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ORIGINAL ARTICLE

Polymorphisms in *CYP2A13* and *UGT1A7* genes and head and neck cancer susceptibility in North Indians

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OBJECTIVES: To examine role of genetic variants of *CYP2A13* and *UGT1A7* genes, involved in activation and detoxification of tobacco carcinogens, with risk of head and neck cancer as well as to assess the potential modifying role of gene-gene and gene-environment interactions.

METHODS: 203 head and neck cancer patients and 201 healthy controls were genotyped for functional polymorphisms of *CYP2A13* and *UGT1A7* genes using polymerase chain reaction-restriction fragment length polymorphism, denaturing high-performance liquid chromatography and sequencing.

RESULTS: We identified two novel polymorphisms T478C and T494C in *CYP2A13* gene which were associated with significantly reduced risk of cancer (OR 0.37; 95% CI 0.19–0.71; $P < 0.05$). A *CYP2A13* haplotype carrying variant alleles of T478C/T494C was found to be associated with reduced risk of head and neck cancer (OR 0.42; 95% CI 0.22–0.78; $P = 0.005$). Mutant 'T' allele of *CYP2A13* C578T polymorphism was found to be present in cancer patients only. A sevenfold increased risk of cancer was observed in smokers with *UGT1A7* low activity genotypes (OR 7.01; 95% CI 1.02–48.37; $P < 0.05$). *UGT1A7* haplotype carrying C allele (T622C) showed 10-fold increased risk of cancer (OR 10.12; 95% CI 1.29–79.4; $P < 0.05$).

CONCLUSION: Interplay between genetic variants of *CYP2A13* and *UGT1A7* genes and smoking may modulate susceptibility to head and neck cancer.

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Keywords: *CYP2A13*; *UGT1A7*; smoking; head and neck cancer; genetic polymorphisms; haplotypes

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Introduction

Head and neck cancer represents the most common malignancy among males and third among females in India (Parkin *et al*, 2005). Epidemiological differences reveal that smoking and alcohol consumption are major risk factors in western countries (Choi and Kahyo, 1991) whereas tobacco consumption is the most implicated etiological factor in South East Asia, including India (Notani, 2001). Although, tobacco consumption has been consistently linked with head and neck cancer, all the exposed individuals do not develop the fatal disease, indicating interplay between genetic and environmental factors in the etiology of head and neck cancer.

The *CYP2A13*, a phase I enzyme, catalyzes the metabolism of most deleterious carcinogens present in tobacco such as 4-methylnitroso-1-(3-pyridyl)-1-butanone (NNK), hexamethylphosphoramide, N-nitrosomethylphenylamine and N,N-dimethylaniline (Su *et al*, 2000). Till date, more than 20 polymorphisms have been identified in this gene, but only three of them, C578T (Arg101Stop) in exon 2, the C3375T (Arg257Cys) in exon 5 and C7520G in 3′ untranslated region, are known to exhibit functional consequences (Cauffiez *et al*, 2005). The 3′ UTR polymorphism C7520G has been correlated with a 10-fold decrease in *CYP2A13* expression (Day and Tuite, 1998; Zhang *et al*, 2004). A transition from C to T at nucleotide 3375 in exon 5 has been shown to result in catalytically less active *CYP2A13* protein (Zhang *et al*, 2002) while C578T leads to formation of truncated non-functional protein (Zhang *et al*, 2003). These *CYP2A13* variants confer protection toward tobacco carcinogens and subsequent reduced cancer risk (Cauffiez *et al*, 2005).

UDP-glucuronosyltransferases (UGTs) represent an important family of detoxification enzymes that catalyze the conjugation of tobacco carcinogens; NNK and polycyclic aromatic hydrocarbons (Tephly and Burchell, 1990). Three allelic variants of wild type *UGT1A7**1 allele have been described. Two of the polymorphisms N129K and R131K, present in *UGT1A7**2 and *UGT1A7**3 alleles are genetically linked and result in

asparagine to lysine and arginine to lysine amino acid substitutions at codons 129 and 131 respectively (Guillemette *et al*, 2000). T to C transition at nucleotide 622 which is present in *UGT1A7**3 and *UGT1A7**4 alleles, results in substitution of arginine for tryptophan. The *UGT1A7* enzyme encoded by wild type allele (*UGT1A7**1) is two to fourfold more catalytically active than the enzymes coded by variant alleles (*UGT1A7**2, *UGT1A7**3 & *UGT1A7**4) (Guillemette *et al*, 2000). An additional polymorphism T-57G, present in TATA box element of *UGT1A7* has been shown to result in 70% reduction of promoter activity and consequent reduction of *UGT1A7* enzyme activity (Lankisch *et al*, 2005). These variants of *UGT1A7* which lead to reduced capacity to detoxify the tobacco carcinogens have been reported to be associated with increased risk of tobacco related cancer (Nagar and Remmel, 2006).

Till date, epidemiological studies of cancer risk in relation to polymorphisms of *CYP2A13* and *UGT1A7* genes have revealed conflicting findings. Besides different study designs, prevalence of genetic polymorphisms and linkage disequilibrium in different ethnic populations and effect modification by environmental or other genetic factors may contribute to varying results obtained (Strassburg *et al*, 2002; Wang *et al*, 2003; Feng *et al*, 2005; Piepoli *et al*, 2006; Song *et al*, 2009). Moreover, studies examining the effect of genetic polymorphisms as well as haplotypes of these genes on cancer susceptibility are lacking in Indian population. We have thus concerted our focus on identification of polymorphisms in *CYP2A13* and *UGT1A7* genes and disease haplotypes in an ethnically homogenous North Indian population. We also evaluated the role of gene-gene and gene-environment interactions in the susceptibility to tobacco related head and neck cancer.

Materials and methods

Study subjects

A total of 203 consecutive head and neck cancer patients were enrolled from department of Otolaryngology, Post Graduate Institute Medical Education Research (PGIMER), Chandigarh, India after informed consent during the period from April, 2005 to June, 2008. We enrolled patients with primary head and neck cancer prior to any chemotherapy and radiotherapy and patients with metastatic head and neck cancer were excluded from the present study. All the cases were confirmed by histopathology for the presence of cancer of head and neck region consisting of oral cavity, pharynx, and larynx. The control group consisted of 201 healthy individuals having no evidence of malignancy or any other chronic disease. We enrolled healthy blood donors as controls, obtained through the blood bank situated in the referral region of our hospital. All the study subjects were of North Indian origin and the origin was confirmed on the basis of their mother language and ancestral history. The research protocol was approved by Institute Research Ethics Committee, PGIMER.

All the subjects were interviewed using a standardized questionnaire regarding the demographic information, lifestyle factors such as use of tobacco and alcohol consumption (including frequency and duration), family history of cancer and medical history prior to diagnosis date of cancer. The response rate for the interview was greater than 85% from the respondents taken as controls and cases. The lifetime exposure to smoking and alcohol was measured as described by Buch *et al* (Buch *et al*, 2002).

Lifetime exposure to smoking = No. of Cigarettes/beedis per day × Duration of smoking (years)

Lifetime exposure to alcohol = No. of shots per day × Duration of alcohol intake (years)

Subjects were classified on the basis of their tobacco and alcohol consumption as smokers and alcohol consumers. The median value (400) of lifetime exposure of smoking in controls was used as cutoff value for stratification of smokers into heavy smokers (> 400) and light smokers (≤400).

Genotyping

Genomic DNA was isolated from peripheral blood lymphocytes using the proteinase K digestion and phenol chloroform method. The C7520G and C3375T polymorphisms of *CYP2A13* were identified using PCR-RFLP methods (Polymerase chain reaction-restriction fragment length polymorphism) as described by Cauffiez *et al* (Cauffiez *et al*, 2005). To detect Arg101Stop polymorphism, exon 2 of *CYP2A13* was screened by denaturing high-performance liquid chromatography using the Transgenomic WAVE instrument (Transgenomic Inc., San Jose, CA, USA), which is based on ion-paired reversed phase high-performance liquid chromatography at melting temperature of 64.2°C (Stenirri *et al*, 2004). The variants identified were confirmed by direct sequencing by automated fluorescent sequencing using Big Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA) and ABI 3730 sequencer (Applied Biosystems). *UGT1A7* exon 1 polymorphic variants were identified by PCR-RFLP methods, as described by van der Logt *et al* (van der Logt *et al*, 2004).

Depending on combinations of different *UGT1A7* genotypes at codons 129/131 and 208, the predicted *UGT1A7* enzyme activity was classified as low, intermediate and high according to Guillemette *et al* (Guillemette *et al*, 2000). The promoter polymorphism T-57G resulting in creation of HpyCH4IV restriction site was detected using PCR RFLP method as explained by Lankisch *et al* (Lankisch *et al*, 2005). Positive and negative controls were used in each genotyping assay and 5% of randomly selected samples were re-genotyped by other lab personal with 100% concordance.

Statistical analysis

Statistical analysis was performed using the SPSS software (SPSS software version 12.0, SPSS Inc., Chicago, IL, USA). Discrete and continuous variables were compared between cases and controls using Pearson's χ^2 test and unpaired *t*-test as appropriate. The risk to head and neck cancer in relation to polymorphic prevalence was evaluated using logistic regression to

calculate odds ratio (OR) and 95% confidence intervals (CI) after adjustment for potential confounders with wild type as reference. In case of UGT1A7 genotypes stratified on basis of predicted enzyme activity, high activity genotype associated with normal enzyme activity was taken as reference category in statistical analysis. Power analysis was performed using Quanto (Version 1.0, http://www.hydra.usc.edu/GxE/ provided in the public domain by the University of Southern California, Los Angeles, CA) and HWE calculator was used to check for deviation from Hardy–Weinberg equilibrium (HWE). Haplotype analysis was performed using PHASE (version 2.1, http://www.stephenslab.uchicago.edu/software.html/ provided in the public domain by the University of Washington, Seattle, WA, USA). Chi square test was performed to determine significance and odds ratio between cases and controls. Linkage disequilibria (LD) were also estimated for the polymorphisms of *CYP2A13* and *UGT1A7* genes in the study population, using Haploview software (version 3.2, http://www.broadinstitute.org/haploview/haploview-downloads/ provided in the public domain by the Broad Institute of MIT and Harvard, Cambridge, MA, USA). The assessment of gene-gene and gene-environment interactions was carried out using Multifactor dimensionality reduction (MDR) which employs data reduction approach to identify combinations of genotypes and environmental factors that are associated with either high or low risk of disease (Hahn *et al*, 2003). In this study, we used 10-fold cross validation and 1000-fold permutation testing during MDR analysis. A two sided *P*-value of <0.05 was considered statistically significant.

Results

Demographic characteristics of head and neck cancer patients and controls are given in Table 1. There was no significant difference of age, gender, mean lifetime exposure of smoking and alcohol consumption among patient and control group. All the patients were histopathologically diagnosed as squamous cell carcinoma, with majority of tumors (56.7%) belonging to stage IV according to clinical staging for head and neck cancer.

All the polymorphisms in present study were in Hardy–Weinberg equilibrium (each *P*-value >0.05). The frequency distribution of *CYP2A13* and *UGT1A7* genotypes and alleles among cases and controls is summarized in Table 2a and 2b. Our study revealed the presence of two novel linked polymorphisms in exon 2 of *CYP2A13*; T478C and T494C (Figure 1). The frequency of variant alleles as well as genotypes of these linked polymorphisms were found to be significantly higher in controls as compared with cases and conferred protection to head and neck cancer (T/T allele OR, 0.44; 95% CI 0.24–0.82; $P = 0.007$; Heterozygous genotype OR, 0.37; 95% CI 0.19–0.71; $P = 0.003$). We observed non-sense mutation C578T of *CYP2A13* in heterozygous condition in five cancer patients while none of healthy controls carried this mutation (Figure 1). The distribution of C3375T and C7520G polymorphisms was found to be similar in patient and control group in our cohort. There was no significant difference in distribution of *UGT1A7* variant genotypes between cases and controls.

When we studied correlation between anatomic subsites within head and neck region and polymorphisms of *CYP2A13* and *UGT1A7*, it was seen that heterozygous genotype of *CYP2A13* T478C/T494C conferred protection to cancer of pharynx (OR, 0.24; 95% CI 0.08–0.71; $P = 0.005$) and larynx (OR, 0.22; 95% CI 0.05–0.96; $P = 0.029$) but not in patients with cancer of oral cavity. Intermediate activity *UGT1A7* genotypes were found to be associated with significantly reduced risk of laryngeal cancer (OR, 0.08; 95% CI 0.03–0.19; $P < 0.001$). Low activity *UGT1A7* genotypes showed threefold increased risk of cancer of pharynx (OR, 3.18; 95% CI 1.1–10.03; $P = 0.04$), however, the same genotypes offered protection to laryngeal cancer (OR, 0.28; 95% CI 0.18–0.78; $P = 0.01$) (Table 3). No association was observed between variants of *CYP2A13* and *UGT1A7* genes and tumor size, nodal involvement and clinical stage of head and neck cancer (data not shown).

Table 1 Demographic characteristics of study population

Characteristics	*Patients (n = 203)*	*Controls (n = 201)*	P-*value*
Mean age (years)	53.26 ± 11.69	51.10 ± 11.17	0.06[a]
Gender			
Males	183 (90.1)	190 (94.5)	0.142[b]
Females	20 (9.9)	11 (5.5)	
Lifetime exposure smoking	483.67 ± 341.68	479.06 ± 400.90	0.439[c]
Smoking index	408	400	
Smoking			
Yes	176 (86.7)	153 (76.2)	0.013[b]
No	27 (13.3)	48 (23.8)	
Heavy smokers	95 (53.9)	76 (49.7)	
Light smokers	81 (46.1)	77 (50.3)	
Lifetime exposure alcohol	286.67 ± 321.14	162.17 ± 290.39	0.083[c]
Alcohol consumption			
Yes	114 (56.2)	100 (49.8)	0.234[b]
No	89 (43.8)	101 (50.2)	

[a]Student's *t*-test; [b]Pearson's χ^2-test; [c]Mann–Whitney test.

Table 2a Allele frequencies of *CYP2A13* and *UGT1A7* variants in cases and controls

Polymorphisms	*Patients (2n = 406)*	*Controls (2n = 402)*	*OR(95% CI)*	P-*value*
CYP2A13: T478C/T494C	T/T = 390 (0.96)	T/T = 368 (0.92)	1.0	
	C/C = 16 (0.04)	C/C = 34 (0.08)	0.44 (0.24–0.82)	0.007
CYP2A13: C578T	C = 401 (0.988)	A = 402 (1.00)	1.0	
	T = 5 (0.012)	G = 0 (0.0)	–	
CYP2A13: C3375T	C = 394 (0.97)	C = 383 (0.953)	1.0	
	T = 12 (0.03)	A = 19 (0.047)	0.61 (0.29–1.28)	0.189
CYP2A13: C75205G	C = 388 (0.96)	C = 393 (0.98)	1.0	
	G = 18 (0.04)	G = 9 (0.02)	2.03 (0.89–4.56)	0.08
UGT1A7:T-57G	T = 249 (0.61)	T = 252 (0.63)	1.0	
	C = 157 (0.39)	C = 150 (0.37)	1.06 (0.79–1.41)	0.68
UGT1A7	*UGT1A7*1* = 104 (0.256)	*UGT1A7*1* = 103 (0.256)	1.0	
	*UGT1A7*2* = 130 (0.321)	*UGT1A7*2* = 143 (0.356)	0.90 (0.63–1.29)	0.57
	*UGT1A7*3* = 156 (0.384)	*UGT1A7*3* = 143 (0.356)	1.08 (0.76–1.54)	0.67
	*UGT1A7*4* = 16 (0.039)	*UGT1A7*4* = 13 (0.032)	1.29 (0.56–2.66)	0.62

OR, odds ratio; CI, confidence intervals.

Table 2b Genotype frequencies of *CYP2A13* and *UGT1A7* variants among cases and controls

Polymorphisms	*Patients (n = 203)*	*Controls (n = 201)*	*Crude OR(95% CI)*	*Adjusted OR(95% CI)*[a]
CYP2A13: T478C/T494C	W = 187 (92.1)	TT = 167 (83.1)	1.0	1.0
	Het = 16 (7.9)	TC = 34 (16.9)	0.42 (0.22–0.78), 0.005	0.37 (0.19–0.71), 0.003
CYP2A13:	CC = 198 (97.5)	CC = 201 (100.0)	1.0	1.0
C578T	CT = 5 (2.5)	CT = 0 (0.0)	–	–
CYP2A13: C3375T	CC = 191 (94.1)	CC = 182 (90.5)	1.0	1.0
	CT = 12 (5.9)	CT = 19 (9.5)	0.60 (0.28–1.27), 0.18	0.67 (0.31–1.44), 0.31
CYP2A13: C7520G	CC = 186 (94.1)	CC = 192 (90.5)	1.0	1.0
	CG = 16 (5.9)	CG = 9 (4.5)	1.83 (0.79–4.25), 0.15	1.99 (0.85–4.69),0.11
	GG = 1 (0.5)	GG = 0 (0)	–	–
UGT1A7:	TT = 79 (38.9)	TT = 80 (39.8)	1.0	1.0
T-57G	TG = 91 (44.8)	TG = 92 (45.8)	1.01 (0.65–1.53), 1.0	0.99 (0.65–1.54) 0.99
	GG = 33 (16.3)	GG = 29 (14.4)	1.15 (0.64–2.07), 0.63	1.12 (0.62–2.03) 0.70
UGT1A7	High = 14 (6.9)	H = 13 (6.5)	1.0	1.0
	Intermediate = 145 (71.4)	I = 161 (80.1)	0.84 (0.38–1.84), 0.66	0.93 (0.42–2.02) 0.75
	Low = 44 (21.7)	L = 27 (13.4)	1.51 (0.62–3.70), 0.36	1.29 (0.54–3.59) 0.47

[a]Adjusted OR, odds ratio adjusted for age, gender, smoking and alcohol with wild type as reference; OR, odds ratio; CI, confidence intervals.

Logistic regression analysis to study interactions between smoking, genotypes and cancer risk, revealed that heterozygous variant (T478C/T494C) of *CYP2A13* was associated with reduced risk of head and neck cancer regardless of smoking. *CYP2A13* C578T was found to be present in four smokers. We did not observe any significant interaction between smoking and *UGT1A7* intermediate and low activity genotypes. However, on stratification of smokers into heavy and light smokers, the low activity *UGT1A7* genotypes were found to be associated with sevenfold increased risk of head and neck cancer among heavy smokers (OR, 7.01; 95% CI 1.02–48.37; $P < 0.05$) (Table 4a). As for the effect of modification by alcohol consumption, we did not observe any interaction between alcohol consumption and variant genotypes of *CYP2A13* and *UGT1A7* in modulating risk of head and neck cancer (Table 4b).

Five major haplotypes of *CYP2A13* were observed in our cohort. CCCCC haplotype carrying variant alleles of linked polymorphisms T478C/T494C was found to be more prevalent among controls as compared with cases and showed trend for protection (OR, 0.42; 95% CI 0.22–0.78; $P = 0.005$). A total of six haplotypes were generated for *UGT1A7* gene, using PHASE version 2.1. The frequency of TTCC haplotype was significantly higher among patients as compared with controls and was associated with fivefold increased risk of head and neck cancer (OR, 4.91; 95% CI 1.05–22.9; $P = 0.03$). The results of haplotype analysis are summarized in Tables 5a and 5b.

Linkage disequilibria values were generated to look for association among polymorphisms of *CYP2A13* and *UGT1A7* genes. Significant LD was observed among T478C and T494C variants of *CYP2A13* gene. The T-57G, N129K/R131K and T622C polymorphisms of *UGT1A7* gene were also found to be linked in our study population. A pair wise comparison of the polymorphisms depicting LD measures is shown in Table 6.

Potential interactions between *CYP2A13* and *UGT1A7* genes were assessed using MDR analysis (Table 7). We found that the four way combination of T622C, N129K/R131K, T-57G (*UGT1A7*) and T478C/T494C (*CYP2A13*) polymorphisms was the best model for prediction of head and neck cancer risk. The testing accuracy of the selected model was 0.56

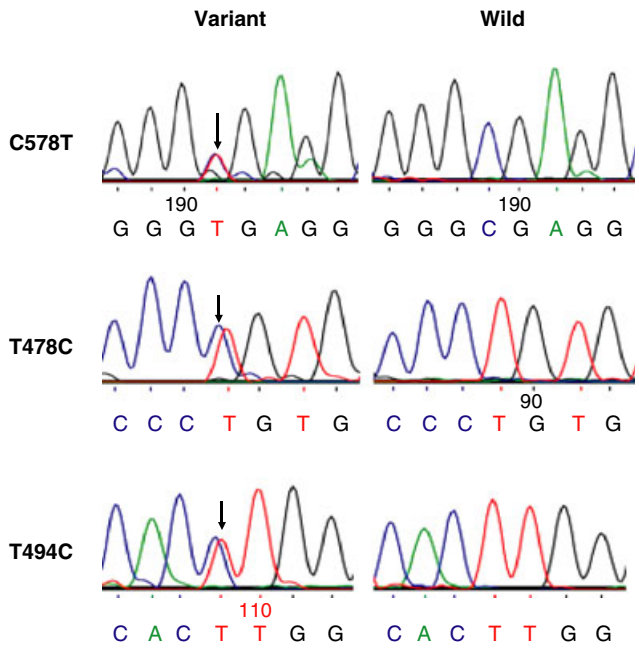

Figure 1 Nucleotide sequences of *CYP2A13* gene in exon 2. Arrows indicate the variant nucleotide positions

($P < 0.05$; based on 1000-fold permutations) and was associated with threefold elevated risk of head and neck cancer (OR, 3.4; 95% CI 2.15–5.5; $P < 0.0001$). The combination of T622C and T-57G polymorphism of *UGT1A7* was the best two factor model, with the testing accuracy of 0.49. The three factor model added T478C/T494C (*CYP2A13*) to combination of T622C and T-57G polymorphisms of *UGT1A7* and increased the testing accuracy to 0.53. However, the permutation testing showed that the prediction error for two and three factor models were non-significant ($P > 0.05$; based on 1000-fold permutations).

Discussion

In the present study, we report an association between *CYP2A13* and *UGT1A7* genes, involved in metabolism of major classes of tobacco derived procarcinogens and susceptibility to head and neck cancer. This is the first study to report an association of polymorphisms of *CYP2A13* and *UGT1A7* genes with head and neck cancer in Indian population.

CYP2A13 is highly active in metabolic activation of major tobacco specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Su *et al*, 2000). Till date, there are no reports available on association of *CYP2A13* genetic variants with head and neck cancer. We found linked two novel polymorphisms (T478C/T494C) in exon 2 of *CYP2A13*, to be associated with reduced risk of head and neck cancer. Since these polymorphisms are synonymous in nature, their implication for decreased risk of head and neck cancer is intriguing. Although it needs to be investigated, one

Table 3 Distribution of *CYP2A13 and UGT1A7* genotypes among the study subjects based on the site of cancer

Polymorphisms	*Controls* (n = *201*)	*Oral cavity* (n = *73*)	*Pharynx* (n = *84*)	*Larynx* (n = *46*)
CYP2A13: T478C/T494C				
Wild (Reference)	167 (83.1)	63 (86.0)	80 (95.2)	44 (95.7)
Heterozygous	34 (16.9)	10 (14.0)	4 (4.8)	2 (4.3)
OR(95% CI)[a]; *P*		0.78 (0.36–1.67); 0.52	0.24 (0.08–0.71); 0.005	0.22 (0.05–0.96); 0.03
CYP2A13: C578T				
CC(Reference)	201 (100.0)	71 (97.2)	81 (96.4)	46 (76.1)
CT	0 (0.0)	2 (2.8)	3 (3.6)	–
OR(95% CI)[a]; *P*		0.99 (0.49–2.01); 1.0	2.5 (1.39–4.5); 0.001	1.3 (0.59–2.9); 0.5
CYP2A13: C3375T				
CC(Reference)	182 (90.5)	67 (91.8)	79 (94.0)	45 (97.8)
CT	19 (9.5)	6 (8.2)	5 (6.0)	1 (2.2)
OR(95% CI)[a]; *P*		0.86 (0.32–2.24); 0.75	0.61 (0.22–1.68); 0.33	0.21 (0.03–1.63); 0.13
CYP2A13: C7520G				
CC(Reference)	192 (90.5)	67 (91.8)	78 (92.9)	41 (89.1)
CG	9 (4.5)	5 (6.8)	6 (7.1)	5 (10.9)
OR(95% CI)[a]; *P*	0 (0)	1.59 (0.51–4.92); 0.29	1.64 (0.56–4.76); 0.26	2.60 (0.82–8.17); 0.15
GG		1 (1.4)	–	–
UGT1A7:T-57G				
TT(Reference)	80 (39.8)	31 (42.5)	29 (34.5)	19 (41.3)
TG	92 (45.8)	31 (42.5)	40 (47.6)	20 (43.5)
OR(95% CI)[a]; *P*		0.87 (0.49–1.55); 0.63	1.19 (0.68–2.10); 0.52	0.91 (0.45–1.83); 0.81
GG	29 (14.4)	11 (15.0)	15 (17.9)	7 (2.2)
OR(95% CI)[a]; *P*		0.98 (0.44–2.19); 1.0	1.43 (0.67–3.03); 0.35	1.01 (0.38–2.67); 1.0
UGT1A7				
High(Reference)	13 (6.5)	6 (8.2)	5 (5.9)	17 (36.9)
Intermediate	161 (80.1)	50 (68.5)	62 (73.8)	17 (36.9)
OR(95% CI)[a]; *P*		0.67 (0.24–1.86); 0.57	1.0 (0.34–2.92); 1.0	0.08 (0.03–0.19); < 0.001
Low	27 (13.4)	17 (23.3)	33 (39.3)	10 (2.2)
OR(95% CI)[a]; *P*		1.36 (0.43–4.27); 0.59	3.18 (1.1–10.03); 0.04	0.28 (0.10–0.78); 0.01

[a]ORs, odds ratio adjusted for age and gender with wild type as reference; CI, confidence intervals.

Table 4a Association of *CYP2A13* and *UGT1A7* genetic variants with head and neck cancer, stratified by smoking

Genotype	*Exposure*	*Patients (*n*/ref)*[a]	*Controls (*n*/ref)*[a]	*OR*[b] *(95% CI)*	P-*value*
CYP2A13:	Non-smokers	1/25	6/41	0.08 (0.01–0.92)	< 0.05
T478C/T494C	Smokers	14/163	28/126	0.38 (0.18–0.75)	< 0.05
Heteozygous	Heavy smokers	4/91	11/66	0.24 (0.07–0.80)	< 0.05
	Light smokers	10/71	17/59	0.48 (0.19–1.14)	0.09
CYP2A13: C578T	Non-smokers	1/25	–	–	
	Smokers	4/173	–	–	
CT	Heavy smokers	2/93	–	–	
	Light smokers	2/79	–	–	
CYP2A13: C3375T	Non-smokers	–	5/42	–	
	Smokers	12/165	14/139	0.78 (0.35–1.78)	0.56
CT	Heavy smokers	7/88	10/66	1.72 (0.45–6.53)	0.42
	Light smokers	5/76	4/72	0.46 (0.15–1.44)	0.18
CYP2A13: C7520G	Non-smokers	1/24	2/45	1.19 (0.09–14.33)	0.89
	Smokers	15/162	7/147	2.06 (0.8–5.27)	0.13
CG	Heavy smokers	10/85	5/72	1.87 (0.58–5.97)	0.29
	Light smokers	5/76	2/74	2.39 (0.44–12.97)	0.31
CYP2A13: C7520G	Non-smokers	1/24	–	–	
	Smokers	–	–	–	
GG	Heavy smokers	–	–	–	
	Light smokers	–	–	–	
UGT1A7:T-57G	Non-smokers	9/11	24/18	1.08 (0.30–3.84)	0.9
	Smokers	82/68	68/62	1.04 (0.64–1.71)	0.87
TG	Heavy smokers	41/38	33/33	1.10 (0.56–2.19)	0.78
	Light smokers	41/30	35/29	1.15 (0.55–2.39)	0.71
UGT1A7:T-57G	Non-smokers	7/11	6/18	1.12 (0.19–6.58)	0.89
	Smokers	26/68	23/62	0.64 (0.28–1.47)	0.29
GG	Heavy smokers	16/38	11/33	0.57 (0.15–1.66)	0.26
	Light smokers	10/30	12/29	0.78 (0.22–2.73)	0.69
UGT1A7	Non-smokers	17/3	38/4	0.68 (0.11–4.05)	0.67
	Smokers	128/11	123/9	0.82 (0.31–2.14)	0.68
Intermediate	Heavy smokers	72/3	67/4	1.52 (0.31–7.46)	0.60
	Light smokers	56/8	55/5	0.61 (0.17–2.14)	0.44
UGT1A7	Non-smokers	7/3	5/4	1.86 (0.19–17.96)	0.59
	Smokers	37/11	22/9	1.73 (0.54–5.52)	0.355
Low	Heavy smokers	20/3	6/4	7.01 (1.02–48.37)	< 0.05
	Light smokers	17/8	16/5	0.76 (0.16–3.57)	0.73

[a]genotype/reference; [b]ORs, odds ratio adjusted for age, gender and alcohol consumption.

Table 4b Association of *CYP2A13* and *UGT1A7* genetic variants with head and neck cancer, stratified by alcohol consumption

Genotype	*Exposure*	*Patients (n/*ref*)*[a]	*Controls (n/*ref*)*[a]	*OR*[b] *(95% CI)*	P-*value*
CYP2A13: T478C	Drinkers	11/103	17/83	0.51 (0.22–1.14)	0.10
TC	Non-drinkers	4/85	17/83	0.15 (0.04–0.52)	< 0.05
CYP2A13: C578T	Drinkers	1/25	–	–	
CT	Non-drinkers	4/173	–	–	
CYP2A13: C3375T	Drinkers	7/108	7/98	0.90 (0.30–2.70)	0.85
CT	Non-drinkers	5/83	12/89	0.54 (0.18–1.63)	0.28
CYP2A13: C7520G	Drinkers	10/105	7/93	1.29 (0.47–3.58)	0.62
CG	Non-drinkers	6/81	2/99	3.54 (0.69–18.2)	0.13
CYP2A13: C7520G	Drinkers	–	–	–	
GG	Non-drinkers	1/81	–	–	
UGT1A7:T-57G	Drinkers	54/44	38/45	1.20 (0.65–2.23)	0.55
TG	Non-drinkers	37/35	54/35	0.94 (0.47–1.86)	0.86
UGT1A7:T-57G	Drinkers	17/44	17/45	0.63 (0.23–1.76)	0.38
GG	Non-drinkers	16/35	12/35	0.68 (0.21–2.14)	0.51
UGT1A7	Drinkers	89/5	73/11	2.43 (0.78–7.59)	0.12
Intermediate	Non-drinkers	56/9	88/2	0.16 (0.03–0.83)	< 0.05
UGT1A7	Drinkers	21/5	16/11	3.51 (0.86–14.29)	0.08
Low	Non-drinkers	23/9	11/2	0.64 (0.10–4.11)	0.64

[a]genotype/reference; [b]ORs, odds ratio adjusted for age, gender and smoking.

may speculate possible linkage of these polymorphisms to one or more functional genetic variants within CYP2A locus that are important in genetic susceptibility to head and neck cancer. A similar linkage phenomenon has been described in a previous study in which a silent mutation C1358T in PNLIP gene was found to be

Table 5a *CYP2A13* gene haplotype frequency distribution in patients and controls

Haplotype[a]	*Patients* 2n *(frequency)*	*Controls* 2n *(frequency)*	*OR (95%CI)*	P-*value*
TTCCC	356 (0.88)	340 (0.84)	1.00 (Reference)	
TTCCG	18 (0.04)	10 (0.02)	1.72 (0.78–3.77)	0.18
CCCCC	15 (0.036)	34 (0.085)	0.42 (0.22–0.78)	0.005
TTCTC	12 (0.03)	18 (0.04)	0.64 (0.30–1.34)	0.23
TTTCC	5 (0.01)	–	–	–

[a]Order of polymorphisms in *CYP2A13* gene haplotypes: T478C, T494C, C578T, C3375T, C7520G.

Table 5b *UGT1A7* gene haplotype frequency distribution in patients and controls

Haplotype[a]	*Patients* 2n *(frequency)*	*Controls* 2n *(frequency)*	*OR (95%CI)*	P-*value*
TTCT	114 (0.28)	112 (0.26)	1.0 (Reference)	
GGAC	140 (0.341)	116 (0.268)	1.18 (0.83–1.69)	0.35
TGAT	102 (0.252)	99 (0.25)	1.02 (0.69–1.48)	1.0
TGAC	24 (0.06)	38 (0.11)	0.62 (0.35–1.10)	0.10
GGAT	16 (0.04)	18 (0.05)	0.87 (0.42–1.79)	0.71
TTCC	10 (0.03)	2 (0.005)	4.91 (1.05–22.9)	0.03

[a]Order of polymorphisms in *UGT1A7* gene haplotypes: T-57G, N129K/R131K, T622C.

Table 6 Pair wise comparison of measures of LD (D', LOD and r^2) for the polymorphisms of *CYP2A13* and *UGT1A7* genes

Gene	*Variant 1*	*Variant 2*	*D'*	*LOD*	r^2
CYP2A13	T478C	T494C	1.0	65.51	1.0
	T478C	C578T	1.0	0.14	0.0
	T478C	C3375T	0.048	0.1	0.0001
	T478C	C7520G	0.187	0.01	0.0
	C578T	C3375T	1.0	0.09	0.0
	C578T	C7520G	1.0	0.07	0.0
	C3375T	C7520G	1.0	0.17	0.001
UGT1A7	T-57G	T387G	0.719	12.61	0.14
	T-57G	T622C	0.708	47.26	0.44
	T387G	T622C	0.778	18.37	0.19

associated with complex plasma lipid traits (Hegele *et al*, 2001).

A transition from C to T at 578 nucleotide position results in introduction of premature stop codon and synthesis of non-functional *CYP2A13* protein (Zhang *et al*, 2003). We observed this non-sense mutation to be present only in cancer cases and none of the controls carried this mutation, suggesting the pathogenic nature of this mutation. Our results are supported by an earlier study in French population in which this deleterious mutation was most frequent and associated with elevated risk of lung cancer (Cauffiez *et al*, 2004). In contrast to our findings, Jiang *et al*, found no association of this mutation with risk of nasopharyngeal cancer in Chinese population (Jiang *et al*, 2004). The observed discrepancy in these findings could be because of ethnic differences or might be as a result of tissue specific expression of *CYP2A13* (Ling *et al*, 2007). Lack of *CYP2A13* activity caused by C578T mutation may be involved in tobacco related head and neck cancer risk, either as a result of a reduced capacity to bioactivate procarcinogens into genotoxic compounds or on the other hand, as a result to detoxify carcinogens.

We also investigated two functional polymorphisms C3375T and C7520G for their association with head and neck cancer risk and did not find either polymorphism to be associated with risk of head and neck cancer. There have been inconsistent reports on association of C3375T polymorphism with cancer of different organs (Wang *et al*, 2003; Jiang *et al*, 2004; Song *et al*, 2009). For example, the variant genotypes of this polymorphism were shown to be associated with substantially reduced risk of lung adenocarcinoma, whereas no significant association of this polymorphism was observed with risk of lung squamous cell carcinoma (Wang *et al*, 2003) and nasopharyngeal carcinoma (Jiang *et al*, 2004). Our results suggest that *CYP2A13* C3375T and C7520G variants may not be associated with risk of head and neck cancer in our cohort.

CYP2A13 haplotype containing rare allele C/C (T478C/T494C) was found to be protective to cancer in our cohort indicating a very strong allelic, genotypic

Table 7 Interactions between genetic and environmental factors and head and neck cancer risk prediction by multifactor dimensionality reduction method

No. of factors	*Best model*	*Testing accuracy*	*CVC*[*]	*OR (95%CI)*	*Permutation test:* P *value*[**]
2	*UGT1A7* T622C *UGT1A7* T-57G	0.49	4/10	1.64 (1.11-2.44)	0.62
3	*UGT1A7* T622C *UGT1A7* T-57G *CYP2A13* T478C/T494C	0.52	6/10	2.94 (1.80-4.72)	0.16
4	*UGT1A7* T622C *UGT1A7* T-57G *UGT1A7* N129K/R131K *CYP2A13* T478C/T494C	0.56	7/10	3.42 (2.15-5.47)	<0.05

(version 1.0, http://www.hydra.usc.edu/GxE/ provided in the public domain by the University of Southern California, Los Angeles, CA).
(version 2.1, http://www.stephenslab.uchicago.edu/software.html/ provided in the public domain by the University of Washington, Seattle, WA, USA).
(version 3.2, http://www.broadinstitute.org/haploview/haploview-downloads/ provided in the public domain by the Broad Institute of MIT and Harvard, Cambridge, MA, USA).

and haplotypic association with head and neck cancer. This is the first report of *CYP2A13* haplotype associated with head and cancer risk in Indians.

UGT1A7 catalyzes the glucuronidation and detoxification of benzo[a]pyrene, a tobacco procarcinogens (Strassburg *et al*, 1998). There have been inconsistent reports available on association *UGT1A7* polymorphisms with head and neck cancer risk (Zheng *et al*, 2001; Lacko *et al*, 2009). The predicted low activity genotypes of *UGT1A7* were found to be significantly associated with increased risk of orolaryngeal cancer in Caucasians and African Americans (Zheng *et al*, 2001), whereas in a recent study, significant association of high activity *UGT1A7* genotypes were reported to be associated with head and neck cancer (Lacko *et al*, 2009). We did not observe any significant association of *UGT1A7* genetic variants with risk of head and neck cancer in our study population. These findings suggest that UGT1A7 low catalytic activity as a result of polymorphisms may cause shift to alternative metabolic pathways which result in efficient detoxification of carcinogens. The reason for deviation between our results and those of Lacko *et al*, could be because of ethnic differences or as a result of variation in the allele frequency distributions of UGT1A7 variant genotypes between the control populations enrolled in these studies (The frequency of high activity UGT1A7 genotypes is 6.5% in our study whereas, it is 35% in report by Lacko *et al* 2009,) while in case of report by Zheng *et al*, these differences may be attributed to increased sample size of the present study (203 North Indian subjects vs 125 caucasian and 69 African American patients enrolled in study by Zheng *et al*, 2001).

UGT1A7 haplotype carrying C allele (T622C) showed fivefold increased risk of head and neck cancer and was thus predisposing to head and neck cancer. Our finding differs from a previous study in which a risk haplotype containing rare alleles of N129K/R131K and T622C was associated with an increased risk of orolaryngeal cancer (Zheng *et al*, 2001). These differences may be contributed to ethnic differences in population enrolled in two studies.

Promoter *UGT1A7* polymorphism, T-57G was found to be in strong linkage disequilibrium with T622C polymorphism as reported in an earlier study (Lankisch *et al*, 2005). The promoter polymorphism T-57G was also found to be linked to N129K/R131K polymorphism. This polymorphism is in linkage with T622C and is thus associated with *UGT1A7**3 and *UGT1A7**4 genotypes which explains the observed coincidence of T-57G with N129K/R131K.

We observed differential genetic susceptibility to head and neck cancer among different anatomic sites. For example, the predicted low activity *UGT1A7* genotypes were found to be associated with significantly reduced risk of laryngeal cancer whereas the same genotypes showed threefold increased risk of pharyngeal cancer. *CYP2A13* T478C/T494C showed low odds ratio for cancers of pharynx and larynx but not for cancers of oral cavity. But these results should be interpreted with caution since the heterozygous genotype of these novel polymorphisms was observed at a very low frequency and further studies with larger sample size are required to confirm these findings. Similar heterogeneity in genetic susceptibility with respect to high activity *UGT1A7* genotype has been observed in Caucasians (Lacko *et al*, 2009). These findings suggest that there may be tissue specific differences in expression and regulation of phase I and phase II enzymes which can alter cancer risk attributed by carcinogens.

Further, on examining gene environment interactions, we found significant interaction between smoking, low activity *UGT1A7* variants and cancer risk; however the increased risk was limited to heavy smokers carrying these variants. Low activity *UGT1A7* genotypes conferred sevenfold enhanced risk of head and neck cancer among heavy smokers. These low activity *UGT1A7* variants are associated with decreased detoxification activities and thus may lead to accumulation of tobacco derived carcinogens and consequently to an increased risk of cancer. Similar results have also been reported in African- American and Caucasian cohorts (Zheng *et al*, 2001). However, in a recent study by Lacko *et al*, high activity *UGT1A7* genotypes were reported to increase the risk of head and neck cancer among heavy smokers (Lacko *et al*, 2009). These observed differences may be attributed to differences in type of tobacco exposure and/or ethnic differences. Beedi is the most prevalent form of smoking in India (Choi and Kahyo, 1991; Notani, 2001).

We found significant interaction between smoking and *CYP2A13* C578T variant, which results in an inactive *CYP2A13* enzyme as the mutation results in a premature truncation of the protein (Zhang *et al*, 2003). Thus, non-functional enzyme may lead to increased risk of tobacco associated cancer as a result of reduced capacity for detoxification. In contrast to earlier reports, which found *CYP2A13* C3375T and C7520G polymorphisms to confer protection to lung adenocarcinoma at low dose of smoking (Wang *et al*, 2003) in Chinese, we did not observe interaction between smoking and head and neck cancer risk in our cohort. These differences may be because of differences in the etiology of the two cancers with different pathological characteristics (squamous cell carcinoma vis a vis adenocarcinoma).

A combination of T622C, N129K/R131K, T-57G (*UGT1A7*) and T478C/T494C (*CYP2A13*) polymorphisms was associated with sixfold increased risk of head and neck cancer indicating strong synergistic interactions between low activity *UGT1A7* variants and *CYP2A13* T478C/T494C polymorphisms. Functional studies are required to confirm these epistatic interactions.

A potential limitation of our study is relatively medium size of our cohort which could introduce type 1 errors. However, our cohort consisted of ethnically homogenous and well phenotyped cancer subjects enrolled from single center which may help overcome this limitation. As the polymorphisms in XMEs may be potentially involved in pathogenesis of other diseases, hospital based controls were avoided to exclude potential selection bias in this group. Thus we enrolled population based controls which may further help to overcome this

limitation. Moreover, the present study fulfills most of the prerequisites for a good genetic association study as suggested by Bird *et al* (Bird *et al*, 2001).

In conclusion, the present study highlights epistatic interactions between tobacco metabolizing gene variants, smoking habits and alcohol consumption in modulating the risk of head and neck cancer in our population.

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References

Bird TD, Jarvik GP, Wood NW (2001). Genetic association studies. *Neurology* **57:** 1153–1154.

Buch SC, Notani PN, Bhisey RA (2002). Polymorphisms at GSTM1, GSTM3 and GSTT1 gene loci and susceptibility to oral cancer in an Indian population. *Carcinogenesis* **23:** 803–807.

Cauffiez C, Lo-Guidice JM, Quaranta S *et al* (2004). Genetic polymorphism of the human cytochrome *CYP2A13* in a French population: implication in lung cancer susceptibility. *Biochem Biophys Res Commun* **317:** 662–669.

Cauffiez C, Pottier N, Tournel G *et al* (2005). *CYP2A13* genetic polymorphism in French Caucasian, Gabonese and Tunisian populations. *Xenobiotica* **35:** 661–669.

Choi SY, Kahyo H (1991). Effect of cigarette smoking and alcohol consumption in the aetiology of cancer of the oral cavity, pharynx and larynx. *Int J Epidemiol* **20:** 878–885.

Day DA, Tuite MF (1998). Post-transcriptional gene regulatory mechanisms in eukaryotes: an overview. *J Endocrinol* **157:** 361–371.

Feng FY, Liang G, Lu WF *et al* (2005). Correlation of polymorphisms of UDP-glucuronosyltransferase 1A7 gene to genetic susceptibility of lung cancer. *Ai Zheng* **24:** 1085–1090.

Guillemette C, Ritter JK, Auyeung DJ *et al* (2000). Structural heterogeneity at the UDP-glucuronosyltransferase 1 locus: functional consequences of three novel missense mutations in the human *UGT1A7* gene. *Pharmacogenetics* **10:** 629–644.

Hahn LW, Ritchie MD, Moore JH (2003). Multifactor dimensionality reduction software for detecting gene–gene and gene–environment interactions. *Bioinformatics* **19:** 376–382.

Hegele RA, Ramdath DD, Ban MR *et al* (2001). Polymorphisms in PNLIP, encoding pancreatic lipase and associations with metabolic traits. *J Hum Genet* **46:** 320–324.

Jiang JH, Jia WH, Chen HK *et al* (2004). Genetic polymorphisms of *CYP2A13* and relationship to nasopharyngeal carcinoma in Cantonese population. *J Transl Med* **2:** 24–31.

Lacko M, Roelofs HM, Te Morsche RH *et al* (2009). Genetic polymorphisms in the tobacco smoke carcinogens detoxifying enzyme *UGT1A7* and the risk of head and neck cancer. *Head Neck* **31:** 1274–1281.

Lankisch TO, Vogel A, Eilermann S *et al* (2005). Identification and characterization of a functional TATA box polymorphism of the UDP glucuronosyltransferase 1A7 gene. *Mol Pharmacol* **67:** 1732–1739.

Ling G, Wei Y, Ding X (2007). Transcriptional regulation of human *CYP2A13* expression in the respiratory tract by CCAAT/enhancer binding protein and epigenetic modulation. *Mol Pharmacol* **71:** 807–816.

van der Logt EM, Bergevoet SM, Roelofs HM *et al* (2004). Genetic polymorphisms in UDP-glucuronosyltransferases and glutathione S-transferases and colorectal cancer risk. *Carcinogenesis* **25:** 2407–2415.

Nagar S, Remmel RP (2006). Uridine diphosphoglucuronosyltransferase pharmacogenetics and cancer. *Oncogene* **25:** 1659–1672.

Notani PN (2001). Global variation in cancer incidence and mortality. *Curr Sci* **81:** 465–474.

Parkin DM, Bray F, Ferlay J *et al* (2005). Global cancer statistics, 2002. *CA Cancer J Clin* **55:** 74–108.

Piepoli A, Gentile A, Valvano MR *et al* (2006). Lack of association between *UGT1A7*, UGT1A9, ARP, SPINK1 and CFTR gene polymorphisms and pancreatic cancer in Italian patients. *World J Gastroenterol* **12:** 6343–6348.

Song DK, Xing DL, Zhang LR *et al* (2009). Association of NAT2, GSTM1, GSTT1, CYP2A6, and *CYP2A13* gene polymorphisms with susceptibility and clinicopathologic characteristics of bladder cancer in Central China. *Cancer Detect Prev* **32:** 416–423.

Stenirri S, Fermo I, Battistella S *et al* (2004). Denaturing HPLC profiling of the ABCA4 gene for reliable detection of allelic variations. *Clin Chem* **50:** 1336–1343.

Strassburg CP, Nguyen N, Manns MP *et al* (1998). Polymorphic expression of the UDP-glucuronosyltransferase UGT1A gene locus in human gastric epithelium. *Mol Pharmacol* **54:** 647–654.

Strassburg CP, Vogel A, Kneip S *et al* (2002). Polymorphisms of the human UDP-glucuronosyltransferase (UGT) 1A7 gene in colorectal cancer. *Gut* **50:** 851–856.

Su T, Bao Z, Zhang Q-Y *et al* (2000). Human cytochrome P450 *CYP2A13*: predominant expression in the respiratory tract and its high efficiency metabolic activation of a tobacco specific carcinogen, 4-methylnitroso-1-(3-pyridyl)-1-butanone. *Cancer Res* **60:** 5074–5079.

Tephly TR, Burchell B (1990). UDP-glucuronosyltransferases: a family of detoxifying enzymes. *Trends Pharmacol Sci* **11:** 276–279.

Wang H, Tan W, Hao B *et al* (2003). Substantial reduction in risk of lung adenocarcinoma associated with genetic polymorphism in *CYP2A13*, the most active cytochrome P450 for the metabolic activation of tobacco-specific carcinogen NNK. *Cancer Res* **63:** 8057–8061.

Zhang X, Su T, Zhang QY *et al* (2002). Genetic polymorphisms of the human *CYP2A13* gene: identification of single-nucleotide polymorphisms and functional characterization of an Arg257Cys variant. *J Pharmacol Exp Ther* **302:** 416–423.

Zhang X, Chen Y, Liu Y *et al* (2003). Single nucleotide polymorphisms of the human *CYP2A13* gene: evidence for a null allele. *Drug Metab Dispos* **31:** 1081–1085.

Zhang X, Caggana M, Cutler TL *et al* (2004). Development of a real-time polymerase chain reaction-based method for the measurement of relative allelic expression and identification of *CYP2A13* alleles with decreased expression in human lung. *J Pharmacol Exp Ther* **311:** 373–381.

Zheng Z, Park JY, Guillemette C *et al* (2001). Tobacco carcinogen-detoxifying enzyme *UGT1A7* and its association with orolaryngeal cancer risk. *J Natl Cancer Inst* **93:** 1411–1418.