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ORIGINAL ARTICLE

CYP2A6 and CYP2A13 Genetic Variants are Associated with Decreased Risk of Esophageal Squamous Cell Carcinoma– A Case-control Study Based Assessment

Gulzar Ahmad BHAT,[1] Nasir Ab SHEIKH,[2] Gowhar RASHID,[2] Farooq A GANIE,[3] Tariq Rasool MALIK,[4] Mudassar SYED,[5] Nazir A DAR[1]

[1]Multidisciplinary Research Unit, Sher-i-Kashmir Institute of Medical Sciences Srinagar, *India*
[2]Department of Clinical Biochemistry, University of Kashmir, Srinagar-*India*
[3]Department of Cardio Vascular and Thoracic Surgery, Sher-i-Kashmir Institute of Medical Sciences Srinagar, *India*
[4]Department of Radiation Oncology, Sher-i-Kashmir Institute of Medical Sciences, Srinagar-*India*
[5]Department of Biochemistry, Sher-i-Kashmir Institute of Medical Sciences, Srinagar-*India*

OBJECTIVE

Xenobiotic metabolising enzymes (XMEs) play an important role in carcinogenesis. However, in the case of oesophagal cancer, the association of XMEs in general and Phase-1 XMEs in particular remained largely inconclusive. The current study aimed to explore the association of the genetic variants of cytochrome P450 (CYP) 2A6 and CYP2A13 with oesophagal squamous cell carcinoma (ESCC) and the potential effects of environmental factors on such association.

METHODS

The genetic variants of CYP2A6 and CYP2A13 genes were investigated by polymerase chain reaction- restriction fragment length polymorphism, allele-specific PCR and sequencing methods in 492 histopathologically confirmed ESCC cases and an equal number of matched controls. Gene-gene and gene-environment interactions were calculated by logistic regression analysis.

RESULTS

Inverse association was found between the variant genotypes of CYP2A6 (OR=0.6; 95% CI, 0.4–0.9) and CYP2A13 (OR=0.5; 95% CI, 0.31–0.8) with ESCC risk. Individually, the inverse association of variant genotypes of the three studied CYP2A6 genes was retained when harboured by a participant in combination with CYP2A13 variant genotype (OR=0.3; 95%CI, 0.1–0.8). Participants who were smokers, consumed alkaline beverage, had used biomass fuel for cooking, lived in adobe houses and had a positive family history of cancer showed a strong ESCC risk when harbouring homozygous wild genotypes of CYP2A6 and CYP2A13. Among the different gene environmental interactions, only CYP2A6b (OR=1.5; 95%CI, 1.1–2.0; $P_{interaction}$=0.018), and CYP2A13 (OR=1.4; 95%CI, 1.1–2.0; $P_{interaction}$=0.021) genotypes showed statistically significant interactions with smoking.

CONCLUSION

Normal genotypes of CYP2A6 and CYP2A13 considerably increase ESCC risk in subjects who also had exposure to environmental risk factors.

Keywords: Cytochrome P450 gene variants; gene-environment interaction; Kashmi; oesophagal squamous cell carcinoma.

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Dr. Gulzar Ahmad BHAT
Multidisciplinary Research Unit,
Sher-i-Kashmir Institute of Medical Sciences Srinagar,
India
E-mail: gulzar.bhat@skims.ac.in

INTRODUCTION

The incidence of Oesophagal Squamous Cell Carcinoma (ESCC) varies dramatically across the globe.[1] The two main kinds of esophageal cancer, adenocarcinoma and squamous cell carcinoma, are geographically, etiologically, and histologically distinct.[1] This extensive global variation in oesophagal cancer incidence is attributed to the presence or absence of several risk factors in different populations.[2–7] In general, the industrialised world provides a favourable environment for the development of adenocarcinoma, whereas in underdeveloped nations, risk factors for ESCC, such as those associated with poor socioeconomic situations, predominate.[8] The presence or absence of a number of risk variables in various populations is responsible for the wide global diversity in oesophagal cancer incidence. However, under similar exposures, only a subset of individuals develops cancer, reflecting inter-individual differences in cancer risk. The difference in ESCC risk among individuals with similar exposures. [9] and consistent evidence from a prospective twin cohort, [10] segregation and migration studies,[11] as well as findings such as ESCC onset at a younger age[12] and familial clustering of ESCC,[13] suggest that inter-individual genetic variations may contribute to elevating the ESCC risk.[14,15]

Polymorphisms in xenobiotic metabolising enzymes (XME) are among the most important genetic differences in carcinogenesis. The Phase I and Phase II XMEs biotransform the xenobiotic to make it more water-soluble, and the Phase III transporter then aids in the elimination of the changed intermediate from the body via urine. The intermediates produced during biotransformation are more reactive and, if not eliminated, can bind biomolecules, including DNA. Inter-individual genetic variants in XMEs cause differences in XME expression and activity, as well as the ability to eliminate reactive intermediates from the body, thereby modulating the risk of cancer caused by xenobiotics in food or the environment.[16–19]

Kashmir, with a high incidence of ESCC in both genders,[20] is reportedly exposed to toxic chemicals, including PAHs and N-nitrosamines.[21–23] Cytochrome P450 (CYP) 2A6 and CYP2A13 are among the most common enzymes involved in the activation of pro-carcinogens like PAHs and nitrosamines present in tobacco smoke and in some dietary foods.[24–26] The biological and biochemical evidence has consistently revealed that different polymorphic variants of these enzymes have different levels of activity towards these substrates.[27,28] Previously, our analyses of the association of some XMEs polymorphisms with ESCC risk showed mixed results. Polymorphisms in CYPIB1 and GSTM1 showed no association,[29,30] while polymorphisms in CYP1A1, CYP2E1, CYP2C19, CYP2D6, CYP1A2, GSTTI and Sulpho transferases were associated with ESCC risk in the Kashmiri population.[29,31–33] In most of the polymorphic variants of these studied genes, we observed a slight to strong ESCC risk when harboured by subjects individually or in combination. To the best of our knowledge, only a few studies have reported the relationship between genetic variants of CYP2A6 and ESCC risk, and no study has been available to date to have assessed the relationship between the genetic polymorphism of CYP2A13 and ESCC risk. Given the reported exposure to the xenobiotic substrates of CYP2A6 and CYP2A13, it will be interesting to assess the association of their polymorphisms with ESCC risk in Kashmir, which has not been studied in this high-risk region of ESCC yet.

MATERIALS AND METHODS

Subject Recruitment and Data Collection

A hospital-based case-control study comprising a total of 492 histopathologically confirmed ESCC cases and equal number of age (±5years), gender and residence-matched controls was carried out in Sher-i-Kashmir Institute of Medical Sciences (SKIMS) from September 2008 to January 2012. The incident cases of ESCC were recruited in the Department of Radiation Oncology, SKIMS, Srinagar. All the cases had no prior history of any malignancy. For most of the cases, controls were enrolled from inpatient wards of district hospitals in the respective districts from where these cases were referred. The selection of the controls was not limited to a particular set of diseases; however, the patients who were admitted for diseases related to tobacco smoking and alcohol drinking, the two leading etiological factors for ESCC, were excluded as controls. The details of wards in which controls were recruited and the reasons of hospitalisation are provided elsewhere.[21] The controls were recruited within six months after their respective cases were recruited, and no proxies were used during subject recruitment. The participation rate for both cases and controls was high (96% for cases and 98% for controls). Informed consent was obtained from all participants, and the study was reviewed and approved by the Institutional Ethics Committee of SKIMS, Srinagar and the study was conducted according to the Helsinki Declaration.

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Data Collection

Detailed information on age, sex, place of residence, ethnicity, religion, education, dietary data, including intake of fresh fruits and vegetables and other potential confounding factors of interest was collected using a questionnaire specifically designed for the study population. Detailed information on the life-long history of use, with starting and stopping ages and daily amount of use, was obtained for several tobacco products. Any change in the type of tobacco products and amount of use was also recorded. Ever use of traditional hookah, nass, cigarette and gutkha, was defined as the use of the respective product (s) daily or at least weekly for a period of 6 months or more. Information on family history of any cancer (FHC) was obtained from all the participants. To assess the socioeconomic status (SES) of the subjects, information on potential parameters of SES was obtained including education level (highest level attained), monthly income (INR), house type, cooking fuel, and ownership of several household appliances. Similarly, the information regarding oral hygiene, house type, second hand smoking was also acquired from all the subjects.

Genetic analysis

Five millilitres of venous blood was collected from each subject in sterilised plastic vials containing EDTA (0.5M; pH=8.0) and stored at -80ºC before DNA extraction. Genomic DNA was extracted from the collected blood samples using the phenol-chloroform method.[34] The extracted DNA was quantified and stored at 4°C until used for further analysis.

For identification of the CYP2A6 genotypes, PCR-RFLP analysis was performed as described previously.[32] The single PCR and RFLP methods were used to identify the wild CYP2A6 allele (*1/*1), heterozygous alleles (*1/*6) and CYP2A6 homozygous gene deletion (*6/*6).

Allele Specific-Polymerase Chain Reaction (AS-PCR) was employed for the CYP2A6b gene, and whole gene deletion genotyping was based on a 2-step PCR method. The first PCR reaction produced a 1,961bp fragment of the CYP2A6b from all individuals with or without the deleted CYP2A6b gene. The second PCR, which specifically detected the deleted CYP2A6b gene, used the product resulting from the first PCR amplification as a template. 1.5% ethidium bromide-stained agarose gel was run to check the amplified products. The presence of the CYP2A6b-specific 1,181bp product amplified with the first primer pair indicated the CYP2A6b wild genotype (*1/*1). The presence of the product resulting from amplification with the second primer pair indicated the deleted CYP2A6b genotype (*4/*4). The presence of the product in both reactions indicated the heterozygote genotype (*1/*4). It is important to mention that 10% of the samples of cases and controls were randomly tested twice for experimental validation; however, the results were similar for all duplicate sets.

PCR amplification of CYP2A6c genotypes, including homozygous for the wild-type (*1A/*1A), heterozygous type (*1A/*4C) and deletion-type (*4C/*4C) were determined by PCR-RFLP assay as previously described.[35]

The details of PCR conditions, primers, restriction enzyme, and length of expected fragments on digestion, mutant alleles and change in nucleotide position of the above genes are given in Table 1.

It is pertinent to mention that three different SNPs were simultaneously studied in the case of the CYP2A6 gene based on their substrate specificity with nitrosamines and PAHs to which the study population is frequently exposed through various exogenous exposures.

Similarly, in the case of CYP2A13, the three genotypes wild homozygous (C/C), heterozygous (C/T) and homozygous mutant (T/T) were determined by PCR-RFLP assay as previously described.[36] The details of PCR conditions, primers, restriction enzyme, and length of expected fragments on digestion, mutant alleles and change in nucleotide position of the studied gene are given in Table 1.

PCR-RFLP results were validated by sequencing 10% of the randomly picked samples. For sequencing, unpurified PCR products were directly sent to SciGenome Private Limited, Cochin Kerala-India. The resulting sequence chromatograms were then compared with the original gene sequences for the expected results. Sequence scanner software (Finch TV Geospiza 1.4.0) was used for comparing sequences for the possible sequence variations due to gene polymorphisms.

Statistical Analysis

Categorical variables were set for presenting and calculating numbers and percentages for different genotypes of CYP2A6 and CYP2A13. Tests for Hardy-Weinberg Equilibrium (HWE) were conducted by comparing observed and expected genotype distributions by the χ^2 goodness of fit. Statistical significance for the departure of a genotype frequency from its expected frequency under the HWE model was set at $p \leq 0.05$. Conditional logistic regression models were used to calculate odds ratios (ORs) and corresponding 95% confidence intervals (95%CIs) to assess the

Table 1 Details of the studied genes

Gene	Primers	PCR conditions	RE	DP (bp)	N.C	R. no
CYP2A6a	FP5′- CCT GAT CGA CTA GGC GTG GTA -3′ RP5′- TCG TCC TGG GTG TTT TCC TTC -3′	95°C 40sec 58°C 35sec (35 cycles) 72°C 35sec	MspI	AP=215 W=116, 99 H=215,116, 99 M=215	C > A at 406	[68]
CYP2A6b*	FP15′- CCA AGA TGC CCT ACA TG-3′ RP15′- TTG TGA GAC ATC AGA GAC AA -3′ FP25′- CAC TTC CTG AAT GAG -3′ OR FP25′- CAT TTC CTG GAT GAC -3′ RP25′ - AAA ATG GGC ATG AAC GCC C -3′	95°C 40sec 55°C 45sec (25cycles) 72°C 2min 94°C 30sec 50°C 30sec (15cycles) 72°C 2min	AS-PCR	AP=1961 (WMH=1181)	Whole gene deletion	[48]
CYP2A6c	FP5′- CAC CGA AGT GTT CCC TAT GCTG-3′ RP5′- TGT AAA ATG GGC ATG AAC GCCC-3′	95°C 60sec 63°C 50sec (35cycles) 72°C 50sec	Eco81I	AP=1259 W=789, 470 H=1259, 789, 470 M=728	Whole gene deletion	[35]
CYP2A13	FP5′- TAA CTC CGT TCC TTC CTT GCT -3′ RP5′- TAA TTT GAA TGG GCC TGT GTC -3′	94°C 60sec 63°C 30sec (35cycles) 72°C 30sec	HhaI	AP=375 W=217, 158 H=375,217,158 M=375	C > Tat 3375	[36]

*: CYP2A6b is allele specific PCR genotyping. RE: Restriction enzyme; DP: Digestion products; N.C: Nucleotide change; R. No: reference number; FP: Forward Primer, RP: Reverse primer; AP: Amplified product; W: Homozygous wild genotype; H: heterozygous genotype; M: Homozygous mutant genotype; AS-PCR: allele specific PCR; WHM: Wild, heterozygous or mutant genotype, depends upon the type of primer pair resulting product amplification

association of the genotypes with ESCC risk and to assess the possible gene-gene and gene-environment interaction (GEI). Subjects were stratified into various groups based on smoking habit, FHC, type of fuel used for cooking, house type and various possible genotypic combinations. Adjustment was made with known ESCC risk factors like age, sex, residence, education level, SES, fruit and vegetable consumption, oral hygiene, animal contact, salted tea consumption and smoking in different forms. For genetic analysis, wild homozygous, heterozygous, mutant homozygous genotypes, as well as a variant group (a combination of heterozygous and mutant homozygous with at least one and/or both defective alleles) were analysed separately. However, for gene-gene or GEI analysis, genotypes were restricted to homozygous wild and a variant genotype only. All statistical analysis was done using STATA software, version 12 (STATA Corp., College Station, TX, USA). Two-sided P values <0.05 were considered statistically significant.

RESULTS

A total of 492 ESCC cases and the same number of individually matched controls were recruited in the study. The mean ages (standard deviation) of cases and controls were 60.88 (±11.25) and 61.26 (±11.17) years, respectively. Formal education, wealth score, fruit and vegetable intake, salt tea beverage consumption, tobacco smoking in various forms, snuff chewing, FHC, contact with animals and oral hygiene were significantly different in cases and controls ($p<0.05$) (Table 2).

Allelic Frequencies and Genotype Analysis

The minor allele frequency differences observed among cases and controls were statistically significant ($p=<0.05$) except for CYP2A6b ($p=0.230$), and the genotype frequencies were in agreement with Hardy-Weinberg Equilibrium (Appendix 1). PCR-RFLP results of CYP2A6a, CYP2A6c and CYP2A13 genes are presented in Appendices 2, 3 and 4, respectively.

As shown in Table 3, the various CYP2A6 (a, b, and c) and CYP2A13 genes with ESCC risk showed a tendency towards inverse association as compared to respective wild type genotypes and even some of the relationships are significant. This relationship of various genotypes with ESCC risk did not change when their combinatorial effect was analysed (Table 4).

On analysing the modulating effect of various known risk factors of ESCC in the study population, the association of the various genotypes with ESCC risk changed reasonably in participants with wild-

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Table 2 Characteristics of esophageal squamous cell carcinoma cases and controls

Characteristics	Cases		Controls		p*
	n[a]	%	n[a]	%	
Total	492	100	492	100	
Age (years, mean±SD)	60.88±11.2		61.26±11.2		**<0.001**
Fruit and vegetables (median g/day IQR)	1.84 (1.2)		3.23 (1.2)		
Ethnicity					**0.199**
Kashmiri	476	96.7	484	98.4	
Other	16	3.3	08	1.6	
Gender					**<0.001**
Male	287	58.3	287	58.5	
Female	205	41.7	205	41.5	
Place of residence					**0.001**
Urban	19	3.9	42	8.5	
Rural	473	96.1	450	91.5	
Education					**<0.001**
No formal schooling	433	88.0	315	64.0	
Primary (less than 5th)	23	4.7	60	12.2	
Middle (5th-8th)	19	3.9	30	6.1	
High school (9th-12th)	14	2.8	51	10.4	
College or above	03	0.6	36	7.3	
Religion					**1.00**
Muslim	487	98.9	489	99.4	
Other	05	1.1	03	0.6	
mcacat[b]					**<0.001**
Quintile 1 (lowest)	281	57.1	96	19.5	
Quintile 2	77	15.6	94	19.1	
Quintile 3	42	8.5	101	20.5	
Quintile 4	51	10.4	96	19.5	
Quintile 5	41	8.4	105	21.4	
Hookah					**<0.001**
Never	174	35.4	288	58.5	
Ever	318	64.6	204	41.5	
Cigarette[#]					**<0.001**
Never	439	89.2	423	86.0	
Ever	53	10.8	69	14.0	
Second hand smoking					**0.028**
Yes	110	65.1	160	70.5	
No	59	34.9	67	29.5	
Naas					**<0.001**
Never	354	71.9	429	87.2	
Ever	137	27.9	59	12.0	
Ghutka					**0.292**
Never	483	98.2	487	99.0	
Ever	09	1.8	05	1.0	
Alcohol					**1.000**
Never	486	98.8	492	100.0	
Ever	06	1.2	0	0.00	
Family history of cancer (FHC)					**<0.001**
FHC^{+}	173	35.2	38	7.7	
FHC^{-}	319	64.8	454	92.3	
Salted tea consumption					**0.033**
Twice or thrice a week	61	12.4	105	21.8	
Daily at least ones	64	13.1	123	25.6	
Animal contact					**<0.001**
No contact	64	13.0	141	28.7	
Yes contact	42	87.0	351	71.3	

[a]: Cases and controls were individually matched, however variation in number or in percentages may not be always equal because of some missing numbers; [b]: Wealth score and quintile 1 represent highest category; *: P-values calculated using χ-tests for categorical variables; #: Cigarette smokers also include few subjects which are hookah users as well. SD: Standard deviation; IQR: Interquartile range

type genotypes as compared to variant genotype referents (heterozygous and homozygous mutant genotypes clubbed together as variants).

Tobacco Smoking and Nass Use

The smoker participants had higher risk of ESCC when carry the wild type genotypes of CYP2A6a (OR=2.7; 95% CI, 1.3–5.3); CYP2A6b (OR=2.9; 95% CI, 1.3–6.9); CYP2A6c (OR=3.0; 95% CI, 1.6–5.6) and CYP2A13 (OR=2.2; 95% CI, 1.2–4.0). Similarly, the use of nass increased risk in participants when carrying wild-type genotypes of CYP2A6 or CYP2A13 (Table 5).

Salt Tea Consumption

Unlike the other genotypes, moderately higher ESCC risk was found in CYP2A6C and CYP2A13 wild genotype harbouring subjects with salt tea consumption.

Family History

The presence of a family history of cancer was strongly associated with ESCC risk. The OR and 95% CI for wild genotypes of CYP2A6a, CYP2A6b, CYP2A6c and CYP2A13 were (OR=8.8; 95% CI, 4.5–17.9), (OR=5.2; 95% CI, 2.7–9.7), (OR=7.6; 95%CI, 4.0–14.6) and (OR=7. 95%CI, 4.1–14.1), respectively (Table 5).

Biomass Fuel Use and House Type

High risk of ESCC was found in participants who carried the wild homozygous genotype of CYP2A6a and used biomass as cooking fuel (OR=7.8; 95% CI, 2.1–29.0) and lived in adobe houses (OR=4.7; 95%CI, 2.1–10.7). Similarly, higher risk was found in participants who lived in adobe house and used biomass fuel for cooking in other CYP2A6 and CYP2A13 analyzed wild genotypes (Table 5).

Table 3 Genotypic distribution of CYP2A6 and CYP2A13 genes in ESCC cases and matched controls

	Genotype	Cases (%)	Controls (%)	UAOR (95%CI)	AOR[#] (95% CI)
CYP2A6a	Wild	405 (82.3)	369 (75.00)	1.0 (Referent)	1.0 (Referent)
	Heterozygous	76 (15.5)	114 (23.2)	0.6 (0.4–0.8)	0.6 (0.3–1.1)
	Mutant	11 (2.2)	09 (1.8)	1.1 (0.4–2.6)	1.8 (0.3 - 9.7)
	Variant3	87 (17.7)	123 (25.0)	0.6 (0.4–0.8)	0.6 (0.4–1.1)
CYP2A6b	Wild	385 (78.2)	365 (74.2)	1.0 (Referent)	1.0 (Referent)
	Heterozygous	93 (18.90)	114 (23.2)	0.8 (0.5–1.0)	0.6 (0.3–1.1)
	Mutant	14 (2.9)	13 (2.6)	1.02 (0.48–2.18)	1.8 (0.5–6.5)
	Variant3	107 (21.8)	127 (25.8)	0.78 (0.58–1.06)	1.0 (0.7–1.6)
CYP2A6c	Wild	387 (78.7)	320 (65.0)	1.0 (Referent)	1.0 (Referent)
	Heterozygous	79 (16.0)	143 (29.1)	0.50 (0.37- 0.67)	0.5 (0.3–0.9)
	Mutant	26 (5.3)	29 (5.9)	0.8 (0.4–1.4)	1.05 (0.4–3.1)
	Variant3	105 (21.34)	172 (35.0)	0.5 (0.4–0.7)	0.60 (0.4–0.9)
CYP2A13	Wild	385 (78.2)	347(70.5)	1.0 (Referent)	1.0 (Referent)
	Heterozygous	92 (18.7)	127(25.8)	0.8 (0.4 - 1.6)	0.50 (0.28–0.8)
	Mutant	15 (3.1)	18 (3.7)	0.6 (0.5 - 0.8)	0.74 (0.17–3.3)
	(Variant)3	107 (21.8)	145 (29.5)	0.7 (0.5–0.8)	0.50 (0.31–0.8)

ESCC: Oesophagal squamous cell carcinoma; UAOR: Unadjusted odds ratio; AOR: Adjusted odds ratio and 3variant indicates combined genotype, which has at least one variant allele. ORs (95% CIs) were obtained from conditional logistic regression models; CI: Confidence interval; [#]: Adjusted for age, ethnicity, gender, place of residence, religion, education level, wealth score, animal contact, oral hygiene, log of fruits and vegetables, tobacco smoking, nass consumption, alcohol drinking, family history of any cancer and salted tea

Table 4 Combined effect of CYP2A6 and CYP2A13 genotypes on ESCC risk

Genotype combinations	Cases n (%)	Controls n (%)	UAOR (95% CI)	AOR[#](95% CI)
Total	492(100)	492(100)		
2A6 wild + 2A13 wild	477 (96.9)	452 (91.9)	1.0 (Referent)	1.0 (Referent)
2A6 variants[C] + 2A13 variants	15 (3.1)	40 (8.1)	0.4 (0.2–0.7)	0.3 (0.1–0.8)
2A6a wild + 2A6b wild	323 (94.7)	303 (85.1)	1.0 (Referent)	1.0 (Referent
2A6a variants + 2A6b variants	18 (5.3)	53 (14.9)	0.3 (0.2–0.7)	0.4 (0.1–1.1)
2A6a wild + 2A6c wild	311 (93.7)	248 (86.1)	1.0 (Referent)	1.0 (Referent)
2A6a variants + 2A6c variants	21 (6.3)	40 (13.9)	0.44 (0.2–0.8)	0.2 (0.1–0.8)
2A6b wild + 2A6c wild	324 (94.7)	245 (85.6)	1.0 (Referent)	1.0 (Referent)
2A6b variants + 2A6c variants	18 (5.3)	41 (14.4)	0.3 (0.1–0.6)	0.4 (0.1–1.2)

[C]: variant indicates combined genotype, which has at least one variant allele. ESCC: Oesophagal squamous cell carcinoma; UAOR: Unadjusted odds ratio; AOR: adjusted odds ratio; ORs (95% CIs) were obtained from conditional logistic regression models. [#]: Adjusted for age, ethnicity, gender, place of residence, religion, education level, wealth score, animal contact, oral hygiene, log of fruits and vegetables, tobacco smoking, nass consumption, alcohol drinking, family history of any cancer and salted tea

Gender Wise Risk

On analysing the gender wise risk, males showed increased risk while harboring wild genotypes of either CYP2A6b (OR=2.1; 95% CI, 1.0–4.8); CYP2A6c (OR=2.5; 95% CI, 1.1–5.4) and CYP2A13 (OR=3.5; 95%CI, 1.6–7.7) (Table 5) as compared to females.

Gene Environment Interaction

Among the various gene environment combinations, only CYP2A6b ($P_{interaction}$=0.018) and CYP2A13 ($P_{interaction}$=0.021) genes showed synergistically significant associations with smoking (Table 6).

DISCUSSION

Genetic variants of different genes play an important role in the development of different diseases, including cancer. Among these, XMEs in general and CYP2A6 and CYP2A13 genes in particular are of critical importance in carcinogenesis. In the presence of inactive or deleted gene, enzyme activity or expression of these genes is reduced resulting impaired metabolism or inactivation of lethal compounds and hence the inverse association of such gene variants towards ESCC seems plausible. The increased risk

Table 5 OR and 95% CI of CYP2A6 and CYP2A13 genotypes in ESCC cases and control stratified by various risk factors

Genotypes/variable	CYP2A6a			CYP2A6b			CYP2A6c			CYP2A13		
	Cases n (%)	Controls n (%)	AOR[1] (95% CI)	Cases n (%)	Controls n (%)	AOR[1] (95% CI)	Cases n (%)	Controls n (%)	AOR[1] (95% CI)	Cases n (%)	Controls n (%)	AOR[1] (95%CI)
Tobacco smoking							62 (12.6)	73 (14.8)				
Variant + smokers$^-$	34 (6.9)	61 (12.4)	1.0 (Referent)	48 (9.7)	62 (12.6)	1.0 (Referent)	44 (8.9)	89 (18.1)	1.0 (Referent)	45 (9.1)	72 (14.6)	1.0 (Referent)
Variant + smokers$^+$	53 (10.8)	62 (12.6)	1.6 (0.7–3.6)	59 (12.0)	65 (13.2)	1.4 (0.5–2.4)	61 (12.4)	83 (16.8)	1.7 (0.8–3.5)	62 (12.6)	73 (14.8)	1.0 (0.5–2.2)
Wild + smokers$^-$	130 (26.4)	188 (38.2)	1.4 (0.7–2.7)	116 (23.6)	187 (38.0)	0.8 (0.3–2.0)	120 (24.4)	160 (32.5)	1.5 (0.8–2.7)	119 (24.4)	177 (35.9)	0.9 (0.5–2.7)
Wild + smokers$^+$	275 (55.9)	181 (36.8)	2.7 (1.3–5.3)	269 (54.7)	178 (36.2)	2.9 (1.3–6.9)	267 (54.3)	160 (32.5)	3.0 (1.6–5.6)	266 (54.1)	170 (34.6)	2.2 (1.2–4.0)
Snuff (Nass) consumption												
Variant + snuff$^-$	64 (13.0)	108 (21.9)	1.0 (Referent)	71 (14.3)	115 (23.4)	1.0 (Referent)	74 (15.0)	151 (30.7)	1.0 (Referent)	84 (17.1)	125 (25.4)	1.0 (Referent)
Variant + snuff$^+$	23 (4.7)	15 (3.0)	1.4 (0.5–3.7	36 (7.3)	12 (2.4)	3.7 (1.5–9.3)	31 (6.3)	21 (4.3)	2.4 (1.1–5.4)	23 (4.7)	20 (4.1)	0.8 (0.3–1.9)
Wild + snuff$^-$	290 (58.9)	321 (65.2)	1.5 (0.9–2.4)	283 (57.5)	314 (63.8)	1.1 (0.7–2.0)	280 (56.6)	278 (56.5)	1.8 (1.1–2.8)	270 (54.9)	304 (61.8)	1.1 (0.7–1.8
Wild + snuff$^+$	115 (23.4)	48 (9.8)	3.0 (1.6–5.7)	102 (20.7)	51 (10.4)	1.9 (1.1–3.5)	107 (20.7)	42 (8.5)	3.1 (1.7–5.8)	115 (23.4)	43 (8.7)	2.8 (1.5–5.2)
Salted tea consumption												
Variant + salted tea	86 (17.8)	119 (25.3)	Referent	107 (22.1)	124 (26.3)	Referent	103 (21.3)	165 (35.0)	1.0 (Referent)	1 (0.2)	6 (1.2)	1.0 (Referent)
Wild + salted tea	397 (82.2)	352 (74.7)	1.5 (0.9–2.6)	376 (77.9)	347 (73.7)	0.9 (0.6–1.5)	380 (78.7)	306 (65.0)	1.6 (1.0–2.6)	337 (76.6)	332 (67.5)	1.7 (1.0–3.4)
Family history of any malignancy												
Variant + FHC$^-$	55 (11.2)	111 (22.6)	Referent	69 (14.2)	120 (24.4)	Referent	68 (13.8)	162 (32.9)	1.0 (Referent)	65 (13.2)	134 (27.2)	1.0 (Referent)
Variant + FHC$^+$	32 (6.5)	12 (2.4)	2.8 (1.1–7.7)	38 (7.7)	7 (1.4)	8.2 (2.2–30.7)	13 (7.5)	10 (2.0)	9.6 (3.3–27.6)	42 (8.5)	11 (2.2)	6.5 (2.4–17.6)
Wild + FHC$^-$	264 (53.7)	343 (69.7)	1.3 (0.8–2.2)	250 (50.8)	334 (67.9)	1.0 (0.6–1.6)	251 (51.1)	292 (59.4)	1.8 (1.2–2.8)	254 (51.6)	320 (65.0)	1.5 (0.9–2.3)
Wild + FHC$^+$	141 (28.6)	26 (5.3)	8.8 (4.5–17.9)	135 (27.3)	31 (6.3)	5.2 (2.7–9.7)	136 (27.6)	28 (5.7)	7.7 (4.2–14.1)	131 (26.6)	27 (5.5)	7.6 (4.0–14.1)
Fuel												
Variant + other	17 (4.1)	79 (20.1)	Referent	20 (5.1)	84 (20.8)	Referent	32 (7.7)	98 (26.3)	1.0 (Referent)	21 (5.2)	88 (22.8)	1.0 (Referent)
Wild +Biomass	395 (95.2)	308 (78.4)	7.8 (2.1–29.0)	376 (94.2)	313 (77.7)	4.2 (1.6–10.9)	381 (91.8)	269 (70.1)	3.7 (1.5–9.1)	376 (94.00)	292 (75.6)	5.4 (1.7 –17.0)
House type												
Variant +concrete	74 (16.7)	148 (32.2)	Referent	72 (17.0)	150 (32.4)	Referent	83 (19.1)	184 (40.9)	1.0 (Referent)	74 (17.4)	163 (35.2)	1.0 (Referent)
Wild +Adobe	269 (60.6)	94 (20.5)	4.7 (2.1–10.7)	248(58.5)	98 (21.2)	3.7 (1.8–7.5)	251(16.1)	83 (18.4)	3.0 (1.5–6.1)	246 (57.9)	99 (21.4)	4.3 (2.1–9.0)
Gender												
Variant + male	49 (17.1)	75 (26.0)	Referent	62 (21.6)	81 (28. 1)	1.0 (Referent)	57 (19.9)	98 (34.3)	1.0 (Referent)	230 (80.1)	196 (68.1)	1.0 (Referent)
Wild+male	238 (82.9)	213 (74.0)	1.5 (0.6–3.3)	225 (78.4)	207 (71.9)	2.1 (1.0–4.8)	230 (80.1)	190 (65.9)	2.5 (1.1–5.4)	57 (19.9)	92 (31.9)	3.5 (1.6–7.7)
Variant + female	38 (18.3)	48 (23.5)	Referent	45 (21.9)	45 (22.5)	1.0 (Referent)	48 (23.4)	74 (36.3)	Referent	155 (75.6)	151 (74.0)	1.0 (Referent)
Wild + female	167 (81.5)	156 (76.5)	1.4 (0.6–3.4)	160 (78.1)	158 (77.5)	1.1 (0.4–2.6)	157 (76.6)	130 (63.7)	1.2 (0.6–2.6)	50 (24.4)	53 (26.0)	0.9 (0.4–2.2)

[1]: Adjusted for age, ethnicity, gender, place of residence, religion, education level, wealth score, animal contact, oral hygiene, log of fruits and vegetables, tobacco smoking, nass chewing, alcohol drinking, family history of any cancer and salted tea. The variable under consideration was not additionally adjusted for it. ORs (95% CIs) were obtained from conditional logistic regression models. Numbers may not add up to the total numbers due to missing data in some variables. ESCC: Oesophagal squamous cell carcinoma; CI: confidence interval

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Table 6 Gene-gene and gene-environment interaction results

Genotype+exposure	SE	p	OR	95% CI
CYP2A6b+ tobacco smoking	0.24	0.018	1.5	1.1–2.0
CYP2A13+ tobacco smoking	0.27	0.021	1.4	1.1–2.0

P value: Statistically significant results are in bold (p≤0.05). SE: Standard error; OR: Odds ratio; CI: Confidence interval

among subjects harbouring normal genotypes of the above genes in combination with different environmental and lifestyle exposures like tobacco smoking and diet, etc. reveals that the wild form of these genotypes is completely active for enzyme activity, hence leading to a substantial increase in production of PAHs and nitrosamine-specific DNA adduct.[37,38] PAHs and nitrosamines have been experimentally proven to be carcinogenic and have been associated with different cancers, including gastrointestinal malignancies.[39–42]

In case of the CYP2A6 gene, 23 variants are currently known that reduce its enzymatic activity, and 5 variants were shown to completely abolish enzymatic function.[43–46] Similarly, the variant genotype of CYP2A13 was 37 to 56% less active than the wild-type genotype towards almost all tested substrates, including nitrosamines and PAHs.[47] Some mutations in this gene were observed to provide some protection against xenobiotic toxicity in xenobiotic-exposed organs.[47] A C/T variation in exon 5 of the CYP2A13 gene leads to an Arg257Cys amino acid change. This change in amino acids from Arg to Cys results in a genotype with significantly reduced activity towards its substrates. The reduction in CYP2A13 variant enzyme activity towards PAHs, N-nitrosamines and other substrates leads to less DNA adduct formation, suggesting a protective role against carcinogenicity in the target tissue of an individual.[47]

In this study, we observed an inverse association of some variant genotypes of CYP2A6 and CYP2A13. However, the ESCC risk increased significantly with normal genotypes in the presence of different ESCC risk factors in the study population.

The reduced risk of ESCC due to CYP2A6 gene variants in our study is in agreement with the previous studies on oesophagal and lung malignancies.[48–50] A study with a large sample size showed significantly decreased associations of the CYP2A6 variant (*4) genotype towards lung cancer in Asian population with pooled analysis (OR=0.761; 95%CI, 0.67–0.86). After stratifying Asian samples on smoking status, significant decrease in risk was noted in smokers carrying a variant genotype (OR=0.71; 95%CI, 0.61–0.84).[37] Another meta-analysis from January, 1966 to August 2011 observed the inverse association of one (OR=0.82; 95%CI, 0.73–0.92) or both mutant alleles (OR=0.57; 95%CI, 0.48–0.68), in comparison with the wild-type CYP2A6 gene.[51] Furthermore, reduced risk was strengthened among lung cancer cases who were smokers as well as carrying one (OR=0.71; 95%CI, 0.58–0.87) and/or both mutant alleles (OR=0.47; 95%CI, 0.35–0.62).

Similarly, frequencies of the CYP2A6*4 allele in three regions of China, in Han (N=120), Uighur (N=100), Bouyei (N=100) and Tibetan (N=100) (p<0.0001) were 7.9%, 15%, 0% and 2%, respectively.[52] This suggests that different ethnic populations might have different environmental and lifestyle exposure and hence different xenobiotic response. Among African American ever-smokers, drawn from two independent case-control studies of lung cancer, reduced activity CYP2A6 alleles showed lower risk as compared to normal metabolizers (OR=0.44; 95%CI, 0.26–0.73).[50] The association was replicated in an independent study (n=407) from MD Anderson Cancer Centre, USA (OR=0.64; 95% CI, 0.42–0.98), and on pooling ethnically different populations, an OR of 0.64 (95%CI, 0.48–0.86) was yielded. These findings support a contribution of genetic variation in CYP2A6 to lung cancer risk among African American smokers, particularly men, whereby CYP2A6 genotypes associated with reduced metabolic activity confer a lower risk of developing lung cancer.[50] Additionally, an increased cancer risk (OR=2.65; 95%CI, 1.84–3.81, p<0.001) was noted among individuals harboring a wild homozygous (*1/*1) genotype of CYP2A6, in one of the large sample-sized Asian study.[53] while another similar study (2524 cases and 2258 controls) reported the decreased frequency of the mutant (*4/*4) genotype in Asians while no *4/*4 genotype was detected in Caucasians.[54]

Besides a huge body of literature supporting our findings, still a few studies that have not shown any significant relationships between CYP2A6 genotypes and lung cancer in both never and ever smokers.[51] Similarly, a study in the case of gastric malignancy could not repeat our findings when CYP2A6 gene deletion was studied alone; however, reduced risk was noted when subjects were smokers as well as carrying a CYP2A6 gene in variant form as compared to wild genotype carrying smoking subjects.[55]

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In case of CYP2A13 gene, our results were in line with the previous reports which have revealed a 2–3 fold reduction in the metabolic activation of tobacco specific nitrosamine - 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in subjects with variant genotype of CYP2A13 than subjects carrying a wild genotype, [47] hence supports the decreased risk of ESCC among individuals who harbored variant allele of CYP2A13 gene. Reduced risk for lung adenocarcinoma was observed in the case of variant CYP2A13 genotype (CT + TT) than wild (CC) genotype (OR=0.41; 95%CI, 0.23–0.71), but not for squamous cell carcinoma (OR=0.86; 95%CI, 0.57–1.29) or other types of lung malignancies (OR=0.58; 95%CI, 0.32–1.09).

Stratification analysis showed that the reduced risk of lung adenocarcinoma related to the variant CYP2A13 genotype was limited to smokers, especially light smokers (OR=0.23; 95% CI, 0.08–0.68) but not non-smokers or heavy smokers. The two novel polymorphisms T478C and T494C in the CYP2A13 gene were associated with a significantly reduced risk of head and neck cancer (OR=0.37; 95%CI, 0.19–0.71). A CYP2A13 haplotype carrying variant alleles of T478C/T494C was associated with the reduced risk of (OR 0.42; 95%CI, 0.22–0.78).[56] Similarly, the CYP2A13 R257C variant carrier was associated with substantially reduced risk for lung adenocarcinoma (OR=0.41; 95%CI, 0.23–0.71).[57]

The retention of inverse effect due to the combination of CYP2A6 and CYP2A13 variants in our study is consistent with the earlier studies, but other than oesophagal malignancy.[37,50] These studies reported a decreased cancer risk in subjects harbouring both genes in variant form. This decreased risk again could be a result of an additional decrease in enzyme activity of these genotypic combinations.

One of the interesting observations of the current study is that the ESCC risk is more common among men than women. The biological mechanism for such association is still not known, but one of the plausible explanations could be that males have a higher prevalence of active tobacco smoking as compared to females. The combination of tobacco smoking exposure in males with susceptible genotypes puts them at higher risk and hence male predominance towards ESCC risk in our population. Interestingly, a reduction in the consumption of smoking among the carriers of variant alleles of the CYP2A6 gene could also be the probable reason for male dominance with CYP2A6 wild genotype carrying subjects, and hence an increased risk than female participants.[58] Although females are comparatively less active smokers, they are at increased ESCC risk in our population. The possible reason for this finding could be their comparatively greater exposure to second-hand smoke from poorly ventilated adobe houses and cooking fumes generated from biomass fuels. These results are in agreement with the already published reports from high ESCC regions.[59–61]

A limited number of studies are available regarding the modifying effect of the CYP2A6 and CYP2A13 genes in subjects with a positive history of cancer among relatives. The plausible reason for increased risk in our study could be either similar exposure to ESCC risk factors within the family and/or similar genetic setup among the relatives.[62,63] Similarly, due to a lack of any reports regarding the association of study genes with ESCC risk with respect to dwelling and indoor air pollution among subjects, we could infer from our results that indoor air pollution from poor ventilated/adobe houses could increase the exposure of different toxic chemicals and hence risk of different malignancies.[64–66]

The synergistic association of the CYP2A13 wild genotype in the presence of smoking could reflect the biological feature of CYP2A13–257Cys, which exhibits a decreased catalytic efficiency toward N-nitrosamines as compared to CYP2A13–257Arg.[47,57,67] This observation may also emphasise an enhanced risk of ESCC among tobacco smokers in our population and hence the reduced risk of variant genotypes.[21] However, to the best of our knowledge, the relevance of this functional polymorphism in CYP2A13 to ESCC has not been investigated to date.

Limitations

Though, no study has reported the exposure of these genotypes with ESCC under such environmental combinations and confounding of the results with the probable ESCC risk factors, selection or recall bias could be one of the weak points of this study, although the same hospital setting and limited number of interviewers lessen this type of bias to some extent.

CONCLUSION

The study suggests that CYP2A6 and CYP2A13 gene variants are associated with decreased ESCC risk, and exposure to different potential ESCC risk factors proved more lethal in subjects with normal activity gene variants.

Ethics Committee Approval: The study was approved by the Sher-i-Kashmir Institute of Medical Sciences Ethics Committee (no: SIMS 1 31/IEC-SKIMS/2013, date: 17/01/2013).

Informed Consent: Informed consent was obtained from all participants.

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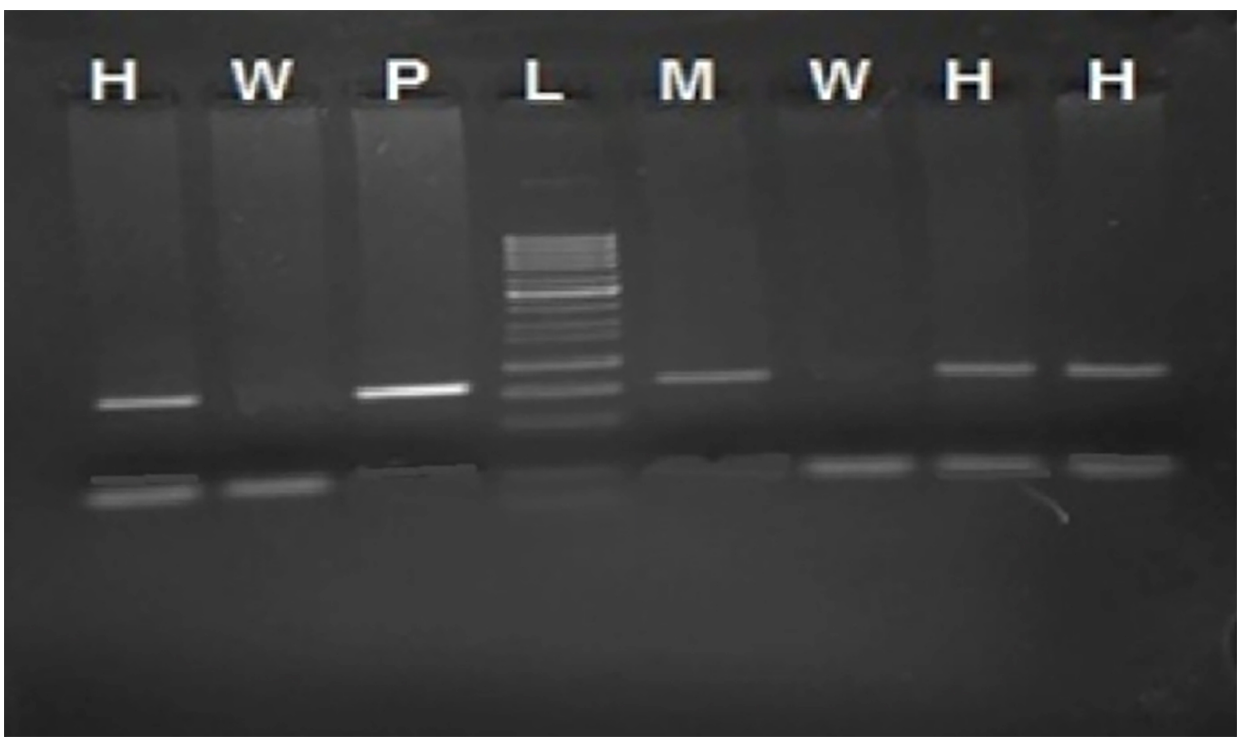
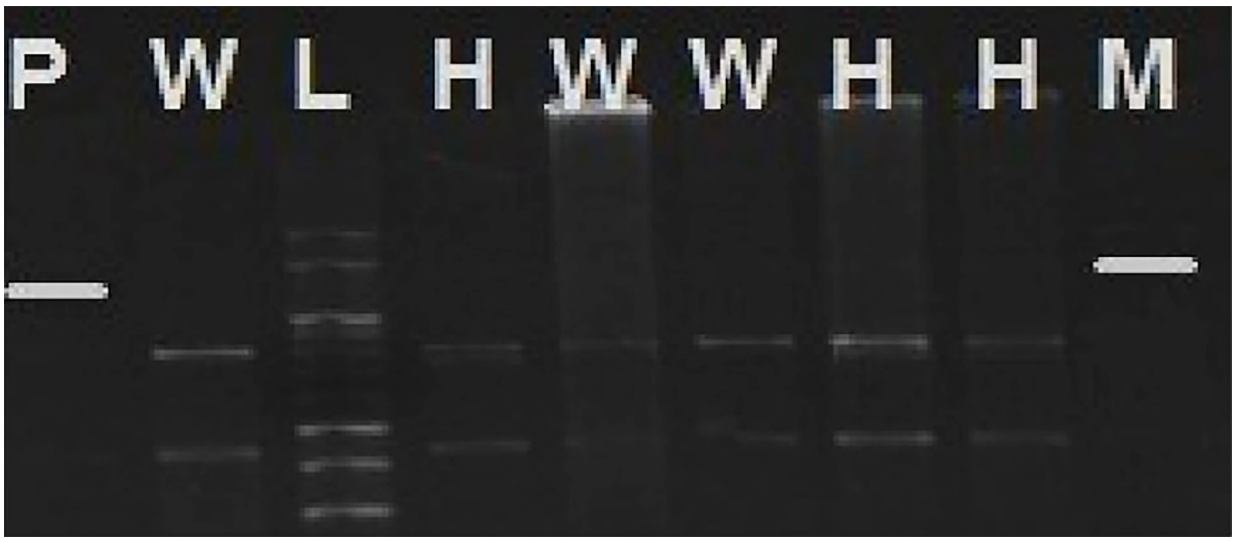
Appendix 1 CYP2A6a, CYP2A6b, CYP2A6c and CYP2A13 allele frequencies, crude odds ratios and 95% confidence interval in ESCC cases (2n=984) and controls (2n=984)

Allele	Case n (%)	Control n (%)	OR (95% CI)[a]	p
CYP2A6a*1	886 (90.0)	738 (86.6)	Referent	<0.0001
CYP2A6a *6	98 (13.4)	246 (10.0)	3.0 (2.3–3.9)	
CYP2A6b *1	863 (87.7)	844 (85.8)	Referent	0.230
CYP2A6b *4	121 (12.3)	140 (14.2)	1.2 (0.9–1.5)	
CYP2A6c*1A	853 (86.7)	783 (79.4)	Referent	<0.0001
CYP2A6c*4C	121 (12.3)	201 (20.4)	1.7 (1.3–2.1)	
CYP2A13 C	862 (87.6)	821 (83.4)	Referent	0.010
CYP2A13 T	122 (12.4)	163 (16.6)	1.4 (1.1–1.8)	

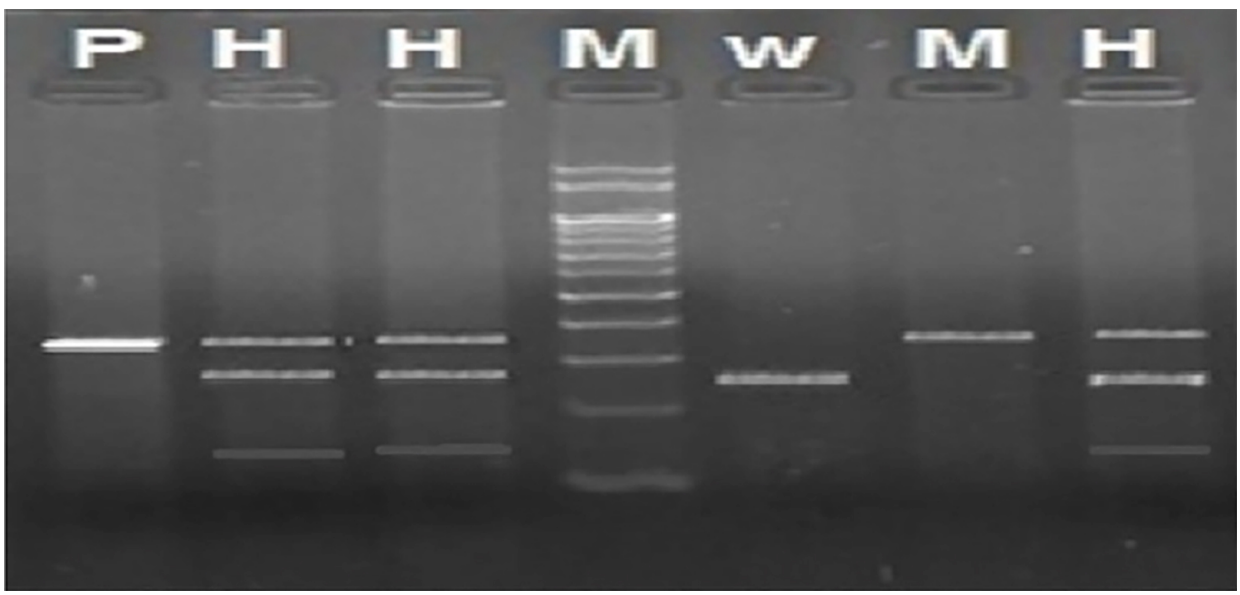
ESCC: Oesophagal squamous cell carcinoma; OR: Odss ratio; CI: Confidence interval



Appendix 2. PCR-RFLP analysis of CYP2A6a polymorphism.
"P" is the PCR product, "W" represents the 116bp and 99bp CYP2A6 *1/*1 (homozygous wild) genotype; "M" represents the undigested parent band (215bp) indicating the CYP2A6 *1/*1 (homozygous mutant) genotype; "H" is the CYP2A6 *1/*6 (heterozygous) genotype which all the three bans i.e 215p, 116p and 99bp and "L" represent the 50bp marker.



Appendix 3. PCR-RFLP analysis of CYP2A6c polymorphism.



Appendix 4. PCR-RFLP analysis of CYP2A13 polymorphism.
"W" represents the 217bp and 158bp CYP2A613 C/C (homozygous wild) genotype; "M" represents the undigested parent band (375bp) indicating the CYP2A613 T/T (homozygous mutant) genotype while as "H" is the CYP2A613 C/T (heterozygous) genotype with all the three bands i.e 375bp, 217 and 158bp and "L" and "P" represent the 50 bp marker and PCR product respectively.