

Association between polymorphisms at N-acetyltransferase 1 (*NAT1*) & risk of oral leukoplakia & cancer

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Background & objectives: N-acetyltransferases 1 and 2 (*NAT1* and *NAT2*) are important enzymes for metabolism of tobacco carcinogens. Due to polymorphisms, improper activities of these enzymes might lead to the formation of DNA adducts that may modulate risk of tobacco related oral precancer and cancer. Previously, it was shown that *NAT2* polymorphisms did not modulate the risk of oral precancer and cancer. We undertook this study to check whether polymorphisms at *NAT1* can modulate the risk of oral leukoplakia and cancer either alone or in combination with *NAT2*.

Methods: Genotypes at four SNPs on *NAT1* were determined by TaqMan method in 389 controls, 224 leukoplakia and 310 cancer patients. Genotype data were analyzed to know haplotypes and acetylation status of individuals and, then to estimate the risk of diseases. Using our previously published *NAT2* data, combination of *NAT1* and *NAT2* acetylation genotypes of patients and controls were also analyzed to estimate the risk of diseases.

Results: Analysis of *NAT1* genotype data revealed that *1088T* and *1095C* alleles exist in strong linkage disequilibrium ($r^2=0.97$, $P<0.0001$) and SNPs are in Hardy-Weinberg Equilibrium ($P=0.1$). Wild type or normal acetylating and variant or rapid acetylating alleles were two major alleles (frequencies 0.62 and 0.36, respectively) present in the control population. *NAT1* rapid acetylation could not modulate the risk of leukoplakia and cancer (OR=0.9, 95% CI: 0.6-1.3; OR=1.0, 95% CI: 0.7-1.4, respectively). Analysis of combined *NAT1* and *NAT2* acetylating data also showed no significant enhancement of the risk of diseases.

Interpretation & conclusions: *NAT1* rapid acetylation alone as well as combination of *NAT1* rapid-*NAT2* slow acetylation did not modulate the risk of oral precancer and cancer in our patient population. So, *NAT1*/*NAT2* metabolized carcinogen products may not be involved in tobacco related oral precancer and cancer. It may be interpreted that large sample size as well as combination of polymorphisms at other candidate loci may be important to estimate the risk of a complex disease like oral cancer.

Key words Combination of polymorphisms - leukoplakia - *NAT1* - *NAT2* - oral cancer

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Annually about 270,000 cases of oral cancer are reported worldwide and of these about 80,000 are diagnosed in India¹. Annual incidence of oral leukoplakia has been reported to be 0.2-11.7 per cent in different populations of India and about 2-12 per cent of the leukoplakia cases become malignant within several years^{2,3}. As an early sign of damage to oral mucosa, tobacco smokers and chewers often develop different precancerous lesions, such as leukoplakia, erythroplakia, submucous fibrosis, *etc.*, and these lesions are easily accessible to diagnosis. As leukoplakia is one of the good predictors of oral cancer, knowledge of genetic risk factors for leukoplakia may provide useful strategy to control oral cancer incidence.

Tobacco carcinogens undergo different kinds of modifications by Phase I (such as cytochrome P450, CYPs *etc.*) and Phase II (such as GSTs, NAT1, NAT2 *etc.*) enzymes either for detoxification or activation depending on the specific enzymes. It has been reported earlier that polymorphisms at *GSTs*, *CYPs*, *NAT2* and different DNA repair loci, either independently or in combination, could modify the risk of oral cancer and precancer in different Indian populations⁴⁻¹². But, no report is available on association between polymorphism at *NAT1* locus and risk of oral precancer and cancer in Indian population. NAT1 can catalyze O-acetylation (activation) and N-acetylation (detoxification) reactions with aromatic and heterocyclic amines present in tobacco. Polymorphisms at *NAT1* may result in the production of NAT1 proteins with variable enzyme activity or stability¹³ and *NAT1* is highly polymorphic with more than 25 variant alleles in different population¹⁴. Wild type or normal allele (called as *NAT1*4*) contains wild-type nucleotides at all polymorphic sites but the most common variant *NAT1*10* allele contains two variant nucleotides (*A*¹⁰⁸⁸ and *A*¹⁰⁹⁵) at 1088 and 1095nps in the 3'-untranslated region of the gene. These changes in nucleotides modify mRNA stability which, in turn, increases the activity of the NAT1 enzyme. So, this allele has been assigned as rapid acetylating allele¹⁵. Other variant alleles such as *NAT1*3*, *NAT1*11*, *NAT*15*, *NAT1*14*, *etc.* could have different acetylating activities but implication of these variant alleles in association study is not known since frequencies of these alleles are very low in the population¹⁶. Some studies have reported both presence and absence of association between the risk of oral/head and neck cancer¹⁷⁻¹⁹ and *NAT1*10* haplotype/allele (*i.e.* rapid acetylating allele). Investigators have also reported that NAT1 rapid acetylation status

could be a modulating factor for the risk of other cancers²⁰⁻²².

Two recent studies could not confirm the previous observations on association between cancer and NAT1 acetylation status^{16,23}. In an analytical study, highest levels of DNA adducts were detected in individuals who had both *NAT1* rapid and *NAT2* slow acetylation activity²⁴. A study, with low sample size, also reported significant association between fast acetylators at both NAT1 and NAT2 (*i.e.* with *NAT2*4/NAT1*10* diplotype) and risk of head and neck cancer even though *NAT1* and *NAT2* could not modify risk of cancer independently²⁵.

In Indian scenario, except one report on frequencies of *NAT1* alleles in a small number of south Indian people²⁶, there is no report of polymorphism at *NAT1* in other Indian populations. The objective of this study was, therefore, to find out extent of polymorphism at *NAT1* in healthy controls and estimate the risk of oral leukoplakia and cancer. Using previously published⁷ *NAT2* acetylation data from the same patients and controls, combination of NAT1 and NAT2 acetylation status of patients and controls was also analyzed to estimate risk of leukoplakia and cancer.

Material & Methods

The study was conducted in the Human Genetics Unit, Biological Sciences Division, Indian Statistical Institute, Kolkata, India.

Patients, controls and tobacco habit: Oral leukoplakia and cancer patients were recruited from the R. Ahmed Dental College and Hospital (a primary referral hospital at Kolkata, India) during 1999-2005 with the help of an oral pathologist from the same hospital. Individuals, without any lesions in oral cavity, were recruited from outpatient department as "controls". Patients and controls were unrelated. Information on age, sex, occupation, type of tobacco habit, tobacco use frequency and duration, alcohol intake and duration were recorded. Individuals, who had family history of any type of cancer and exposed to any carcinogen or toxic chemicals in work place, were excluded from the study. For inclusion, individuals should have any types of tobacco habits, be older than 25 yr considering the diseases have been manifested due to gene-environment interaction rather than due to genetic component/s only. The cases were confirmed as oral squamous cell carcinoma (SCC) and leukoplakia by histopathology⁷. Overall, 310 cancer, 224 leukoplakia and 389 control

individuals were included in the study. Written informed consent was obtained from each subject. Blood sample (5 ml) was drawn and stored for DNA analysis. This study protocol was approved by the Institutional Committee for Protection of Research Risk to Humans as also Ethics Committee of ISI, Kolkata.

All individuals 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*) and/or chewing/dipping of tobacco in different forms⁷. Patients and controls reported tobacco habits of either smoking or chewing/dipping or both. Lifetime tobacco chewing/dipping exposure was measured as chewing-year (CY): taking smokeless tobacco once in a day for 1 year=1 CY²⁷ and similarly, dose of tobacco smoking was measured as pack-years (PY)²⁸: 1 packet per day for 1 year=1 PY (1 pack=20 cigarettes or 40 *bidies*), since amount of tobacco present in one cigarette (750-1000 mg) is similar to that present in two *bidies* (850-1050 mg)⁵.

Genomic DNA was isolated from blood samples⁷ and study of histopathology was performed using cancer and precancer biopsy materials.

Genotypes at NAT1: Four single nucleotide polymorphisms (SNPs) at nucleotide positions (np) 445 (*G>A*), 559 (*C>T*), 1088 (*T>A*) and 1095 (*C>A*) on exon 2 (having rs#4987076, #4986782, #1057126, #15561) were genotyped by TaqMan method according to the published procedure²⁹.

Haplotypes and acetylation status: Frequencies of haplotypes at *NAT1* in all patients and controls were estimated from genotype data by a maximum-likelihood method using the expectation maximization algorithm (<http://www.broad.mit.edu/mpg/haploview>). Acetylation status of each individual was determined by genotype of that individual's haplotype data¹⁴.

Genotypes at NAT2: Polymorphic data at five SNPs at nucleotide positions (np) 341 (*T>C*), 481 (*C>T*), 590 (*G>A*), 803 (*A>G*) and 857 (*G>A*) (having rs#1801280, #1799929, #1799930, #1208, #1799931, respectively) and acetylation status of each individual were taken from our previous study⁷.

Combination of NAT1 and NAT2 acetylation data: All patients and controls were divided into sub-groups depending on the *NAT1* and *NAT2* acetylation status of each patient and control. Each sub-group of patients and controls was also compared to check whether a

particular combination of *NAT1* and *NAT2* acetylation status could modify risk of diseases.

Re-sequencing of PCR products: PCR products (~15% of total samples) from *NAT1* and *NAT2* were re-sequenced (ABI 3100 Genetic Analyzer; Applied Biosystem, USA) to confirm the genotypes determined by TaqMan method.

Detection of "9bp deletion" polymorphism at NAT1: This insertion/deletion polymorphism is spread over the sequence between np 1065-1090 and was determined by resequencing. For analyses, Phred, Phrap, Consed (<http://www.phrap.com/>) were used to determine the sequence of the PCR product and "9bp insertion/deletion" polymorphism.

Statistical analysis and power calculation: Age-, sex-, and tobacco dose-adjusted risk of oral cancer and leukoplakia was calculated as odds ratios (ORs) with 95% confidence intervals (CIs) by binary logistic regression analysis using SPSS statistical package (version 10.0, SPSS Inc., Chicago, USA). Chi-square test was used for comparison of categorical variables, such as genotype/allele frequencies, sexes, smokers, chewers, *etc.* and t-test was used to compare continuous variables such as age, CY, PY, *etc.* between patient and control groups.

Power of the sample sizes used in this study was calculated using the software "Power for Genetic Association Version 2"³⁰. The expected risk estimate (relative risk=2.0) was calculated specifying the values for a number of parameters such as minor allele frequency of 0.36, disease prevalence 0.0002, statistical power 80 per cent, marker allele frequency 0.36 (assuming disease allele is marker allele), $r^2=1.0$, case-control ratio =1.

Results

Demographic and tobacco habit information of patient and control populations are summarized elsewhere⁷. About 90 per cent of smokers had habits of both cigarettes and *bidis*, so it was not possible to analyze *bidi* and cigarette smokers separately. In patient and control groups, only a few (6 controls, 10 leukoplakia and 14 cancer patients *i.e.*, <5%) had occasional alcohol-drinking habit, therefore, alcohol consumption was also not considered in the analyses. All cancer and leukoplakia patients were incident cases and none of the controls had family history of cancer. Leukoplakia mostly affected buccal mucosa and commissural area in 146 cases (65%), buccal mucosa and alveolar sulcus in 45 (20%) and other sites including lip, tongue, *etc.* in

33 (15%). Most of the patients suffered from ulcerative lesion in 137 (61%) followed by homogeneous in 80 (36%) and nodular in 7 (3%) types of leukoplakia. Cancer affected sites were buccal mucosa and alveolar sulcus in 161 (52%), lip in 47 (15%), tongue in 37 (12%), buccal sulcus in 34 (11%), and retro molar area in 31 (10%). Morphologically these were classified as well in 201 (65%), moderately in 53 (17%) and poorly in 56 (18%) differentiated SCC.

The power of the study was >80 per cent with the sample sizes of 389 controls, 224 leukoplakia and 310 cancer samples. Polymorphic data at SNP on *NAT1* were checked for Hardy-Weinberg equilibrium³¹ but no deviation was observed ($P=0.1$ for SNP at

np1095). Analysing genotype data for pair-wise linkage disequilibrium (LD), it was observed that the 1088T allele and 1095C allele were in strong positive LD ($r^2 = 0.97$, $P<0.0001$). Since the homozygote variant genotypes at np445 and 559 were absent in the populations, LD was not estimated at these two SNPs. Some genotypes (~15% of total samples), determined by TaqMan method were cross-checked by sequencing method to ensure that genotypes were correctly determined.

Variant homozygotes at 445 np, 559 np and “9 bp del” polymorphic sites were absent and frequencies of heterozygotes at these SNP sites were very low. So, risk of disease was not observed to be modified

Table I. Distribution of genotypes in leukoplakia, cancer and control populations and risk of diseases

Genotypes at <i>NAT1</i>	Control	Leukoplakia	*Adjusted OR, 95% CI Control vs. Leukoplakia	Cancer	*Adjusted OR, 95% CI Control vs. Cancer
<i>np 445</i>	N=389 (%)	N=224 (%)		N=309 (%)	
G/G (Ile/Ile)	368 (95)	215 (96)	1.0 (reference)	301(97)	1.0 (reference)
G/A (Ile/Val)	21 (5)	9 (4)	0.8 (0.3-1.8)	8 (3)	0.9 (0.9-1.0)
A/A (Val/Val)	0	0		0	
<i>np 559</i>	N=389 (%)	N=224 (%)		N=310 (%)	
G/G (Gln/Gln)	388 (99)	224 (73)		308 (99)	
G/A (Gln/Arg)	1 (1)	0	ND	2 (1)	ND
A/A (Arg/Arg)	0	0		0	
<i>np 1088</i>	N=368 (%)	N=212 (%)		N=298 (%)	
T/T	171 (47)	102 (48)	1.0 (reference)	131 (44)	1.0 (reference)
T/A	146 (40)	78 (37)	0.9 (0.6-1.3)	123 (41)	1.0 (0.7-1.5)
A/A	51 (13)	32 (15)	1.0 (0.8-1.3)	44 (15)	1.0 (0.8-1.3)
9 bp del in between <i>np 1065-1090</i>	N=389 (%)	N=223 (%)		N=308 (%)	
Non-del	369 (95)	211 (94)	1.0 (reference)	298 (96)	1.0 (reference)
Heterozygous deletion	20 (5)	12 (6)	1.1 (0.5-2.4)	10 (3)	0.6 (0.3-1.3)
Homozygous deletion	0	0		0	
<i>np 1095</i>	N=388 (%)	N=223 (%)		N=309 (%)	
C/C	167 (43)	98 (44)	1.0 (reference)	128 (41)	1.0 (reference)
C/A	164 (42)	88 (39)	0.9 (0.8-1.1)	130 (42)	1.0 (0.9-1.0)
A/A	57 (15)	37 (17)	0.9 (0.9-1.1)	51 (17)	1.0 (0.9-1.0)

“9 bp del allele” is spread over between np 1065-1090. *Odds ratio was adjusted for age, sex, and tobacco dose.
Np, nucleotide position; ND, not determined
Some genotypes could not be determined so values of “n” are different at different polymorphic sites

Table II. Distributions of different *NAT1* haplotypes and acetylation phenotypes in patients and controls

@ Haplotype sequence	Allele	Acetylation phenotype	Frequency in control n (%)	Frequency in leukoplakia n (%)	# OR, 95% CI control vs. leukoplakia	Frequency in cancer n (%)	# OR, 95% CI control vs. cancer
<i>G-G-T-C</i>	<i>*4</i>	Normal	482 (62)	285 (64)	1.0 (reference)	393 (64)	1.0 (reference)
<i>G-G-A-A</i>	<i>*10</i>	Rapid	276 (36)	144 (32)	0.9 (0.7-1.1)	197 (32)	0.9 (0.7-1.1)
<i>G-G-T-A</i>	<i>*3</i>	Normal	6 (1)	7 (2)	ND	4 (1)	ND
<i>G-G-Δ9-A</i>	<i>*11A</i>	Rapid	10 (1)	9 (2)	ND	16 (3)	ND
<i>G-A-A-A</i>	<i>*14A</i>	Rapid	0	0	ND	1	ND
	Total		774	445		612	

@Genotypes at np 445 (*G/A*), 559 (*G/A*), 9bp deletion ($\Delta 9$ present/absent), 1088 (*T/A*) and 1095 (*C/A*) on *NAT1* were used to estimate haplotype frequencies. Other haplotypes were absent

Crude odds ratio

$\Delta 9$ allele: 9bp deletion allele is spread over the sequence between np 1065-1090

NAT1 *4 is wild type allele with normal acetylation activity; *NAT1* *10, *11A are rapid acetylation alleles. ND (not determined); since the sample sizes are very low under these categories, so they were not compared

by these SNPs. Frequencies of major, heterozygous and variant genotypes at each of 1088 and 1095np polymorphic sites were observed to be similar among control, leukoplakia and cancer samples and, hence, risk of diseases was not modulated by these genotypes (Table I). Alleles present in each genotype have been arranged into haplotypes in the order: np 445(*G/A*)-np 559(*G/A*)-np 1088 (*T/C*)-np 1095 (*C/A*). The

*G*⁴⁴⁵-*G*⁵⁵⁹-*T*¹⁰⁸⁸-*C*¹⁰⁹⁵ haplotype, which contains major alleles at all loci, was most common in control and patient populations followed by *G*⁴⁴⁵-*G*⁵⁵⁹-*A*¹⁰⁸⁸-*A*¹⁰⁹⁵ haplotype with variant alleles at np 1088 and 1095. Each haplotype corresponds to different acetylation status, such as normal or rapid or slow acetylating allele. Haplotype *G*⁴⁴⁵-*G*⁵⁵⁹-*T*¹⁰⁸⁸-*C*¹⁰⁹⁵, designated as *4 allele, is a reference haplotype with normal acetylation

Table III. Distribution of *NAT1* diplotypes and acetylation phenotypes in leukoplakia, cancer and control populations and risk of diseases

Acetylation phenotype	<i>NAT1</i> Genotypes/ diplotypes	Control N=381 (%)	Leukoplakia N=215 (%)	+ Adjusted OR, 95% CI control vs. leukoplakia	Cancer N=299 (%)	+ Adjusted OR, 95% CI control vs. Cancer
Normal	<i>*4/*4</i>	166 (43)	98 (45)		126 (42)	
	Total	166 (43)	98 (45)	1.0 (reference)	126 (42)	1.0 (reference)
Slow	<i>*4/*16</i>	2 (0.5)	1 (0.5)		2 (0.6)	
	Total	2 (1)	1 (1)	ND	2 (1)	ND
Rapid	<i>*4/*10</i>	145 (38)	77 (35)	0.9 (0.6-1.2)	121 (40)	1.1 (0.7-1.5)
	<i>*4/*11A</i>	14 (4)	5 (2)	ND	1(0.3)	ND
	<i>*10/*10</i>	50 (13)	32 (15)	1.1 (0.6-1.8)	43 (14)	1.1 (0.6-1.7)
	<i>*10/*11A</i>	4 (1)	2 (1)	ND	6 (2)	ND
	Total	213 (56)	116 (54)	0.9 (0.6-1.3)	171 (57)	1.0 (0.7-1.4)

+Age, sex and tobacco dose (*i.e.* smoking, smokeless, mixed habit) adjusted OR

NAT1 *4 is wild type allele with normal acetylation activity; *NAT1* *16 slow acetylation allele; *NAT1* *10, *11A are rapid acetylation alleles.

A pair of alleles makes a genotype/diplotypes and determines the acetylation status in an individual

ND (not determined): Since the frequencies of **4/*16*, **4/*11A* and **10/*11A* were very low so these were not compared, individually

NAT1 *16 allele had [AAA] insertion immediately after np 1091 and variant *A* allele at np1095

Table IV. Distributions of *NAT1* acetylators having different types of tobacco habit and risk of disease

Acetylators with different tobacco habit		Controls N (%)	Leukoplakia N (%)	*Adjusted OR, 95% CI control vs. leukoplakia	Cancer N (%)	*Adjusted OR, 95% CI control vs. cancer
Smoking	Normal acetylators	51 (35)	60 (46)	1 (Reference)	20 (38)	1 (Reference)
	Rapid acetylators	93 (65)	72 (54)	0.7 (0.4-1.1)	33 (62)	0.8 (0.4-1.6)
Total		144	132		53	
Smokeless tobacco	Normal acetylators	80 (47)	14 (44)	1 (Reference)	75 (43)	1 (Reference)
	Rapid acetylators	89 (53)	18 (56)	1 (0.4-2.2)	99 (57)	1.1 (0.7-1.7)
Total		169	32		174	
Mixed Habit	Normal acetylators	38 (51)	26 (46)	1 (Reference)	35 (44)	1 (Reference)
	Rapid acetylators	36 (49)	31 (54)	1.2 (0.6-2.5)	44 (56)	1.1 (0.6-2.2) 1.2
Total		74	57		79	

Only two individuals are *NAT1* slow acetylators so was not included in this Table.
*Adjusted for age, sex, and tobacco dose.

Table V. Comparison of distribution of *NAT1* acetylators with different *NAT2* acetylation status in patients and controls

<i>NAT1</i> acetylators	<i>NAT2</i> acetylation status	Controls N (%)	Leukoplakia N (%)	Control vs. leukoplakia <i>P</i> values	Cancer N (%)	Control vs. cancer <i>P</i> values
Normal	Slow	101 (61)*	62 (63)*		82 (65)*	
	Intermediate	56 (34)	28 (29)	0.5	37 (29)	0.7
	Rapid	9 (5)	8 (8)		7 (6)	
	Total	166	98		126	
Slow	<i>NAT2</i> Slow	1 (50)	1 (100)	ND	2 (100)	ND
	Intermediate	1 (50)	0		0	
	Rapid	0	0		0	
	Total	2	1		2	
Rapid	<i>NAT2</i> Slow	116 (55)*	63 (56)*		107 (63)*	
	Intermediate	86 (40)	43 (38)	0.8	57 (33)	0.2
	Rapid	11 (5)	7 (6)		7 (4)	
	Total	213	113		171	

*Individuals having *NAT1* normal-*NAT2* slow and *NAT1* rapid-*NAT2* slow acetylation status in cases and controls were also compared to estimate risk of diseases
OR=0.88, 95% CI: 0.57-1.37, for control vs. leukoplakia samples
OR=1.14, 95% CI: 0.77-1.68, for control vs. cancer samples

status and G⁴⁴⁵-G⁵⁵⁹-A¹⁰⁸⁸-A¹⁰⁹⁵ haplotype allele is variant *10 allele with rapid acetylation status^{14,16}. Other haplotypes frequencies were very low or absent in the samples. None of the haplotypes modified the risk of leukoplakia and cancer in the population (Table II). Each individual depending on the diplotype (*i.e.* haplotype in the pair of chromosomes) was designated as normal or rapid or slow acetylators in all three populations. Distribution of diplotypes or acetylators was not significantly different in controls, leukoplakia and cancer samples (Table III).

Among the patients and controls, some had only smoking habit, some of them had only tobacco chewing habit and remaining had mixed tobacco habit. Stratifying individuals on the basis of tobacco habit, it was observed that NAT1 rapid acetylators having smoking or chewing or mixed tobacco habits were not susceptible to leukoplakia or cancer in this study (Table IV). Working on these patients and controls, previously it was shown that NAT2 slow acetylation could not modulate the risk of leukoplakia and cancer⁷. Risk of the diseases could also be estimated using combination of NAT1 and NAT2 acetylation data of each individual in patients and controls. In this way, patients and controls were sub-grouped as having NAT2 slow, intermediate and rapid acetylation status in NAT1 normal, slow and rapid acetylators (Table V). Distribution of combined NAT1 normal-NAT2 slow, NAT1 normal-NAT2 intermediate and NAT1 normal-NAT2 rapid acetylators in cases and controls were not significantly different. Similarly, distribution of NAT1 rapid-NAT2 slow, NAT1 rapid-NAT2 intermediate and NAT1 rapid-NAT2 rapid acetylators in cases and controls were also not significantly different. Acetylators with NAT1 rapid-NAT2 slow combination were also not significantly susceptible to leukoplakia and cancer when compared with NAT1 normal-NAT2 slow combination (OR=0.88, 95% CI: 0.57-1.37 for leukoplakia; OR=1.14, 95% CI: 0.77-1.68 for cancer, Table V).

Discussion

Numbers of males and females, age and number of smokers and chewers were not similar in controls and leukoplakia and cancer patients (data not shown)⁷. So, adjustments for age, sex, and tobacco doses were done to estimate the risk of disease. In India, males use both smoking and smokeless tobacco whereas females use mostly smokeless tobacco. Although smokers and smokeless tobacco users are affected by leukoplakia and cancer but comparatively more male patients as

well as smokers were present in leukoplakia population. One of the reasons might be that leukoplakia is not life threatening initially, so females (mostly housewives from low income families) did not visit hospital due to several procedural steps required in the hospital. As a result, females, who were mostly smokeless tobacco users, were less represented in leukoplakia population.

Distribution of normal (43%), rapid (56%) and slow acetylators (1%) in the control population was observed to be similar to those (39, 60 and 1%, respectively) in Caucasian population¹⁹, but rapid acetylation status was different from those in Japanese and Turkish populations^{18,32}. Frequencies of *NAT1**4 and *NAT1**10 alleles in this control population (0.62 and 0.36, respectively) were different from those in a south Indian population (0.51 and 0.17, respectively) settled in Singapore²⁶.

Similar to a study on head and neck cancer in Turkish population²⁵, we also did not find any association between polymorphism at *NAT1* and risk of oral cancer. But unlike previous studies on Japanese and Western populations^{18,19}, rapid acetylation (*i.e.* *NAT1**10 allele) did not modulate the risk of oral cancer in this Indian population. Although sample sizes became low, NAT1 rapid acetylation could not modify risk of disease among tobacco habit-based stratified samples such as smokers, chewers and mixed habituals. This difference in result between Indian and Western and Japanese populations might be explained by small sample sizes studied in those populations, so possibility of false positive result could not be ruled out. Other explanations for this difference in observation might be attributed to the facts that majority of the cancer patients in our study had tobacco chewing/dipping habit (data not shown)⁷ whereas Western and Japanese populations had mostly tobacco smoking and alcohol abuse habit.

In a study, acetylation of *N*-hydroxy-4-aminobiphenyl was measured for analyses of carcinogen-DNA adducts and the highest levels of DNA adducts were found in individuals who had both *NAT1* rapid and *NAT2* slow acetylation activity²⁴. *NAT1* is expressed in the oral cavity itself; so *NAT1* rapid acetylators might have higher levels of *NAT1*-catalyzed O-acetylated carcinogens to produce DNA adducts. Although *NAT2* is not expressed in oral tissue, the effect of *NAT2* is thought to be mediated by metabolism in liver or gut and part of the modified carcinogens might be excreted through saliva. In

another study with low sample size, significant association was observed between combined NAT1-NAT2 fast acetylation (*i.e.* NAT2*4 and NAT1*10) and risk of head and neck cancer²⁵. We also considered NAT1 and NAT2 acetylation data of each individual to analyze the risk of disease. Since, acetylation status at NAT1 and NAT2 of each individual was assigned on the basis of genotypes, each patient or control was grouped into sub-groups with different combinations of NAT1-NAT2 acetylation status. Different combinations of NAT1-NAT2 acetylation sub-groups among patients and controls were compared to estimate the risk of disease. But, no association was observed between the risk of disease and different combinations of NAT1-NAT2 acetylation genotypes. One of the possibilities might be low sample size in each sub-group after combination of NAT1-NAT2 acetylation phenotypes. Since the sample size in each NAT1-NAT2 acetylation sub-group among patients and controls became low, they were not further stratified on the basis of tobacco habits to estimate risk of disease.

In conclusion, our results showed that NAT1 and/or NAT2 phenotypes did not enhance the risk of oral cancer and leukoplakia either alone or in combination. So, potential public health impact of this result is minimal at the moment as there is insufficient evidence implicating NAT polymorphism in the aetiology of oral cancer. Patients and controls could not be recruited matching sex, age and tobacco habits due to logistic problems and this was one of the limitations of the study. Another limitation was that the same control population was used to compare the data of leukoplakia and cancer patients but the number of smokers was more in the leukoplakia patients whereas number of smokeless tobacco users was more in cancer patients compared to controls. It would have been better to recruit two matched control populations for leukoplakia and cancer patients groups. In any association study, sample sizes play an important role to dissect out a real picture, thus a study with large sample size from an ethnic population is desirable.

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