45, XO karyotype in women with atypical Turner’s syndrome presentation

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Case Report

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45, XO karyotype in women with atypical Turner’s syndrome presentation

R Venkatesan¹*, S Balaji², K Suresh¹, R Jayakumar¹, BL Kumar³, R Chandrasekar¹, D Nedumaran², K Sasikala¹

¹Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore, TN, India.  
²Department of Medicine, Coimbatore Medical College Hospital, Coimbatore, TN, India.  
³Department of Biotechnology, Kongu Nadu Arts and Science College, Coimbatore, TN, India.

*Corresponding Author: venkatesangene@gmail.com

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Abstract
Turner’s syndrome (TS) is one of the important chromosomal disorders with loss of one sex chromosome in females. The characteristic features include short stature, webbed neck and poorly developed secondary sexual characters. Here we report four cases of TS (one asymptomatic and three symptomatic) who were admitted at Coimbatore Medical College Hospital with general health complaints. Further diagnosis was followed and discussed. Karyotyping was performed, which confirmed the presence of TS with presentation of 45, XO karyotype.

Keywords: Turner’s syndrome; short stature; secondary sexual character.

Introduction
Classical Turner’s syndrome (TS) is characterized by sexual infantilism, webbed neck, short stature, peripheral edema, lymph edema, renal and cardiovascular anomalies, gonadal dysplasia and learning disability (Zinn et al., 1993; Gicquel et al., 1998; Sybert, 1998; Ross et al., 2000). It affects at least 1:2000 live born girls and is therefore one of the most common sex chromosome abnormalities in humans (Nielsen and Wohlert, 1991). More than half of the TS patients have mosaic complement (Simpson, 1975; Saenger, 1993). The frequency of physical abnormalities in TS varies with the pattern of karyotype (Ranke, 1989). Women with TS show specific cognitive deficits in visuo-spatial processing as well as in selective aspect of attention, executive processing and memory functions. Specific neurophysiologic deficit that may affect adaptation includes four interacting areas of functioning: visual–spatial deficit (e.g., difficulty in driving), social–cognitive defect (e.g., failure to appreciate subtle social clues), problem with nonverbal solving (e.g., mathematics) and psychomotor deficit (e.g., clumsiness) (Rovet, 2004). One half of clinically identified cases possess part of a second X chromosome too, which is structurally abnormal, usually in association with some 45, XO cells (Jacobs et al., 1997).

Case 1
A 45-year-old woman was admitted in Coimbatore Medical College (CMC) Hospital with complaints of cough with sputum production and fever of 1 week duration. Fever was intermittent with no other symptoms such as rashes and joint pain. Bowel and bladder habits were normal. On physical examination she was found to be of short stature, with height of 140 cm and weight of 40 kg. She was febrile, and pallor was present. In this case, the woman had no webbed and short neck that forms a characteristic of TS. Breasts were underdeveloped and there was lack of pubic and axillary hair growth (Figure 1). Infantile external genitalia were present and no skeletal abnormalities were observed. Her IQ was normal with normal mental status. All physical and clinical investigations were carried out and the results were found to be normal. On ultrasound analysis, abdomen showed infantile uterus. Echocardiography was found to be normal. Skeletal survey was done and it showed mild scoliosis. The resting position was also found to be normal. Karyotyping study was carried out at Human Genetics Laboratory, Bharathiar University, Coimbatore and every metaphase plate was found to represent the 45, XO karyotype (Figure 2).

Case 2
A 27-year-old woman with severe abdominal pain for 2 weeks was admitted in CMC Hospital,
Coimbatore. Features observed included short stature, absence of secondary sexual characters, lack of menstruation cycle (primary amenorrhea) within puberty stages, severe abdominal pain and underdeveloped ovaries. Finally, karyotyping revealed the presence of 45, XO (TS) aberration (Figure 2).

Case 3
A 17 year female born to non-consanguineous parent had short stature, primary amenorrhea, absence of secondary sexual characters, developed pulmonary tuberculosis and took anti-tubercular treatment for 6 months. She then developed backache and spinal abnormality. She presented with anemia. Patient had exaggerated lordosis along with Gibbus deformity in lumbar region. Chest X ray postero-anterior (PA) view was normal; X ray thoraco-lumbar spine showed wedge compression and collapse of L3, L4, and L5 vertebrae. Sputum was acid-fast bacilli (AFB) negative, ultrasonography (USG) of abdomen showed streak ovaries. Karyotyping report showed 45, XO (TS) karyotype (Figure 2).

Case 4
A 26-year-old woman with complaint of abdominal pain for past 2 weeks was admitted in the above-mentioned hospital. Further investigations revealed absence of secondary sexual characters, primary amenorrhea, widely spaced nipples, hypoplastic uterus, underdeveloped ovary, cardiac examination including the echocardiography revealed atrial septal defect of ostium secundum type, but no webbed neck. Karyotyping showed mosaic type of TS 45, XO/46, XX in metaphase plates (Figure 2).

Figure 1: Typical presentation of underdeveloped breasts with wide spaced nipples in a patient diagnosed with TS.

Figure 2: 45, XO/46, XX (mosaic) one of the cases confirmed by karyotype.
Discussion
Turner’s syndrome is the result of complete or partial X chromosomal monosomy in phenotypic females and is associated with characteristic clinical features. The most consistent characteristics found in the cases studied were short stature and gonadal dysgenesis. The cases presented here lacked some of the other typical phenotypic characteristics like broad shield-like chest, webbed neck, high-arched palate, and puffiness of the dorsum of the fingers found in TS (Williams, 1992; Pasquino et al., 2000). In such presentations, the diagnosis is subsequently confirmed by chromosome study, with a karyotype of 45, XO. Turner’s syndrome should be suspected when there is a combination of short stature, infantile genitalia, and infantile uterus. Although there is no pathognomonic clinical feature of this syndrome, any woman with short stature along with underdeveloped genitalia and uterus should be considered for diagnosis of TS with assistance of chromosomal analysis. The psychosocial impact of TS may be substantial in young girls and women. These effects may be caused by infertility, short stature and impaired development of sexual characteristics and most importantly, by lack of libido (Sutton et al., 2005). Physicians should elicit specific concerns from patients, addressing them individually and should recommend comprehensive school-based psycho-educational assessment (Noor et al., 2007).

Conclusion
The present study again reemphasizes the need for chromosomal analysis in a syndrome like Turner’s, where more often the classical phenotypic manifestations are not found resulting in diagnostic dilemma.

Acknowledgement
We wish to thank all the faculties of the Department of Medicine, Coimbatore Medical College Hospital, Coimbatore, TN, India.

Ethical Approval
The patients’ consent was taken by the authors before conducting this study.

Conflict of Interests
No funds were received for this study. Authors do not have any conflicting interests.

References


46, XY, t (4q–; 7q+) Translocation in Laurence-Moon-Bardet-Biedl Syndrome: A Case Report

R. Venkatesan1, S. Balaji2, D. Nedumaran2, R. Chandirasekar1 and K. Sasikala1

1Unit of Human Genetics, Department of Zoology, School of Life Sciences, Bharathiar University, Coimbatore 641 046, Tamil Nadu, India
2Department of Medicine, Coimbatore Medical College Hospital, Coimbatore 641 014, Tamil Nadu, India


ABSTRACT A sixteen year old boy was brought to the hospital with complaints of poor vision. A few cardinal features of Laurence Moon Bardet Biedl syndrome were observed viz., central obesity, hypogonadism, retinitis pigmentosa, mental retardation, delay of the speech and polydactyly. Emphasis was placed on finding out the chromosomal aberrations that might serve as a diagnostic marker for the disease at a state when some of the characteristic features of the syndrome may be lacking. The significance of diagnosis and its importance in genetic counseling are discussed pertaining to the recent literatures.

INTRODUCTION

Laurence-Moon-Bardet-Biedl Syndrome (MIM 209900), first described in 1920, is an autosomal recessive condition with a wide spectrum of clinical features. LMBBS is a rare, genetically heterogeneous, autosomal recessive disorder characterized by early onset of retinitis pigmentosa, post axial polydactyly, central obesity, mental retardation, hypogonadism and renal anomalies (Schachat and Maumenee 1982).

The cardinal manifestations are retinal pigmentary dystrophy (previously termed retinitis pigmentosa), postaxial polydactyly, central obesity, mental retardation, and hypogonadism. Minor features include hepatic fibrosis, diabetes mellitus, reproductive abnormalities, endocrinological disturbances, short stature, hearing loss, developmental delay, and speech deficit.

Other features that vary in frequency include diabetes mellitus, hypertension and congenital heart disease (Nishimura et al. 2001). Main aim of the present study was to find out the chromosomal aberrations if any that might help in diagnosis of the syndrome.

CASE REPORT

A sixteen year old boy was brought to the outpatient ward of Coimbatore Medical College Hospital with complaint of excessive weight gain noticed since early childhood and poor vision (Fig. 1). He was born out of non-consanguineous marriage of who were asymptomatic of the condition. He was born at term, with no antenatal or perinatal complications. The patient had a younger brother who was normal but the patient’s sister had the same complications as of the patient. The patient had progressive deterioration in vision for the last five to six years, starting with difficulty in seeing at night.

Systemic evaluation revealed truncal obesity (Fig. 1), polydactyly (post axial, present only in hands and not in legs) (Fig. 2a, b), truncal obesity, hypogonadism, micropenis (Fig. 3) and speech disorder. Laboratory investigations were normal. Serum tests were unrewarding.

Hence a diagnosis of Laurence-Moon-Bardet-Biedl syndrome was made based on physical observations (Table 1). It was decided to go for genetic analysis in the form of karyotyping to determine abnormal chromosomal aberrations if any.

To disclose the association of genetics with this disorder, the chromosomes of the patient were analyzed karyotyping on peripheral blood lymphocyte culture. Interestingly, a translocation of deleted long arm portion of 4th chromosome to the long arm of chromosome 7 (46, XY, t (4q–; 7q+) was observed (Fig. 4).
DISCUSSION

The patient of our study is a classical case of Laurence-Moon-Bardet-Biedl syndrome.

The case presented with several characteristic features of Laurence-Moon-Bardet-Biedl syndrome including the five cardinal features namely central obesity, hypogonadism, retinitis pigmentosa, mental retardation, delay of the speech and systemic abnormalities like polydactyly. Karyotypic analysis revealed a translocation of deleted long arm portion of 4th chromosome to the long arm of chromosome 7 (46, XY, t (4q-; 7q+)). Badano et al. (2003) represented a deletion of 4q27 and Nishimura et al. (2005) demonstrated

Table 1: Characteristic features observed in the patient

<table>
<thead>
<tr>
<th>Features</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary Features</td>
<td>+</td>
</tr>
<tr>
<td>Red cone dystrophy</td>
<td>+</td>
</tr>
<tr>
<td>Post axial Polydactyl</td>
<td>+</td>
</tr>
<tr>
<td>Truncal obesity</td>
<td>+</td>
</tr>
<tr>
<td>Hypogonadism</td>
<td>+</td>
</tr>
<tr>
<td>Renal anomalies</td>
<td>-</td>
</tr>
<tr>
<td>Secondary Features</td>
<td>+</td>
</tr>
<tr>
<td>Speech disorder/delay</td>
<td>+</td>
</tr>
<tr>
<td>Developmental delay</td>
<td>+</td>
</tr>
<tr>
<td>Behaviour</td>
<td>+</td>
</tr>
<tr>
<td>Ataxia/imbalance</td>
<td>-</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>-</td>
</tr>
<tr>
<td>Congenital heart defects</td>
<td>-</td>
</tr>
<tr>
<td>Liver disease</td>
<td>-</td>
</tr>
<tr>
<td>Hearing loss</td>
<td>-</td>
</tr>
<tr>
<td>Facial features</td>
<td>+</td>
</tr>
<tr>
<td>Situs inversus</td>
<td>-</td>
</tr>
<tr>
<td>Hirschprung disease</td>
<td>-</td>
</tr>
<tr>
<td>Polyuria/polydipsia</td>
<td>-</td>
</tr>
<tr>
<td>Dental crowding</td>
<td>+</td>
</tr>
<tr>
<td>Anosmia</td>
<td>-</td>
</tr>
<tr>
<td>Vision on left and Right eyes</td>
<td>Affected during night time.</td>
</tr>
</tbody>
</table>

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the deletion of a region 7p14 chromosome in Bardet-Biedl syndrome. Bardet-Biedl syndrome (BBS) is also a genetically heterogeneous disorder, the primary features of which include obesity, retinal dystrophy, polydactyly, hypogonadism, learning difficulties, and renal malformations (Badano et al. 2003).

Hence the present study results reveal that a chromosomal translocation between chromosomes 4 and 7 may aid in diagnosis for LMBBS in patients lacking the characteristic features of LMBBS. But a larger number of cases need to be studied in order to confirm this finding. Further, the genes residing on the long of chromosome 4 and 7 should be probed further for association with this genetic disorder.

ACKNOWLEDGEMENTS

We wish to thank all the faculties of the Department of Medicine, Coimbatore Medical College Hospital, Coimbatore, India.

REFERENCES


Identification of an Aberrant Karyotype in Bronchiectasis with Congenital Anomalies - A Case Report

Venkatesan Ramachandran*, Sasikala Keshavarao†, Nedumaran Dooraisamy‡, Jayakumar Rajarajeswaran*, Chandirasekar Ramachandran*, Balaji Sivasubramaniam* and Suresh Kuppanan*.

*Unit of Human Genetics, Department of Zoology, Bharathiar University, Coimbatore – 641 046.
†Department of Medicine, Coimbatore Medical College Hospital, Coimbatore – 641 018, TN, India.

Keywords:
Bronchiectasis
Polydactyly
Short stature
Chromosome.

1. Introduction

Bronchiectasis is defined by permanent and abnormal widening of the bronchi [1]. Bronchiectasis, as a clinical syndrome, has the classical “symptoms triad” of cough, excess sputum production and repeated infection [2]. Polydactyly is one of the most common congenital deformities of the hands. It can occur as an isolated disorder, in association with other malformations of the hands or feet, or as part of a syndrome. It can occur sporadically but it can also be inherited with a mainly autosomal dominant inheritance [3]. A common and conspicuous congenital hand anomaly, polydactyl commonly involves only the hand or the foot. Polydactyly involving both hands and feet is rare [4]. Genetic defects in cilia, motile and sensory organelles with important roles in human development, also has been found to produce a host of disease symptoms, including polycystic kidney disease, hydrocephalus, retinal degeneration, chronic bronchiectasis, infertility, and polydactyly [5].

2. Case Report

We present a case of 33 yr old female, who presented with history of cough and breathlessness of one month duration. The cough was productive, with mucoid sputum. The patient had no complaints of chest pain and had no significant past history of any heart disease. On examination, patient was conscious, oriented, and afebrile. Patient had no pallor/ jaundice/ cyanosis/ clubbing/ lymphadenopathy/ pedal edema or goitre. She was short statured, with height of 142cm, and was moderately nourished. She had polydactyly in all four limbs. The limbs exhibited short broad digits with dysplastic nails. The examination of the respiratory system had features of bilateral lower lobe bronchiectasis with above congenital abnormalities. Other system examinations were normal.

Chromosomal aberration analysis was carried out by the following standard procedure (Hoyos et al., 1996). Briefly, 0.5 ml of whole blood was added to 5.0 ml RPMI 1640 medium (Hyclone), supplemented with 20% fetal bovine serum (PAA Laboratories), 2 mM L-glutamine (Himedia), 1% streptomycin-penicillin antibiotic (Himedia) and 0.2 ml of phytohemagglutinin (Gibco). The mixture was incubated at 37rC for 72 hours. After 71 hours, the cells were treated with 0.01% Colchicine (Hi media) to arrest cells in mitosis. Lymphocytes were harvested upon completion of 72 hours by centrifuging the cells at 1800 rpm for 7 minutes. About 6mL of pre-warmed (37°C) hypotonic solution (Kd 0.075 M) was added and left aside for 20 minutes at room temperature.
After removing the hypotonic solution by centrifugation, the cells were fixed in Carnoy’s fixative. Slides were prepared and stained in 2% Giemsa stain. For the chromosomal aberration analysis, 100 well spread metaphase plates were examined per subject under a microscope (100X) to identify numerical and structural chromosomal aberrations [6].

3.2. Clinical Investigations

Chest X-Ray showed ectatic changes in both lower lung fields. Blood complete hemogram showed mild anaemia of 10.2 gm%. Other parameters were normal. Basic biochemical investigations like renal function tests, liver function tests were normal. Urine analysis was normal. Sputum microscopic examination for Acid Fast Bacilli was negative. Sputum Culture had NO growth. Further patient was subjected for karyotypic study, which revealed the following Karyotype (GTG banding technique at 450 -550 band resolution) – 46,XX 15q-.

3.3. Clinical Diagnosis

Congenital bilateral lower lobe bronchiectasis with short stature and Polydactyly

4. Discussion and conclusion

Bronchiectasis is a structural abnormality characterized by abnormal dilation and distortion of the bronchial tree, resulting in chronic obstructive lung disease. There is good evidence for believing that the disease is usually of acquired origin, but in a few cases the presence of other developmental anomalies favours the view that there may also be a congenital factor. Here the additional anomalies like polydactyly support a congenital development of the condition. The proper formation of alveoli does not occur in portions of atelectatic lung, so that the evacuating mechanism of an expulsive blast of air through the bronchi on expiration or coughing is not established. Though secreted mucus may become infected if not removed adequately, here sputum was not infected though bronchial weakening and dilatation was found. The presence of an abnormal karyotype also points towards a genetic defect but it needs a larger study for confirmation.

Primary ciliary dyskinesia (PCD), previously known as immotile cilia syndrome, is an autosomal recessive hereditary disease that includes various patterns of ciliary ultrastructural defects. The literature shows that sinusitis, bronchiectasis, and digital clubbing are late complications of PCD that can progress to chronic cor pulmonale and its consequences. There have been reports in the literature that other malformations, such as hydrocephalus, deformed palate, cardiac malformations, polydactyly, hypospadia, can accompany PCD [7]. Hence this case points to such defects but the clinical diagnosis needs confirmation through transmission electron microscopy.

Altogether this case report presents one of the rare conditions of congenital bilateral lower lobe bronchiectasis along with other congenital anomalies in form of polydactyly and short stature along with a previous unreported chromosomal abnormality - 46,XX 15q-.

5. Acknowledgement

We wish to thank all the faculties the Department of Medicine, Coimbatore Medical College Hospital, Coimbatore, Tamil Nadu, India.

Figure 1. Short stature of the patient

Figure 2. Polydactyly condition in Feet
Figure 3. Polydactyly condition in Hand

Figure 4. Dysplastic Nail Conditions

6. References


Association of Trp53 arg72pro polymorphic variants with breast cancer – a case control study in south Indian population

*Suresh K1, Venkatesan R1, Chandirasekar R1, Kumar BL2, Sasikala K1

1 Unit of Human Genetics, Department of Zoology, Bharathiar University, Coimbatore, India.
2 Department of Biotechnology, Kongunadu Arts and Science College, Coimbatore, India.

*Corresponding Author: ksureshbio@live.com

Abstract

The common single nucleotide polymorphism at Trp53 codon 72 is extensively analysed for its association with breast cancer, but conflicting evidences were obtained. We tested the hypothesis that the SNP at Trp53 codon 72 contributes to the development of breast cancer. Using the polymerase chain reaction-restriction fragment length polymorphism method, the genotype and allele distribution of the Trp53 codon 72 was determined among breast cancer patients (n=35) and healthy normal controls (n=37). Genotypic analysis revealed that the heterozygous (arg/pro) individuals were higher among breast cancer patients than in the controls. Overall, although there was no statistically significant difference in the allelic and genotypic distribution, higher number of arg allele was observed in breast cancer patients when compared to the controls suggesting that the arg allele may be associated with predisposing women to breast cancer.

Keywords: Breast cancer; First-degree female relatives; Single nucleotide polymorphism; Trp53 codon 72 polymorphism; Trp53 gene mutation.

Introduction

Breast cancer is the most frequent cancer in woman worldwide with 1.05 million new cases every year and represents over 20% of all malignancies among females (Parkin et al., 2001). It is also the primary cause of death among women globally, and in 2001, there were approximately 80,000 new breast cancer cases in India (Siddiqi et al., 2001). It is believed that oral contraceptive use (Kahlenborn et al., 2006), obesity (Carmichael, 2006) and hyperinsulinemia (Gunter et al., 2009) are probable factors increasing risks of developing breast carcinoma. In addition, DNA damage caused by carcinogenic agents, such as IR, ROS and estrogen metabolites, may contribute to genetic alterations that are critical in breast carcinogenesis (Smith et al., 2003). Although exposure to these risk factors causes the development of breast cancer only in a small group of exposed people, implying that genetic factors might contribute to the carcinogenic mechanisms and complex interactions between many genetic and environmental factors might be the major cause of breast cancer.

The genetic variants (arg, pro) of Trp53 have received attention as possible modifiers of cancer risk due to their critical role in cell cycle control, DNA repair and apoptosis and possible interaction with the breast cancer susceptibility genes BRCA1 and BRCA2. The Trp53 arg72pro polymorphism refers to the Ex4+199 G>C variant rs1042522, which results in the non-synonymous amino acid substitution of an arginine (72arg) amino acid at codon 72 with a proline (72pro) amino acid. Extensive studies have been carried out to reveal the association of arg72pro variants with breast cancer susceptibility (Wang-Gohrke et al., 1998; Papadakis et al., 2000; Suspitsin et al., 2003; Damin et al., 2006; Khaliq et al., 2000), however, inconsistent results were obtained and the association remains largely uncertain. The aim of the present work was to evaluate the association of the Trp53 arg72pro polymorphism with breast cancer, through assessing the genotype and subsequently, comparing the frequency of Trp53 arg72pro genotypes between breast cancer patients and controls.

Materials and Methods

The study consisted of 35 clinically confirmed breast cancer patients and 37 controls. The cancer patients included in the study were from various territory cancer care hospitals at Coimbatore and Erode districts of Tamilnadu State, South India. The controls were from general population employed in various professions. The objectives of the study were explained to the participants. They have answered for an interviewer-administered questionnaire covering standard demographic
questions as well as questions pertaining to their medical history and smoking habit. All the participants gave their written consent prior to inclusion in the study. DNA was extracted from the peripheral blood cells using standard procedure, involving SDS / Proteinase K digestion followed by ethanol precipitation.

**PCR amplification and polymorphism analysis of Trp53 arg72pro**
The primer sequences were obtained commercially (F1 base, Singapore) and verified using UCSC In-silico PCR (http://genomemirror.duhs.duke.edu/cgi-bin/hgPcr) to eradicate the possibility of amplification of any non-specific DNA sequences. Purified genomic DNA was amplified by PCR for exon 4 codon 72 of Trp53 gene. The PCR contained a quantity of 200ng of genomic DNA, 1µM of each forward 5' - TTG CCG TCC CAA GCA ATG GAT GA - 3' and reverse 5' - TCT GGG AAG GGA CAG AAG ATG AC - 3' primers, 10 mM of Tris-HCl, 50 mM of KCl, 2 mM MgCl₂, 0.2 mM of each dNTPs (Fermentas, Germany) and 1.25 U of Taq DNA polymerase (Fermentas, Germany) in a final reaction volume of 50µl. The PCR amplification involved an initial denaturation at 94°C for 4 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min and a final cycle of extension at 72°C for 5 min. The polymorphisms were identified by digesting the PCR products (199bp long) with 5 U of Bsh1236I (Fermentas) for 4-16 hours. The digested PCR product was resolved on 8% polyacrylamide gel (29:1) electrophoresis for 21/2 hours at 65V/cm in 1X TBE buffer. Electrophoresis of the digestion product revealed that, the appearances of two bands correspond to arg/arg (113bp and 86bp long), three bands correspond to arg/pro (199bp, 113bp, and 86bp long) and one undigested band corresponds to pro/pro (199bp long).

**Statistical analysis**
Chi-square analysis ($\chi^2$) was applied to test the association between the genotypes and alleles between patients and controls. The odds ratio (OR) and their confidence intervals (CI) were calculated to estimate the strength of the association between polymorphism genotype alleles of patients and controls (Martin Bland and Douglas, 2000).

**Results**

**Characteristics of breast cancer patients**
The mean age, height and weight of the breast cancer patients was 54.05±5.89, 154.57±4.86, 52.94±6.76, respectively. Breast cancer was seen among 11.42% of first-degree relatives (FDFRs). The prevalence of breast cancer was high among the postmenopausal women (74.28%, mean age 53.61±7.25) and low among the premenopausal women (25.71%, mean age 51.00±4.94). Active smoking did not exist among breast cancer patients, but 25.71% of breast cancer patients were exposed to passive smoking. Meanwhile, tobacco-chewing habit was seen among 22.85% of breast cancer patients. Diabetes, high blood pressure, jaundice, epilepsy and anemia were seen among 11.42%, 5.71%, 2.85%, 2.85% and 2.85% of breast cancer patients respectively.

**Characteristics of controls**
The mean age, height and weight of controls was 53.48±7.42, 155.70±5.81, 53.08±5.89 respectively. Regarding the menopausal status of the controls, 35.13% were premenopausal woman and 64.86% were postmenopausal woman. Active smoking was not seen, whereas passive smoking and tobacco chewing habit was seen among 21.62% and 13.51% of controls respectively. Diabetes in 8.10%, high blood pressure in 2.70%, migraine in 2.70% and arthritis in 8.10% of controls, were observed.

**Trp53 arg72pro analysis**
The distribution of three genotypes namely, arg/arg, arg/pro and pro/pro, observed in the breast cancer patients were 28.57%, 62.85% and 8.57% respectively. The controls showed 29.72%, 51.35% and 18.91% of arg/arg, arg/pro and pro/pro respectively. There was no significant difference in the distribution of genotypes between breast cancer patients and controls ($\chi^2$=0.31, df=1, P=0.57). The allele frequencies of breast cancer patients and controls were fitted in the Hardy-Weinberg Equilibrium with an allele frequencies of 0.56 (Controls) and 0.60 (breast cancer patients) for arg-coding alleles and 0.44 (Controls) and 0.40 (breast cancer patients) for pro-coding alleles. No significant difference in allele frequencies between breast cancer patients and controls were observed ($\chi^2$=0.31, df=1, P=0.57). The combined analysis of arg/arg and arg/pro genotypes in breast cancer patients and controls revealed no significant association with cancer, but showed an increased breast cancer risk.
[Odd ratio (OR) = 1.27, 95% Confidence Interval (CI) 0.44-3.65, P=0.65]. Meanwhile, the combined analysis of arg/pro and pro/pro genotypes showed no significant association with breast cancer risk (OR=0.37, CI=0.08-1.63, P=0.17) (Table 1, Figure 1).

Table 1. showing the genotypic distribution of Trp53 Arg72Pro in breast cancer cases and control subjects.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Controls (%)</th>
<th>Breast cancer patients (%)</th>
<th>P (X²)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>arg/arg</td>
<td>11 (29.72)</td>
<td>10 (28.57)</td>
<td>0.40</td>
<td>1.27 (0.44-3.65)</td>
<td>0.65</td>
</tr>
<tr>
<td>arg/pro</td>
<td>19 (51.35)</td>
<td>22 (62.85)</td>
<td>0.05</td>
<td>0.37 (0.08-1.63)</td>
<td>0.17</td>
</tr>
<tr>
<td>pro/pro</td>
<td>7 (18.91)</td>
<td>3 (8.57)</td>
<td>0.57</td>
<td>0.82 (0.42-1.60)</td>
<td></td>
</tr>
<tr>
<td>arg allele frequency</td>
<td>0.56</td>
<td>0.60</td>
<td></td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>pro allele frequency</td>
<td>0.44</td>
<td>0.40</td>
<td></td>
<td>0.82</td>
<td></td>
</tr>
</tbody>
</table>

OR – Odds Ratio; CI – Confidence Interval
* P > 0.05

Figure 1. Digested PCR products with the restriction enzyme Bsh12361.

8% Polyacrylamide gel, Electrophoretic conditions: 2:15 hours in 1X TBE buffer, current 65V/cm.
From left to right: Lane 1, 2, 3 and 6: arg/pro; Lane 4: pro/pro, Lane 5: arg/arg; Lane 7: 20bp marker.
Discussion

In our study, about 11.42% of breast cancer patients were FDFRs. Family history is a well established risk factor for breast cancer, with the familial relative risk (FRR) being approximately two-fold for first degree relatives of breast cancer patients compared with controls from the general population (Familial Breast Cancer, 2001). Rajeswari et al. (2000) found that the DNA damage significantly increased from controls to FDFRs and from FDFRs to breast cancer patients. Who further observed that the FDFRs showed ~ 2.5 times higher DNA damage as compared with the controls. Decreased DNA repair synthesis in leukocytes has also been reported in breast cancer patients (Jaloszynski et al., 1997) and healthy FDFRs of breast cancer patients (Rao et al., 1998).

In the general population, reproductive factors including age at menarche, late age at first full-term pregnancy, null and low parity and late age at menopause are established risk factors for an increased risk of breast cancer (Key et al., 2001). The patterns of risk associated with reproductive history suggest that the lifetime estrogen exposure (Steiner and Klubert, 2008) and late age at menopause (Kelsey et al., 1993) are associated with increased rates of breast cancer. According to Meshram et al. (2009) menopause (≥50 years of age) was observed to be associated with increased risk and the risk was 7.9 times more among women who had menopause at or after 50 years of age compared to women who had menopause before 45 years. The present study is consistent with the previous studies; we found that breast cancer was prevailed among 74.28% (Mean age = 53.61±7.25) of postmenopausal woman and the 25.71% (Mean age = 51.00±4.94) of premenopausal woman.

The study of genetic polymorphisms in genes associated with the repair of DNA damage and those involved in cell cycle control are particularly attractive targets, since an inability to tightly regulate either of these two processes is likely to result in less than the most favorable outcome. Even though studies correlated Trp53 arg72pro variants with breast cancer, conflicting evidences are available over the influence of this genetic variation on manifestation of breast cancer.

Several case control studies suggested that pro allele was associated with breast cancer risk in Swedish (Sjalander et al., 1996), American (Weston et al., 1997), German (Wang-Gohrke et al., 1998, 2002), Russian (Suspectsin et al., 2003), Japanese (Noma et al., 2004) and Slovakian (Franekova et al., 2007) populations. A few investigations supported that arg allele was associated with breast cancer risk in Greek (Papadakis et al., 2000), Turkish (Buyru et al., 2003), Italian (Bonafe et al., 2003), Israeli (Ohayon et al., 2005), Chinese (Ma et al., 2006), and Brazilian (Damin et al., 2006) populations. No association of Trp53 arg72pro polymorphism with breast cancer risk was reported in Japanese (Kawaijiri et al., 1993), Pakistani (Khaliq et al., 2000), Tunisian (Mabrouk et al., 2003), Finnish (Tommiska et al., 2005) and Iranian populations (Khadang et al., 2007).

The present work investigated the association of Trp53 arg72pro polymorphism with breast cancer outcome and found that neither arg nor pro was significantly associated with breast cancer outcome. However, in our study arg allele frequency was found to be higher (0.63) in breast cancer patients than the controls (0.53). Sharp ethnic differences in the arg and pro allele frequencies have been observed. In the Northern hemisphere, the pro72 allele shows a North-South gradient, from 0.17 [Swedish Saamis] to 0.63 [African Blacks-Nigerians] (Beckman et al., 1994). In Western Europe (France, Sweden, and Norway), North America (USA), Central and South America (Mexico, Costa-Rica and Peru) and Japan, the most common allele is arg72, with frequencies ranging from 0.60 to 0.83. (IARC, 2010).

The two polymorphic variants of the wild type Trp53 have been shown to have different biochemical properties; Trp53 pro form was suggested to activate transcription much more efficiently than the Trp53 arg variant (Thomas et al., 1999), the Trp53 arg variant is more efficiently induces cell death than the Trp53 pro variant (Dumont et al., 2003), the Trp53 pro variant was shown to induce cell cycle arrest better than the Trp53 arg form (Pim and Banks, 2004). These data highlight that both the polymorphic variants of Trp53 might have involved for selectively regulating specific cellular functions. Besides these functions, there is emerging evidence for Trp53 role in regulating the various DNA-repair processes (Sengupta and Harris, 2005) among other functions.

Using several cellular systems, Siddique et al. (2005) reported that cells expressing Trp53 pro form are able to repair DNA-damage much more efficiently than the Trp53 arg-expressing cells, thus it is inferred that Trp53 pro variant
Trp53 plays critical role [preferentially by inducing Trp53 dependent DNA-repair target gene promoters (p53R2)] in the Trp53 dependent DNA-repair process, which may influence cancer risk. These data indicated that Trp53 arg might be a predisposing allele to cancer, probably owing to its reduced ability to repair damaged DNA, a process that has been shown to be important in cancer formation. Trp53 arg is only slightly less efficient than Trp53 pro in its ability to repair damaged DNA. Subtle differences manifested owing to combination of polymorphic effects in several gene loci may result in the overall sensitivity to cancer development. In this respect, it was recently shown that the polymorphism in the Mdm2 promoter (SNP309) was involved in cancer susceptibility (Bond et al., 2004). Further, Siddique et al. (2005) reported that Trp53 arg-expressing cells were less effective in removing micronuclei (small nuclei, arise from acentric chromatids or chromosome fragments induced by radiation or other DNA damaging agents), suggesting that Trp53 arg might be less potent in reducing genomic instability and perhaps cancer predispositions.

In this context, recent evidences have shown that healthy Asian heterozygous individuals (Trp53 arg/pro) tend to preferentially express the Trp53 arg allele at the RNA level (Siddique et al., 2005). By contrast, the Trp53 arg allele was preferentially expressed in most heterozygote breast cancer patients, suggesting that Trp53 arg expression correlates with breast cancer development (Siddique et al., 2005). These findings together with many other reports indicating that the Trp53 arg allele was associated with cancer predisposition (Sjalander et al., 1996; Weston and Godbold, 1997; Papadakis et al., 2000; Bergamaschi et al., 2003), suggested that even if the Trp53 arg form might be capable of inducing apoptosis better, it might not be efficient in preventing cancer formation. In our study, analysis of genotypic distributions revealed that about 62.5% of breast cancer patients and 51.35% of controls were heterozygous (Trp53 arg/pro), moreover the arg allele frequency was slightly higher in breast cancer patients 0.63 when compared to controls 0.53. Even though the distribution of genotypes and allele frequency between breast cancer patients and controls were statistically insignificant, the presence of more number of heterozygous individuals and arg alleles in breast cancer patients made it reasonable to suspect the role of arg allele in predisposing woman to breast cancer in our study. To add more strength to our result, several experimental evidences support the notion that arg allele was associated with breast cancer predisposition in woman. Keshava et al. (2002) observed a high prevalence of the Trp53 arg genotype in breast cancer patients among Caucasian women of New York. In this context, it was shown that Caucasians are primarily arg-expressers (Siddique et al., 2005) and they are about 2-fold more prone to cancer than the Asians (Oliver et al., 2002). Aoki et al. (2009) reported that breast cancer patients presented a significantly over representation of Trp53 arg homozygosity (55.5%) compared with the healthy control group (33.3%), who also suggested that it is possible that Trp53 arg homozygosity is associated with breast cancer and may represent a potential risk factor for breast tumorigenesis. Papadakis et al. (2000) observed higher frequency of arg/arg (61%) in breast cancer patients than the controls (20%). It is suggested that Trp53 arg homozygosity could represent a risk factor for the tumorigenesis of the breast. Langerod et al. (2002) reported a growth advantage of breast carcinoma cells carrying the Trp53 arg allele in a Norwegian population. Ohayon et al. (2005) reported that the arg allele was significantly associated with breast cancer in non-Ashkenazi-Jews. Similarly, Ma et al. (2006) found a significant association of arg allele with breast cancer risk in Chinese population.

We found that the Trp53 pro allele was less prevalent (8.57%) in the breast cancer patients when compared to controls (18.91%). Our results are in contrast with the previous reports (Sjalander et al., 1996; Weston et al., 1997; Wang-Gohke et al., 1998, 2002; Suspitsin et al., 2003; Nomura et al., 2004; Franekova et al., 2007). Our study suggested that the Trp53 pro allele was not associated or may confer a decreased risk to development of breast cancer. Alawadi et al. (2010) studied the Trp53 gene polymorphism with breast cancer risk in Arab women and found that the pro homozygosity at codon 72 was associated with decreased breast cancer risk.

Siddique et al. (2005) showed that there is a significant increase in the number of Trp53 arg expressers in the Chinese breast cancer cohort, indicating that there is a strong correlation between carcinogenesis and the expression of the Trp53 arg allele in the Chinese population. However, there was no significant
increase in the numbers of Trp53 arg homozygotes between the Chinese healthy and cancer populations, supporting many previous studies and the present study which reports a lack a correlation between a particular genotype and cancer predisposition (Weston and Godbold, 1997). According to Siddique et al. (2005), it may be possible that the Trp53 arg allele may not be functionally involved, but its expression may simply relate to tumorigenesis.

Conclusion

In conclusion, our study suggests that an association may exist between the Trp53 arg72pro polymorphism and breast cancer development. However, the study’s sample size limits the possibility to confirm the association of Trp53 72 codon polymorphism with breast cancer. In order to obtain a more definitive conclusion concerning the association of this polymorphism with breast cancer, a careful study in different ethnics with large number of cases versus controls should examine the expression status of the Trp53 gene, in addition to determining the genotype.

References


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XRCC1 gene variants and possible links with chromosome aberrations and micronucleus in active and passive smokers

R. Chandirasekar, K. Suresh, R. Jayakumar, R. Venkatesan, B. Lakshman Kumar, K. Sasikala

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1. Introduction

Smoking is a major cause for cancer, cardiovascular diseases and chronic obstructive pulmonary diseases (USDHHS, 2004; IARC, 2004). Cigarette smoking is the single most preventable cause of debilitating illness and premature death (US, 1989). Cigarette smoke constitutes 4500 chemical compounds and some of them are known to be mutagens (DeMarini, 1983; Obe et al., 1984). Investigations reported that there are 1.1 billion smokers worldwide and 182 million (16.6%) of them live in India (WHO, 1997; Gajalakshmi et al., 2000; Jha et al., 2002; Shimkhada and Peabody, 2003). Smoking related cancers accounted for 22% of all cancers (AIHW, 2003) and smoking alone is responsible for five million deaths in the world every year more over 50 per cent of these deaths occur in the middle age (35–69 years) population. In India, mortality ascribed by tobacco has been estimated to be one million every year (Pai, 2002). According to a prediction by the World Health Organization, more than 500 million people alive today will be killed...
by tobacco by 2030 and tobacco consumption will become the single leading cause of death (World Bank, 1999). Cigarette smoke is one among the most important mutagenic factors which causes damage to human genetic material (Jin et al., 1997). To find out the effect of smoking on the genetic material of actively and passively exposed subjects, the present investigation was performed.

Cytogenetic endpoints such as chromosome aberrations (CAs) and micronuclei (MN) frequency have been proposed as sensitive parameters for assessing genotoxic effects of chemical or physical mutagens (Adhvaryu et al., 1991). The present study employed CAs analysis, buccal epithelial and blood lymphocyte MN analysis to study the genotoxic effect among subjects actively and passively exposed to tobacco. As cytogenetic biomarkers may reflect genotoxic exposures that occurred months before the cell sampling, single measurements of urinary metabolites or ambient concentrations may not be representative of the relevant exposure period. Information about the possible influence of genetic polymorphism of DNA repair genes on cytogenetic end-points are emerging but more information is still needed to verify the findings.

The aim of this investigation was to ascertain whether cigarette smoking modulates the frequency of CAs and MN in peripheral blood lymphocytes and MN in buccal epithelial cells, and the influence of XRCC1 arg399gln polymorphic variants on chromosome damage and micronuclei frequency in control and cases of exposure to cigarette smoking.

2. Materials and methods

2.1. Subjects

Subjects for the present study were recruited based on answers to a standard questionnaire comprising demographic questions (age, gender, etc.) as well as questions pertaining to medical history (genetic disorders, vaccination, medication, etc.), smoking status (no. of cigarettes consumed/day, years of exposure) and alcohol intake (no. of times/month) from the general public residing in Dharmapuri and Salem districts in Tamilnadu, South India. The purpose of the work was explained to the participating subjects. All the study subjects gave their written consent to obtain blood. About 2–3 ml of venous blood was drawn from the subjects into a heparin coated vaccutainer. Great care was taken not to harm subjects and appropriate medical procedures were followed during blood collection by a skilled medical nurse. About 110 subjects were selected as exposed subjects, of them 27 were active smokers (Directly exposed to cigarettes smoke), all of them were males and 83 passive smokers (indirectly exposed to cigarette smoke in places like home, canteen and work place) included males and females. Equal numbers of unexposed (not exposed to tobacco smoke either actively or passively) individuals were selected to serve as controls. The exposed subjects were categorized into active smokers and passive smokers. Further grouping was done among the active and passive smokers based on the age of subjects. Group I subjects age ranges from 20 to 30 years; Group II from 31 to 40 years; Group III from 41 to 50 years and above (characteristics of the study population was presented in Table 1).

2.2. Chromosomal analysis

Lymphocytes cultures were initiated with the following standard procedures (Hoyos et al., 1996). A volume of 0.5 ml blood was added to 4.5 ml RPMI 1640 medium supplemented with 15% fetal bovine serum, 2 mM l-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and 0.2 ml of phytohemagglutinin. The whole culture was incubated at 37 °C for 72 h and the culture tube received periodical shaking twice a day to aid proper mixing of the medium and cells in the culture. After 71 h, cultures were treated with 0.01 mg/ml colcemid to arrest the cells at mitotic stage. Lymphocytes were harvested after 72 h by centrifuging the cells to remove culture medium (800–1000 rpm), thereafter added pre-warmed (37 °C) hypotonic solution (KCl 0.075 M) and left undisturbed for 20 min for hypotonic treatment. The cells were treated twice with Carnoy’s fixative (3:1 ratio of methanol:acetic acid). Slides were prepared and carefully dried on a hot plate (56 °C, 2 min). Later, slides were stained using giemsa stain. For the CAs analysis, 50 well spread metaphase plates were analyzed for each subject under oil immersion lens by means of Leica light microscope (100×) and well spread metaphases were photographed.

2.3. Micronuclei analysis in peripheral blood lymphocytes

Lymphocyte cultures were set up according to the following standard method of Fenech and Morley (1986). Cytochalasin B was added to the cultures at a final concentration of 6 µg/ml after 44 h. At the end of incubation time (72 h), cells were harvested by centrifugation and hypotonic solution (0.075 M KCl) was added then left undisturbed for a minute. The cells were fixed in fresh fixative solution (methanol:acetic acid, 3:1) and this fixation step was repeated twice. About 500 cells were scored from each subject.

2.4. Micronuclei analysis in buccal epithelial cells

After a mouth wash with sterile water, oral mucosal cells were obtained by scraping the right/left cheek mucosa with a moist wooden spatula. Cells were transferred to a tube containing saline solution (0.09%) and centrifuged at 800 rpm for 5 min. The cells were fixed in 3:1 methanol/acetic acid 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. (1987).

2.5. Genotyping of XRCC1 arg399gln polymorphism

Genomic DNA was isolated according to the standard protocol (Miller et al., 1988). XRCC1 (codon 399) genotype was analyzed through PCR–RFLP method. The primer sequences were as follows. Forward primer sequence: 5′-TTG TGC TTT CTC GTC CA-3′; reverse primer sequence: 5′-TCC TCC AGC CTG TTC TGA TA-3′. The final reaction volume for PCR was 25 µl, it contained 0.25 µl of forward and 0.25 µl of reverse primers, 200 ng of template DNA, 1.25 U of taq DNA polymerase, 0.2 mM dNTPs and
Table 1 – The demographic details in control and exposed subjects.

<table>
<thead>
<tr>
<th>Particulars</th>
<th>No. of sample and %</th>
<th>Year of exposure</th>
<th>Age</th>
<th>Gender</th>
<th>No. of cigarettes (average/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>110</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>33(30)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
<td>Nil</td>
<td>24.18 ± 2.59</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td></td>
<td>23.65 ± 2.60</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>52(47.27)</td>
<td></td>
<td>25.18 ± 2.20</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>43(39.09)</td>
<td></td>
<td>35.55 ± 2.57</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>21</td>
<td></td>
<td>34.47 ± 2.40</td>
<td>M</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td></td>
<td>36.59 ± 2.34</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>43(39.09)</td>
<td></td>
<td>36.01 ± 2.46</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td>Group III</td>
<td>34(30.90)</td>
<td></td>
<td>55.79 ± 7.29</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>11</td>
<td></td>
<td>59.18 ± 10.05</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>23</td>
<td></td>
<td>54.17 ± 5.02</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>


2 mM MgCl2. Temperature cycles involved initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. The PCR product (615 bp) was digested using 5 U of HpaII (Fermentas, Germany) restriction enzyme. After 4–16 h, 0.5 M EDTA was added to a final concentration of 20 mM to stop enzymatic digestion. The digested PCR products were run on 6% polyacrylamide gel electrophoresis in 1× TBE buffer at 65 V/cm for 1:45 h. An undigested fragment (615 bp) represents gln/gln, two fragments (375 bp and 240 bp) represent arg/arg, and three bands (615 bp, 375 bp and 240 bp) represent arg/gln polymorphism.

3. Results

3.1. Chromosome analysis

Among all the three groups studied, CAs was found to be higher in exposure cases of group III (active smokers 3.00 ± 0.36, passive smokers 1.64 ± 1.06) when compared to group I and group II exposed subjects and controls. Among the exposed groups, active smokers of group III showed significantly (P < 0.05) increased CAs when compared to other groups of active smokers. Likewise group III of passive smokers showed significantly (P < 0.05) higher CAs when compared to group I and group II passive smokers. On the whole, exposed subjects (include active and passive) showed significant increase in CAs than the controls (Table 2).

3.2. Micronuclei analysis in buccal cells and peripheral blood lymphocytes

A total of 1000 buccal epithelial cells and 500 blood lymphocytes were screened for the MN. In the buccal epithelial cells, the buccal micronuclei (BMM) frequency was found to be higher (P < 0.05) in group III passive smokers. When compar-
ison was made between active and passive smokers, passive smokers of group III showed a significantly ($P < 0.05$) elevated MN frequency. Both the BMN and blood lymphocyte micronuclei (BLMN) showed a significant ($P < 0.05$) increase in MN frequency among active and passive smokers when compared to their respective control groups (Table 2).

In the active smokers, the confounding factors like age and no. of cigarettes influenced CAs, frequency of BMN and BLMN. However, only the frequency of CAs was found to be significantly ($P < 0.05$) increased as the age of the subjects increase and the number of cigarettes increase, an insignificant increase was observed in BMN and BLMN frequency irrespective of age and number of cigarettes. In passive smokers, it was observed that the age of the subjects significantly ($P < 0.05$) contributed to the increase in the CAs, meanwhile an insignificant increase was observed for the BMN and BLMN frequency (Table 3).

### 3.3. Genotyping of XRCC1 arg399gln polymorphism

The allele and genotype distribution of XRCC1 are shown in Table 4 the Gln allele frequency ranged from 0.37 to 0.38 among controls. Gln allele frequency was from 0.33 to 0.43 among the exposed cases. The genotype frequency was almost similar among the controls and exposed subjects. Chi-square analysis revealed that no significant difference in genotype distribution between the controls and exposed subjects.

The occurrence of chromosomal aberrations among the XRCC1 genotypes was analyzed (Table 5). Among the genotypes, gln/gln carriers harbored significantly ($P < 0.05$) a higher number of aberrations than the arg/gln and arg/arg genotypes in both the controls and exposed subjects. Similarly, the MN frequency in buccal cells as well as blood lymphocytes was significantly ($P < 0.05$) higher among the homozygous variant gln/gln of controls, whereas, the increase in the BMN and BLMN frequency among the gln/gln carriers of exposed subjects was insignificant.

### 4. Discussion

There is an increasing effort world-wide to determine the impact of environmental, genetic and life-style factors on genomic stability in human populations. Tobacco smoke induces an array of genetic aberrations, including gene mutations, chromosome aberrations (CAs), micronuclei (MN), sister chromatid exchanges, DNA strand breaks, and oxidative DNA adduct in various models (DeMarini, 2004).

Many of the substances contained in tobacco are genotoxic and therefore cytogenetic damage seems to be an excellent biomarker for determining the effect of exposure to chromosome-damaging agents in tobacco. In our study, CAs like gaps, breaks and dicentrics were scored in the peripheral blood lymphocytes of active and passive smokers. Active smokers of group III exhibited significantly higher CAs than the rest of the active smokers groups. A similar of situation exists in the passive smokers, where, the group III showed significantly higher CAs when compared to rest of the passive smokers groups. The duration of exposure to tobacco smoke and age of the subjects in the group III was higher than the group I and group II; this may be the reason for this unusual high CAs. Likewise, group III controls also showed higher CAs, this suggested us that age might be contributed to the increased CAs. Fenech et al. (1998) suggested that chromosomal damage has been shown to increase progressively with age. The influence of smoking on the level of structural CAs has been addressed in both large-and small scale population studies, however, no clear trend has emerged. A few studies found that smoking induced a 10–20% fold increase in CAs frequency (Nordic Study Group on the Health Risk of Chromosome Damage, 1990), whereas, smoking caused a sig-

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**Table 2 – Comparison of cytogenetic parameters between and within groups.**

<table>
<thead>
<tr>
<th>Particulars</th>
<th>No. of subjects studied</th>
<th>Chromosome aberrations</th>
<th>Micronuclei frequency (buccal cells/1000)</th>
<th>Micronuclei frequency (blood cells/500)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp</td>
<td>110</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>10</td>
<td>$2.00 \pm 0.33^{a,b}$</td>
<td>$1.90 \pm 0.27^{b}$</td>
<td>$1.00 \pm 0.14^{b}$</td>
</tr>
<tr>
<td>Group II</td>
<td>11</td>
<td>$2.36 \pm 0.20^{b}$</td>
<td>$2.09 \pm 0.09^{b}$</td>
<td>$1.36 \pm 0.20^{b}$</td>
</tr>
<tr>
<td>Group III</td>
<td>06</td>
<td>$3.00 \pm 0.36^{c,b}$</td>
<td>$2.50 \pm 0.34^{b}$</td>
<td>$1.50 \pm 0.22^{b}$</td>
</tr>
<tr>
<td>Passive</td>
<td>83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>23</td>
<td>$0.82 \pm 0.49^{b}$</td>
<td>$1.69 \pm 0.13^{b}$</td>
<td>$0.95 \pm 0.13^{b}$</td>
</tr>
<tr>
<td>Group II</td>
<td>32</td>
<td>$1.03 \pm 0.12^{b}$</td>
<td>$1.81 \pm 0.07^{b}$</td>
<td>$1.12 \pm 0.11^{b}$</td>
</tr>
<tr>
<td>Group III</td>
<td>28</td>
<td>$1.64 \pm 1.06^{d,b}$</td>
<td>$2.14 \pm 0.21^{c,b}$</td>
<td>$1.35 \pm 0.17^{b}$</td>
</tr>
<tr>
<td>Control</td>
<td>110</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I</td>
<td>33</td>
<td>$0.06 \pm 0.04$</td>
<td>$0.51 \pm 0.08$</td>
<td>$0.30 \pm 0.08$</td>
</tr>
<tr>
<td>Group II</td>
<td>43</td>
<td>$0.11 \pm 0.04$</td>
<td>$0.51 \pm 0.08$</td>
<td>$0.39 \pm 0.07$</td>
</tr>
<tr>
<td>Group III</td>
<td>34</td>
<td>$0.26 \pm 0.07^{*}$</td>
<td>$0.70 \pm 0.07$</td>
<td>$0.50 \pm 0.08$</td>
</tr>
</tbody>
</table>

Duration of exposure: Group I – <10 years; Group II – 10 and below 20 years; Group III – >20 years

Age: Group I – <30 years; Group II – >30 and <40 years; Group III – >40 years.

* $P < 0.05$ compared to active smokers of Group II and group III as estimated by ANOVA followed by Bonferroni’s correction for multiple comparisons.

$P < 0.05$ compared to respective groups of controls as estimated by ANOVA.

$P < 0.05$ compared to Active smokers of group I with group III as estimated by ANOVA.

$P < 0.05$ compared to passive smokers of group I, group II as estimated by ANOVA.

$P < 0.05$ compared to group I as estimated by ANOVA.
Table 3 – Age, no. of cigarettes versus CAs and MN (buccal and blood).

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Smoking status</th>
<th>Age (mean ± SD)</th>
<th>No. of cigarettes (average/day)</th>
<th>CA (mean ± SD)</th>
<th>MN buccal (mean ± SD)</th>
<th>Mn blood (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>Active smokers</td>
<td>25.80 ± 1.61</td>
<td>4.6</td>
<td>2.00 ± 0.33</td>
<td>1.90 ± 0.27</td>
<td>1.00 ± 0.14</td>
</tr>
<tr>
<td>Group II</td>
<td>Active smokers</td>
<td>35.45 ± 1.59</td>
<td>6.27</td>
<td>2.36 ± 0.20</td>
<td>2.09 ± 0.09</td>
<td>1.36 ± 0.20</td>
</tr>
<tr>
<td>Group III</td>
<td>Active smokers</td>
<td>58.33 ± 1.63</td>
<td>6.33</td>
<td>3.00 ± 0.36a</td>
<td>2.50 ± 0.34</td>
<td>1.50 ± 0.22</td>
</tr>
<tr>
<td>Group I</td>
<td>Passive smokers</td>
<td>25.52 ± 2.46</td>
<td>–</td>
<td>0.82 ± 0.49</td>
<td>1.69 ± 0.13</td>
<td>0.95 ± 0.13</td>
</tr>
<tr>
<td>Group II</td>
<td>Passive smokers</td>
<td>36.96 ± 1.95</td>
<td>–</td>
<td>1.03 ± 0.12</td>
<td>1.81 ± 0.07</td>
<td>1.12 ± 0.11</td>
</tr>
<tr>
<td>Group III</td>
<td>Passive smokers</td>
<td>55.03 ± 2.87</td>
<td>–</td>
<td>1.64 ± 1.06b</td>
<td>2.14 ± 0.21</td>
<td>1.35 ± 0.17</td>
</tr>
</tbody>
</table>

a P<0.05 compared to group I active smokers as estimated by ANOVA.
b P<0.05 compared to group I and group II passive smokers as estimated by ANOVA.

A significant 1.5-fold increase in stable aberrations in newborns whose mothers smoked during pregnancy (Ramsey et al., 1995). Several studies have found increased frequencies of CAs in lymphocytes from smokers as compared to non-smokers (Sinues et al., 1990; Tawn and Whitehouse, 2001; Monica et al., 2004) supports the results of our study. Chen et al. (1989) suggested that the higher CAs in the smokers may be due to lower folate levels in their blood erythrocytes. Sasikala et al. (2003) reported that the CAs in active smokers were higher than the passive smokers. Moreover, a few studies identified smoking habit as an important factor that induces significant alterations in the genetic material (Kopjar et al., 2006; Moacir et al., 2010) and the genotoxic effects in lymphocytes of active and passive smokers are most likely caused by cigarette smoke constitutions (Bhatia and Vijayan, 1994).

Tobacco smoke has been associated with the formation of micronuclei in diverse types of human and animal cells. In our study, buccal MN frequency in active and passive smokers were significantly (P<0.05) increased when compared to controls. Among the active and passive smokers, active smokers harbored a slightly higher BMN. Wu et al. (2004) reported that, prolonged smoking was associated with increased buccal micronuclei frequency. A significantly higher MN frequency was observed in the buccal cells of smokers than the non-smokers by Konopacka (2003). We also observed a progressive and insignificant increase in MN frequency from the group I to group III controls. As a possible explanation for this, large intra-individual and day-to-day variation in buccal cell strand breaks might have contributed to these changes (Szeto et al., 2005).

It has been previously proved that the tobacco particulate matter has been shown to induce micronuclei in cultured mammalian cells (Jones et al., 1991). The MN frequency in lymphocytes of active and passive smokers were significantly (P<0.05) higher than the controls. This suggested us that, cigarette smoking contributed to the formation of MN among

Table 4 – The XRCC1 (arg399gl) gene polymorphisms identified in controls and exposed subjects.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Polymorphism</th>
<th>Controls (%)</th>
<th>Exp (%)</th>
<th>P(χ²)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>XRCC1-arg399gln Allele frequency</td>
<td>gln 25(37.87) 0.37</td>
<td>22(33.33) 0.33</td>
<td>0.58(0.29)</td>
<td>1.21(0.6-2.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>arg 41(62.12) 0.73</td>
<td>44(66.67) 0.74</td>
<td>0.84(0.42-1.71)</td>
<td>1.35(0.7-2.3)</td>
</tr>
<tr>
<td></td>
<td>Genotype</td>
<td>arg/arg 12(36.36) 0.35</td>
<td>14(42.42) 0.35</td>
<td>1.00</td>
<td>0.8(0.29-2.26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>arg/gln 17(51.51) 0.41</td>
<td>16(48.48) 0.41</td>
<td>1.00</td>
<td>0.8(0.29-2.26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gln/gln 04(12.12) 0.12</td>
<td>03(9.09) 0.12</td>
<td>0.84(0.32)</td>
<td>0.79(0.15-4.13)</td>
</tr>
<tr>
<td>II</td>
<td>XRCC1-arg399gln Allele frequency</td>
<td>gln 33(38.37) 0.38</td>
<td>37(43.02) 0.43</td>
<td>0.53(0.38)</td>
<td>0.82(0.45-1.52)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>arg 53(61.62) 0.62</td>
<td>49(56.97) 0.62</td>
<td>0.84(0.42-1.71)</td>
<td>1.35(0.7-2.3)</td>
</tr>
<tr>
<td></td>
<td>Genotype</td>
<td>arg/arg 15(34.88) 0.35</td>
<td>13(30.23) 0.35</td>
<td>1.00</td>
<td>0.8(0.29-2.26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>arg/gln 23(53.48) 0.41</td>
<td>23(53.48) 0.41</td>
<td>1.00</td>
<td>0.8(0.29-2.26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gln/gln 05(11.62) 0.12</td>
<td>07(16.27) 0.12</td>
<td>0.78(0.47)</td>
<td>1.04(0.39-5.06)</td>
</tr>
<tr>
<td>III</td>
<td>XRCC1-arg399gln Allele frequency</td>
<td>gln 26(38.23) 0.38</td>
<td>29(42.67) 0.42</td>
<td>0.60(0.27)</td>
<td>0.83(0.42-1.65)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>arg 42(61.76) 0.62</td>
<td>39(57.35) 0.62</td>
<td>0.81(0.40)</td>
<td>1.49(0.39-5.74)</td>
</tr>
<tr>
<td></td>
<td>Genotype</td>
<td>arg/arg 13(38.23) 0.38</td>
<td>12(35.29) 0.38</td>
<td>1.00</td>
<td>0.8(0.29-2.26)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>arg/gln 16(47.05) 0.41</td>
<td>15(44.11) 0.41</td>
<td>1.01(0.35-2.92)</td>
<td>1.49(0.39-5.74)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gln/gln 05(14.70) 0.14</td>
<td>07(20.58) 0.14</td>
<td>0.81(0.40)</td>
<td>1.49(0.39-5.74)</td>
</tr>
</tbody>
</table>

Statistically significance level <0.05; OR (CI): odd ratio and confidential interval.
the active and passive smokers. Of the three groups of active and passive smokers, group III holds higher number of MN counts than the group II and Group I. The influence of tobacco smoking on the frequency of MN in human lymphocytes has been examined in many population studies (Bonassi et al., 2003) where mixed results have been obtained. In most studies, no effect of smoking on MN frequency has been observed, while in many instances smokers had lower MN frequencies as compared to non-smokers. Aiming at understanding the influence of smoking habit, Bonassi et al. (2003) performed a pooled analysis and the analysis showed that MN was significantly increased among non-exposed heavy smokers and the MN frequency was slightly higher than the controls. This finding is consistent with a recent investigation (Neri et al., 2003) which suggested that the MN frequency was significantly increased in passive smokers when compared to unexposed. We found no evidence for the effect of age and number of cigarettes on the frequency of MN. Recently, investigations revealed that the MN frequency is associated with the level of micronutrients. Several studies (Fenech and Rinaldi, 1995; Fenech et al., 1998) showed a clear increase in the level of MN with a decrease in the folic acid concentration. The subjects of our study were from a lower socio-economic background, hence the subjects are completely lack awareness about the balanced and nutrition’s diet.

In our study, the genotype distribution was almost equal in both controls and exposed subjects. We did not find any significant difference in the allele frequency and genotype distribution between controls and exposed subjects. Several studies have suggested that genetic polymorphisms can affect the level of chromosome damage associated with genotoxic exposures. In both the controls and exposed subjects, the level of chromosomal damage was significantly \((P < 0.001\) higher in subjects homozygous for variant glu/glu, when compared to arg/arg and glu/arg. This suggested us that glu/glu genotype may influence the extent of genotoxic damage due to cigarette smoke than the rest of the arg/arg and glu/arg genotypes.

The arg399glu substitution at exon 10 of XRCC1 (X-ray repair cross-complementing group 1) gene has been suggested to be associated with reduced DNA repair efficiency (Lunn et al., 1999; Duell et al., 2000). Skjelbred et al. (2006) studied the influence of XRCC1 gene polymorphism on chromosomal aberration frequencies and found that irrespective of age and smoking habit individuals carrying XRCC1 399glu allele had an increased risk of chromosomal damage. Two more studies found a positive association with XRCC1 399glu allele and CAs (Vodicka et al., 2004; Au et al., 2004). Similarly, smokers with the XRCC1 399glu variant allele displayed a higher frequency of CAs than carriers of the normal allele (http://ethesis.helsinki.fi/julkaisut/bio/bioja/vk/tuimala/inheritet.pdf). A recent cohort study showed that the variant XRCC1 399glu allele had a significant impact on chromosomal damage induction in both smokers and non-smokers, with

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Control ((n = 110))</th>
<th>Exp ((n = 110))</th>
<th>Mean frequency of MN ± SD buccal cells</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>arg/arg</td>
<td>40</td>
<td>39</td>
<td>0.47 ± 0.50</td>
<td>0.89 (0.47–1.71)</td>
</tr>
<tr>
<td>arg/gln</td>
<td>56</td>
<td>54</td>
<td>0.57 ± 0.49</td>
<td>0.90 (0.43–1.90)</td>
</tr>
<tr>
<td>glu/gln</td>
<td>14</td>
<td>17</td>
<td>0.92 ± 0.82</td>
<td>2.29 ± 1.31</td>
</tr>
</tbody>
</table>

OR (95% CI): odd ratio and confidential interval value.

\(^a\) \(P < 0.001\) compared to arg/arg and arg/gln as estimated by ANOVA (\(P\) value 0.0000).

\(^b\) \(P < 0.05\) compared to arg/arg and glu/gln as estimated by ANOVA (0.041).

\(^c\) \(P < 0.05\) compared to arg/arg and glu/gln as estimated by ANOVA.

\(^{**}\) \(P < 0.05\) compared to arg/arg as estimated by ANOVA.
smokers having slightly higher frequency ratio for cells with chromosomal aberrations, chromosome-type aberrations and chromatid gaps (Skjelbred et al., 2006).

We found a significant increase in the buccal and lymphocyte MN frequency among gln/gln genotypes of controls, meanwhile an insignificant increase was noted among gln/gln subjects of exposed subjects. In this context, a few investigations (Au et al., 2003; Aka et al., 2004; Goddersis et al., 2004) reported that the presence of at least one variant allele (gln instead of arg for XRCC1) is associated with increased MN frequencies. Recently, the effect of three XRCC1 polymorphisms on DNA damage and DNA repair in EM9 cells using the CBMN assay was reported. It had been observed that only the arg399gln polymorphism influenced the ability of XRCC1 to repair DNA (Qu et al., 2005). Guven et al. (2007) assessed the effect of XRCC1 codon 399 polymorphisms on MN frequency in coronary artery disease patients. Results indicated that an association existed between the frequencies of MN and XRCC1 399 polymorphism, subsequently, showed that the gln variant allele had an increased frequency of MN. Lunn et al. (1999) suggested that the arg to gln change at codon 399 may alter the phenotype of the XRCC1 protein, resulting in deficient DNA repair. It can be inferred from the findings of our study that gln/gln genotype is associated with increased frequencies of CAs and MN.

5. Conclusion

In our study a higher number of chromosome aberrations and micronuclei were found to be associated with homozygous variant of the gln allele of XRCC1. Accumulating evidences suggested that gln allele of XRCC1 is associated with the risk of various cancers. Cytogenetic end points such as chromosome aberrations and micronuclei are good indicators of genotoxic insults. Hence cytogenetics and molecular genetic analyses should be carried out together for risk assessment in various categories of smokers, though this study has added some valuable information to this ever increasing problem, but in order to validate the present findings a larger sample size needs to be considered.

Conflict of interest statement

Nothing declared.

REFERENCES


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What is This?
Genotoxicity assessment in smokeless tobacco users: a case–control study

R Chandirasekar1, K Suresh1, K Sasikala1, B Lakshman Kumar2, R Venkatesan1, G Karthik Ganesh3 and Raichel Jacob1

Abstract
India has a long history of tobacco, which includes chewing tobacco and smoking tobacco in various forms. Initially, the smokeless tobacco chewing habit was seen among the majority of the farmers who cultivated tobacco; but in recent years, smokeless tobacco is available in many forms and is cheaper as well and hence it is widely being used among literate and illiterate people. The subjects of our study are living in hilly regions of Yerkaud in Salem district, South India. Most of the inhabitants of our study area are illiterate and more particularly they are unaware of the health effects due to tobacco use. Recent epidemiological reports have strongly indicated the association of cancer risk with usage of smokeless tobacco. The prime aim of our study is to evaluate the genotoxic effects of tobacco use by analysing the cytogenetic end points such as chromosome aberrations in peripheral blood and micronucleus in peripheral blood and buccal cells. About 85 smokeless tobacco users were enrolled for the study and same numbers of age- and sex-matched nontobacco users were also enrolled to serve as controls. The result of our study revealed that tobacco users displayed varied levels of elevated chromosomal damage and micronucleated cells than nontobacco users. The variation in the extent of genetic damage was dependent on the duration of the tobacco use. In conclusion, this study might be helpful in creating awareness on the hazards of the smokeless tobacco products among the global population as a whole for those who chose such products as a cheap alternative to tobacco smoke.

Keywords
Smokeless, tobacco products, genotoxicity, chromosome aberration, micronucleus assay

Introduction
Tobacco use is one of the leading preventable causes of morbidity and mortality in the world (WHO, 2001). It is estimated that 4.9 million deaths occur annually due to tobacco and expected to rise to about 10 million by 2030 (WDR, 1993). It means that tobacco will cause more deaths in the next 30 years than malaria, tuberculosis, maternal and major childhood diseases all together and 70% of these tobacco-related deaths are expected to occur in the developing countries (Da Costa et al., 1998; The World Health Report, 2002). In India, traditionally dried raw tobacco leaf strands and snuffs were used. But, the later form of smokeless tobacco (SLT) use although still practiced but not quite common. Nowadays, these dried raw tobacco leaves are being gradually replaced by the more convenient chewing tobacco products under Indian trade names such as Hans, Chaini Khaini, Ganesh, and so on, and the use of these is now quite common irrespective of men or woman and illiterate or literate. Usually, the SLT users place an amount of tobacco between their cheek and gum, retaining it for a longer or shorter time.
during which it is sucked slowly. The quantity and frequency of use per day differs among different persons. In India, according to literatures SLT forms are mostly the species of *Nicotiana rustica*, while most smoking tobacco is *Nicotianan tabacum* (Chari and Rao, 1992; ICMR, 2001). The samples of *Nicotiana rustica* have been found to contain higher concentrations of tobacco-specific nitrosamines than *Nicotianan tabacum* (Bhide et al., 1989). Nearly 3000 chemical constituents have been identified in SLT; these include alkaloids, phytosterols, alcohols, phenolic compounds, chlorogenic acid, rutin, carboxylic acids and several free amino acids (IARC, 1985).

In addition, a wide range of toxic metals like arsenic, nickel and cadmium have also been found in tobacco (NCFS, 1997). According to a working group of the IARC, SLT is classified as group I ‘carcinogenic to humans’ (IARC, 2007). The most prevalent strong carcinogens in SLT products are the tobacco-specific nitrosamines (IARC, 2007). Tobacco usage in any form is associated with aetiology of many diseases for many decades. SLT is well recognized as a cause of cancer of the oral cavity (Baron et al., 1996) in addition to other cancers like, lung cancer, stomach, pancreas, kidney, ureter, bladder, colon and uterine cervix (IARC, 2004). Many of the substances in tobacco are genotoxic and therefore cytogenetic damage seems to be a good biomarker for determining the effect of exposure to genotoxic agents in tobacco (IARC, 1985). In addition to that, the chromosomal aberrations and micronuclei have been used as an important biological end point in the study population at risk (Boffetta and Trichopoulos, 2002). Therefore, we performed the assays like chromosome aberration (CA) assay and micronucleus (MN) assay aimed at predicting the population subgroups at increased risk.

**Materials and methods**

Our study area was the hilly region of Eastern Ghats, Yercaud, Salem, South India. The study group comprised 35 males and 50 females who chewed dried raw tobacco strands and an equal number of controls (nontobacco chewers) matched for age, gender and socioeconomic conditions. Prior to venous blood collection, informed consent was obtained from all the individual donors. About 2 ml of venous blood was collected aseptically in heparinised vials. While, the exfoliated buccal mucosa cells were also collected in 0.9% saline solution.

**Chromosome aberration assay on peripheral lymphocytes**

Chromosomal aberration analysis was carried out by the following standard procedures (Hoyos et al., 1996). Briefly, 0.5 ml whole blood was added to 5.0 ml of RPMI 1640 medium (Hyclone) supplemented with 20% foetal bovine serum (PAA Laboratories, Austria), 2 mM L-glutamine (Himedia, Mumbai, India), 1% streptomycin–penicillin (Himedia) and 0.2 ml of phytohaemagglutinin (Gibco, USA). The mixture was incubated at 37°C for 72 h. After 71 h, the cells were treated with 0.01% Colchicine (Himedia) to arrest cells in mitosis. Lymphocytes were harvested upon the completion of 72 h by centrifugation of the cells at 1800 r/min for 7 min. About 6 ml of prewarmed (37°C) hypotonic solution (KCl 0.075 M) was added and left aside for 20 min at room temperature. After removing the hypotonic solution by centrifugation, the cells were fixed in Carnoy’s fixative. Slides were prepared and stained in 2% Giemsa stain. For the chromosomal aberration analysis, 100 well-spread metaphase plates were examined per subject under a microscope (×100) to identify numerical and structural CA.

**Micronucleus in peripheral lymphocytes**

Cytokinesis-blocked MN assay was carried out following the procedures of Fenech and Morley (1986). In brief, whole blood (0.5 ml) was added to 4.5 ml of RPMI-1640 medium (Hyclone) supplemented with 20% foetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), and 0.2 ml of phytohaemagglutinin (Gibco). The mixture was incubated at 37°C for 72 h. Cytokinesis was blocked by the addition of cytochalasin B at a final concentration of 6 μg/ml 44 h after stimulation with phytohaemagglutinin. After 72 h of incubation, cells were harvested by centrifugation, given 1 min hypotonic treatment (0.075 M KCl) and fixed in fresh fixative solution (methanol/acetic acid, 3:1). This fixation step was repeated twice after 20 min storage at 4°C and eventually stained in Giemsa stain.

**Micronucleus test in buccal mucosa cells**

After rinsing the mouth well with tap water, exfoliated buccal mucosa cells were collected by scraping the right/left cheek mucosa with a moist wooden spatula. Cells were transferred to a tube containing saline solution (0.9% NaCl). The cells were centrifuged (800 r/min) for 5 min, fixed in 3:1 methanol/acetic
acid, and dropped onto precleaned slides. Later, the air-dried slides were stained in Feulgen plus fast green. The identification of micronuclei was based on the criteria proposed by Sarto et al. (1987). About 1000 cells were screened for calculating the frequency of micronucleated cells from each subject.

Statistical analysis
Statistical analysis was carried out using the statistical software programme for windows (SPSS Version 16). Analysis of variance (ANOVA) was performed to compare the frequency of chromosomal aberrations and micronuclei between tobacco chewers and controls. p < 0.05 was used as the criterion of significance.

Results
Our study comprised males and females of different age groups and they were categorised as I, II and III in accordance with the duration of SLT usage, the results are mentioned in Table 1.

In our study we observed that, the SLT users exhibited varied levels of chromosomal aberrations (chromatid type aberrations included chromatid gaps and breaks while chromosome type aberrations included chromosome gaps, dicentrics and acentric fragments). The SLT users in groups I, II and III have significantly (p < 0.05) higher chromosomal aberrations when compared to controls. Group I SLT users have low level of chromosomal aberrations than the SLT users of group II and group III in terms of chromatid and chromosome type. But the extent of chromosomal aberrations gradually increased from group I to group II and from group II to group III. The observations suggest that the frequency of chromosomal aberrations increases with the increase in the duration of SLT usage. We also found that the chromatid type aberrations are prevalent than the chromosome type aberrations, suggesting that the chromosomal aberrations caused by the tobacco chemical constituents are mainly of chromatid type. Among all the three groups studied, the chromatid type aberration was found to be predominant in group III (p < 0.0001) when compared to other groups and controls (Table 2).

Table 3 shows the frequencies of MN cells scored in both blood and buccal cells of controls and experimentals (Exps). A total of 1000 and 500 cells were screened in buccal and blood lymphocytes, respectively, for MN frequency. In both the buccal and blood lymphocytes, the frequencies of MN were higher when compared to controls. In buccal MN analysis, the Exp subjects of group I Exp A and Exp B showed a mean ± SD value of 1.5 ± 0.7 and 1.6 ± 0.5, respectively. Group II Exp A and Exp B were found to be 2.3 ± 0.5 and 2.7 ± 0.3, respectively, and group III Exp A and Exp B showed values of 2.6 ± 0.9 and 2.9 ± 1.1, respectively. Whereas in control subjects, a value of 0.3 ± 0.2 was found in group I, 0.4 ± 0.3 in group II and 0.9 ± 0.1 in group III. MN frequency in blood lymphocytes was also evaluated for control and Exp subjects. The control subjects of group I exhibited 0.2 ± 0.1, group II exhibited 0.3 ± 0.2 and group III exhibited 0.5 ± 0.3. On the other hand, the Exp group I showed the values 1.2 ± 0.4 for Exp A and 0.9 ± 0.3 Exp B. In groups II and III, significant alterations (t test, p < 0.0001) were observed. Among the three groups, high frequency of MN was identified in group III active smokers of both Exp A and B (2.9 ± 1.1 in buccal and 1.6 ± 1.0 in blood cells). Among the female subjects of Exp group high frequency of MN was identified in group III. A gradual increase in the frequency of MNs was also found from group I to group III in both buccal and blood cells. Statistically significant differences were observed between the Exp subjects and control subjects (p < 0.0001).

Table 1. Frequencies of age and year of exposure in smokeless tobacco users and nonusers

<table>
<thead>
<tr>
<th>Particulars</th>
<th>Smokeless tobacco users, mean ± SD</th>
<th>Nonusers (controls), mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total samples</td>
<td>85</td>
<td>85</td>
</tr>
<tr>
<td>Male</td>
<td>35 (41.17%)</td>
<td>35 (41.17%)</td>
</tr>
<tr>
<td>Female</td>
<td>50 (58.82%)</td>
<td>50 (58.82%)</td>
</tr>
<tr>
<td>Age and year of exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I, 5 &lt; 10</td>
<td>19 (23.26 ± 2.88)</td>
<td>19 (24.36 ± 2.62)</td>
</tr>
<tr>
<td>Group II, 10 &lt; 20</td>
<td>27 (39.48 ± 6.30)</td>
<td>27 (35.51 ± 2.15)</td>
</tr>
<tr>
<td>Group III, above 20</td>
<td>39 (59.69 ± 6.76)</td>
<td>39 (57.15 ± 6.94)</td>
</tr>
</tbody>
</table>

SD: standard deviation; group I: year of exposure 5–10 years; group II: 10–20 years; group III: above 20 years.
The present study demonstrated that the mean value of MN was significantly higher at \( p < 0.05 \) level in SLT users than in nonsmokers/non-SLT users. The control subjects who were healthy subjects with no apparent nutritional deficiency showed a minimal number of micronuclei when compared to Exp subjects. A comparison was made between all controls which showed nonsignificant result. This can be attributed to the fact that the controls were healthy volunteers with no habitual chewing of tobacco. However, a minor degree of aberrations were observed with statistical insignificance. The results are indicated in Table 4. On the other hand, when a comparison was made for aberrations between all Exp and control groups, statistical significance was achieved depending upon the duration of exposure. When a comparison was made between groups, based upon duration of exposure, significant differences were observed in Exp subjects. The frequency of aberrations increased with increase in duration of exposure.

**Discussion**

In Southeast Asia over 250 million people are using SLT products, about 17\% of total population in Southeast Asia use oral tobacco; of which 95\% belong to India (82\%) and Bangladesh (13\%; WHO, 2004). In some parts of India, such as the states of Bihar and Maharashtra, SLT use is more common than smoking. Apart from regional preferences due to differing

---

**Table 2.** Frequencies of age and chromosome aberrations in smokeless tobacco users and nonusers (mean ± SD)

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Year of exposure</th>
<th>Number of metaphase</th>
<th>Chromated-type aberrations</th>
<th>Chromosome-type aberration</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls, N = 85</td>
<td>Nil</td>
<td>100</td>
<td>0.76 ± 0.73</td>
<td>0.28 ± 0.45</td>
<td>1.04 ± 0.42</td>
</tr>
<tr>
<td>Group I, N = 19</td>
<td></td>
<td>100</td>
<td>0.86 ± 0.41</td>
<td>0.56 ± 0.98</td>
<td>1.40 ± 0.41</td>
</tr>
<tr>
<td>Group II, N = 27</td>
<td></td>
<td>100</td>
<td>0.92 ± 0.71</td>
<td>0.61 ± 0.55</td>
<td>1.53 ± 0.42</td>
</tr>
<tr>
<td>Group III, N = 39</td>
<td></td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>5–10</td>
<td>100</td>
<td>2.26 ± 1.48</td>
<td>1.36 ± 0.49</td>
<td>3.57 ± 1.67</td>
</tr>
<tr>
<td>Group I, N = 19</td>
<td></td>
<td>100</td>
<td>3.51 ± 1.42</td>
<td>1.85 ± 0.98</td>
<td>5.37 ± 1.69</td>
</tr>
<tr>
<td>Group II, N = 27</td>
<td></td>
<td>100</td>
<td>4.53 ± 1.75</td>
<td>2.82 ± 0.41</td>
<td>7.35 ± 2.20</td>
</tr>
</tbody>
</table>

Group I: 5–10 years of exposure; group II: 10–20 years of exposure; group III: above 20 years of exposure.

*Means in column followed by common superscripts for the experiments are significant at \( p < 0.05 \) level compared with control groups.

---

**Table 3.** Micronuclei frequency identified in controls and smokeless tobacco users (buccal and blood cells)

<table>
<thead>
<tr>
<th>Group</th>
<th>Particulars</th>
<th>No. of subjects studied</th>
<th>Mean frequency of micronuclei ± SD of MN scored in buccal cells/1000</th>
<th>Mean frequency of micronuclei ± SD of MN scored in blood cells/500</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CS</td>
<td>19</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.0001a 0.0001a</td>
</tr>
<tr>
<td></td>
<td>Exp A</td>
<td>11</td>
<td>1.5 ± 0.7a</td>
<td>1.2 ± 0.4a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exp B</td>
<td>8</td>
<td>1.6 ± 0.5a</td>
<td>0.9 ± 0.3a</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>CS</td>
<td>27</td>
<td>0.4 ± 0.3</td>
<td>0.3 ± 0.2</td>
<td>0.0001a 0.0001a</td>
</tr>
<tr>
<td></td>
<td>Exp A</td>
<td>15</td>
<td>2.3 ± 0.5a</td>
<td>1.5 ± 0.7a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exp B</td>
<td>12</td>
<td>2.7 ± 0.3a</td>
<td>1.4 ± 0.2a</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>CS</td>
<td>39</td>
<td>0.9 ± 0.1</td>
<td>0.5 ± 0.3</td>
<td>0.0000a 0.0000a</td>
</tr>
<tr>
<td></td>
<td>Exp A</td>
<td>9</td>
<td>2.6 ± 0.9a</td>
<td>1.6 ± 1.1a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exp B</td>
<td>30</td>
<td>2.9 ± 1.1a</td>
<td>1.6 ± 1.0a</td>
<td></td>
</tr>
</tbody>
</table>

CS: control subjects for both males and females; Exp A: experimental A for male; Exp B: experimental B for female; SD: standard deviation.

*Values are significant at \( p < 0.001 \) level compared to control groups and with experimentals.
Table 4. Assessment of within and between the groups of experimentals and controls

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Df</th>
<th>Sum of squares</th>
<th>Mean squares</th>
<th>F ratio</th>
<th>p (value)</th>
<th>Df</th>
<th>Sum of squares</th>
<th>F ratio</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between</td>
<td>5</td>
<td>408.8</td>
<td>81.8</td>
<td>55.53</td>
<td>0.0000</td>
<td>5</td>
<td>151.0</td>
<td>65.342</td>
<td>0.0001b</td>
</tr>
<tr>
<td>Within</td>
<td>164</td>
<td>241.3</td>
<td>1.5</td>
<td></td>
<td></td>
<td>164</td>
<td>75.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>169</td>
<td>650.1</td>
<td></td>
<td></td>
<td></td>
<td>169</td>
<td>22.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Post-hoc t test

<table>
<thead>
<tr>
<th>Group</th>
<th>Chromatid-type aberrations</th>
<th>Chromosomal type aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t value</td>
<td>Df</td>
</tr>
<tr>
<td>Control group I</td>
<td>C group II</td>
<td>0.275</td>
</tr>
<tr>
<td>Control group I</td>
<td>C group III</td>
<td>0.471</td>
</tr>
<tr>
<td>Control group I</td>
<td>Exp group I</td>
<td>-3.811</td>
</tr>
<tr>
<td>Control group I</td>
<td>Exp group II</td>
<td>-7.570</td>
</tr>
<tr>
<td>Control group I</td>
<td>Exp group III</td>
<td>-11.108</td>
</tr>
<tr>
<td>Control group II</td>
<td>C group III</td>
<td>-0.198</td>
</tr>
<tr>
<td>Control group II</td>
<td>Exp group I</td>
<td>-3.854</td>
</tr>
<tr>
<td>Control group II</td>
<td>Exp group II</td>
<td>-8.026</td>
</tr>
<tr>
<td>Control group II</td>
<td>Exp group III</td>
<td>-12.084</td>
</tr>
<tr>
<td>Control group III</td>
<td>Exp group I</td>
<td>-3.948</td>
</tr>
<tr>
<td>Control group III</td>
<td>Exp group II</td>
<td>-8.528</td>
</tr>
<tr>
<td>Control group III</td>
<td>Exp group III</td>
<td>-13.141</td>
</tr>
<tr>
<td>Exp group I</td>
<td>Exp group II</td>
<td>-3.441</td>
</tr>
<tr>
<td>Exp group I</td>
<td>Exp group III</td>
<td>-6.688</td>
</tr>
<tr>
<td>Exp group II</td>
<td>Exp group III</td>
<td>-3.358</td>
</tr>
</tbody>
</table>

Df: degree of freedom; C: control; Exp: experimental.

\(^a\)Chromatid-type and chromosomal-type aberrations multivariate comparison using ANOVA. Statistically significant at p value <0.0001 level (one or more mean and SD compared to ANOVA).

\(^b\)p value <0.01.

\(^c\)p value <0.0001.
sociocultural norms, the preference for SLT is inversely related to education and income (Gupta and Ray, 2003). Our study was conducted in the SLT users of south India in Eastern Ghats tribal people. It is well established that the smoking is responsible for a substantial number of human health problems worldwide (Weir et al., 2003).

Hundreds of millions of people are addicted to SLT, and its use among the young people is increasing in many countries. Overall, there is sufficient evidence that SLT causes oral cancer and pancreatic cancer in humans, and sufficient evidence of carcinogenicity from animal studies. A previous study concluded that SLT is ‘carcinogenic to humans’ (Winn et al., 1981). With regard to age, most of the Exps were old female consumers (40–60 years old). The majority of human cancers are caused by tobacco, synthetic and natural chemicals of occupational, environmental, medical and dietary origin. The chemical carcinogens cause structural alterations in the DNA of target cells, leading to genomic instability in the form of chromosomal abnormalities (Jagetia et al., 2001). The present study was designed to test the validity among the two cytogenetic end points namely MN (buccal and blood) and CA, as biomarkers of early effect and as predictive tools for subsequent risk of SLT-related genotoxicity. The rationale for using these biomarkers is based on the hypothesis that the extent of genetic damage in peripheral blood lymphocytes and oral epithelial cells can be used as effective risk assessment tools of genotoxicity leading to carcinogenesis. However, the causal relationship between SLT users, the induction of biological effects, and the extent of the disease burden among smokers has not been fully documented. In the present study, higher frequency of CAs was observed among SLT users compared to controls using various cytogenetic end points.

Sierra-Torres et al. (2004) reported a significantly higher frequency of CAs among smokers compared to nonsmokers. Our CAs data are in agreement with other cytogenetic studies which have found significantly increased CA frequencies in smokers compared to nonsmokers (Tawn and Whitehouse, 2001). The increase in CA frequencies over the nonsmokers is much higher than those reported in the literature (Bender et al., 1988). In this study, we observed that female subjects used SLT more frequently than males. However, a high level of expression of CAs in SLT users was found when compared to controls. In group I controls, few minor aberrations were identified. The use of the CA assay in the surveillance of the populations exposed to genotoxic carcinogens originates from the observation that most human carcinogens induce chromosome damage in genotoxicity tests (Norppa et al., 2006). Our results suggest that the genotoxic effects in lymphocytes from smokers are most likely caused by tobacco providing a prospective mechanism for oral cancer development.

Among cytogenetic markers, MN is studied from exfoliated buccal mucosa of tobacco chewers. MNs are fragments or whole chromosomes, which did not reach spindle poles during mitosis and remained encapsulated at telophase in a separate nucleus. Whereas CA assay detects only the genome damage, MN assay additionally detects chromosome loss or malfunction of mitotic spindle caused by aneugenic mechanisms (Znaor et al., 2003). MN assay is a simple and rapid screening test applied for an early detection of cancer (Tates et al., 1980) and the test is used on exfoliated cells to identify the genotoxic damage in human tissues, which are targets for carcinogens and from which carcinomas develop. In the present study, a mean and SD occurrence of 0.3 ± 0.2, 0.4 ± 0.3 and 0.9 ± 0.1 buccal micronucleated cells was obtained for the control groups I, II and III, respectively. In this study, group III of SLT users showed a highly significant increase in micronucleated cells with value of 2.9 ± 1.1. Micronucleated cells in the unaffected site could be due to the distribution of genotoxic agents through saliva (Kamboj and Mahajan, 2007). MN can be stated as an early indicator and an upcoming marker for diagnosing oral precancer and cancer. Abnormal oral habits too significantly increase the counts of MN (Kamboj and Mahajan, 2007). Adverse health effects are associated with the use of oral snuff and chewing tobacco (Winn et al., 1981). Potent carcinogens have been measured in SLT products, with levels of tobacco-specific nitrosamines 100-fold greater than legal limits for nitrosamines in foodstuffs (The Health Consequences of Using Smokeless Tobacco, 1986). India is the second most densely inhabited country in the world and the third largest producer and consumer of tobacco (Bhonsle et al., 1992). In recent years, consideration has been given to the use of oral noncombustible or SLT among cigarette smokers who are unable or indisposed to quit using tobacco (Stratton et al., 2001). Formulation is undoubtedly an important mechanism associated with chromosome loss (Hazare et al., 1998). In conclusion, awareness of the hazards of SLT use is very low in rural populations. The
results of this study show that while both tobacco chewers exhibit elevated chromosomal damage, although differences exist in the type of aberrations (chromatid and chromosome). These differences appear to be mediated by the extent of tobacco exposure although the buccal MN is a best site of measurement for early deducting genotoxicity assessment in SLT users. Stringent measures need to be taken to educate the rural population on the ill effects of SLT usage that is rampant among all age groups with no socioeconomic differences.

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**References**


National Cancer Institute Fact Sheet (1997 November) Questions and answers about Smokeless Tobacco and Cancer.


RESEARCH COMMUNICATION

No Association Between the Trp53 Codon 72 Polymorphism and Head and Neck Cancer: A Case-Control Study in a South Indian Population

K Suresh*, R Chandirasekar, B Lakshman Kumar, R Venkatesan, K Sasikala

Abstract

Genetic polymorphisms are important for predispositions to several human cancers. In the tumour suppressor Trp53 gene, a codon 72 polymorphism is frequent in the form of a single nucleotide polymorphism that leads to substitution of an arginine for a proline. In the present study, we analysed the association of Trp53 codon 72 polymorphs with head and neck cancer through a case-control study approach with PCR-RFLP of DNA from blood of 47 clinically confirmed patients and 52 healthy controls. The Pro (Trp53^72P) and Arg (Trp53^72R) allele frequencies in the healthy controls were 0.44 and 0.56, and not significantly different from those in the cancer patients at 0.56 and 0.44. The genotype distribution in the controls was 32.7% Arg/Arg, 46.2% Arg/Pro and 21.2% Pro/Pro and in the cancer patients 17.0% Arg/Arg, 53.2% Arg/Pro and 29.8% Pro/Pro. No significant difference in the distribution of genotypes between head and neck cancer patients and healthy controls (P=0.18, χ² test) was observed. We conclude no association of Trp53 codon 72 polymorphism was observed with head and neck cancer susceptibility.

Keywords: Trp53 codon 72 polymorphism - cancer of head and neck - India

Asian Pacific J Cancer Prev, 11, 1749-1753

Introduction

The squamous cell carcinoma of the head and neck may occur in the oral cavity, oropharynx, hypopharynx and larynx, it is among the five most common cancers and accounts for >500,000 new cases every year worldwide (Pisani et al., 1999). In India, it accounts for 23% of all cancers in males and of 6% in females (ICMR, 1992). This excessively higher incidence may be due to use of tobacco in various forms, alcohol consumption, low socioeconomic condition related to poor hygiene, poor diet and rampant viral infections (Franceschi et al., 2000). A causal association between this squamous cell carcinoma of the head and neck and exposure to tobacco and alcohol is well established (Lewin et al., 1998).

Since the 18th century, it has been recognized that, exposure to environmental chemicals plays a major role in the etiology of human cancers. The development of cancer is not only due to endogenous or exogenous carcinogens but also their interactions with genes whose products are involved in the detoxification of carcinogens, repair of DNA damage and control of cell signaling and cell cycle. Genetic predisposition due to polymorphisms and mutations in such low penetrance genes facilitate the development of sporadic cancers upon appropriate exposure (Kotnis et al., 2005). Single nucleotide polymorphisms (SNPs) are minor genetic variations in the genome that play an important role in promoting susceptibility to disease and in the response to various carcinogens (Hemminki and Shields, 2002). Altered function of Trp53 gene due to SNPs may affect the gene-environment and gene-gene interaction, thereby increasing the risk of the development of sporadic cancers. The importance of the Trp53 tumor suppressor gene in the process of carcinogenesis is well established (Hussain and Harris, 1998).

The Trp53 codon 72 polymorphism is the most common in the general population and arises from a single-base-pair polymorphism where CCC encodes proline or CGC encodes arginine. These two alleles generate three genotypes, Arg/Arg (Trp53^72R), Pro/Pro (Trp53^72P) and Arg/Pro (Trp53^72R/Trp53^72P). The two polymorphic variants of wild type Trp53 have been shown to have different biochemical properties like differential binding to components of the transcriptional machinery (Thomas et al., 1999), inducing cell death (Dumont et al., 2003; Sullivan et al., 2004), cell-cycle arrest (Pim and Banks, 2004) and besides these functions Trp53 regulates the various DNA-repair processes (Sengupta and Harris, 2005). We have investigated the frequency of this Trp53 codon 72 polymorphism in cancer of head and neck and their association with risk of this cancer.

Patients and Methods

Blood was obtained from 47 clinically confirmed...
patients with cancer of head and neck and from 52 controls. The age of the cancer patients range from 45-70, age of the control subjects range from 42-79. The cancer patients included in the study were from various territory cancer care hospitals at Coimbatore and Erode districts of Tamilnadu State, South India. The control subjects were from general population employed in various professions, living in the same geographical area. The purpose of the study was explained to the participants, all participants gave their written consent prior to inclusion in the study.

Trp53 codon 72 polymorphism determination

DNA was extracted from the peripheral blood cells using standard procedure, involving SDS / Proteinase K digestion followed by ethanol precipitation. The primers were commercially purchased [I^th base, Singapore] and the primer sequences were verified through UCSC In-silico PCR [http://genome-mirror.duhs.duke.edu/cgi-bin/hgPcr] to eradicate the possibility of amplification of any non-specific DNA sequences. Purified genomic DNA isolated from the cancer patients and controls was amplified by PCR for exon 4 codon 72 of Trp53 gene. A total of 100-200ng of genomic DNA was amplified through PCR containing 1µM of each forward 5' - TTG CCG TCC CAA GCA ATG and reverse 5' - TCT GGG AAG GGA CAG primers in a final reaction volume of 50 µl contained 10 mM of Tris-HCl, 50 mM of KCl, 2 mM MgCl₂, 0.2 mM of each dNTPs (Fermentas, Germany) and 1.25 U of Taq DNA polymerase (Fermentas, Germany) for 4-16 hours and the digestion product was resolved on an 8% polyacrylamide gel electrophoresis for 2:15 hours at 65V/cm in 1X TBE buffer. Detection of bands was performed by the silver staining method. An undigested PCR product (199 bp long) with 5 U of Bsh12361 (Fermentas, Germany) for 4-16 hours and a final cycle of extension at 72°C for 5 min. The polymorphisms were identified by digesting the PCR products (199 bp long) with 5 U of Bsh12361 (Fermentas, Germany) for 4-16 hours and the digestion product was resolved on an 8% polyacrylamide gel electrophoresis for 2:15 hours at 65°C in 1X TBE buffer. Detection of bands was performed by the silver staining method. An undigested PCR product (199 bp) was representing the homozygous Trp5372R, two fragments of 113-bp and 86-bp representing homozygous Trp5372P, three fragments of 19-bp, 113-bp and 86-bp representing heterozygous Trp5372R/Trp5372P for codon 72.

Statistical analysis

Chi-square analysis (χ²) was used to test the association of the genotypes and alleles in cancer patients and controls. The odds ratio (OR) and their confidence intervals (CI) were calculated to estimate the strength of the association of polymorphism genotype alleles in patients and controls (Martin Bland and Douglas, 2000).

Table 1. Genotype Distribution and Allele Frequency of Trp53 Genotypes in Head and Neck Cancer Patients and Controls

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Controls n=52 (%)</th>
<th>Breast cancer patients n=47 (%)</th>
<th>P (χ²)</th>
<th>OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg/Arg</td>
<td>17 (32.69)</td>
<td>8 (17.02)</td>
<td></td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>Arg/Pro</td>
<td>24 (46.15)</td>
<td>25 (53.19)</td>
<td>0.18</td>
<td>(3.37)</td>
<td>0.21 (0.80-6.07) 0.11</td>
</tr>
<tr>
<td>Pro/Pro</td>
<td>11 (21.15)</td>
<td>14 (29.78)</td>
<td>1.22</td>
<td>(4.6-3.21)  0.68</td>
<td></td>
</tr>
<tr>
<td>Pro allele frequency</td>
<td>0.44</td>
<td>0.56</td>
<td>0.47</td>
<td>(5.1)</td>
<td>0.81 (0.46-1.42)</td>
</tr>
</tbody>
</table>

Significance level P<0.01

Results

The mean age of the control subjects and cancer patients was 56.7±9.00 and 56.9±7.12 respectively. The mean height of the controls was 159.4±7.80, whereas cancer patients have shown 163.6±7.80. The mean weight of the controls was 53.3±8.92, cancer patients showed 51.3±8.92. The number of subjects who used tobacco in controls was 19 and 34 in cancer patients. Alcohol use was seen among 8 controls and 14 cancer patients. No family history of cancer was seen among cancer patients. Types, stages and sites of cancer observed among the patients were, squamous cell carcinoma, primary stage squamous cell carcinoma, buccal mucosa squamous cell carcinoma, adenoid cystic carcinoma, invasive squamous cell carcinoma, infiltrating squamous cell carcinoma, secondary deposit of squamous cell carcinoma, squamous cell carcinoma moderately differentiated, submandibular node cancer supraglossis and posterior tongue cancer.

Smoking was significantly associated with cancer (χ² =12.72, P=0.0003), but not alcohol use (χ² = 2.96, P=0.08). The frequency of the genotypes Trp5372R, Trp5372R/Trp5372P and Trp5372P of the controls was 32.7%, 46.2% and 21.2% respectively. Of the cancer patients 17.0%, 53.2% and 29.8% had Trp5372R, Trp5372R/Trp5372P and Trp5372P respectively. The allele frequency of both groups fitted in the Hardy-Weinberg equilibrium with allele frequencies of 0.56 (controls) and 0.44 (cancer) for Trp5372R-coding alleles and 0.44 (controls) and 0.56 (cancer) for Trp5372P-coding alleles. Allele frequency did not differ significantly between the cancer patients and healthy controls (χ² =0.512, P=0.47). Overall, there was no
significant difference in the genotype distribution between controls and cancer patients. \(P=0.18, \chi^2=3.37\). Further, combined analysis of Trp5372R/Trp5372P and Trp5372P/Trp5372P genotypes \(P=0.685, \chi^2=0.165, \text{OR}=1.22, 95\% \text{CI}=0.46-3.21\) and Trp5372R/Trp5372R and Trp5372R/Trp5372P genotypes \(\chi^2=2.424, P=0.119, \text{OR}=1.22, 95\% \text{CI}=0.464-3.21\) revealed no significant association of this polymorphism with head and neck cancer (Table 1; Figure 1).

Discussion

It is well-known that the role of tobacco and alcohol in the etio-pathogenesis of head and neck cancer (Brennan et al., 1995; Lewin et al., 1998; Zhang et al., 2000; Khandekar et al., 2006; Freedman et al., 2007). In our study, we found that the tobacco use was significantly associated with the incidence of head and neck cancer \(P=0.0003, \chi^2=12.72\), in addition, an increased risk \(\text{OR}=4.54, 95\% \text{CI}=1.93 to 10.65\) of tobacco use with head and neck cancer was seen, but an insignificant association was found for alcohol use with head and neck cancer incidence \(P=0.08, \chi^2=2.96, \text{OR}=2.33, 95\% \text{CI}=0.87-6.21\).

Variations in the function of genes responsible for DNA repair mechanisms and cell-cycle control is an attractive mechanism for explaining any inter-individual variation in cancer susceptibility (Matakidou et al., 2003). In the Trp53 gene, a common genetic variant at codon 72 has been extensively studied for its association with cancer risk, but the findings have ranged from conflicting (Suspirsin et al., 2003) to conclusive (Hildesheim et al., 1998; Rosenthal et al., 1998; Soulitzis et al., 2002; Gemignani et al., 2004). The polymorphism is balanced, varies with latitude and race, and is maintained at different allelic frequencies across the population (Sjalander et al., 1996). The distribution of allele Trp5372P in different world populations is, Swedish Saamis (0.17), Finns (0.24), Sweeds (0.29) and Caucasians (0.21) in which a lower frequency of Trp5372P allele was found (Sjalander et al., 1995; Sjalander et al., 1996). A higher frequency of Trp5372P allele was observed in African-Americans (0.63) (Jin et al., 1995). In Western Europe (France, Sweden, Norway), North America (USA), Central and South America (Mexico, Costa-Rica, Peru) and Japan, the most common allele is Trp5372R, with frequencies ranging from 0.60 to 0.83 (IARC-TP53 Database, 2010).

In Asian populations, the distribution of the heterozygous form (Trp5372R/Trp5372P) was more common than the homozygous genotypes and this distribution pattern was different from Caucasian populations which showed higher Trp5372R and lower Trp5372P homozygous genotypes (Shen et al., 2002).

The two polymorphic variants (Trp5372R and Trp5372P) of the wild type Trp53 have been shown to have different biochemical properties such as, (i) the Trp5372R variant was a more active transcriptional activator than the Trp5372P variant (Thomas et al., 1999), (ii) the Trp5372R variant is more efficient in inducing cell death than the Trp5372P variant in some cell types (Dumont et al., 2003), (iii) the Trp5372P variant was shown to induce cell-cycle arrest better than the Trp5372R variant (Pim and Banks, 2004). These data suggested that both the polymorphic variants of Trp53 might have involved for selectively regulating specific cellular functions and the functional differences between the two forms of Trp53 suggest that their expression status may thus influence cancer risk.

The association of Trp53 codon 72 polymorphism with head and neck cancer in different ethnic backgrounds remains uncertain. In our study, we compared the genotype and allele frequencies of the Trp53 codon 72 polymorphism between head and neck cancer patients and healthy controls in South Indian population. We found no significant difference in the distribution of genotypes, and they were similarly represented in cancer patients and healthy controls \(P=0.18, \chi^2=3.37\). Even though the distribution of genotypes were not significantly different, an increased frequency of Trp5372P alleles in cancer patients \(P=0.47, \chi^2=0.512\) over the controls was observed.

The genotype frequencies observed in our study is consistent with the previous observations in oral cancer patients of Indian population (Tandle et al., 2001; Mitra et al., 2005). However, the presence of higher number of the Trp5372P/Trp5372P genotype and the Trp5372P allele in the cancer patients made it reasonable to suspect the susceptibility of Trp5372P allele to head and neck cancer. Hiyama et al., (2008) reviewed 20 epidemiological studies and suggested that individuals with Trp5372P genotype showed a higher risk for head and neck cancer than individuals with Trp5372R genotype in 15 of 20 studies. Two studies showed a significantly higher risk for head and neck cancer in Trp5372P homozygotes than in Trp5372R homozygotes.

By contrast, recent evidences (Siddique and Sabapathy, 2006) confirmed that, Trp5372P allele reduces genomic instability better than the Trp5372R allele in many respects. First, the Trp5372P variant transcriptionally activates Trp53-dependent target genes involved in DNA-repair better than the Trp5372R form. Consequently, cells expressing Trp5372P form are able to repair DNA-damage much more efficiently than the Trp5372R-expressing cells (preferentially by inducing Trp53 dependent DNA-repair target gene promoters (Trp53R2)) in the Trp53 dependent DNA-repair process, which may influence cancer risk. Second, the efficiency of the unscheduled DNA synthesis, that is DNA synthesis owing to repair replication in non-S-phase cells, revealed that, Trp5372P-expressing cells consistently and reproducibly incorporated significantly more thymidine (\(^{3}H\)) (at both UV doses 25 and 50 J/m\(^2\) at 32°C) compared to Trp5372R-expressing cells, which indicate that NER occurred much more efficiently in Trp5372P-expressing cells compared to Trp5372R-expressing cells. Third, cyclobutane pyrimidine dimers (CPDs) are the predominant product of photo damage in DNA after exposure of cells to UV light. CPDs are recognized and removed by NER, and defects in this process often lead to predisposition to cancer (Hanawalt et al., 2003). Trp5372R-expressing cells remove CPDs more rapidly than the Trp5372P-expressing cells. Fourth, Trp5372P-expressing cells are less able to remove the micronuclei (acentric chromatids or chromosome fragments) which are induced by radiation or other DNA damaging agent, suggesting
that Trp53<sup>72R</sup> might be less potent in reducing genomic instability, and perhaps cancer predisposition. In this context, it can be assumed that the Trp53<sup>72R</sup> allele might not be a predisposing allele to cancer. In our study, the Trp53<sup>72P</sup> allele showed a higher prevalence in the cancer group (0.56), it might be due to the natural selection. A few investigations, examined the Trp53 codon 72 polymorphism in Indian population and reported that the Trp53<sup>72P</sup> allele frequency in different parts of India vary from 0.45-0.56 (Tandle et al., 2001; Katiyar et al., 2003; Mitra et al., 2003). Hence, we report that the presence of higher number of Trp53<sup>72P</sup> allele (0.56) in cancer patients is not associated with susceptibility to cancer of the head and neck; it might be due to the natural selection.

Siddique et al., (2005) indicated that healthy Asian (Chinese) heterozygote individuals (Trp53<sup>72R/72P</sup>) tend to preferentially express the Trp53<sup>72P</sup> allele at the RNA level. By contrast, Trp53<sup>72R</sup> allele was preferentially expressed in most heterozygote breast cancer patients (73.4%), suggesting that the Trp53<sup>72R</sup> allele is selectively activated and the Trp53<sup>72P</sup> allele is silenced in heterozygote cancers. It indicated that the Trp53<sup>72R</sup> was associated with cancer predisposition (Sjalander et al., 1996; Bergamaschin et al., 2003), although the Trp53<sup>72P</sup> form might be capable of inducing apoptosis better than the Trp53<sup>72P</sup> form (this apoptotic effect might be of cell type specific), it might not be efficient in preventing cancer formation. Taken together, suggest that the Trp53<sup>72P</sup> polymorph of Trp53 has a selective advantage over Trp53<sup>72R</sup> and the Trp53<sup>72P</sup> form might be more efficient in other Trp53-related functions in inhibiting malignancy.

In conclusion, from the genotypic analysis of our study, an equal distribution of the genotypes and no over-representation of either Trp53<sup>72P</sup> or Trp53<sup>72R</sup> genotypes in the head and neck cancer patients as compared to the normal healthy controls were seen. Hence, we support the hypothesis that either Trp53<sup>72P</sup> or Trp53<sup>72R</sup> variant is not associated with predisposition to cancer of the head and neck in South Indian population. To add more strength to the conclusion of our study, several recent investigations found no association of Trp53 codon 72 polymorphic variants with head and neck cancer (Hamel et al., 2000; McWilliams et al., 2000; Summersgill et al., 2000; Shen et al., 2002; Kietthubthew et al., 2003), including one study from Indian population (Tandle et al., 2001). Even though, our study found higher frequency of Trp53<sup>72P</sup> allele in cancer patients, it is not associated with head and neck cancer. However, for a more definitive and appropriate conclusion, the study should be performed with larger sample size, in the mean time, the expression status of the Trp53 polymorphs should be determined.

References


No Association Between Trp53 Codon 72 Polymorphism with Head and Neck Cancer in South India


