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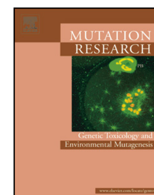

Assessment of genotoxic and molecular mechanisms of cancer risk in smoking and smokeless tobacco users

R. Chandirasekar[a,*], B. Lakshman Kumar[b], K. Sasikala[a], R. Jayakumar[c], K. Suresh[a], R. Venkatesan[a], Raichel Jacob[a], E.K. Krishnapriya[a], H. Kavitha[b], G. Karthik Ganesh[d]

[a] *Unit of Human Genetics, Department of Zoology, Bharathiar University, Coimbatore 641 046, Tamilnadu, India*
[b] *Departments of Biotechnology and Zoology, Kongunadu Arts and Science College, Coimbatore 641 029, India*
[c] *Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia*
[d] *Department of Bioinformatics, Bharathiar University, Coimbatore 641 046, Tamilnadu, India*

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ABSTRACT

Inexpensive forms of tobacco are widely used in developing countries such as India. We have evaluated genotoxicity endpoints (chromosome aberrations, micronucleus frequency, comet assay) and polymorphisms of the *XRCC1* and *p53* genes among smokers and smokeless tobacco (SLT) users in rural Tamilnadu, South India. Cytogenetic, DNA damage and SNP analyses were performed on peripheral blood samples; micronucleus frequency was measured in peripheral blood and buccal mucosa exfoliated cells. Both categories of tobacco users had elevated levels of genotoxic damage. SNP analysis of tobacco users revealed that 17% carry the *XRCC1* gln399gln genotype and 19% carry the *p53* pro72pro genotype. Both genotypes are associated with increased risk of cancer.

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1. Introduction

Smokeless tobacco (SLT) use is a growing health problem, particularly in Asia and Africa. There are about 100 million users of SLT products in India and Pakistan [1]. Smoking is a major cause of cancer, cardiovascular diseases, and chronic obstructive pulmonary diseases [2]. Annual mortality ascribed to tobacco use in India, has been estimated to be 1 million [3].

Epidemiological data have shown a correlation between the use of SLT products, premalignant lesions of the oral cavity, and incidence of oral cancer [4]. It is estimated that some 600 million people use BQ worldwide. The International Agency for Research on Cancer has identified tobacco smoking and betel quid (BQ) chewing, with or without tobacco, as group 1 human carcinogens. Some studies have investigated the effects of BQ chewing and cigarette smoking on OSF (oral sub mucous fibrosis) and OL (oral leukoplakia) [5–7]. SLT use is genotoxic and may affect DNA repair pathways [8–11].

One of the best techniques for studying the effects of environmental factors on genetic stability in human cells is the micronucleus (MN) test [12]. MN may be products of early events in carcinogenesis, especially in the oral cavity, which is directly exposed to cigarette smoke [13].

Chromosomal instability is a common feature of human tumors, and may result from defects in chromosome segregation, telomere stability, cell cycle checkpoint regulation, and DNA repair [14]. More than 130 gene products are involved in DNA repair. Several polymorphisms in DNA repair genes may be associated with cancer risk [15].

The comet assay is widely used for assessment of DNA damage in occupationally and environmentally exposed populations [16]. The aim of this investigation was to test whether smoking and SLT affect the frequencies of CA and MN in peripheral blood lymphocytes, and of MN in buccal epithelial cells, and also to analyze specific genetic polymorphisms in smokers and SLT users.

2. Materials and methods

The study group comprised 183 smoking (beedi and cigarette) and SLT-exposed subjects and an equivalent number of healthy control subjects, matched for age (±5 y), gender, and socio-economic conditions. Samples were collected from persons residing in the Eastern Ghats of Tamilnadu region, South India. The study

Abbreviations: CAs, chromosomal aberrations; MN, micronucleus; SLT, smokeless tobacco; BQ, betel quid; TL, tail length; TM, tail moment.

* Corresponding author at: Human Genetics Laboratory, Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore 641 046, Tamil Nadu, India. Tel.: +91 9942520304.

E-mail addresses: genechandru@gmail.com, geneticschandru.sekar@gmail.com (R. Chandirasekar).

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was approved by the Institutional Review Committee, Bharathiar University, and informed written consent was obtained from all participating subjects. Information regarding exposure duration, types of tobacco product used, health status, habit and occupation was recorded using a standard questionnaire. 183 individuals were selected as exposed subjects: 71 males and 103 females (smokers or smokeless tobacco users). Equal numbers of unexposed (i.e., not exposed to tobacco smoke) individuals served as controls. The exposed subjects were categorized based on duration of exposure and health condition. Further grouping was done based on age of the subjects.

A sample of venous blood (2–3 ml) was drawn into heparin-coated vacutainers. The exfoliated buccal mucosal cells were collected into 0.9% saline solution. Great care was taken not to harm the subjects and appropriate medical procedures were followed during blood collection.

2.1. Chromosomal analysis

A volume of blood (0.5 ml) was added to RPMI 1640 medium (4.5 ml) supplemented with 15% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.2 ml phytohemagglutinin, in a sterile culture tube. The culture tube was incubated at 37 °C for 72 h. After 71 h, the cultures were treated with colcemid (0.01 mg/ml) to arrest the cells at mitotic stage. Lymphocytes were harvested after 72 h by centrifuging to remove culture medium (800–1000 rpm). Then, pre-warmed (37 °C) hypotonic solution (KCl 0.075 M) was added and the sample was left undisturbed for 20 min. The cells were treated twice with Carnoy's fixative (methanol:acetic acid, 3:1). The cells were sprayed on microscopic slides and dried on a hot plate (56 °C) for 2 min. The slides were stained with Giemsa and visualized under the microscope [17]. For the chromosomal aberration analysis, 50 well-spread metaphase plates were analyzed for each subject, under the oil immersion lens of a Leica light microscope (100×), and photographed.

2.2. Micronucleus analysis in peripheral blood lymphocytes

Lymphocyte cultures were set up according to the following standard method of Fenech and Morley [18]. Cytochalasin B was added to the cultures at final concentration 6 μg/ml after 44 h. At the end of the incubation time (72 h), cells were harvested by centrifugation, hypotonic solution (0.075 M KCl) was added, and then the samples were left undisturbed for 1 min. The cells were fixed in fresh fixative solution; this step was then repeated. About 500 cells were scored from each subject.

2.3. Micronucleus analysis in buccal epithelial cells

After a mouthwash with sterile water, oral mucosal cells were obtained by scraping the right/left cheek mucosa with a moist wooden spatula [12,19]. Cells were transferred to a tube containing saline solution (0.09%) and centrifuged at 800 rpm for 5 min. The cells were fixed and dropped onto a pre-cleaned slide. Later, the air-dried slides were stained using the Feulgen/Fast-Green method and examined under a light microscope at 400× magnification to determine the frequency of micronucleated cells. For each sample, 1000 cells were scored according to the criteria described by Sarto et al. [20].

2.4. Single cell gel electrophoresis (SCGE)

SCGE was performed as described by Tice et al. [21] with some modifications. Briefly, after lysis (2.5 M NaCl, 100 mM EDTA, 10 mM Tris and 1% sodium sarcosinate, pH 10) at 4 °C for 48 h, the cells were dropped into a new lysis solution with 140 μl of proteinase K (10 mg/ml) at 37 °C for 2 h. After electrophoresis, the slides were gently removed and the alkaline pH was neutralized with 0.4 M Tris–HCl, pH 7.5. Ethidium bromide solution (20 mg/ml, 75 μl) was added to each slide, and a cover glass was placed over the gel.

DNA migration was analyzed on a Nikon microscope with fluorescence equipment and measured with a scaled ocular. For the evaluation of DNA migration (total image length), 100 cells were scored for each individual. The length of the DNA migrated in the comet tail, which is an estimate of DNA damage, was measured using an ocular micrometer. Quantification of the DNA damage for each cell was calculated as: comet tail length (μm) = (maximum total length) − (head diameter). DNA damage was further quantified by visual classification of cells into categories of 'comets' corresponding to the amount of DNA in the tail. Images of 100 randomly selected cells were analyzed from each individual. Comet tail lengths (nuclear region + tail) were measured in arbitrary units. One unit was approximately 5 μm at 200× magnification. The fluorescence microscope was equipped with a BP546/12-nm excitation filter and a 590-nm barrier filter. 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 center of the cell. Tail moment was calculated as the product of the tail length and the fraction of DNA in the comet tail.

2.5. Gene polymorphism analysis

Genomic DNA was isolated according to the standard protocol [22]. XRCC1 (codon 399) genotype was analyzed through PCR-RFLP was carried out according to methods previously described [23,24]. The XRCC1 PCR product (615 bp) was digested using 5 U of *Hpa*II (Fermentas, Germany) restriction enzyme. The digested products were resolved on an 8% polyacrylamide gel electrophoresis for 135 min at 65 V/cm in 1× TBE buffer. Detection of bands was performed by the silver-staining method. An undigested fragment (615 bp) represents gln/gln; a pair of fragments (375 and 240 bp) represents arg/arg; presence of three bands (615, 375, and 240 bp) represents an arg/gln heterozygote. The p53 polymorphisms were identified by digesting the PCR products (199 bp) with 5 U *Bsh*12361 (Fermentas, Germany) for 4–16 h according to the method of Ara et al. [25] An undigested PCR product (199 bp) represents homozygous Trp5372P; two fragments (113 and 86 bp), represent homozygous $Trp53^{72R}$; three fragments (199, 113, and 86 bp) represent heterozygous Trp53 ^{72R}/$Trp53^{72P}$ for codon 72.

2.6. Statistical analysis

Statistical analysis was carried out using the statistical software program for windows (SPSS Version 16). Analysis of variance (one way-ANOVA) was performed to compare the frequency of chromosomal aberrations and micronuclei between tobacco users and controls. Gene polymorphic variant were analyzed using χ^2 test. $p < 0.05$ was used as the criterion of significance.

3. Results

3.1. Demographical details

The aim of the present study was to carry out the cytogenetic and genotoxic analysis in individuals with tobacco usage and to compare the data with healthy controls. Table 1 shows the age and years of usage of smoke and smokeless tobacco in the population studied. Among the 183 subjects enrolled in this study, 91 individuals were smokeless tobacco users, 58 were smokers and 34 had both smoking and smokeless tobacco habits. From the data, it is observed that the numbers of smokeless tobacco users are greater than the numbers of smokers in the population.

3.2. Chromosome aberration analysis assay

Chromosome aberration analysis was carried out in both experimental and control subjects where chromatid-type and chromosome-type aberrations were observed. Among the chromatid-type aberrations (CAs), gaps and breaks were scored in the metaphase chromosomes. The tested population was divided into three categories: individuals with only smoke, only SLT, or both smoking and SLT usage (Table 2). The presence of aberrant cells was counted in all of the individuals. The control subjects showed the fewest aberrant cells; however, the tobacco users displayed a high number of aberrant cells. Individuals with both smoke and SLT use showed a high number of aberrant cells (23.85 ± 5.13) when compared to smoke (16.29 ± 5.91) and SLT users (17.07 ± 7.46). An increased level of chromatid-type aberrations (3.55 ± 1.28) was seen in the 34 individuals with both smoking and SLT usage and the difference was statistically significant at $p < 0.05$ when compared with only smokers and only SLT users. The chromosome-type aberration was higher (1.42 ± 0.68) in individuals with only smokeless tobacco usage and statistically significantly higher than in only smoking and both smoking and SLT users.

Groups I and II correspond to individuals aged 15–30 and over 30, respectively. Group II individuals showed an increased level of chromosomal aberrations when compared to their respective controls. Among the 126 individuals of group II, 2.48 ± 1.60 chromatid-type and 1.23 ± 0.98 chromosome-type aberrations were identified. The differences were statistically significant at $p < 0.05$ when compared with controls, where 0.93 ± 0.51 chromatid-type and 0.56 ± 0.38 chromosome-type aberrations were identified. This result confirms that increased levels of chromosomal aberrations are found in individuals with both smoking and SLT users and age above 30 y.

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