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Sequence and expression variations in 23 genes involved in mitochondrial and non-mitochondrial apoptotic pathways and risk of oral leukoplakia and cancer☆

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ABSTRACT

Oral cancer is usually preceded by pre-cancerous lesion and related to tobacco abuse. Tobacco carcinogens damage DNA and cells harboring such damaged DNA normally undergo apoptotic death, but cancer cells are exceptionally resistant to apoptosis. Here we studied association between sequence and expression variations in apoptotic pathway genes and risk of oral cancer and precancer. Ninety nine tag SNPs in 23 genes, involved in mitochondrial and non-mitochondrial apoptotic pathways, were genotyped in 525 cancer and 253 leukoplakia patients and 538 healthy controls using Illumina Golden Gate assay. Six SNPs (rs1473418 at *BCL2*; rs1950252 at *BCL2L2*; rs8190315 at *BID*; rs511044 at *CASP1*; rs2227310 at *CASP7* and rs13010627 at *CASP10*) significantly modified risk of oral cancer but SNPs only at *BCL2*, *CASP1*and *CASP10* modulated risk of leukoplakia. Combination of SNPs showed a steep increase in risk of cancer with increase in "effective" number of risk alleles. *In silico* analysis of published data set and our unpublished RNAseq data suggest that change in expression of *BID* and *CASP7* may have affected risk of cancer. In conclusion, three SNPs, rs1473418 in *BCL2*, rs1950252 in *BCL2L2* and rs511044 in *CASP1*, are being implicated for the first time in oral cancer. Since SNPs at *BCL2*, *CASP1* and *CASP10* modulated risk of both leukoplakia and cancer, so, they should be studied in more details for possible biomarkers in transition of leukoplakia to cancer. This study also implies importance of mitochondrial apoptotic pathway gene (such as *BCL2*) in progression of leukoplakia to oral cancer.

1. Introduction

Oral cancer affects more than 263,000 people and kills about 127,000 of them worldwide annually. In India alone, almost 70,000 people develop oral cancer and 50,000 die yearly (Ferlay et al., 2010). Oral cancer almost always involves a two-step process — pre-cancerous lesion followed by carcinoma (Reibel, 2003). A survey on Indian populations found that about 80% of oral cancers were preceded by pre-cancerous lesions and oral leukoplakia being the most common among them (Gupta et al., 1989). The relationship between tobacco abuse (both smoking and smokeless forms) and damage to mucosal cells and DNA, leading to pre-cancerous and cancerous lesions, is now well-established (Proia et al., 2006). Cells harboring severe DNA damage normally enter a programmed cell death pathway known as apoptosis. However, cancer cells are known to be exceptionally resistant to apoptotic death (Hanahan and Weinberg, 2000; Zhivotovsky and Orrenius, 2006). Apoptosis, an intricately complex mechanism involving a large number of gene products, is mediated by two distinct (mitochondrial i.e. intrinsic and non-mitochondrial i.e. extrinsic) pathways leading to the activation of a set of effecter caspases. DNA sequence variations in the apoptotic pathway genes have been reported to be

Abbreviations: MAF, minor allele frequency; SNP, single nucleotide polymorphism; UTR, untranslated region.

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associated with the risk of several kinds of cancer and most of these polymorphisms were located in the functionally important gene regions viz. exons, promoters, and 3′ and 5′ UTRs (Enjuanes et al., 2008; Frank et al., 2006; Ghavami et al., 2009; Kim et al., 2009; Sun et al., 2007; Yang et al., 2008). Though many studies have reported association of oral cancer with polymorphisms in the genes of the carcinogen metabolism and DNA repair pathways, research elucidating the association of apoptotic gene polymorphisms with the risk of oral cancer and precancer has been few (Chen et al., 2007; Hopkins et al., 2008; Scully and Bagan, 2009).

Here, we report results of a case–control association study, based on genotyping of tag SNPs from functionally important regions of 23 genes involved in mitochondrial and non-mitochondrial apoptotic pathways, involving oral leukoplakia and cancer patients and healthy controls.

2. Materials and methods

2.1. Study population

Procedures for blood sample collection and written informed consent were reviewed and approved by the Institutional Ethical Committee. Cancer and leukoplakia patients with history of tobacco use were recruited from Dr. R Ahmed Dental College and Hospital, a tertiary referral center in Kolkata, India, with active help of dentists. All patients were confirmed for leukoplakia or oral squamous cell carcinoma (OSCC) histopathologically. Disease-free healthy individuals with regular tobacco habit were recruited as controls (Datta et al., 2007). Cancer (n = 525) and leukoplakia patients (n = 253) and healthy controls (n = 538) voluntarily donated blood for this study. All patients and controls were personally interviewed to get information on age, sex, occupation, alcohol consumption, type of tobacco habits, daily tobacco use frequency and duration of habits.

Since about 90% of smokers used both cigarettes and *bidis*, data on *bidi* and cigarette smokers were not analyzed separately. In both patient groups, only a few (<3%) individuals consumed alcohol occasionally. Therefore, alcohol consumption was also not considered in analysis. Individuals who smoke tobacco are termed 'smokers' and those who dip/chew tobacco are termed 'chewers'. Dose of tobacco smoking was measured as pack-years (PY) and 1PY is defined as smoking 1 pack cigarette/bidi per day for one year. Lifetime smokeless tobacco exposure was measured in terms of frequency of chewing/dipping per day multiplied by the duration of habit. This is termed as chewing-year (CY) and 1CY is defined as chewing tobacco once a day for one year. Some of the patients and controls had both tobacco smoking and chewing habits (Datta et al., 2007).

2.2. SNP selection

Candidate tag SNPs were selected in a set of 23 genes (Table 1) that were obtained from a systematic Medline search for genes involved in apoptosis. Tag SNPs are defined as variants with $r^2 \geq 0.8$ with other SNPs in HapMap individuals with European ancestry (CEU) (HapMap project, 2003) and these tag SNPs were chosen using Tagger software. Selection of candidate SNPs was done in three ways, mainly focused to include SNPs with putative functional effect in protein structure and gene expression. Firstly, tag non-synonymous SNPs were selected from the exonic region of the gene. Secondly, tag SNPs from putatively functional regions of the gene viz. 5′ UTR, 3′ UTR and 2 kb regions flanking the gene were selected. Thirdly, SNPs previously reported to be associated with cancer or other diseases were included. We selected only SNPs with a minor allele frequency ≥0.01 in Caucasian population of the HapMap (CEU). All the selected SNPs were then filtered by Illumina technology criteria (score ≥0.6 or Golden Gate validated status, Illumina, Inc.).

A total of 99 SNP loci from 23 genes satisfied the above mentioned criteria and were selected for final genotyping (Table 1). These 99 SNPs were distributed in 5′ UTR and near gene regions (n = 26), coding regions (non-synonymous changes, n = 28), and in 3′ UTR and near gene regions (n = 45).

Table 1
Genes included in the study and distribution of SNPs in different functional regions.

Gene (n = 23)	Chromosome	No. of SNPs (n = 99)	Exonic non-synonymous (n = 28)	5′ UTR/near gene (n = 26)	3′ UTR/near gene (n = 45)
BAX	19	2		1	1
BCL10	1	4	1		3
BCL2	18	6		2	4
BCL2L2	14	4	1		3
BID	22	5	1		4
BIRC4	X	2	1		1
BIRC5	17	8	1	2	5
CASP1	11	4	1	1	2
CASP3	4	3	1	1	1
CASP10	2	5	3	1	1
CASP5	11	8	4	2	2
CASP6	4	3	2		1
CASP8	2	6	1	2	3
CASP7	10	6	2	3	1
CASP9	1	1		1	
FAS	10	8		3	5
FASLG	1	4	1	2	1
HTRA2	2	1		1	
NFKB1	4	3	2	1	
PARP1	1	4	2		2
TNF	6	6	1	1	4
TNFRSF1A	12	4	1	2	1
TNFRSF10A	8	2	2		

2.3. Genotyping

2.3.1. Illumina platform

Genomic DNA, isolated from peripheral blood lymphocytes (Datta et al., 2007), was quantified using Pico Green and diluted to a final concentration of 50 ng/μL. Genotyping was carried out on Illumina Golden Gate genotyping platform and genotype clustering was performed using Illumina Bead Studio (Version 3). Illumina established an assay score based on the nucleotide composition of the DNA region and on the presence of duplicated or highly repetitive sequences, palindromes, and neighboring polymorphisms. Polymorphisms showing an Illumina quality score <0.6 were rejected for the final pool of genotyped SNPs. Eight DNA intra-assay duplicates were included in each of 96-well plate assay system.

2.3.2. TaqMan platform

Seven SNPs chosen randomly were re-genotyped in 300 individuals on TaqMan platform (Applied Biosystems Inc.) for confirmation. These 300 individuals consisted of 100 randomly chosen individuals from each of the control, leukoplakia and cancer groups. The assay was done at the default annealing and extension temperature, recommended by the manufacturer, (a denaturing step of 15 s at 95 °C, followed by annealing and extension for 1 min at 60 °C for 40 cycles).

2.4. Data analysis

Quality control of data and tests for association were performed using PLINK v1.07 (Purcell et al., 2007) and SPSS version 16.0 (SPSS, Chicago, IL). An initial pruning of the data based on the following criteria was performed: (i) individuals who had genotype data at less than 90% of the loci, (ii) SNP loci that had genotype data in less than 90% of the individuals, (iii) SNP loci that had a minor allele frequency (MAF) less than 0.01 in cases and controls taken together; and (iv) SNP loci that deviated significantly from HWE (Fisher's exact test) at level of significance 0.001 in controls. Genotypic test of association (at 2 degrees of freedom) and allelic test of association (at 1 degree of freedom) were