Comparative distribution of Lysyl Oxidase (G473A) and NQO1 (C609T) polymorphism among tea-garden workers (habitual chewers of betel quid) of Darjeeling district and Kolkata city of West Bengal

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Abstract

Context: Chewing of processed arecanut products with tobacco and betel quid has been attributed to many oral pathological conditions. These products are very popular among the youngsters of lower economic groups. Genetic predisposition has been now identified as a major risk factor for increasing the susceptibility toward the disease among these chewers. **Aims:** Our study mainly aims to find out the predisposition of LOX (G473A) and NQO1 (C609T) polymorphisms and present a comparison between the population (habitually exposed to processed arecanut and smokeless tobacco products) of a metro-city Kolkata and the tea-garden workers of Darjeeling district of West Bengal. **Settings and Design:** Subjects for the study was recruited from various oral health check-up camps organized in the tea-gardens of Darjeeling district and Kolkata city. **Materials and Methods:** Genotyping analysis was done through a Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR-RFLP)-based approach. **Statistical Analysis Used:** A two-way contingency table analysis software (*JAVASTAT:* http://statpages.org/ctab2 × 2.html) using 95% confidence interval was used to study the distribution of genotypes among the populations. A $P < 0.05$ was considered to be significant. **Results:** The results indicates both the heterozygous and homozygous carriers of NQO1 C > T (609) was found to be significantly higher among the north Bengal tea-garden workers [OR 0.480 (0.280-0.82) $P = 0.01$; 0.218 (0.091–0.524) $P = 0.0001$], respectively. Interestingly CT (21% in both) and TT (8% and 7%, respectively) were found to be equally distributed in the two populations. For LOX G > A (473) a significantly higher number of Kolkata individuals were found to carry the heterozygous GA allele in individuals aged <30 years [OR 3.779 (1.684-6.547) $P = 0.001$]. However, none were carrier of heterozygous GA allele of Kolkata population as compared with 29% north Bengal tea-garden workers aged above 31 years. **Conclusions:** A close observation of occurrence of oral diseases over time among such a population will be helpful to identify risk genotypes responsible for betel quid-induced oral diseases.

Keywords: Genotype, Kolkata city, LOX G > A (473), NQO1 C > T (609), tea-garden workers

INTRODUCTION

The incidence of various oral potentially malignant conditions such as leukoplakia (LKP), lichen planus (LP), erythroplakia, oral submucous fibrosis (OSF) and oral squamous cell carcinoma (OSCC) is alarmingly rising in India.[1] Mostly these conditions are attributed by chewing of various processed arecanut products either alone or with flavoring agents (pan masala) or with smokeless tobacco and lime (gutkha) or a mixture of these with betel leaf (betel quid).[2] Increased selling of these products in attractive sachets and extreme popularization of these products among the people belonging to lower economic status mainly the youngsters have increased the rate of occurrence of these oral pathological conditions. Genetic predisposition has also been identified as one of the major factors that increase the susceptibility of a population toward risk of development of these conditions if exposed to the habitual chewing of "pan masala" and "gutkha."[3-6]

Lysyl oxidase and its family is the key enzyme in biosynthesis of collagen by oxidizing lysine residues of the procollagen molecules,[7] hence plays a major role in maintaining the structural integrity of the extracellular matrix (ECM). Abnormal expression of this enzyme may lead to connective tissue disorders mainly fibrotic diseases including liver fibrosis, scleroderma, kelosis, and pulmonary fibrosis. Evidences suggest LOX plays a major role in tumor progression by promoting remodeling of tumor tissue microenvironment thus helping in metastasis.[8,9] Recently a single nucleotide polymorphism (SNP) of LOX at position 473 of exon 1, which

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results in an Arg158Gln substitution at the peptidase cutting site has been reported to be associated with cancers of lung,[10] ovary,[11] breast,[12] osteosarcoma,[13] OSCC,[14] etc., in several populations.

Chewing of tobacco with betel quid (BQ) increases the concentrations of carcinogenic tobacco specific nitrosamines and reactive oxygen species in mouth and often results in oral carcinogenesis. The human NAD (P) H: Quinone oxidoreductase 1 gene (*NQO1*; DT-diaphorase) is a cytosolic flavoenzyme that detoxifies quinones to hydroquinones.[15] The enzyme NAD (P) H: Quinone oxidoreductase 1 (NQO1) acts as an antioxidant by catalyzing a 2-electron reduction that bypasses the need for two 1-electron reductions that can result in the production of DNA and protein-damaging reactive oxygen species. A C-to-T substitution at position 609 of NQO1 at exon 6, which results in a proline-to-serine change at residue 187 has been reported where no or lesser NQO1 activity has been detected. This lack of activity corresponds to a lack of NQO1 protein, which undergoes rapid turnover via the ubiquitin proteasomal pathway.[15] Previously NQO1 (C609T) polymorphisms has been found to be associated with chronic myeloid leukemias,[16,17] cancer following benzene toxicity,[18] bladder cancer,[19] esophageal, and gastric cardiac carcinomas.[20]

Our study mainly aims to find out the predisposition of LOX (G473A) and NQO1 (C609T) polymorphisms and present a comparison between the population (habitually exposed to processed arecanut and smokeless tobacco products) of a metro-city Kolkata and the tea-garden workers of Darjeeling district of West Bengal.

MATERIALS AND METHODS

Sample selection

Three hundred and twelve workers from various tea-gardens of Darjeeling districts who had the habit of chewing arecanut in some form or the other for at least a period of 1 year or more and gave consent to participate were recruited for the present study out of which blood samples could be collected from 200 individuals for evaluation of NQO1 C609T and LOX G473A polymorphism status.

Unrelated patients ($N = 992$) who reported to the department of Oral Pathology, Dr. R. Ahmed Dental College and Hospital (Kolkata, India) and other dental camps in and around Kolkata city, with a primary complaint of dental ailments and were found to be habitually exposed to oral habits and consented to collect blood samples ($N = 450$) were recruited for this study.

After obtaining informed written consent, all individuals were personally interviewed using a questionnaire. Information on age, sex, occupation, alcohol consumption, type of tobacco habit if any, arecanut use, its form and frequency, duration of habit, and economic status were recorded. The study was approved by the institutional ethical committee.

Blood collection

About 5 ml of blood was collected in tubes containing EDTA (anticoagulant) by vein puncture from all patients and control individuals and stored at 20°C until DNA isolation. Genomic DNA was isolated from whole blood according to standard procedure.

Genotyping analysis

Genomic DNA was isolated from venous blood samples of patient and control individuals by proteinase-K treatment and salt extraction procedure.[5] Genotyping was done using PCR-RFLP analysis. The PCR reaction-mix contained 100 pg DNA, 2 µl 2.5 mM dNTPs, 1 pg of each primer, 3U Taq Polymerase, 3 µl 10X Taq buffer, 0.6 µl 50 mM MgCl2 and 21.1 µl PCR-water in 30 µl.

For detection of NQO1 (C609T) forward: 5′AAG CCCAGACCAACTTCT-3′ and reverse: 5′-ATTTGAAT TCGGGCGTCTGCTG-3′ primers were used with an initial denaturation of 95°C for 2 min followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final elongation of 72°C for 10 min. Subsequently, the PCR products were digested with 20 U of HinfI (New England Biolabs, USA) for 3 h at 37°C and separated on a 6% polyacrylamide gel. The NQO1 wild-type allele shows a 172 bp PCR product resistant to enzyme digestion, whereas the null allele shows a 131 and 41 bp band. The frequency of the NQO1 genotypes was compared in the patient and control groups.

LOX gene encompassing the polymorphic region (G473A) was amplified using primers forward: 5′-CAC TGGTTCCAAGCTGGCTA-3′ reverse: 5′-GGAAGTAG CCAGTGCCGTAT-3′. The reaction conditions were: Initial denaturation at 95°C (2 min), followed by 35 cycles of 94°C (30 s), 60°C (30 s), 72°C (1 min), and final extension at 72°C (1 min). For genotyping, the PCR product was digested with restriction enzyme Pst1 (CTGCAG) and resolved on 6% polyacrylamide gel. Wild (G/G), homozygous mutant (A/A) and heterozygous mutant (G/A) gave one (259 bp), two (149 and 110 bp), and three (259, 149, 110 bp) bands, respectively.

Statistical analysis

A two-way contingency table analysis software (*JAVASTAT*: http://statpages.org/ctab2X 2.html) using 95% confidence interval was used to study the distribution of genotypes among the populations. A $P < 0.05$ was considered to be significant.

Results

Demographic distribution of the population studied

A total of 1304 individuals were included in the present study out of which a total of 312 workers were randomly selected

from different tea-gardens, out of which 282 were males and 30 females. The habit of chewing pan masala/gutkha was seen in almost 60% of males among all age groups. Clinical examination revealed signs and symptoms of OSF in about 28 individuals besides other mucosal disorders like LKP, tobacco pouch keratosis, and lichenoid lesions, but none of them gave consent for obtaining tissue for histopathological confirmation of the above mentioned mucosal disorders. The distribution of demographic status and pathological condition of the said population is described in Table 1 and Figure 1, respectively.

A total of 992 individuals with no oral pathological conditions were randomly selected from the out patients department of the Dr. R Ahmed Dental College and Hospital and other dental camps in and around Kolkata city. The total population was stratified according to age, gender, and habit. Majority of the males (544 out of 992) of the Kolkata and suburbs population were either pan masala/gutkha chewers or had the habit of chewing BQ with or without (+/-) tobacco, out of this 544 males 340 had the habit of chewing pan masala/gutkha only. Among the Kolkata population, the male pan masala/gutkha chewers in all age groups outnumbered the females except for the age group of 31-40 years, where only 25% of the

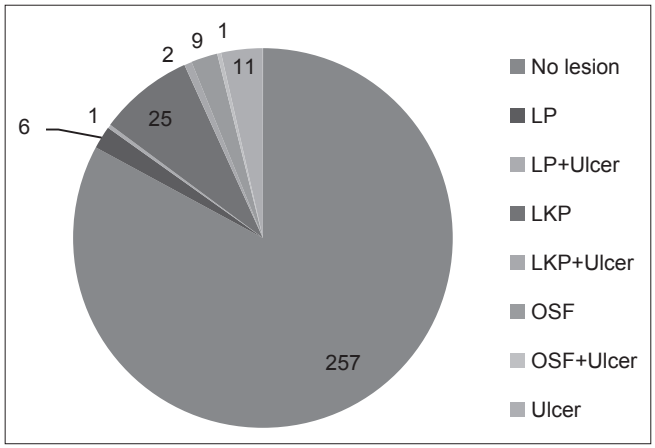

Figure 1: Prevalence of various oral lesions among pan masala or gutkha chewers of tea-garden workers

males were gutkha chewers as compared with 38% of females. However, the male BQ chewers (with or without tobacco) were greater in number (25.5% compared with 10.7% females). Mostly males of age group <30 years and >50 years had the sole habit of chewing pan masala/gutkha. The prevalence of chewing BQ with or without tobacco was found in only 6% in all age group among the tea-garden workers as compared with 34% in the Kolkata population.

Distribution of NQO1 C609T and LOX G473A genotypes among areca-chewers of Kolkata and North Bengal tea-garden workers

Comparative analysis for the distribution of NQO1 C609T and LOX G473A genotype was done to assess the variables present among the control subjects in Kolkata and north Bengal tea-garden workers [Table 2, Figure 2]. With the above distribution pattern, it was now possible to identify the individuals who could be at a higher risk of developing arecanut associated oral mucosal disorders.

The distribution of NQO1 C609T polymorphism was studied in the stratified population of Kolkata and north Bengal tea-garden workers. About 20% of the tea-garden workers were found to carry the minor T allele [OR 0.492 (0.27-0.897) $P = 0.02$]. About 22% of the Kolkata population was found to carry heterozygous T allele as compared with that of north Bengal tea-garden workers (17.5%). When the population was further stratified according to age both the heterozygous and homozygous carriers was found to be significantly higher among the north Bengal tea-garden workers [OR 0.480 (0.280-0.82) $P = 0.01$; 0.218 (0.091-0.524) $P = 0.0001$], respectively. Interestingly CT (21% in both) and TT (8% and 7%, respectively) were found to be equally distributed in the two populations.

When stratified according to gender, though not significant, but higher percentage of males of north Bengal tea-garden workers was found to be heterozygous carrier of NQO1 CT allele (27% compared with 20%) and homozygous TT allele (12% compared with 8%) compared to Kolkata population. No females of North Bengal tea-garden workers were found to be carrying TT allele but a higher percentage of females were heterozygous carrier of NQO1 CT allele (30%) as compared

Table 1: Demographic data of 992 individuals from Kolkata and 312 individuals of tea-garden workers

Age range (Years)	Kolkata population (*n*=992)				North Bengal tea garden workers (*n*=312)			
	Panmasala/Gutkha		Betelquid +/- tobacco		Panmasala/Gutkha		Betelquid +/- tobacco	
	Male (*n*=340)	Female (*n*=311)	Male (*n*=214)	Female (*n*=127)	Male (*n*=223)	Female (*n*=23)	Male (*n*=59)	Female (*n*=7)
<20	31 (38)	18 (21.2)	16 (20.7)	14 (18.1)	32 (76)	6 (14)	4 (10)	0
21-30	110 (41.6)	87 (32.9)	40 (15.2)	27 (10.3)	85 (76.5)	6 (5.4)	15 (13.5)	5 (4.5)
31-40	79 (25.8)	116 (38)	78 (25.5)	33 (10.7)	64 (75.3)	6 (6.9)	15 (17.2)	2 (2.3)
41-50	64 (30)	60 (28.2)	51 (24)	38 (17.8)	27 (55)	2 (4.0)	20 (40.8)	0
51-60	42 (39)	26 (24)	25 (23)	15 (14)	15 (65.2)	3 (13)	5 (21.8)	0
>60	14 (63.6)	4 (18.2)	4 (18.2)	0	0	0	0	0

Table 2: Comparative distribution of NQO1 C609T and LOX G473A genotypes among control subjects in Kolkata and tea-garden workers

Category/ parameter	Genotype	NQO1 C 609 T*		OR (95% CI)	*P* value	LOX G 473 A*		OR (95% CI)	*P* value
		Control *N*=450	TG Worker *N*=200			Control *N*=450	TG Worker *N*=200		
Total	W	324 (72)	124 (62)	Ref		392 (87)	154 (77)	Ref	
	H	99 (22)	55 (17.5)	0.689 (0.467-1.015)	0.06	58 (13)	36 (18)	0.633 (0.402-0.0996)	0.048
	M	27 (6)	21 (20.5)	0.492 (0.27-0.897)	0.02	0	10 (5)	-	
Age									
<31	W	171 (71)	72 (72)	Ref		182 (76)	83 (83)	Ref	
	H	51 (21)	21 (21)	1.023 (0.576-1.814)	0.9	58 (24)	7 (7)	3.779 (1.684-6.547)	0.001
	M	18 (8)	7 (7)	1.083 (0.443-2.636)	0.8	0	10 (10)	-	
≥31	W	153 (73)	52 (52)	Ref		210 (100)	71 (71)	Ref	
	H	48 (23)	34 (34)	0.480 (0.280-0.82)	0.01	0	29 (29)	-	
	M	9 (4)	14 (14)	0.218 (0.091-0.524)	0.0001	0	0	-	
Male	W	144 (72)	110 (61)	Ref		164 (82)	134 (74)	Ref	
	H	40 (20)	49 (27)	0.624 (0.384-1.012)	0.06	36 (18)	36 (20)	0.817 (0.49-1.364)	0.44
	M	16 (8)	21 (12)	0.582 (0.293-1.157)	0.15	0	10 (6)	-	
Female	W	180 (72)	14 (70)	Ref		228 (91)	20 (100)	Ref	
	H	59 (23.6)	6 (30)	0.765 (0.289-2.013)	0.59	12 (9)	0	-	
	M	11 (4.4)	0 (0)	-		0	0	-	
Oral habit									
Pan masala	W	130 (83.3)	68 (80)	Ref		136 (87)	69 (81)	Ref	
	H	19 (12.1)	17 (20)	0.585 (0.288-1.187)	0.187	20 (13)	12 (14)	0.846 (0.395-1.80)	0.67
	M	7 (4.6)	0 (0)	-		0	4 (5)	-	
Combined	W	194 (65)	56 (49)	Ref		256 (87)	85 (74)	Ref	
	H	80 (27)	38 (33)	0.608 (0.374-0.987)	0.04	38 (13)	24 (21)	0.526 (0.299-0.922)	0.025
	M	20 (8)	21 (18)	0.275 (0.140-0.539)	0.0001	0	6 (5)	-	

*HW *P* value: NQO1 C609T Control=0.77, TG workers=0.8, LOX G473A Control=0.65, TG workers=0.85, **TG: Tea garden

26.3% of the Kolkata population. About 4.4% of the females of the Kolkata population were found to carry homozygous minor T allele.

When stratified according to habit though a greater percentage (20%) of pan masala/gutkha chewers of north Bengal tea-garden workers presented heterozygous carrier of NQO1 CT allele compared with Kolkata (12%) but about 4.6% of pan masala/gutkha chewers of Kolkata presented homozygous TT allele compared with none of north Bengal tea-garden workers. Among BQ chewers with or without tobacco, both the heterozygous and homozygous carrier of NQO1 CT allele were found to be significantly higher in number in north Bengal tea-garden workers as compared with Kolkata [OR 0.608 (0.374-0.987) $P = 0.04$; 0.275 (0.140-0.539); $P = 0.0001$].

The distribution of LOX G473A polymorphism was studied in the stratified population of Kolkata and north Bengal tea-garden workers. One of the most interesting observations was that none of the individuals among Kolkata population was carrier of homozygous AA allele. A significantly higher number of individuals of north Bengal tea-garden workers were carriers of LOX GA allele [OR 0.633 (0.402-0.0996) $P = 0.048$].

When stratified according to age, a significantly higher number of Kolkata individuals were found to carry the heterozygous GA allele in individuals aged <30 years [OR 3.779 (1.684-6.547) $P = 0.001$]. However, none were carrier of heterozygous GA allele of Kolkata population as compared with 29% north Bengal tea-garden workers aged above 31 years.

When stratified according to gender, a higher percentage of the females were found to be heterozygous carrier as compared with none among the north Bengal tea-garden workers, but about 6% of the male north Bengal tea-garden workers was found to carry homozygous AA allele compared with that of Kolkata.

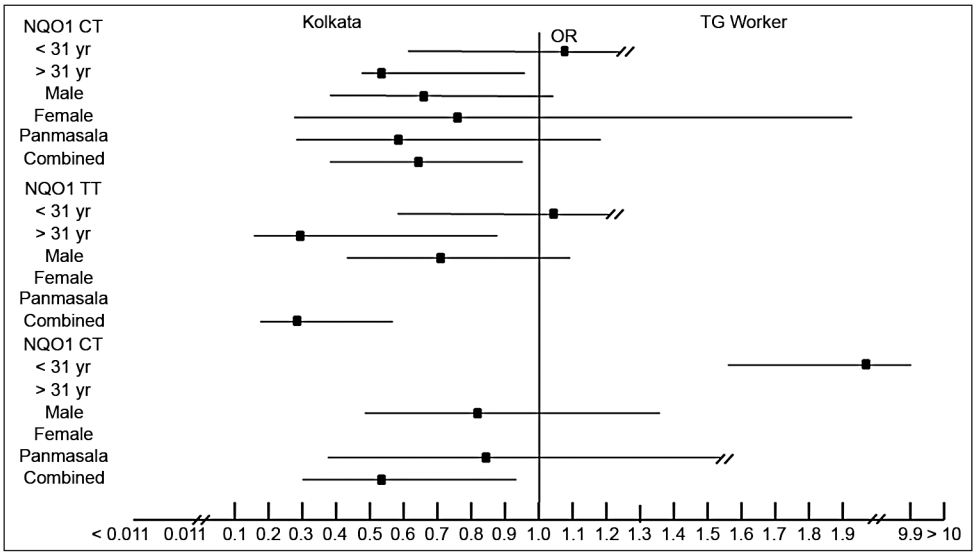

Figure 2: Odd's Ratio distribution of LOX GA and NQO1 CT polymorphisms among stratified populations of Kolkata and tea-garden workers

When stratified according to habit a significantly higher number of heterozygous carrier of GA allele was found who had the habit of chewing BQ with or without tobacco [OR 0.526 (0.299-0.922), $P = 0.025$].

Discussion

Existence of various oral habits has long been a part of human social life. Addiction to various habits such as smoking or chewing is in some case more psychological than a physical need. Many individuals irrespective of their nature of work like auto rickshaw drivers, bus drivers and conductors, factory labors, tailors, farmers, tea-garden workers get used to certain deleterious oral habits possibly because of their peer group, monotonous pattern of work, and socio economic background to which they belong. Discussion with various habitual smokers or chewers revealed that due to some ingredients in the composition or taste, or smell or the sense of well-being that they experience after using the product makes them addicted to such products. Traditionally BQ was the most popular and prevalent habit in ancient Indian culture, which was widely practiced and inculcated but since early 1980, both processed arecanut products such as pan masala and gutkha (arecanut + tobacco + lime + catechu + flavoring agents) were introduced in Indian market as commercial preparations.[21] Since then there has been an increase in its consumption, especially by the younger age groups due to its easy availability and competitive pricing even in remote areas. The rapidly increasing popularity of this habit can be judged from the reports that the Indian market for pan masala and gutkha is worth 25 billion (US$ 500 million).[22] Long hours of mucosal contact due to placing of tobacco (khaini) or arecanut-related products (utkha) have caused several oral disorders like LKP, erythroplakia, verrucous carcinoma, and OSF.[23] Though all of them are potentially malignant, erythroplakia has the highest malignant turnover potential (15-50%) while OSF has (7-14%), OSF causes much more incontinence and morbidity in patients as compared with other disorders due to its overall effect on the underlying connective tissue and muscles, which becomes highly inelastic causing trismus.[24]

Genetic predisposition and association of SNPs in various genes with increased risk of developing oral malignant and potentially malignant conditions is gaining importance. Lysyl oxidase, a major enzyme in collagen biosynthesis and its SNP at 473 (G > A), and NQO1, a major antioxidant gene and its SNP at 609 (C > T), has been found to be associated with various forms of cancers as discussed earlier. However, fewer or no data regarding distribution of NQO1 C609T and LOX G473A exist in India especially in the eastern region; hence it was necessary to have an epidemiological survey done. Our major aim was to observe the predisposition of this polymorphism among the tea-garden workers as these workers were purely endemic to that region and would not have to move out for treatment of any medical ailment, and had least effect of environmental pollution.

The mechanism underlying the correlation of NQO1 C609T polymorphism with the increased risk for developing various tumors likely resides in the different enzyme activities encoded by the NQO1 alleles.

The determination of the NQO1 C609T genotype may be valuable as a stratification marker in future intervention trials for OSF and OSCC. This finding may be particularly important in our country as most of the common people are habituated to areca-chewing in different modes and are susceptible to development of OSF and eventually OSCC. Moreover, since NQO1 C609T polymorphism was found to be positively

associated with many solid as well as blood malignancies, therefore, a practical approach for cost-effective tumor screening may be designed taking other such polymorphisms into account. In contrast, due to the relatively rare occurrence of the T/T genotype in the population, it is clear that in clinical practice NQO1 genotyping may be of importance only in combination with other risk factors.

Previously, a study on Taiwanese population reported LOX Arg158Gln allelotype in approximately 20% of areca-chewers while a higher prevalence of the same alleotype was noted in OSF cases >50 years age.[25] We found a significantly higher number of tea-garden workers to carry the heterozygous genotype. A close observation of occurrence of oral diseases over time among such a population will be helpful to identify risk genotypes responsible for BQ-induced oral diseases.

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