPES IN LEUKOPLAKIA AND ORAL CANCER PATIENTS

Genotypes	Control subjects n (%)	Leukoplakia patients		Oral cancer patients	
		n (%)	OR (95% CI)	n (%)	OR (95% CI)
Intron 3					
1/1	226 (66.1)	150 (78.5)	1.0 (Referent)	218 (71.0)	1.0 (Referent)
2/2 + 1/2	116 (33.9)	41 (21.5)	0.5 (0.4–0.8) ¹	89 (29.0)	0.8 (0.5–1.1)
Codon 72					
1/1 + 1/2	257 (75.2)	124 (64.9)	1.0 (Referent)	221 (71.7)	1.0 (Referent)
2/2	85 (24.8)	67 (35.1)	1.6 (1.1–2.3) ²	87 (28.3)	1.2 (0.9–1.8)
Intron 6					
2/2	212 (61.6)	132 (69.8)	1.0 (Referent)	217 (70.5)	1.0 (Referent)
1/1 + 1/2	132 (38.4)	57 (30.2)	0.7 (0.5–1.1)	91 (29.5)	0.6 (0.5–0.9) ³

Genotypes of patients and controls were compared. OR, odds ratio; CI, confidence interval. Age-, sex-, PY- and CY- adjusted ORs were calculated at the p53 loci intron 3, codon 72 and intron 6, respectively. ¹p = 0.004. ²p = 0.02. ³p = 0.008. The number of samples genotyped are less than the total number of samples in Table I due to failure in PCR for one or more locus in 2 few samples even after repeated attempts.

TABLE IV – DISTRIBUTION OF p53 INTRON 3, CODON 72 AND INTRON 6 GENOTYPES IN LEUKOPLAKIA, ORAL CANCER AND CONTROLS WITH RESPECT TO TOBACCO HABIT AND DOSE

Individual status (n)	p53-int3 genotypes		
	2/2 + 1/2	1/1	OR (95% CI)
Exclusive smokers, control (113)	38	75	0.5 (0.3–0.9) ¹
Exclusive smokers, leukoplakia (109)	22	87	
Light dose (< 24 PY) of smoking in control (52)	19	33	0.3 (0.1–0.8) ²
Light dose (< 24 PY) of smoking in leukoplakia (67)	12	55	
	p53-codon 72 genotypes		
	2/2	1/1 + 1/2	OR (95% CI)
Exclusive smokeless tobacco users, control (159)	35	124	2.7 (1.2–6.3) ³
Exclusive smokeless tobacco users, leukoplakia (30)	13	17	
	p53-int6 genotypes		
	1/1 + 1/2	2/2	OR (95% CI)
Exclusive smokers, control (113)	49	64	0.5 (0.3–0.9) ⁴
Exclusive smokers, leukoplakia (107)	31	76	
Exclusive smokers, control (113)	49	64	0.3 (0.1–0.7) ⁵
Exclusive smokers, oral cancer (53)	10	43	
Light dose of smoking in control (< 24 PY) (52)	24	28	0.3 (0.1–0.9) ⁶
Light dose of smoking in oral cancer (< 24 PY) (22)	4	18	

OR, odds ratio; CI, confidence interval. Age-, sex- and PY- adjusted ORs. ¹p = 0.02. ²p = 0.01. ³p = 0.02. ⁴p = 0.03. ⁵p = 0.002. ⁶p = 0.03. Light and heavy tobacco users are defined as the '< median' and '≥ median', respectively for PY (*i.e.*, 24) in the control group, thereafter used in the other 2 subject groups (both oral cancer and leukoplakia).

patients ($P_{\text{trend}} = 0.06$, Table II). For the other 2 intronic loci, the variant allele 2 at intron 3 and allele 1 at intron 6 were present at a significantly lower proportion in the patients compared to the controls, thereby exhibiting a trend for protection from leukoplakia ($P_{\text{trend}} = 0.002$) and from oral cancer ($P_{\text{trend}} = 0.03$), respectively.

Next, we estimated the risk or protection from leukoplakia and oral cancer associated with each p53 polymorphism. The genotypes arising from the protective alleles (2/2 and 2/1 genotypes at intron 3 and 1/1 and 2/1 genotypes at intron 6) were combined at each loci, and the protection conferred was estimated by taking the remaining homozygous genotype as the referent genotypes (1/1 at intron 3 and 2/2 at intron 6, respectively). The age-, sex-, PY- and CY-adjusted distribution of intron 3 16 bp genotypes of p53 between leukoplakia patients and controls was statistically significant (OR = 0.5, 95% CI 0.4–0.8; Table III) and the 2/2 and 1/2 genotypes in combination were found to protect from leukoplakia development. Similar analysis with genotype distribution of p53 at the intron 6 NciI locus between cancer patients and controls was also significant (OR = 0.6, 95% CI 0.5–0.9; Table III). The combination of 1/1 and 1/2 genotypes at the intron 6 NciI locus were found to protect from oral cancer development. The risk associated with the 2/2 genotype at codon 72 Arg/Pro locus was estimated in comparison to the other 2 genotypes taken together (Table III). The age-, sex-, PY- and CY-adjusted distribution of codon 72 Arg/Pro 2/2 genotype of p53 between leukoplakia

patients and controls was statistically significant (OR = 1.6, 95% CI 1.1–2.3; Table III), suggesting a risk for leukoplakia development conferred by this genotype.

To get a better insight about the tobacco-related oral carcinogenesis, the samples were classified according to tobacco habit and dose. Results suggest that exclusive smokers with p53 2/2 and 1/2 genotype at the intron 3 16 bp locus were protected from leukoplakia (OR = 0.5, 95% CI 0.3–0.9; Table IV). The effect was more apparent with light smokers (<24 PY) when subjects were stratified according to tobacco dose (OR = 0.3, 95% CI 0.1–0.8; Table IV). We did not get any significant association of these genotypes of intron 3 in cancer patients with smoking habit or smokeless tobacco users of both patient groups (data not shown). A 2.7 times higher risk was observed in leukoplakia patients carrying the p53 codon 72 2/2 genotype who were exclusive users of smokeless tobacco (OR = 2.7, 95% CI 1.2–6.3; Table IV). No significant association of the risk genotype at codon 72 was observed with smoking habit or smoking/smokeless tobacco dose in either of the 2 patient groups (data not shown). We also observed that exclusive smokers with p53 1/1 and 1/2 genotype at the intron 6 NciI locus were protected from cancer (OR = 0.3, 95% CI 0.1–0.7; Table IV). The effect was again evident with light smokers (<24 PY) when subjects were stratified according to tobacco dose (OR = 0.3, 95% CI 0.1–0.9; Table IV). This genotype combination was also found to be protective against leukoplakia among

TABLE V – PAIRWISE HAPLOTYPE FREQUENCIES AND LINKAGE DISEQUILIBRIUM ANALYSIS OF *p53* POLYMORPHISMS IN CONTROL AND 2 PATIENT GROUPS

Population	No. of chromosomes	Estimated pairwise haplotype frequency				D	p-value
		1-1	1-2	2-1	2-2		
16 bp - codon 72							
Control	682	0.328	0.481	0.191	0.000	0.0917	<0.0001
Leukoplakia	378	0.294	0.593	0.114	0.000	0.0674	<0.0001
Oral cancer	612	0.309	0.533	0.158	0.000	0.0844	<0.0001
16 bp- <i>Nci</i> I							
Control	682	0.101	0.708	0.114	0.076	0.0733	<0.0001
Leukoplakia	378	0.114	0.772	0.058	0.056	0.0386	<0.0001
Oral cancer	612	0.054	0.788	0.113	0.046	0.0863	<0.0001
Codon 72- <i>Nci</i> I							
Control	682	0.170	0.349	0.045	0.435	0.0582	<0.0001
Leukoplakia	378	0.103	0.304	0.069	0.524	0.0331	=0.0008
Oral cancer	612	0.136	0.332	0.031	0.502	0.0577	<0.0001

D, linkage disequilibrium.

TABLE VI – ESTIMATED *p53* HAPLOTYPE FREQUENCIES OF PATIENTS AND CONTROLS (INTRON 3-CODON 72-INTRON 6)

Subjects (no. of chromosomes)	<i>p53</i> haplotypes						p-value
	1-2-2 (%)	1-1-2 (%)	2-1-1 (%)	2-1-2 (%)	1-1-1 (%)	1-2-1 (%)	
Control (682)	296 (43.5)	186 (27.3)	78 (11.4)	52 (7.7)	37 (5.4)	33 (4.8)	Referent
Leukoplakia (380)	200 (52.7)	93 (24.4)	23 (6.0)	21 (5.6)	18 (4.8)	25 (6.6)	0.008 ¹
Oral cancer (608)	307 (50.5)	173 (28.4)	70 (11.4)	27 (4.5)	16 (2.7)	15 (2.5)	0.001 ²
Exclusive smokers, control (222)	90 (40.5)	66 (29.7)	32 (14.4)	10 (4.5)	10 (4.5)	14 (6.3)	Referent
Exclusive smokers, leukoplakia (216)	110 (50.9)	57 (26.4)	11 (5.1)	13 (6.0)	10 (4.6)	15 (6.9)	0.02 ³
Exclusive smokers, oral cancer (104)	54 (51.9)	30 (28.8)	9 (8.7)	7 (6.7)	3 (2.9)	1 (1.0)	0.09 ⁴
Exclusive smokeless, control (318)	133 (41.8)	85 (26.7)	39 (12.3)	28 (8.8)	22 (6.9)	11 (3.5)	Referent
Exclusive smokeless, oral cancer (346)	173 (50.0)	104 (30.0)	45 (13.0)	8 (2.3)	8 (2.3)	8 (2.3)	0.0002 ⁵

p-values were obtained by comparing the haplotype frequency distribution between. ¹Leukoplakia vs. control. ²Cancer vs. control. ³Exclusive smokers of leukoplakia vs. exclusive smokers of control. ⁴Exclusive smokers of cancer vs. exclusive smokers of control. ⁵Exclusive smokeless of cancer vs. exclusive smokeless of control. –The number of samples haplotyped was less than the total number of samples in Table I due to failure in PCR for one or more locus in 2 few samples even after repeated attempts.

exclusive smokers (OR = 0.5, 95% CI 0.3–0.9; Table IV). There was no significant association of these genotypes in smokeless tobacco users of either patient group (data not shown).

Table V depicts the estimated pairwise haplotype frequencies among the 3 polymorphisms and their linkage disequilibrium values in case and control subjects. The pairwise linkage disequilibria were highly significant in both control and patient population as reported earlier.³² The extended haplotype frequencies consisting of these 3 polymorphisms in total leukoplakia patients, cancer patients and controls as well as when they were subclassified according to tobacco habit are shown in Table VI. The haplotypes constructed have been expressed in the order intron 3-codon 72-intron 6.²⁴ In our study, 6 of 8 possible haplotypes were observed in all groups. The haplotype frequencies were significantly different between leukoplakia and controls ($p = 0.008$; Table VI). Similar differences between cancer patients and controls were also observed, which was statistically significant ($p = 0.001$; Table VI). When haplotype frequencies for exclusive smokers were estimated, the distribution was significantly different between leukoplakia and controls ($p = 0.02$; Table VI), but the same was not significantly different between cancer patients and controls ($p = 0.09$; Table VI). In case of exclusive smokeless tobacco users, the cancer patients showed significant differences in haplotype frequency distribution from the control subjects ($p = 0.0002$; Table VI). The 1-2-2 haplotype was most common in control and patient samples followed by 1-1-2 and 2-1-1 haplotypes (Table VI). The 2-2-2 and 2-2-1 haplotypes were absent in all these 3 groups of subjects (Table VI). The haplotype distribution clearly depicted a higher percentage of the most common haplotype 1-2-2 in the patient groups when compared to controls even after stratifying according to tobacco habit (Table VI). The distribution of 1-2-2 haplotype vs. all other haplotypes was significantly different in leukoplakia and cancer patients when compared to controls (OR = 1.5, 95% CI 1.1–1.9 and OR = 1.3, 95% CI 1.1–1.7, respectively;

Table VII). Again, this haplotype was at risk in exclusive smokers of leukoplakia as well as in cancer patients (OR = 1.5, 95% CI 1.0–2.2 and OR = 1.6, 95% CI 1.0–2.5, respectively; Table VII). The specific haplotype that showed significant risk for cancer patients with exclusive smokeless tobacco habit was also 1-2-2 (OR = 1.4, 95% CI 1.0–1.9; Table VII). Similar analysis was not done for leukoplakia patients because of the small number of smokeless tobacco users in this group.

Discussion

Earlier, we reported lack of association between *p53* genotypes of the polymorphic sites 16 bp duplication at intron 3, codon 72 Arg/Pro and intron 6 *Nci*I RFLP at nt 13494 loci with head and neck squamous cell carcinoma (HNSCC) risk,³² wherein we observed significant variation in haplotype frequency between HNSCC patients and controls. In our present study, we replicated that study by analyzing another set of oral cancer as well as leukoplakia patients with ethnicity-matched control individuals, which did not include the samples from our previous study. The controls as well as the 2 patient groups were taken from the same ethnic group living in the same geographic location. Previously, we reported that various subpopulations within the caste group from eastern India did not differ with respect to allele frequency at several other polymorphic loci.⁴¹ Thus, the possibility of false association due to population stratification has been ruled out. One important aspect of this study is that we attempted to examine the link between risks of oral leukoplakia and cancer and different *p53* polymorphisms in the presence of different types of tobacco habit using satisfactory measurements of exposure. Because controls had either similar or more tobacco exposure than patients, it can be argued that they served as good control because they remained healthy even after sufficient tobacco exposure.

TABLE VII - SUMMARY OF RESULTS SHOWING THE RISK OF p53 1-2-2 HAPLOTYPE IN LEUKOPLAKIA AND ORAL CANCER PATIENTS WITH RESPECT TO TOBACCO HABIT

Tobacco habit	Subjects (no. of chromosomes)	Haplotype (n)		OR (95% CI)
		1-2-2	Rest	
All tobacco users	Control (682)	296	386	1.0 (referent)
	Leukoplakia (380)	200	180	1.5 (1.1-1.9) ¹
	Oral cancer (608)	307	301	1.3 (1.1-1.7) ³
Exclusive smoking	Control (222)	90	132	1.0 (referent)
	Leukoplakia (216)	110	106	1.5 (1.0-2.2) ²
	Oral cancer (104)	54	50	1.6 (1.0-2.5) ⁴
Exclusive smokeless	Control (318)	133	185	1.0 (referent)
	Leukoplakia (62)	Not done	Not done	Not done
	Oral cancer (346)	173	173	1.4 (1.0-1.9) ⁵

The odds of risk for the 1-2-2 haplotype was estimated in comparison to all other haplotypes taken together. OR, odds ratio; CI, confidence interval. ¹ $p = 0.005$. ² $p = 0.03$. ³ $p = 0.01$. ⁴ $p = 0.05$. ⁵ $p = 0.03$.

Among the different p53 polymorphisms, the codon 72 Arg/Pro, 16 bp at intron 3 and NciI at intron 6 (nt 13494) have been used in many studies. Codon 72 allele frequency variation with respect to ethnicity and latitude has been reported.²⁴ The allele 1 (Proline) frequency at codon 72 in different world populations are as follows: 0.17 in Swedish Saamis, 0.24 in Finns, 0.29 in Swedes, 0.38 in Chinese from Singapore, 0.47 in Chinese from Guizhou and 0.63 in African Blacks (Nigerians).^{24,42} Another study with the Caucasians, Hispanics and African Americans reported the frequency of allele 1 at codon 72 as 0.21, 0.49 and 0.63, respectively.²⁵ The allele 1 frequency in Indian population from different geographic regions were reported to vary from 0.45-0.56.^{28,32,34,43} Similar analysis with third generation Dravidian Indians from Singapore reported the codon 72 allele 1 frequency as 0.54.²⁴ In this study, allele 1 frequency in controls was found to be 0.52. The frequency of allele 1 (intron 6 uncut variant) at the NciI locus (nt 13494) has been reported as 0.02 in the Chinese, 0.19 in south Indians and African blacks, 0.22 in Hispanic population and 0.33 in African Americans.^{24,25} At the intron 3 locus, allele 2 (presence of 16 bp duplication) frequencies were reported to be 0.05 in Chinese, 0.23 in south Indians, 0.25 in African blacks, 0.24 in Hispanics and 0.32 in African Americans.^{24,25} In this study, the frequencies of allele 2 at intron 3 and allele 1 at intron 6 in controls were 0.19 and 0.22, respectively.

We estimated the risk of both leukoplakia and oral cancer associated with each p53 genotype for all 3 polymorphic loci separately. The 2/2 genotype of p53 codon 72 was found to be the risk genotype for developing leukoplakia, whereas the combination of 2/2 and 1/2 genotypes at intron 3 and 1/1 and 1/2 genotypes at intron 6 conferred a protective effect against leukoplakia and oral cancer development, respectively. Next, the risk/protection of each genotype was estimated after stratifying the subjects with respect to tobacco habit and dose to understand the tobacco habit and dose-associated susceptibility to leukoplakia or oral cancer development for specific genotypes at the 3 p53 polymorphic loci. As expected, when subjects were stratified according to specific tobacco habit, the risk/protection estimates improved significantly in some cases. For example, leukoplakia patients with 2/2 genotype at codon 72 polymorphism exhibited higher risk when exclusive smokeless tobacco users were considered in comparison to total leukoplakia patients. Previous studies as well as the present study failed to observe any risk of 2/2 genotype at codon 72 of p53 for the oral cancer and HNSCC.²⁶⁻³⁴ Thus, it appears that individuals with specific p53 genotypes are more susceptible to the development of tobacco-assisted oral leukoplakia, indicating a possible role of 2/2 variant of p53 codon 72 in early stages of oral cancer development. We speculate that processing of the signals generated from the tobacco-associated DNA damage is differentially regulated depending on both specific p53 variant present and the developmental stage of the incipient tumor. The supportive evidence to this speculation comes from the study showing that different environmental agents are risk factors for the development of leukopla-

kia and its conversion to oral SCC.⁴⁴ Betelnut chewing and tobacco smoking are major risk factors for the development of leukoplakia from a normal tissue, whereas alcohol consumption is the main risk factor for malignant transformation of the leukoplakia.⁴⁴ Thus, the response of the genetic components to the carcinogen exposure might as well vary with the developmental stage of the tumor. The combination of 2/2 and 1/2 genotypes at the 16 bp locus at intron 3 was found to confer protection from leukoplakia development. Interestingly, the protective effect was evident only at a low dose of smoking. Similarly, combination of 1/1 and 1/2 genotypes at the intron 6 NciI locus protected from oral cancer development that was also obvious at a low smoking dose. This implies that the protection conferred by the particular genetic variants may be compensated at a higher dose of tobacco exposure.

The biochemical and biologic activities of p53 that are affected by the codon 72 polymorphism have been evaluated eloquently in a series of studies. It was first shown that this polymorphism had a profound effect on the primary structure of p53 based on differences in the migration of the protein during SDS polyacrylamide gel electrophoresis.⁴⁵ More recently, it has been shown that the ability of p53 to interact with the TFIIID-associated factors TAFII32 and TAFII70 is much stronger for the Pro-72 form than for the Arg-72 form whereupon the Pro-72 form of p53 has increased transcriptional transactivation capacity.⁴⁶ In contrast, analysis of the ability of the 2 polymorphic forms of p53 to induce apoptosis suggests that the 2 polymorphic varieties, though differing in kinetics, are capable of inducing equal levels of apoptosis.⁴⁶ In another study, the Arg-72 form of the p53 protein was found to be much more susceptible to HPV E6-mediated degradation compared to the Pro-72 form.¹⁰ In addition, analysis of mutants of p53 within a variety of tumors suggests that mutations in the Arg-72 form of p53 are more common in heterozygous individuals and that this enhances interaction with, and subsequent inactivation of, the p53 family member p73.^{47,48} Recent studies using a temperature-sensitive form of the p53 protein further support these observations and show that the Arg-72 form has a much stronger capacity to induce apoptosis than the Pro-72 form of p53 in tumor cells but not in normal cells.⁴⁹ However, in primary head and neck tumors, retention of the Arginine allele in codon 72 of the p53 gene correlates with poor apoptosis.⁵⁰ Finally, the Pro-72 form appears to induce a higher level of G1 arrest compared to the Arg-72 form.⁵¹ In summary, it may be stated that the Arg-72 form p53 induces a lower level of G1 arrest, has reduced transcriptional transactivation capacity of downstream target genes and has a lower apoptotic potential in primary tumors compared to the Pro-72 form. Thus, we argue that individuals with the Arg-72 form of p53 are less efficient in processing tobacco-associated DNA damage signals and hence are more susceptible to develop leukoplakia.

The regulatory role of the intronic sequences is being increasingly recognized.⁵² Several workers reported functional differences in genotypes at introns 3 and 6 polymorphisms of p53 gene.^{15,16,53,54} One study reported low apoptotic index and

less repair capacity of lymphoblastoid cell lines that harbor less frequent allele of intron 3 16 bp duplication and intron 6 *NciI* polymorphism.¹⁵ There were no splicing errors linked to these polymorphisms.¹⁶ However, in cell culture analysis, these 2 intronic polymorphisms did not seem to be sufficient to impair p53 function during the selection process in neoplastic transformation but needed an additional coding region mutation.¹⁶ The intronic change of G→C at nt 13964 in intron 6 either caused or was linked with a variation leading to stabilization and possible inactivation of the p53 protein, thereby contributing to tumorigenesis.⁵⁴ Intron 4 of p53 has been predicted to contain several transcription factor binding sites,⁵⁵ and single base pair substitutions in this intron have been shown to disturb binding of unidentified proteins, resulting in a decreased expression of p53.⁵⁶ Inclusion of intron 3 in murine p53 cDNA has been shown to increase the expression of the gene.⁵⁶ Recently, reduced levels of p53 mRNA has been proposed to be associated with the p53 intron 3 16 bp duplication allele.⁵⁷ Overall, the functional role of the 2 intronic polymorphisms of p53 analyzed and their modulatory role in cancer risk remain uncertain. Further studies need to be conducted to unveil any regulatory role of these intronic sequences. Alternatively, these intronic p53 polymorphisms may confer an increase in cancer risk through linkage disequilibrium with a functional variant of the p53 gene.

It has been proposed that inheritance of specific germline haplotypes based on 3 biallelic polymorphisms of p53 was a better indicator of cancer risk.^{19,32} In concordance with our earlier study, our present study also shows a significant difference in haplotype distribution between the oral cancer as well as leukoplakia patient and normal control (also after subclassification according to tobacco habit; Table VI), raising the possibility of association of specific haplotype with disease risk. Further analysis showed that carriers of specific haplotype 1-2-2 had a higher risk for both leukoplakia and oral cancer development even after stratifying with respect to smoking or smokeless forms of tobacco (Table VII). It is interesting to note that this haplotype consists of allele 2 of the codon 72 polymorphic site, which in homozygous state exhibits tobacco habit-specific association with leukoplakia. The other 2 alleles of this haplotype are allele 1 of intron 3 and allele 2 of

intron 6 polymorphic sites. The homozygous genotypes of these alleles (1/1 at intron 3 and 2/2 at *NciI* loci) were overrepresented in patients compared to the controls. The other alleles in these 2 sites exhibited protection in genotypic analysis. However, this haplotype did not show any association in other cancer types.^{15,19-22} This difference could be due to differences in ethnicity of the populations between these studies.

Despite the useful information that case-control studies provide, one should recognize the limitations inherent in case-control studies, including recall biases and the selection bias that may arise with use of hospital-based controls. We have made every effort to exclude any bias in control samples by maintaining a registry. The allele frequencies of our hospital-based controls were similar to the population allele frequency observed in this part of the country. Thus, the possibility of false association due to biased estimates of allele frequencies has been ruled out. Our results suggest that p53 haplotype 1-2-2 (comprising absence of 16 bp duplication allele at intron 3, Arg at codon 72, presence of *NciI* at intron 6) is a better indicator of tobacco habit and dose-associated leukoplakia and oral cancer risk. The observed association of the haplotype could be either due to strong linkage disequilibrium among these 3 sites or the 2 intronic polymorphisms also contribute functionally to the risk development as discussed above. Further studies are needed to establish the mechanism behind the differential processing of signals generated from tobacco-mediated DNA damage by variant forms of p53.

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