RESEARCH ARTICLE

Leu432Val Polymorphism of *CYP1B1* is Not Associated with Squamous Cell Carcinoma of Esophagus - a Case-Control Study from Kashmir, India

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Abstract

Background: Individual susceptibility to cancer has been attributed to polymorphisms in xenobiotic metabolizing genes. To evaluate the association of the *Leu432Val* polymorphism of cytochrome P4501B1 (*CYP1B1*) with esophageal squamous cell carcinoma (ESCC), we conducted a case control study in Kashmir, India, an area with a relatively high incidence of ESCC. Materials and Methods: We recruited 404 histopathologically confirmed ESCC cases, and an equal number of controls, individually matched for sex, age and district of residence to respective cases. Information was obtained on various dietary, lifestyle and environmental factors in face to face interviews, using a structured questionnaire, from each subject. Genotypes were analysed by polymerase chain reaction, restriction fragment length polymorphism and sequencing of randomly selected samples. Conditional logistic regression models were used to calculate odds ratios (ORs) and 95% confidence intervals (95% CIs). Results: Among the three possible variants, we did not find any *Leu432Leu* genotype of *CYP1B1* in the study population and the genotypic distribution of Val432Val and *Leu432Val* carriers was nearly equal in both cases (89.6% and 10.4%) and controls (88.9% and 11.1%) respectively. We did not find any risk associated with this polymorphism in the current study (OR = 0.64; 95% CI: 0.55 – 1.64). Conclusions: The study indicates that (*Leu432Val*) polymorphism of *CYP1B1*, is not associated with ESCC risk. However, replicative studies with larger sample size are needed to substantiate the findings.

Keywords: *CYP1B1* - esophageal cancer risk - gene polymorphism - kashmir - case control study.

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Introduction

Esophageal cancer is the eight most common incident cancer in the world, and ranks sixth among all cancers in mortality (Kamangar et al., 2006; Ferlay et al., 2010; Jemal et al., 2011). Among its two main histological types, esophageal squamous cell carcinoma (ESCC) and adenocarcinoma, ESCC still remains the most common histological subtype globally (Eslick, 2009; Jemal et al., 2011). It occurs frequently within a geographic region extending from the southern shore of the Caspian Sea on the west to northern China on the east and encompassing parts of Iran, Central Asia, Afghanistan, Siberia, and Mongolia- so called "*Central Asian esophageal cancer belt*" (Islami et al., 2004; Hamrah et al., 2014). The geographical variation in ESCC incidence is attributed to differences in prevalence of environmental or lifestyle risk factors. However, the association between environmental factors and ESCC risk is not same across populations, socioeconomic classes, or even in men and women (Louwman et al., 2010; Thrift et al., 2012; Zhong et al., 2013). This differential risk is attributed to predisposing genetic markers harbored by an individual that modulate the effect of environmental exposure (Su et al., 2003; Guengerich, 2008).

A class of enzymes, Xenobiotic metabolising enzymes (XMEs) are involved in the processing of toxic xenobiotics in our body. Cytochrome P-450 (CYP) enzymes, the major players of the xenobiotic pathway, are involved in activation and detoxification of common xenobiotics/ carcinogens like polycyclic aromatic hydrocarbons (PAHs) (Hakami et al., 2008; Boffetta, 2010) and nitrosamines (Kamangar et al., 2005) present in tobacco and some dietary items (Zhuo and Watanabe, 1999; Roshandel et al., 2012). CYP enzymes oxidize PAHs and aromatic amines in an oxidation reaction in which one atom of a molecule of oxygen is incorporated into the substrate, rendered more soluble and available to further processing (Bartsch et al., 2000; Rose and Hodgson, 2004; Hrycay and Bandiera, 2015). Most of XMEs are polymorphic and their polymorphic variants alter the activity of these enzymes and thus have been attributed to the differential cancer risk of individuals (Garte, 1998; Manfred, 2011). *CYP1B1* is a heme-thiolate monooxygenase; that is

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involved in the NADPH-dependent monooxygenation of a variety of carcinogens like (PAHs) including benzo(a) pyrene and dimethylbenz(a)anthracene (DMBA), (Shimada et al., 1996; Buters et al., 1999; Gajjar et al., 2012). The genetic variations in *CYP1B1* can modify the environmental exposure and subsequent risk of cancer (Li et al., 2007; Bye et al., 2011; Shi et al., 2012). Although *Leu432Val* polymorphism of *CYP1B1* has been associated with different cancers (Liu et al., 2014) , its association with ESCC has not been studied well.

ESCC is the most common cancer among men and women in Kashmir, India (Khuroo et al., 1992; Ayub et al., 2011). The population is reportedly exposed to a wide range of toxic xenobiotic substances through diet (Siddiqi et al., 1988; Siddiqi et al., 1991; Mir and Dar, 2009), hooka (water-pipe) smoking (Dar et al., 2012), smoke from domestic fumes and by using biomass as cooking fuel (Dar et al., 2013). But the data for role of polymorphisms in genes involved in the metabolism of such xenobiotics is sparse. We therefore conducted a hospital-based case-control study to evaluate the risk of ESCC associated with *Leu432Val* polymorphism of *CYP1B1*.

Materials and Methods

Case and control selection: A total of 808 subjects were recruited in the study including an equal number of cases and controls. Cases were recruited in the Radiation Oncology Department of Sher-i-Kashmir Institute of Medical Sciences (SKIMS), Srinagar; while as controls were recruited from different in-patient departments of SKIMS and other district hospitals. Only histopathologically confirmed ESCC cases that were above the age of 18 years and had no previous cancer, were invited to participate in the study. For each case subject, we recruited one hospital-based control individually matched for sex, age (± 5 years), and district of residence. The details of the case and control selection are provided elsewhere (Dar et al., 2012; Dar et al., 2014). The participation rate for cases and control was 96% and 98%, respectively. The majority of those who refused were too ill to participate in the study. Written consent was obtained from each subject before registration. The study was reviewed and approved by the Institutional Ethics Committee of SKIMS.

Data collection. Trained researchers administered structured questionnaires. ESCC cases and controls were interviewed in local language at the hospitals in which they were recruited. The questionnaires collected detailed information on age, sex, ethnicity, religion, place of residence, education and other potential confounding factors of interest. Dietary data including intake of fresh fruits and vegetables were collected using a food frequency questionnaire specifically designed for this population. Detailed information on lifelong history of use of, alcohol, tobacco and related products (gutka, nass, hookah, cigarette, and bidi) was obtained. Ever use of alcohol and tobacco products was defined as the use of the respective product at least weekly for a period of 6 months or more. To assess the socio economic status (SES) of the subjects, information on potential parameters of SES was obtained including education level (highest level attained), occupation, professional work intensity, monthly income (in INR), house type, cooking fuel, and ownership of several household appliances, including personal automobile, motorbike, B/W TV, colour TV, refrigerator, washing machine, vacuum cleaner, computer, and bath in the residence. Information on family history of cancer was also obtained from all the participants. In order to minimize inter-individual variation, a limited number of staff conducted the face-to-face interviews and no proxies were used.

Isolation of Genomic DNA. Five mL of the venous blood was obtained from the subjects in an EDTA coated (0.5M, pH-8.0) sterilized plastic vials and stored at -80oC prior to use. Genomic DNA from the frozen blood samples was isolated within one week of sample collection by Phenol-Chloroform method (Sambrook J and Rusell, 2001). The purity and integrity of extracted DNA was tested by taking absorbance and running electrophoresis, respectively.

Laboratory assays

*CYP1B1**3: 4326C>G or (*Leu432Val*) polymorphism of *CYP1B1* was genotyped using primers: sense, 5´-GCCTGTCACTATTCCTCATGCC-3; antisense, 5´-GTGAGCCAGGAT GGAGATGAAG-3´ (Sigma-Aldrich) after an earlier reported method (Sliwinski et al., 2010). A 283bp PCR product was amplified with Polymerase chain reaction; performed by initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 56°C for 30s, extension at 72 °C for 30s, and a final extension at 72 °C for 7 min. Genomic DNA (150–190 ng) was amplified in a total volume of 25 μL reaction mixture containing 10× PCR buffer, MgCl2 (1.5 mM), each primer (20 pmol), dNTPs (200 μM), and 1 unit of DNA Taq polymerase (Fermantas, MBI, Vilnius, Lithuania). PCR amplicon was digested overnight with 5 Units of the restriction enzyme *BstXI* (Fermentas MBI, Vilnius, Lithuania). The Val allele was digested into 171 and 112 bp fragments, heterozygotes yielded three bands of 283bp 171bp and 112bp on resolving in 2.5%, ethidium bromide stained, agarose gel. For 10 % of randomly picked samples the results were validated by sequencing.

Statistical analysis

Numbers and percentages were calculated and presented for categorical variables, as well as means and standard deviations (SD) or median and inter-quartile range for continuous variables. Fruit and vegetable intake data (g/day) were transformed to logarithmic values following addition of 0.1 to original values. To assess the socioeconomic status (SES), we built a composite score for wealth, based on appliances ownership and other variables by using multiple correspondence analysis (MCA) (Islami et al., 2009). Information on MCA method is provided elsewhere (Dar et al., 2013). By design, case and control subjects were matched by age, sex, and district of residence. Conditional logistic regression was used to calculate unadjusted and adjusted odds ratios (ORs) and corresponding 95% confidence intervals (CIs) for

association of *CYP1B1*3* (*Leu432Val*) polymorphism with ESCC risk. In order to avoid the possible confounding, results were adjusted for age, place of residence, wealth score, monthly income, education level, daily fruit and vegetable consumption, consumption of tobacco (hookah cigarette and bidi) and related products (Gutka and nass a local smokeless tobacco product). All statistical analysis was done using Stata software, version 12 (STATA Corp., College Station, TX, USA). Two sided P values < 0.05 were considered as statistically significant.

Results

Distribution of demographic factors, the wealth score a socioeconomic indicator, tobacco use and allele frequency by case status are shown in Table 1. The mean age of cases and controls was 61.6 and 59.8 years, respectively and ~57% were males. Majority of ESCC cases resided in rural areas. More number of cases were smokers and nass chewers than the respective controls. The fresh fruit and vegetable consumption was higher in controls than respective cases. Wealth score, monthly income and

Table 1. Demographic Characters of Esophageal Cancer Cases and Controls from Kashmir India

Characteristics	Cases n (%)	Controls n (%)	P value
Total	404 (100)	404 (100)	
Mean Age (SD)	61.4 (11.4)	60.4 (11.4)	
Gender			
Male	232 (57.4)	232 (57.4)	
Female	172 (42.6)	172 (42.6)	
Place of residence			< 0.001
Urban	11 (2.7)	35 (8.7)	
Rural	393 (97.3)	369 (91.3)	
Education			< 0.001
No education	363 (89.8)	258 (63.8)	
Primary	21 (5.2)	55 (13.6)	
Middle	12 (3.0)	25 (6.2)	
High school	6 (1.5)	37 (9.2)	
College or above	2 (0.5)	29 (7.2)	
Fruit vegetable mean (SD*)	1.79 (1.25)	3.11 (1.21)	
Smoking			0.004
Never	157 (38.9)	198 (49.0)	
Ever	247 (61.1)	206 (51.0)	
Nass chewing			< 0.001
Never	294 (72.8)	358 (88.6)	
Ever	110 (27.2)	46 (11.4)	
Fuel used			<0.001
Biomass	395 (97.8)	323 (79.9)	
Gas/Electricity	9 (2.2)	81 (20.1)	
House type			<0.001
Adobe made	240 (59.4)	99 (24.5)	
Concrete	164 (40.6)	305 (75.5)	
Monthly Income (INR)			<0.001
≤ 5000	320 (79.2)	253 (62.6)	
5001–10000	52 (12.9)	81 (20.1)	
> 10000	32 (7.9)	70 (17.3)	
Wealth score			< 0.001
Quintile 1 (lowest)	236 (58.4)	77 (19.1)	
Quintile 2	67 (16.6)	72 (17.8)	
Quintile 3	33 (8.2)	88 (21.8)	
Quintile 4	38 (9.4)	87 (21.5)	
Quintile 5	30 (7.4)	80 (19.8)	

n number of subjects; Chi-square test (χ^2) was used to calculate P values for categorical variables; * Standard deviation

Table 2. Distribution of CYP 1B1 Genotypes in ESCC cases and Matched Controls and Risk of ESCC

Genotype	Cases n (%)	Controls n (%)	Un adjusted OR 95% CI	1 Adjusted OR 95% CI	P value 2
CYP1B1*3					0.733
Val432Val	362 (89.6)	359 (88.9)	Referent	Referent	
Leu432Val	42 (10.4)	45 (11.1)	0.79 (0.46 – 1.36)	0.64 (0.55 – 1.64)	
Leu432Leu	–	–	–	–	

CI, confidence interval; ESCC, esophageal squamous cell carcinoma; OR, odds ratio; Numbers may not add up to the total numbers due to missing data in some variables. ORs (95% CIs) were obtained from conditional logistic regression models; 1Adjusted ORs (95% CIs) was obtained in conditional logistic regression models when adjusted for age, ethnicity, religion, gender daily fruit and vegetable consumption, place of residence, education level, income, wealth score, nass chewing and tobacco smoking; 2 P-values calculated using χ^2-tests

education status was higher in controls.

We did not find any *Leu432Leu* genotype of *CYP1B1* in the study population. The genotype distribution of Val432Val and *Leu432Val* carriers was nearly equal in both cases (89.6% & 10.4%) and controls (88.9% & 11.1%). We did not find any risk associated with this polymorphism in the study population (OR = 0.64; 95% CI: 0.55 – 1.64). Table 2

Discussion

The study was conducted in the ethnic population of Kashmir, India. Unlike the well documented role of *Leu432Val* polymorphism of *CYP1B1* in different cancers (Liu et al., 2014) , we did not find any significant association with ESCC in Kashmir.

CYP1B1 is among key enzymes of the xenobiotic pathway involved in the biotransformation of a variety of carcinogens.(Shimada et al., 1996; Buters et al., 1999). It has been detected in a variety of human tissues, including the respiratory and gastrointestinal tracts and is commonly overexpressed in human malignancies (Murray et al., 1997; Go et al., 2015). *CYP1B1* is also induced by PAHs by way of altering the gene transcription by binding and activating the aryl hydrocarbon receptor (AhR) (Murray et al., 2001; Spink et al., 2008). The polymorphic variants of the *CYP1B1* gene have been reported to have 2.4 to 3.4 fold higher catalytic activity than the wild type enzyme (Shimada et al., 1999; Hanna et al., 2000), thus making it an important marker for different cancers. Studies have persistently studied the association of (*Leu432Val*) polymorphism of *CYP1B1* with different cancers including ovarian cancer, lung cancer bladder, and endometrial cancer ,leukaemia laryngeal carcinoma, colorectal carcinoma, renal cell carcinoma risk but overall results are mixed (Wang et al., 2011; Berber et al., 2013; Chang et al., 2014; He et al., 2014; Liu et al., 2014; Liu et al., 2015; Lopes et al., 2015; Yu et al., 2015). In the current study, we also did not find any *Leu432Leu* allele of *CYP1B1**3 polymorphism in the study population suggesting no role of latter in ESCC development, suggesting the role of other possible genetic markers. Our study is in agreement with an earlier report from china which also has not found any significant association of studied polymorphism with ESCC (Wang et al., 2006). The risk for different cancers in distinct ethnic groups could be modulated by interaction between genetic variants and different endogenous and exogenous carcinogens (Kotnis et al., 2008).

Histological verification of ESCC, and adjustments of the results for multiple potential confounding factors are the major strengths of this study. Limitations of the study include its modest sample size. Although a limited staff interviewed the subjects, similar to other case-control studies with retrospective exposure assessments, recall and interviewer bias may be also be a concern in this study but is unlikely to effect the out-come of the study.

This study indicates that *CYP1B1* cannot be an independent marker for ESCC risk. Overall this polymorphism is poorly studied in relation with ESCC and our findings need to be replicated in studies with bigger sample size.

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