RESEARCH COMMUNICATION

XRCC1399 and hOGG1326 Polymorphisms and Frequencies of Micronuclei, Comet and Chromosomal Aberrations among Tobacco Chewers: A South Indian Population Study

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Abstract

DNA repair plays a critical role in protecting the genome of the cell from the insults of cancer-causing agents such as those found in tobacco. Reduced DNA repair capacity may constitute a significant risk factor for cancers. Recently, a number of polymorphisms in several DNA repair genes have been discovered, these polymorphisms may affect DNA repair capacity and thus modulate cancer susceptibility in exposed populations. In the present study, we explored the relationship between polymorphisms in the DNA repair gene XRCC1399 and hOGG1326 genotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR/RFLP) and risk of cancer development. 156 smokeless tobacco users and 70 controls without significant exposure to mutagens were recruited. Questionnaires were completed to obtain detailed occupational, smoking, and medical histories. A standard micronucleus assay, comet assay and chromosomal aberration assays were used as a marker of genetic damage. There were significant differences in the micronucleus (MN), Comet scores and chromosomal aberrations (CA) between smokeless tobacco users and control subjects by Student's t-test ($P<0.05$). These findings provide evidence for the view that polymorphisms in DNA repair genes may modify individual susceptibility to tobacco related cancers and justify additional studies to investigate their potential role in development of cancer.

Key Words: Genotoxicity - micronucleus and comet assays - RFLP - karyotyping - DNA repair enzymes

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Introduction

Tobacco is a uniquely dangerous consumer product that is addictive and harmful to human health when used as intended. Smokeless tobacco practices are common in some parts of the world, especially in India (Pershagen, 1996). Nicotine exposure is similar in smokeless tobacco users and smokers (Ebbert et al., 2006) often leading to strong physical dependence. As a rule, smokeless tobacco products contain high levels of nitrosamines with carcinogenic potency in experimental animals (Phillips et al., 2004). Habitual use of oral tobacco can increase the risk of oral cancer, but the data are insufficient to assess in detail the risks associated with many types of smokeless tobacco. Tobacco induces DNA adducts and oxidative DNA damage in human tissues. Formation of carcinogen–DNA adducts in critical genes can lead to mutations that alter protein function and that cause the carcinogenic progression of cells from normality to neoplasias (Hainaut et al., 2001; Cooper, 2002). Goran Pershagen suggested that smokeless tobacco use is related to genotoxicity affecting DNA repair pathways (Pershagen, 1996).

DNA repair pathways are responsible for maintaining the integrity of the genome in face of environmental insults and general DNA replication errors, playing a role in protecting it against mutations that lead to cancer (Lindahl, 1997). Various DNA alterations can be caused by exposure to environmental and endogenous carcinogens. Most of these alterations, if not repaired, can result in genetic instability, mutagenesis and cell death. DNA repair mechanisms are important for maintaining DNA integrity and preventing carcinogenesis (Friedberg et al., 1995; Qiao et al., 2002).

Chromosomal instability; a common feature of human tumors has its sources such as defects in chromosome segregation, telomere stability, cell cycle checkpoint regulation and repair of DNA damage (Reshmi et al., 2005). More than 130 genes are known to be involved in the repair of different types of DNA damage and the disruption of the transcription of these genes accounts for the lethal effects of DNA damage. Several polymorphisms in DNA repair genes have been reported to be associated with cancer risk (Ishikawa et al., 2005). The repair of DNA damage has a key role in protecting

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the genome from the insults of cancer-causing agents. Tobacco-related carcinogens cause a variety of DNA damage and DNA repair capacity plays an important role in tobacco-induced carcinogenesis (Wu et al., 2004). Cancer affects as many as 2, 74,000 people world wide annually, and the frequency is often indicative of the patterns of tobacco use, which is a major contributor to deaths from chronic diseases (Bhide et al., 1984).

Several polymorphisms in genes that participate in different DNA repair pathways, such as XPD, XPF, ERCC1, XRCC1, XRCC3, hOGG1, XPA, XPB and XPC, have been identified and related to cancer susceptibility (Duarte et al., 2005). Nucleotide excision repair (NER), base excision repair (BER), and double-strand break repair (DSBR) are the main DNA repair pathways. Repair of damaged DNA is an important biological phenomenon and it ensures the integrity of the transcribed genome (Rakhorst et al., 2006).

Base excision repair (BER) operates on small lesions such as oxidized or reduced bases, fragmented or non-bulky adducts, or those produced by methylating agents (Popanda et al., 2004). Base excision repair is a vital response to multiple types of DNA damage, including damage from tobacco exposure. Single-nucleotide polymorphisms (SNP) in these pathways may affect DNA repair capacity and increases the risk for cancer development (Popanda et al., 2004).

XRCC1 plays an important role in the base excision repair pathway and interacts with DNA polymerase, poly (ADP) ribose polymerase, and DNA ligase III. It also contains a BRCA1 COOH terminus domain, which is characteristic of proteins involved in cell cycle checkpoint functions; this domain can be responsive to DNA damage. 8-oxoG DNA glycosylase 1 (OGG1) and DNA repair enzyme X-ray repair cross-complementing group 1 (XRCC1) play a central role in the DNA BER pathway. OGG1 catalyzes the removal of 8-hydrodeoxyguanine (8-OHdG), which has been considered as a key biomarker of oxidative DNA damage (Kohno et al., 1998; Yamane et al., 2004). The hOGG1 codon 326 polymorphism was associated with the risk of lung (Le Marchand et al., 2002) esophagus (Xing et al., 2001) and stomach cancer (Tsukino et al., 2004).

XRCC1, a base excision repair protein that plays a central role in the BER pathway, has multiple roles in repairing ROS-mediated, basal DNA damage and single-strand DNA breaks (Xie et al., 2009). Three polymorphisms of DNA repair genes XRCC1 have been identified at codons 194 (Arg to Trp), 280 (Arg to His) and 399 (Arg to Gln) (Han et al., 1998). In particular, XRCC1 Gln 399 polymorphism resulting in single base substitution, which could affect binding to PARP (Poly (ADP-ribose) polymerase), may lead to deficiency of DNA repair. In cells lacking XRCC1 activity, an increased SCE frequency has been observed (Leia et al., 2002). In addition, defects in XRCC1 Gln 399 are associated with increased smokeless tobacco associated cancers, head and neck cancer and lung cancer (Wei et al., 1996; Divine et al., 2001). Some studies have found that both hOGG1 and XRCC1 polymorphisms are associated with a high risk of lung and other cancers (Sturgis et al., 2000).

Our study was to investigate the relationship between polymorphisms in XRCC1399 and hOGG1326 genotypes and their prognostic role in risk of developing cancer among tobacco chewers in south India.

Materials and Methods

Selection of subjects and collection of specimens

The study includes 226 subjects of both genders, 156 tobacco users and 70 healthy non-tobacco users as controls. Detailed dietary and lifestyle histories were collected mainly through self administered questionnaires according to the protocol published by the International Commission for Protection against Environmental Mutagens and Carcinogens (Carrano et al., 1988). Signed informed consent forms were collected from all participants. The study has been approved by the Institutional and local Ethical Committees.

Individuals having only tobacco chewing/dipping habit are termed as ''smokeless tobacco users'' in this study. In India, the prevalent tobacco chewing habits involve use of betel quid (betel leaf with tobacco, areca nut, and lime), ''gutkha'' (dried mixture of betel quid and tobacco sold in attractive pouches), ''mawa'' and ''zarda'' (flavored tobacco) or ''khaini'' (crude form of dried and ground tobacco with lime). Lifetime smokeless tobacco exposure was measured in terms of the frequency of chewing/dipping per day multiplied by the duration of habit. This is termed as chewing-year (taking smokeless tobacco once in a day for 1 year = 1 CY). All subjects were sub classified into <10yrs and >10years tobacco users. About 5 ml heparinised blood samples were collected from subjects by venepuncture using sterile syringes and stored in vials for Chromosomal aberration (CA) assay and Polymerase chain reaction-Restriction fragment length polymorphism (PCR-RFLP). Buccal cells were collected by cytobrush method in 0.9% saline. The resulting cell suspension was divided into 2 parts; for Micronucleus (MN) assay and Comet assay.

Genes and laboratory analysis

Micronucleus Assay: Microscopical slides (5-15 per sample) for the MN analysis were prepared at the site of sampling. The cell suspension was centrifuged at 2000rpm, the supernatant was removed and a small amount of physiological NaCl was added to obtain a milky cell suspension. The slides were prepared taking 50 ml of sample per slide. The smears were air dried, fixed in methanol: acetic acid (3:1) and were stained with Feulgen. The cells were analysed under a light microscope (Leitz, Germany) with 40X dry-lens objective.

Alkaline Comet Assay: The buccal cell suspension was centrifuged, the pellet obtained was mixed with 0.7% low melting agarose (LMA) and placed on fully frosted roughened slides previously coated with 1% normal melting point agarose. To the solidified agarose, a third layer of 0.1% LMA was applied and were immersed in freshly prepared ice cold lysis solution for 1 hour. The slides were then electrophoresed, neutralized, dried and stained with ethidium bromide.

A total of 100 randomly captured comets from each slide were examined at 400X magnification using an epifluorescence microscope (Zeiss) connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments Ltd, UK). A computerized image analysis system acquires images, computes the integrated intensity profiles for each cell, estimates the comet cell components and then evaluates the range of derived parameters. To quantify the DNA damage tail length (TL) and tail moment (TM) were evaluated. Tail length (length of DNA migration) is related directly to the DNA fragment size and presented in micrometers. It was calculated from the centre of the cell. Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail.

GTG banding :0.5ml of the collected blood was inoculated under asceptic condition into a culture vial containing 5.0ml of Mc Coy's 5A culture medium (Sigma) containing 1.0 ml of serum and 0.2ml of Phytohemagglutinin (PHA) and incubated at 37˚C for 72hrs with periodical shaking. 0.01% of colchicine solution (Sigma) was added to the culture. The cells were centrifuged (1,000g) and 0.75M KCl was added to the cell pellet. After incubation, cells were fixed with 1.0ml of fixative (methanol: acetic acid, 3:1) and centrifuged till we obtained a colorless pellet. A modified technique of previously described procedure (12 article) was employed to obtain chromosomal bands. The slides bearing chromosomal spreads were treated with 0.25% Trypsin (Sigma), stained in 4% Giemsa solution (HIMEDIA), air dried and observed under microscope (100X).

Restriction Fragment Length Polymorphism

The polymorphism of XRCC1 (codon 399) and hOGG1 (codon 326) were analyzed by a PCR-RFLP procedure with the following nucleotide primers,
for XRCC1399, FP: 5' CCC CAA GTA CAG CCA GGT C 3' RP: 5' TGT CCC GCT CCT CTC AGT AG 3'
for hOGG1326 FP: 5' GGA AGG TGC TTG GGG AAT 3' RP: 5' ACT GTC ACT AGT CTC ACC AG 3'
PCR conditions were an initial denaturing step at 94˚C for 3 minutes, followed by 40 cycles at 94˚C for 1 minute, annealing at 55˚C for 1 minute (for XRCC1399), 57˚C for 1 minute (for hOGG1326), initial extension at 72˚C for 1 minute and final extension at 72˚C for 7 minutes. A negative control without template DNA was used in each run. The PCR products were resolved in 2% agarose gel stained with ethidium bromide (0.5 microgram/ml).

The PCR products were then digested with restriction endonuclease, MspI and PstI (Invitrogen Life Technologies) for XRCC1399 and hOGG1326 respectively, as recommended by the manufacturer. The fragments obtained were electrophoresed in 1.2% agarose gel at 100V for 1.5 hrs. Gels were stained with ethidium bromide and photographed under UV light. The profiles obtained were confirmed in triplicate.

Statistical analysis

All statistical analyses were conducted using the SPSS for Windows statistical package, version 11.5 (IL, USA). Distribution of every variable obtained in this study did not depart significantly from normality and therefore parametric tests were considered adequate for the statistical analysis of these data. Samples were coded at the time of preparation and scoring. They were decoded before statistical analysis for comparison. Mean and standard deviation (SD) was calculated for each biomarker. The significance of the differences between controls and the tobacco chewers' end point means were analysed using Students 't' test (mean CA, comet tail length and MN frequency). Mean values and SDs were computed for the scores and statistical significance ($P<0.05$) of effects (exposure and age) and mutated nucleotide and base excision repair gene expression levels were analysed by Students 't' test. We used linear regression analysis to evaluate whether the consumption of smokeless tobacco associated with the mutation of these repair genes.

Results

In the present study, 70 healthy controls and 156 smokeless tobacco users were recruited from the same ethnic population living in the same geographic location from south India. The main characteristics showing the profile of the users and control subjects who took part in the study are presented in Table 1. The studied individuals were classified according to their age, sex, duration of chewing habit and range of tobacco consumption per day. The mean age of the chewers group was 45.4 ± 10.2, ranging from 35 -65 and that of controls was 50.4 ± 8.7 years.

Table 2 shows the data regarding the total number of cells with micronuclei (MN) of both the chewers and controls evaluated in this research. A significant difference ($p<0.05$) was found between the chewers and the control group regarding the average number of MN. There was no considerable difference between the male and female tobacco chewers regarding the averages of MNC, but there

Table 1. Characteristics of Controls and Tobacco Chewers

Characteristics	Controls n=70	Tobacco chewers n=156
Mean age	50.4 ± 8.74	45.4 ± 10.2
Mean duration of consumption	-	16.2 ± 7.32
Range of tobacco consumption	-	5.45 ± 0.5
Females	18	52
Males	52	104

*P<0.05. Values are represented in mean ± SD

Table 2. Frequency of MNC Observed in Controls and Tobacco Chewers

		Subjects	Micronuclei
Control	Male	52	0.86 ± 0.52
	Female	18	1.2 ± 0.91
Tobacco chewers	Male	104	2.2 ± 0.67*
	Female	52	2.0 ± 0.47*

*P<0.05. Values are represented in mean ± SD

Table 3. Mean Comet Tail Length (in mm) by Age and Years of Tobacco Consumption

Parameters		Controls		Tobacco chewers	
		N	Tail length	N	Tail length
Age (Years)	<45	19	32.1 ± 1.14	88	34.3 ± 1.12
	≥45	51	32.5 ± 1.01	68	34.3 ± 1.21
Time of tobacco consumption (Years)					
	<10	-	-	74	35.2 ± 1.16
	≥10	-	-	82	35.7± 1.10
Range of tobacco consumption (Count)					
	<10	-	-	85	34.7 ± 1.05
	≥10	-	-	71	35.0 ± 1.15

Comet tail (mm) mean ± SD

Table 4. Total Chromosomal Aberrations (CA) and Chromatid -type (CTA) and Chromosome-type (CSA)

	Total CA	(CTA)	(CSA)
Controls	1.21 ± 0.91	0.52 ± 0.46	0.69 ± 0.78
Tobacco chewers	2.18 ± 1.31*	1.02 ± 1.14	1.16 ± 1.23

*P<0.05; Data are mean ± SD

Table 5. Genotype and Allele Frequencies for the Gln 399 Arg Polymorphism of the XRCC1 Gene

Group	Gln/Gln	Gln/Arg	Arg/Arg	Gln	Arg
Control	28 (40.0)	19 (27.0)	23 (32.9)	53.5	46.4
Tobacco chewers	78 (50.0)	24 (15.4)	44 (28.2)	57.7	35.9

Data are number (%)

Table 6. Genotype and Allele Frequencies for the Ser 326 Polymorphism of the hOGG1 Gene

Group	Ser/Ser	Ser/Cys	Cys/Cys	Ser	Cys
Control	29 (41.4)	16 (22.9)	25 (35.7)	52.8	47.1
Tobacco chewers	73 (46.8)	25 (16.0)	58 (37.2)	54.8	45.2

Data are number (%)

was a significant difference between each one of these groups and the control group (p<0.05). The male chewer group revealed a significant induction of mean MNC when compared with its controls (2.2 versus 0.86; p<0.05) and in women, a statistical increase of mean MNC (2.0 versus 1.2) was observed when compared with its controls.

The extent of basal DNA damage evaluated by comet assay in buccal cells of all the study subjects as measured by mean comet tail length is presented in Table 3. The mean tail length in the 156 tobacco chewers was significantly increased than that in 70 control subjects. There was a significant increase in the mean tail length of tobacco chewers when compared to controls in both age groups, <45 and >45 (34.3 ± 1.12 vs. 32.1 ± 1.14 and 34.3 ± 1.21 vs. 32.5 ± 1.01). No statistical significance was observed in the mean comet tail length among tobacco users with respect to their time and range of tobacco consumption (analysis of covariance, p>0.05).

We detected higher frequency of total chromosomal aberrations in tobacco chewers group than control (2.18 ± 1.31 and 1.21 ± 0.91). In exposed group we did not detect any difference between chromatid – type (CTA-type) and chromosome – type (CSA – type) - 1.02 ± 1.14 vs. 1.16 ± 1.23 (Table 4).

In PCR-RFLP, normal XRCC1399 gene produced one band whereas heterozygous individuals produced 3 bands when digested with MspI. Likewise when normal hOGG1326 gene was digested with PstI, single band was observed; but in heterozygous individuals, 3 bands were observed.

The XRCC1 genotype distributions in smokeless tobacco chewers are summarized in Table 5. The homozygous Arg/Arg genotype was present in 28.20%, heterozygous genotype in 15.38% and the homozygous Gln/Gln genotype in 50%. The prevalence of the 399Gln genotype was significantly higher in smokeless tobacco chewers than in controls. The distributions of hOGG1 genotype in smokeless tobacco chewers are represented in Table 6. The homozygous Cys/Cys genotype was present in 37.18%, heterozygous genotype in 16.02% and the homozygous Ser/Ser in 46.79%. The presence of polymorphisms in both XRCC1 and hOGG1 genotypes in both control and chewers along with the mean comet score, MN and CA frequency is comparable for further evaluations (Table 7).

Discussion

Genotoxicity biomarkers have received considerable interest as tools for detecting human genotoxic exposure and effects, especially in health surveillance programs dealing with chemical carcinogens. The use of a biomarker as an indicator of disease development is that the marker will translate into a relationship between exposure and disease (Schatzkin et al., 1990). The only cytogenetic biomarker that has been outlined previously is the technique of classical metaphase analysis for measurement of CA in human lymphocytes. While MN assay is one of the most commonly used methods for measuring DNA damage in human populations because it is relatively easier to score MN than CA (Fenech, 2002).

Micronucleus test has been receiving increasing attention as a simple and sensitive short-term assay for detection of environmental genotoxicants (Stich et al., 1982). By applying this test, an elevated incidence of micronuclei has been recorded in the buccal mucosa cells of smokeless tobacco chewers in our previous study

Table 7. Effects ofXRCC1 and hOGG1 Polymorphisms on DNA damage in Smokeless Tobacco Chewers

Group	XRCC1		hOGG1		Comet	MN	CA
	Genotype	N (%)	Genotype	N (%)	Mean ± SD	Mean ± SD	Mean ± SD
Controls	Gln/Gln	28 (40.0%)	Ser/Ser	29 (41.4%)	32.3 ± 1.07	1.03 ± 0.71	1.2 ± 0.91
	Arg/Arg	23 (32.9%)	Cys/Cys	25 (35.7%)			
	Gln/Arg	19 (27.0%)	Ser/Cys	16 (22.9%)			
Chewers	Gln/Gln	78 (50.0%)	Ser/Ser	73 (46.8%)	34.9 ± 1.13	2.1 ± 0.57	2.2 ± 1.31
	Arg/Arg	44 (28.2%)	Cys/Cys	58 (37.2%)			
	Gln/Arg	24 (15.4%)	Ser/Cys	25 (16.0%)			

(Sellappa et al., 2009). The present study has identified increasing frequency of MN among tobacco chewers and controls in response to the specific DNA repair genes. Although the buccal mucosal micronucleus has been applied by other investigators to assess the genotoxic action of several compounds in cigarettes (Stich et al., 1983; Nair et al., 1991; Rosin et al, 1992); none of the studies have been done in tobacco chewers.

The percentage of MN cells is significantly higher in smokeless tobacco chewers than in controls. There was significant difference between the percentage of MN cells in smokeless tobacco chewers and controls. Carcinogenic and mutagenic compounds, including tobacco-specific nitrosamines present in smokeless tobacco forms (Hecht et al., 1988), are believed to be responsible for the induction of micronuclei (Erenmemisoglu et al., 1995; Ozkul, 1995). These compounds are produced from nicotine by bacterial or enzymatic activity. The same formation occurs in the mouth under the influence of saliva (Winn, 1981).

The results found in the tobacco chewers group and the fact that these subjects are in contact mainly through nicotine, a recognized genotoxic agent that cause chromosome breaks, justify the significant increase in the MNC frequency found in this work. These findings prove that the substances to which the tobacco chewers are exposed cause an increase in the number of MN and are, therefore, genotoxic.

Understanding the mechanisms of DNA damage and repair has grown tremendously (Yu, 1999). Comet assay helps us obtain information on inter-individual differences in DNA repair kinetics by the measurement of DNA damage at multiple time points. Due to its good sensitivity for detecting genetic damage at individual cell level, assay has been widely used to assess the tobacco consumption. Numerous studies employing the comet assay for comparative analysis of DNA strands and other end points of genotoxicity have been conducted (Singh et al., 1988; Betti et al., 1995; Speit et al., 1995; Maluf et al., 2000). The association of CA and cancer risk is seen in subjects with non-carcinogenic exposure and in those with no history of exposure to carcinogens through smokeless tobacco usage (Milic et al, 2008). The results of a report have established diverse buccal cell changes indicated by CA assay and its association with smokeless tobacco (Proia1 et al., 2006).

As shown in our results, the control group shows a minimum number of MN comet tail lengths and CA when compared with the tobacco chewers. It indicates that the habit of tobacco chewing cause an increased level of DNA damage. The present study confirms that duration of exposure to smokeless tobacco plays an important role in genetic damage.

The mutagen in the study essentially induce, oxidative DNA damage, single strand breaks and double strand breaks deficiencies in BER (XRCC1399 and hOGG1326) and are expected to influence the frequency of MN harboring accentric chromatid/chromosome fragments. The smokeless tobacco contents may also induce MN through their interaction with spindle tubulins leading to chromosome loss. Several associated studies have addressed the link between DNA repair polymorphisms and MN induction, but the evidence that DNA repair polymorphisms influence MN frequencies remain limited (Aka et al., 2004; Godderis et al., 2004; Mateuca et al., 2008).

We investigated the relationship between polymorphisms of DNA repair genes XRCC1399 and hOGG1326 and smokeless tobacco-induced DNA damage. Interestingly, when gene-exposure interactions are considered, the XRCC1399 Gln/Gln and hOGG1326 Ser/Ser genotypes is associated with DNA damage level in subjects with smokeless tobacco exposure, but we did not find any marked relationship between other XRCC1 and hOGG1 polymorphisms and risk of smokeless consumption. DNA damage is an early and essential step in the process by which genotoxic carcinogens initiate the carcinogenic process. The detection of DNA adducts in human tissues is a useful and appropriate means to assess human exposure to such agents (Phillips, 2005).

In conclusion, the genotoxic effects of smokeless varieties of tobacco should be considered, in addition to their carcinogenic effects and other known risks. Unfortunately, since it was difficult to collect exact exposure data, we cannot evaluate genotypic exposure interactions.

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