RESEARCH ARTICLE

Clinical, Cytogenetic and CYP1A1 exon-1 Gene Mutation Analysis of Beedi Workers in Vellore Region, Tamil Nadu

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Abstract

Background: Beedi rollers are exposed to unburnt tobacco dust through cutaneous and pharyngeal route and it is extremely harmful to the body since it is carcinogenic in nature and can cause cancer during long exposure. This indicates that occupational exposure to tobacco imposes considerable genotoxicity among beedi workers. Materials and Methods: In the present study, 27 beedi workers and age and sex matched controls were enrolled for clinical, cytogenetics and molecular analysis. Clinical features were recorded. The workers were in the age group of 28-67 years and were workers exposure from 8-60 years. Blood samples were collected from workers and control subjects and lymphocyte cultures were carried out by using standard technique, slides were prepared and 50 metaphases were scored for each sample to find the chromosomal abnormalities. For molecular analysis the genomic DNA was extracted from peripheral blood, to screen the variations in gene, the exon 1 of CYP1A1 gene was amplified by polymerase chain reaction (PCR) and then screened with Single Strand Conformation Polymorphism (SSCP) analysis. Results: A statistically significant increase was observed in the frequencies of chromosomal aberrations in exposed groups when compared to the respective controls and variations observed in Exon 1 of CYP1A1(Cytochrome P450, family 1, subfamily A, polypeptide 1) gene. Conclusions: This study shows that, the toxicants present in the beedi that enter into human body causes disturbance to normal state and behavior of the chromosomes which results in reshuffling of hereditary material causing chromosomal aberrations and genomic variations.

Keywords: Beedi workers - cancer - chromosome aberrations - CYP1A1 gene - polymorphism

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Introduction

Beedi is a thin South Asian cigarette wrapped using tendu leaf (Giri, 2010). Tobacco leaves mainly contain specific chemicals such as nitrosamines, formaldehyde, acetaldehyde, crotanaldehyde, hydrazine, arsenic, nickel, cadmium, benzopyrene, and potassium which are cancer causing substances. Raw tobacco dust can contain bacteria, endotoxins, and fungal spores, pollen, mites, insects, particulates, of inorganic materials such as quartz, and residues of pesticides or insecticides (Blair et al., 1983).

Occupational exposure to tobacco dust in workers processing beedi was shown to be associated with an increased cotinine level and urinary mutagenicity (Bagwe et al., 1995) as well increased chromosomal aberrations (Mahimkar et al., 1995). Daily exposure in beedi work ranged from 5 to 10 hours for both males and females. During rolling of beedi nicotine of tobacco powder comes into direct contact with the skin and becomes absorbed through the skin into the blood. Nicotine is harmful to the body since it is carcinogenic in nature and can cause cancer during long exposure (Ghosh et al., 2005). Smoking is one of the major environmental risk factors for breast cancer due to the presence of procarcinogens in tobacco (Berber et al., 2013). In our study most of the workers rolling beedi for more than 10 years and they affected with different types of health issues like headache, back pain, urinary infection, leg pain, shoulder pain, low eye contact, blood pressure, skin allergy.

In india, over five million people engaged in tobacco cultivation, processing and beedi rolling (Shimkhada et al., 2003; Umadevi et al., 2003) researched on the cytogenetic toxicity caused by occupational exposure to tobacco. Srinivasulu (1997) reported that 90% of beedi workers are women. Ranjitsingh and Padmalatha (1995) reviewed that beedi rollers were affected by respiratory disorders, skin diseases, gastrointestinal illness, gynaecological problems, lumbo-sacral pain and are susceptible to fungal diseases, peptic ulcer, haemorrhoids and diarrhoea.

Genetic polymorphisms including single nucleotide polymorphisms (SNPs) that affect xenobiotic metabolism may modulate the individual susceptibility to environmental contaminant exposures and the risk of developing cancers (Bozina et al., 2009). The CYP1A1

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gene, located at 15q22-q24, comprises seven exons and six introns and spans 5,810 base pairs (Masson et al., 2005). CYP1A1 enzyme belong to cytochrome p450 family, and responsible for the activation of procarcinogens to reactive metabolites (Turesky and Le Marchand, 2011) CYP isoforms metabolize estrogens to catecholoestrogens (CEs), semiquinones and quinines by oxidation reactions. These carcinogens metabolites are capable of forming either stable or depurinating DNA adducts, thus having the potential to result in permanent nucleotide mutation (Syamala et al., 2010). CYP1A1 gene polymorphisms have been shown to be associated with moderate to high risk of lung cancer (Hayashi et al., 1991) and DNA damage (Chen et al., 2006; Moretti et al., 2007). The aim of our study was to identify the probable effects of occupational tobacco exposure among vellore region beedi workers using molecular and cytogenetic studies.

Materials and Methods

Blood sampling and workers history

Informed consent was taken from all individuals included in this study. Blood samples were collected from 27 beedi workers in Vellore region, Tamilnadu. Blood collection was done by venipuncture and the blood was collected in sterile heparin coated vacutainers. The age and sex matched control samples were collected simultaneously. The personal history including the details of age, smoking history, health issues and duration of exposure at the work place was recorded.

Chromosome aberration analysis

Chromosome preparations were obtained from phytohaemagglutinin-stimulated peripheral blood lymphocytes by using modified method of (Hungerford, 1965). About 2ml of venous blood sample was collected in a sterile heparinized syringe. 0.5ml of the blood was inoculated into the vials containing 5ml of Hikaryos XL Ready-mix RPMI1640 medium (Himedia) under aseptic condition. The culture vials were then placed in an incubator at 37°C. Cultures were shaken every 24th hour and carbon dioxide was released. At the end of 72nd hour of incubation period, the dividing cells were arrested at metaphase by adding 2 drops of 0.1% colchicine solution to each culture vial and incubated further for 20 minutes at 37°C. After incubation period, the content at 1500rpm for 10 minutes was centrifuged and the supernatant was discarded. Hypotonic treatment was given by addition of 6ml of hypotonic solution (KCl 0.075 M) at 37ºC for 6-7 min to swell the cells, the content at 1500rpm for 10 minutes was centrifuged, the supernatant was discarded and 6 ml of Carnoy's fixative (3:1 ratio of methanol: acetic acid) was added to fix the cells. Continue the washing step until the pellet become white. Slides were prepared and carefully dried on a hot plate (40ºC). Later, the slides were stained with Giemsa.

Scoring criteria for metaphase chromosome

For the cytogenetic analysis, 50 well spread complete metaphase cells were evaluated per subject under a microscope at ×100 magnification to identify numerical and structural Chromosomal aberrations. Chromosome analysis after the metaphase spread is done by observing under inverted microscope which is attached to a camera and this assembly is connected to a computer. The stained cells are photographed and displayed on the screen to visualize the aberrations more clearly.

Statistical analysis

The samples were coded at the time of preparation and scoring. They were decoded before statistical analysis for comparison. Mean and standard deviation (SD) were calculated for biomarker. The significance of the differences was found between control and worker. Mean values and standard deviations were computed for the scores and the values are statistically significant.

Molecular analysis

DNA was extracted from the peripheral blood samples of the workers and the controls using a modified and standardized protocol of (Lotery, 2000) the extracted DNA was quantified by using Biophotometer (Eppendorf) and checked in 1% agarose gel. The gel was observed under UV transilluminator and photographed. The PCR was carried out in Master Gradient Thermal Cycler (Eppendorf) with a specific programme for specific primer. Polymerase reaction consisted of PCR buffer (10X), dNTPs (10mM), Taq Polymerase (1 unit/ul). The product size of the Exon 1 of CYP1A1 gene is 299bp, forward TCCGCCACCTTTCTCTCCAATCCCA and reverse GGCAAGCCAGAA-GTCCCCCAGCAAC Gradient PCR was set up to determine optimum annealing temperature for primer. 5µl of PCR products were electrophoresed at 100V in 2% of agarose gel. PCR conditions used for amplification of Exon 1 of CYP1A1 gene consisted of initial denaturation at 94°C for 6 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute and extension at 72°C for 1 minute with a final extension at 72°C for 10 minutes. The PCR products were then visualized under UV transilluminator and 8µl of PCR products were used SSCP and it is used to find out unknown mutations in CYP1A1 gene in Exon1, SSCP was performed by the modified and standardized protocol of (Orita, 1989) on the samples.

Results

Clinical analysis

This study is carried out with 27 beedi workers. Out of the 27 cases 7 workers were seen to have the habit of smoking and 20 workers were not indulged in the habit of smoking. The chromosomal aberration of workers with smoking is high in range than the workers without smoking and their age ranges from 28-67. Out of the 27 cases 20 workers were men and 7 workers were women (Table 1).

Chromosome aberration analysis

Along with 27 Beedi Workers, equally age and sex matched controls were taken for cytogenetic analysis to study the frequency of chromosome aberrations of which results are presented in Table 2 and Table 3. In workers

Table 1. Age, Gender Distribution, Years of Exposure and Clinical Features of Beedi Workers and Controls

		Number of beedi workers (n=27) (%)	Control (n=27) (%)
	Male	20 (74%)	20 (74%)
	Female	7 (26%)	7 (26%)
Age	28-40	11 (41%)	11 (41%)
	41-60	14 (52%)	13 (48%)
	61-67	2 (7%)	3 (11%)
Years of Exposure			
	8 to 20 years	12 (44%)	0 (0%)
	21 to 40 years	12 (44%)	0 (0%)
	41 to 60 Years	3 (11%)	0 (0%)
	Smoking Habit	7 (26%)	4 (15%)
	Skin allergy	17 (63%)	1 (4%)
	Headache	26 (96%)	6 (22%)
	Urinary Infection	24 (89%)	0 (0%)
	Cough	19 (70%)	2 (7%)
	Leg Pain	25 (93%)	6 (22%)
	Back pain	25 (93%)	9 (33%)
	Shoulder pain	22 (81%)	2 (7%)
	Frequent Cold	15 (56%)	11 (41%)
	Low vision	23 (85%)	6 (22%)
	Stomach burning	21 (78%)	1 (4%)
	BP	23 (85%)	5 (19%)
	Diabetic	6 (22%)	2 (7%)

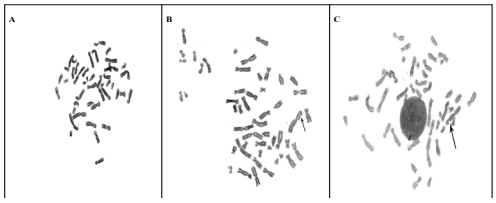

Figure 1. Showing Chromosome Aberrations in Beddi Workers, A Represents Control Metaphase Chromosome, B and C Represents Metaphase Chromosome with Breaks

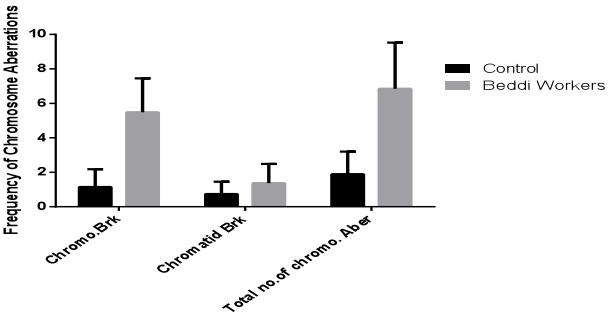

Figure 2. Graph Showing the Mean and SD of Chromosome Break (p<0.0001), Chromatid Break (p=0.02) And Total Chromosome Aberrations (p<0.0001)

observed different types of chromosome aberrations like chromosome breaks as well chromatid breaks (Figure 1). The total chromosome aberrations in controls were found 51 out of 1,350 metaphases, in which percentage of aberrations were 3.77% and the frequency of total aberration is 1.89±1.31 (mean and SD). In workers the total number of chromosome aberration is 185 out of 1,350 metaphases, in which percentage of chromosome aberration is 13.7% and the frequency of total chromosome aberrations is 6.85±2.67. The percentage and frequency

Table 2. Different Types of Chromosome Aberration and Their Percentage, Mean, Standard Deviation in Control Samples

Code	Age/Sex	No*of metaphase analysed	No of chro brk*	No of Chromatid brk	Total no of chr abr*	%Chr abr	No of abr per cell
CC01	32/M	50	2	1	3	6	0.06
CC02	28/F	50	0	1	1	2	0.02
CC03	31/M	50	0	1	1	2	0.02
CC04	33/M	50	1	0	1	2	0.02
CC05	38/M	50	3	1	4	8	0.08
CC06	37/M	50	1	0	1	2	0.02
CC07	35/M	50	2	1	3	6	0.06
CC08	45/M	50	0	0	0	0	0
CC09	47/M	50	2	2	4	8	0.08
CC10	53/M	50	0	0	0	0	0
CC11	52/M	50	0	0	0	0	0
CC12	30/F	50	1	0	1	2	0.02
CC13	57/M	50	1	1	2	4	0.04
CC14	48/M	50	0	2	2	4	0.04
CC15	49/M	50	3	0	3	6	0.06
CC16	41/F	50	1	1	2	4	0.04
CC17	50/F	50	2	0	2	4	0.04
CC18	55/F	50	2	1	3	6	0.06
CC19	40/M	50	1	0	1	2	0.02
CC20	64/M	50	0	1	1	2	0.02
CC21	63/M	50	1	2	3	6	0.06
CC22	60/F	50	0	1	1	2	0.02
CC23	67/F	50	2	0	2	4	0.04
CC24	20/M	50	3	2	5	10	0.1
CC25	50/M	50	2	1	3	6	0.06
CC26	47/M	50	0	1	1	2	0.02
CC27	30/M	50	1	0	1	2	0.02
	Total	1350	31	20	51	3.77	0.04
	Mean±SD		1.15±1.03	0.74±0.71	1.89±1.31		

*No: Number, Chr Brk: Chromosome Break, Chr Abr: Chromosome Aberration

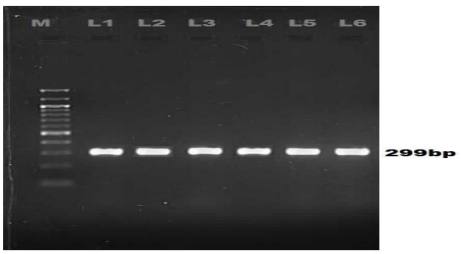

Figure 3. Agarose Gel Electrophoresis of the PCR Products of Exon-1, CYP1A1 Gene. Lane M molecular weight marker, lane 1 represent control sample and lanes 2, 3, 4, 5, 6 represents beedi workers sample

of chromosome aberrations were significantly higher in workers than in controls. Mean values and standard deviations were computed for the scores and it is statistically significant (Figure 2).

Molecular analysis

The SSCP analysis of the PCR products of the exon 1 of CYP1A1 gene in beedi workers is presented in Figure 03. Analysed 27 samples, in that found band variations in 3 (11%) samples showed polymorphism by using Single Strand Conformation Polymorphism (SSCP) in CYP1A1 gene exon1 and presented in Figure 4.

Table 3. Different Types of Chromosome Aberration and Their Percentage, Mean, Standard Deviation in Beedi Workers

Code	Age/Sex	No*of metaphase analysed	No of chro brk*	No of Chromatid brk	Total no of chr abr*	%Chr abr	No of abr per cell
BD 01	30/M	50	9	1	10	20	0.2
BD 02	31/M	50	7	2	9	18	0.18
BD 03	32/M	50	7	2	9	18	0.18
BD 04	41/F	50	3	0	3	6	0.06
BD 05	30/F	50	6	1	7	14	0.14
BD 06	50/F	50	2	1	3	6	0.06
BD 07	60/F	50	4	1	5	10	0.1
BD 08	63/M	50	9	3	12	24	0.24
BD 09	35/M	50	7	2	9	18	0.18
BD 10	28/F	50	3	0	3	6	0.06
BD 11	67/F	50	2	0	2	4	0.04
BD 12	55/F	50	4	3	7	14	0.14
BD 13	47/M	50	5	0	5	10	0.1
BD 14	33/M	50	3	2	5	10	0.1
BD 15	48/M	50	6	3	9	18	0.18
BD 16	52/M	50	4	0	4	8	0.08
BD 17	47/M	50	7	2	9	18	0.18
BD 18	31/M	50	8	1	9	18	0.18
BD 19	53/M	50	7	2	9	18	0.18
BD 20	45/M	50	7	2	9	18	0.18
BD 21	50/M	50	6	3	9	18	0.18
BD 22	49/M	50	7	3	10	20	0.2
BD 23	57/M	50	5	0	5	10	0.1
BD 24	64/M	50	4	1	5	10	0.1
BD 25	38/M	50	5	0	5	10	0.1
BD 26	40/M	50	6	0	6	12	0.12
BD 27	37/M	50	5	2	7	14	0.14
	Total	1350	148	37	185	13.7	0.14
	Mean±SD		5.48±1.97	1.37±1.11	6.85±2.67		

*No: Number, Chr Brk: Chromosome Break, Chr Abr: Chromosome Aberration

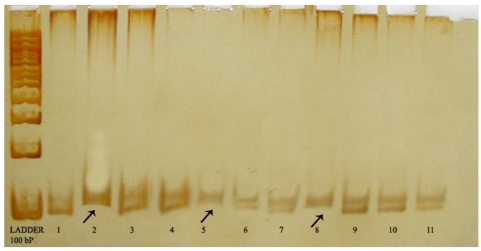

Figure 4.The Photograph Showing DNA Silver Stained SSCP Analysis of CYP1A1 Gene Exon 1 in Beedi Workers. Used 100-bp Marker; 1- Control, 2, 5 and 8 showing band variations and 3, 4, 6, 7, 9, 10, 11 showing no band variations

Discussion

The present study shows high frequency of various chromosomal aberrations in the Beedi workers when compared with the control group. The results are in accordance with the study of (Mahimkar and Bhisey 1995) who reported significant elevation in the frequency of total aberrant metaphases in the non-smoking tobacco processors working in beedi industry as compared to the zero frequency in the respective controls. The chromosomal damage in the smoker exposed group is almost twice that of controls suggesting the multiplicative effect of smoking and exposure to tobacco dust. In our study, we did not observe any significant association of age, sex and consumption habits with genetic damage (Giri et al., 2012).

The biological impact of CYP1A induction can be two fold. Induction of CYP1A in general serves as a means of maintaining the homeostasis of the chemical environment in the cells by increasing the metabolic clearance of substrates. Since CYP1A1/2 catalyzes metabolic activation of PAHs and HHAs ultimate carcinogens, it is expected that induction of the enzymes is detrimental in humans who are exposed to high levels of PAHs and HHAs such as by cigarette smoking. Moreover, induction of the enzymes in humans exhibits large variations (Ma and Lu 2003). Methylation of the CYP1A1 promoter is already associated with prostate and lung cancers (Okino et al., 2006; Tekpli et al., 2012) an association between CYP1A1 polymorphisms and expression may occur through either changes in metabolism of estrogen or through changes in the metabolism of the environmental toxin. With regards to estrogen metabolism, CYP1A1 has been predominantly identified as being involved in the formation of the 2-hydroxyestrogens, which exhibit lower estrogenic activity than the 4-hydroxyestrogens and 16-alpha-hydroxy estrogens, metabolites that are believed to increase cancer risk (Cribb, 2006). In recent case-control study that examined estrogen-metabolizing enzyme polymorphisms in relation to breast cancer incidence among women in Prince Edward Island (PEI) (Cribb, 2011).

However, in the previous study there no risk was found to be associated for the presence of CYP1A1 polymorphism and breast cancer and also found that there is a link between usage of tobacco, exposure to pollution and presence of CYP1A1 polymorphism with breast cancer (Kiruthiga et al., 2011). In this study between tobacco use and presence of CYP1A1 gene polymorphism confirmed that higher incidence of presence of CYP1A1 polymorphism in populations with tobacco intake. Toxicological studies support that environmental toxicants like polychlorinated biphenyls induce CYP1A1 to metabolize environmental carcinogens into highly reactive intermediates, potentially resulting in DNA damage and ultimately carcinogenesis (Zhang et al., 2004).

CYP1A1 polymorphic variants are mostly linked to tobacco related cancers such as lung and esophageal cancer in different ethnic populations (Yang et al., 2005; Dong et al., 2008; Lee et al., 2008). Polymorphism of CYP1A1 gene has been studied with relation to different cancers including head and neck cancer (Sabitha et al., 2010). Environmental factors along with genetic polymorphism of CYP1A1 gene are the main cause of head and neck cancer (Masood et al., 2011) and four different sequence polymorphisms have been reported in CYP1A1 gene, first known as CYP1A1*2 involve a T6235 to C transition in the 3' noncoding region (Jun et al., 2010). Single nucleotide polymorphisms and inherited loss of both alleles are common in these gene superfamilies with varying frequencies among different populations (Senthilkumar and Thirumurugan, 2012; Sharma et al., 2012; Zhou et al., 2012)

The study of (Bhagwe and Bhisey, 1995) from India reported significantly higher levels of total suspended particulate matter and inspirable dust in the ambient air of tobacco processing factories and in the breathing zone of the workers when compared with the general environment outside. Earlier studies reported the mutagenicity of urine samples collected from tobacco chewers and tobacco processors using Ames test. Tobacco dust generated during beedi making was reported to act as an initiator or promoter of skin tumors in S/RV Cri-ba mice (Bagwe et al., 2004). A low prevalence of chronic respiratory symptoms was found in control workers and the prevalence of cough with breathlessness, morning cough were higher among workers exposed to tobacco dust than the control (Chattopadhyay and Kundu, 2006).

The highly inducible forms of CYP1A1 are associated with an increased risk of lung cancer in smokers. Light smokers with the susceptible genotype CYP1A1 have a sevenfold higher risk of developing lung cancer compared to light smokers with the normal genotype (Sobti, 2003). This study presents evidence for the clastogenic effects of chronic exposure to tobacco dust in the beedi workers. Hence, the management is advised to take precautionary measures to minimize occupational exposure to tobacco dust in order to reduce the genetic effects and health problems in the workers. Thus a detailed study by gene sequencing and functional analysis of CYP1A1 gene would ascertain the exact nature of the polymorphism.

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