Published in final edited form as:

Oral Oncol. 2003 July ; 39(5): 483–490.

Epoxide hydrolase genotype and orolaryngeal cancer risk: interaction with GSTM1 genotype

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Summary

The human microsomal epoxide hydrolase (EH) gene contains polymorphic alleles which are associated with altered EH activity and may be linked to increased risk for tobacco-related cancers. The objective was to examine the role of EH polymorphisms in orolaryngeal cancer risk. The prevalence of the EH codons 113 and 139 polymorphisms were examined in 81 African American and 142 Caucasian incident orolaryngeal cancer patients and 335 controls frequency-matched on age, sex, and race. In Caucasians, a significant risk increase was observed for subjects with the EH^{113Tyr} variant (OR=2.1, 95% CI=1.1–4.0) and predicted high-activity EH genotypes in heavy-smokers (35 pack-years; OR=3.4, 95% CI=1.2–9.6). A significant association between predicted high EH activity genotypes and orolaryngeal cancer risk was observed in Caucasian subjects with the GSTM1 null (OR=3.5, 95% CI=1.3–9.3) but not GSTM1 [+] (OR=0.9, 95% CI=0.4–2.1) genotype. These results suggest that EH polymorphisms play an important role in risk for orolaryngeal cancer in Caucasians.

Keywords

Epoxide hydrolase; Glutathione S-transferase; Orolaryngeal cancer; Genetic polymorphism; Cancer susceptibility

1. Introduction

Genetic polymorphisms are associated with a number of genes that code for enzymes involved in the metabolic activation or detoxification of carcinogens. Large differences in the prevalence of certain genetic polymorphisms have been described among racial groups for several metabolizing enzyme genes and it has been shown that enzyme activity-altering polymorphisms may influence individual cancer risk.^{1–5}

EH cleaves a range of alkene and arene oxides to form *trans*-dihydrodiols. For some polycyclic aromatic hydrocarbons including benzo[a]pyrene (BaP), the dihydrodiol derivatives are substrates for additional metabolism to more highly reactive and carcinogenic compounds. Two amino acid-altering polymorphisms have been described in the human EH gene (tyr113his and his139arg) and both have been associated with alterations in EH activity. The EH^{113his} variant is associated with a 40% decrease in EH

activity while the EH^{139arg} variant enhances enzyme activity by 25% via an increase in EH protein stability.⁶ These polymorphic alleles have previously been linked to increases in risk for colon,⁷ lung,^{4, 8–13} and ovarian¹⁴ cancers. Recent studies also have suggested that predicted intermediate and high EH activity genotypes are associated with increased risk for both laryngeal and oral/oropharyngeal cancer.¹⁵ Together with the finding that EH is expressed in oral tissue,¹⁶ these data suggest that EH polymorphisms may play an important role in risk for orolaryngeal cancer.

In previous studies, we reported differences in the association between metabolizing enzyme gene polymorphic profiles and oral cancer risk in Caucasians versus African Americans.^{5, 17} Specifically, a significant association was observed for the GSTM1 null genotype and oral cancer risk in African Americans but not Caucasians. Both GSTM1 and EH code for enzymes that metabolize the same intermediate metabolite (BaP-7,8-epoxide), a metabolite that is critical for activation of BaP to its ultimate carcinogenic metabolite, BaP-7,8-dihydrodiol-9,10-epoxide. In this paper, data are presented examining the effects of EH genotype on orolaryngeal cancer risk in the two racial groups, with the effects of EH genotype on orolaryngeal cancer risk examined both alone and in combination with GSTM1 genotype.

2. Materials and methods

2.1. Study populations and sample processing

All cases were patients diagnosed with primary orolaryngeal squamous cell carcinoma and were identified between 1994 and 2000 from three institutes: Temple University Hospital (Philadelphia, PA; $n=30$), Memorial Sloan-Kettering Cancer Center (New York, NY; $n=63$), and The New York Eye and Ear Infirmary (New York, NY; $n=130$). All cases were diagnosed within 1 year prior to recruitment into the study. All case diagnosis were histologically confirmed by the Pathology Departments at each of the respective institutes. Non-cancer controls were recruited at participating hospitals and were frequency-matched with the age at diagnosis (± 5 years), race and gender.

For cases, buccal cell samples ($n=160$), collected post-surgery at a follow-up examination, or archived non-tumor orolaryngeal tissues ($n=63$) were used for the analysis of polymorphic genotypes, while buccal cells were collected for the analysis in controls ($n=335$). Protocols involving the analysis of buccal cell specimens were approved by the institutional review boards at each of the collaborating hospitals and informed consent was obtained from all subjects.

A questionnaire was administered to all subjects that contained questions on demographics, lifelong smoking habits, and alcohol consumption. Tobacco use was categorized into pack-years (py) for smokers of cigarettes (1 pack/day for 1 year=1 py), cigars (4 cigars/day for 1 year=1 py), and pipe tobacco (5 pipes/day for 1 year=1 py) according to the criteria described by Benhamou et al.¹⁸ Alcohol consumption (consumed over a minimum of 10 years) were calculated as shots per day, where one shot=12.9 g of 43% alcohol. Subjects were defined as alcohol drinkers if they had been drinking a minimum of 1 shot/week for a minimum of 10 years. For regression analysis, subjects were classified as never (1 shot/week), light (1.1–7 shots/week), moderate (7.1–28 shots/week), or heavy-drinkers (>28 shots/week).

DNA was isolated from either buccal cells or tissues by incubation overnight with proteinase K (0.1 mg/ml) in 1% sodium dodecyl sulfate at 50 °C, extracted with phenol:chloroform, and ethanol precipitation as previously described.¹⁹

2.2. Genotyping assays

All subjects were screened for the presence of the EH codon 113 polymorphism by a modified PCR-restriction fragment length polymorphism (RFLP) analysis similar to that described previously¹⁴ using 100 ng of sense (5'-CTTACACCAGAGGATCGAT-3') and antisense (5'-CTTAGTCTTGAAGTGACGGT-3') primers homologous to exon 3 of the EH gene to generate a 172 bp fragment. The antisense primer contains a mismatched nucleotide (underlined) to create a restriction enzyme site for *AspI* in the polymorphic variant EH sequence. The standard PCR was performed in a 50 µl reaction volume containing 50 ng of genomic DNA, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each of the dNTPs, and 2.0 units of *Taq* polymerase. The reaction mixtures underwent the following incubations: 1 cycle of 95 °C for 2 min, 40 cycles of 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s, followed by a final cycle of 10 min at 72 °C. PCRs were incubated with *AspI* (2.5 units, New England Biolabs, Beverly, MA) for 16 h at 37 °C prior to electrophoresis. As there is no internal control for *AspI*/RFLP analysis within the exon 3 PCR product, all experiments were performed with positive control that were previously identified as containing an *AspI* site for RFLP. Three banding patterns were observed by RFLP analysis (Fig. 1A): a 172 bp band that corresponded to the 113^{tyr}/113^{tyr} homozygous wild-type genotype (lanes 1, 2 and 5), 172 bp, 153 bp, and 19 bp bands that corresponded to the 113^{tyr}/113^{his} heterozygous genotype (lane 4), and 153 bp and 19 bp bands that corresponded to the 113^{his}/113^{his} homozygous polymorphic genotype (lane 3).

The genotyping assay for the EH codon 139 polymorphism was performed by PCR-RFLP analysis similar to that described previously,⁶ with 50 ng of sense, (5'-GGCTGGACATCCACTTCATC-3') and antisense, (5'-CACCGGGCCCACCCTTGG-3') primers homologous to exon 4 and intron four sequences in the EH gene utilized to generate a 286 bp fragment (Fig. 1B, lane 7) using a 57 °C annealing temperature during PCR. Differences in RFLP patterns were detected after *RsaI* restriction enzyme digestion (2.5 units, New England Biolabs, Beverly, MA) at 37 °C for 16 h using 10 µl of PCR amplification. In addition to the polymorphic *RsaI* site at codon 139, an additional *RsaI* site is present within the 286 bp EH exon 4 PCR-amplified product, serving as an internal control for restriction enzyme digestion for all EH codon 139 polymorphism analysis. Three banding patterns were observed by RFLP analysis (Fig. 1B): 230 bp and 56 bp bands that corresponded to the 139^{his}/139^{his} homozygous wild-type genotype (lanes 1, 2 and 4), 230, 170, 60 and 56 bp bands that corresponded to the 139^{his}/139^{arg} heterozygous genotype (lanes 5 and 6), and 170, 60 and 56 bp bands that corresponded to the 139^{arg}/139^{arg} homozygous polymorphic genotype (lane 3).

This analysis was repeated for 10% of the specimens and selected PCR-amplified DNA samples ($n=20$) were examined by dideoxy DNA sequencing²⁰ to confirm EH genotyping results. GSTM1 genotyping was performed by multiplex PCR as previously described.⁵

2.3. Statistical analysis

The risks of orolaryngeal cancer in relation to EH and GSTM1 genotypes were estimated using unconditional logistic regression to calculate ORs and 95% CIs. Logistic regression analysis was performed using categorical variables for alcohol consumption as described above. In this study, we designated the four possible EH alleles arising from the codons 113/139 polymorphism analysis as EH*1 (EH^{113tyr/139his}), EH*2 (EH^{113tyr/139arg}), EH*3 (EH^{113his/139his}), and EH*4 (EH^{113his/139arg}; see Fig. 2). Subjects were categorized into three groups based on the predicted activity of their EH genotype as determined by in vitro functional analysis⁶ and as suggested by Benhamou et al.:¹¹ low EH activity (EH*1/EH*3, EH*3/EH*3, and EH*3/EH*4), intermediate EH activity (EH*1/EH*1, EH*2/EH*3, EH*1/EH*4 and EH*4/EH*4) and high EH activity genotypes (EH*1/EH*2, EH*2/EH*2, and

EH*2/EH*4). The chi-square test was utilized for the analysis of allelic frequencies. The Student's *t*-test (2-tailed) was used for comparing smoking (py) variable between cases and controls. The statistical computer software SPSS (ver. 10.1) was used to perform all statistical analysis.²¹

3. Results

A total of 142 Caucasian and 81 African American orolaryngeal cancer patients, and 213 Caucasian and 122 African American frequency-matched control subjects (case:control ratio=1:1.5) were entered into this study. The mean age of cases and controls ranged from 58 to 61 in both races, and approximately 30% of both cases and controls were female in both racial groups (Table 1). As expected, cases had a significantly higher level of cigarette consumption than controls for both racial groups ($P<0.001$ for both Caucasians and African Americans; Table 1). A significantly higher percentage of cases were ever-alcohol drinkers as compared with controls for both African Americans (86% of cases versus 41% of controls, $P<0.001$) and Caucasians (69% of cases versus 50% of controls, $P<0.001$).

The genotyping was determined by the combined data obtained from individual PCR-RFLP analysis of the codons 113 and 139 polymorphisms. Other than the EH*2/EH*3 versus EH*1/EH*4 genotypes, all EH genotypes could be distinguished by this analysis. All subjects exhibiting the EH*2/EH*3 or EH*1/EH*4 genotypes were considered to be EH*2/EH*3 because the prevalence of the EH*4 allele is low in the population (0.035 in Caucasians and 0.02 in African Americans, Table 2) and the predicted phenotypes of these EH genotypes are intermediate in activity based upon functional analysis.⁶ In controls, the prevalence of the EH^{113his} variant was 0.38 (Caucasians) and 0.18 (African Americans), while the prevalence of the EH^{139arg} variant was 0.18 (Caucasians) and 0.30 (African Americans). These polymorphic prevalences were similar to those observed in previous studies of both Caucasians and African Americans.^{4, 6, 9, 11, 15} Differences in EH allelic frequencies were observed between control groups: a significantly ($P<0.001$) lower frequency of the EH*2 allele was observed for Caucasians (0.14) as compared to African Americans (0.29), while the EH*3 allelic frequency was significantly ($P<0.001$) higher in Caucasians (0.34) as compared to African Americans (0.17; Table 2). In addition, significant differences in the prevalence of EH genotypes were also observed between African American and Caucasian controls (Table 2). The prevalence of the EH*1/EH*2 and EH*2/EH*2 genotypes were both significantly higher in African Americans as compared to Caucasians ($P<0.0005$ table and $P<0.01$, respectively) while the prevalence of the EH*3/EH*3 genotype was significantly lower in African Americans ($P<0.005$).

Differences in EH allelic frequencies were observed between Caucasian case and control groups. A significantly ($P<0.005$) lower frequency of the EH*2 allele was observed for controls (0.14) as compared to cases (0.20), while the EH*3 allelic frequency was significantly ($P<0.05$) higher in controls (0.34) as compared to cases (0.27; Table 2). In addition, the prevalence of the EH*3/EH*3 genotype was significantly lower in Caucasian cases (0.09) than in Caucasian controls (0.18; $P<0.025$) (Table 2).

In Caucasians, a significant increase in risk for orolaryngeal cancer was observed for subjects with the EH^{113tyr} variant (OR=2.1, 95%CI=1.1–4.0; Table 3). When subjects were stratified based upon predicted EH activity genotypes, a non-significant increase in risk for orolaryngeal cancer was observed among Caucasian subjects with high EH activity genotypes (OR=1.7, 95%CI=0.9–3.1; Table 4). No association between predicted EH activity genotype and orolaryngeal cancer risk was observed for African Americans (Table 4).

The association between EH genotypes and orolaryngeal cancer risk was examined separately in smokers (due to a lack of statistical power, never-smokers were excluded from this analysis). Ever-smokers (i.e. 100 cigarettes lifetime) were categorized into two groups based upon lifetime smoking history divided at the median number of pack-years of all smokers in the entire cohort. Predicted high EH activity genotypes were significantly associated with increased risk for orolaryngeal cancer in Caucasian smokers with a history of ≥ 35 py (OR=3.4, 95%CI=1.2–9.6) but not in Caucasian smokers with a lesser dose of lifetime smoking (i.e. <35 py; OR=1.0, 95%CI=0.3–2.8). Similar associations with smoking dose were not observed in African Americans (Table 4). No association between predicted EH activity genotypes and orolaryngeal cancer risk was observed for either Caucasians or African Americans after stratifying by alcohol consumption (results not shown).

After stratification with different GSTM1 genotypes, a significant association between EH genotype and orolaryngeal cancer risk was observed in Caucasian subjects with predicted high EH activity genotypes who were GSTM1 null (OR=3.5, 95%CI=1.3–9.3) but not GSTM1 [+] (OR=0.9, 95%CI=0.4–2.1, Table 5). Using the presumed most protective EH (low+intermediate) activity/GSTM1 [+] genotype as the reference group, a significant trend towards increased risk for orolaryngeal cancer was observed with potentially less protective EH/GSTM1 genotypes ($P<0.02$, trend test, Table 5). A similar assessment could not be performed for the African American cohort due to smaller subject numbers for this group and the low prevalence of the GSTM1 null genotype in African Americans (0.16).

4. Discussion

BaP-7,8-epoxide is a critical intermediate metabolite in the BaP carcinogenic pathway and is formed predominantly via oxidation by the CYP1A1²². Once formed, BaP-7,8-epoxide can either be detoxified by the GST family of phase II enzymes, or metabolized by EH to a diol intermediate (BaP-7,8-dihydrodiol), the precursor of ultimate carcinogenic metabolite. Similar to that observed in previous studies of tobacco-related cancer risk,¹⁵ we demonstrate a significant association between EH genotype and risk for orolaryngeal cancer in Caucasians. This association is smoking dose-dependent, with significantly increased risk observed for subjects with predicted high EH activity genotypes who were heavy- but not light-smokers. These results are consistent with a critical role for EH in tobacco-related cancer risk and in the metabolism of BaP-7,8-epoxide. In previous studies, the GSTM1 null polymorphism was shown to play a significant role in risk for oral cancer in African Americans but not Caucasians.⁵ In the present study, no association between EH genotype and orolaryngeal cancer risk was observed for African Americans. Similar to other studies on both lung as well as oral cancer,^{1, 2, 23–26} these data suggest that differences in the importance of metabolizing enzyme polymorphisms on cancer risk are observed between ethnic and/or racial groups.

To assess the role of gene:gene interactions, we have examined combined genotypes for EH and GSTM1 and their role in risk for orolaryngeal cancer in Caucasians. The predicted high EH activity genotypes were significantly associated with increased risk for orolaryngeal cancer in Caucasians who were GSTM1 null but not GSTM1 [+]. These data suggest that EH and GSTM1 gene:gene interactions play a critical role in susceptibility to orolaryngeal cancer. This interaction may be explained as follows: risk associations for genotypes exhibiting small increases in EH activity may potentially be discernable only under circumstances where exposure to BaP-7,8-epoxide is greatest. The fact that EH genotype plays an important role in orolaryngeal cancer risk exclusively in GSTM1 null subjects is consistent with this hypothesis since increased levels of BaP-7,8-epoxide would be present due to decreased rates of detoxification by GST μ . As discussed above, no association between the GSTM1 null polymorphism and oral cancer risk was observed in Caucasians in

previous studies, even after stratification by smoking dose.⁵ However, the data presented in the present study demonstrates that the GSTM1 null genotype is significantly associated with orolaryngeal cancer risk in subjects with predicted high EH activity genotypes but not low or intermediate EH activity genotypes. Therefore, similar to that discussed above for EH genotype, the risk associated with the GSTM1 null polymorphism may only be discernable when the combined net effect of multiple genotypes results in significant increases in levels of BaP-7,8-epoxide. In 2summary, EH genotype appears to play an important role in susceptibility to orolaryngeal cancer, with a race and smoking dose-dependent association that is linked to GSTM1 null polymorphic status.

Acknowledgments

The authors thank Nina Suslina for her participation in the collection of clinical samples and questionnaire data, Abul Elahi for helpful discussions, and Susan Gray for her excellent technical assistance. These studies were supported by NIH Grants: CA91314 (JP), CA084973 (JP), and DE12206 (PL).

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Figure 1.

PCR-RFLP analysis of, EH (A) codons 113, and (B) 139 polymorphisms in controls. Shown in A is a representative PCR-RFLP analysis for the codon 113 polymorphism. Wild type (lanes 1, 2 and 5), homozygous polymorphic EH (lane 3), and heterozygous EH (lane 4). For the codon 139 polymorphism (shown in B), wild type (lanes 1, 2, and 4), homozygous polymorphic samples (lane 3), and heterozygous samples (lanes 5 and 6), undigested PCR product (lane 7).

Figure 2.
Schematic diagram of epoxide hydrolase alleles and their corresponding polymorphisms at codons 113 and 139.

Distribution of orolaryngeal cancer cases and controls according to demographic characteristics

Table 1

	N	Mean age (year)	Sex (M/F)	Smoking mean±S.D. (py)
African Americans	81	57.7	58/23	40.8±23.7 ^{a,b}
Controls	122	59.4	87/35	14.2±21.1 ^c
Caucasians	142	60.6	100/42	45.7±41.4 ^{a,b}
Controls	213	59.6	150/63	20.8±31.1 ^a

^aOne subject with incomplete smoking information was excluded from this analysis.
^bSmoking consumption was significantly higher ($P<0.001$) in cases as compared to controls in both racial groups.
^cTwo subjects with incomplete smoking information was excluded from this analysis.

Table 2

EH allele and genotype prevalence in Caucasians and African Americans

	Caucasians		African Americans	
	Cases	Controls	Cases	Controls
EH allele				
EH*1	0.50 ^a	0.48	0.49	0.53
EH*2	0.20	0.14 ^b	0.33	0.29 ^c
EH*3	0.27	0.34 ^b	0.15	0.17 ^c
EH*4	0.03	0.04	0.03	0.02
EH genotype				
EH*1/EH*1	39 (27) ^d	69 (32)	19 (23)	38 (31)
EH*1/EH*2	30 (21)	30 (14) ^e	30 (37)	38 (31)
EH*2/EH*2	7 (5)	4 (2) ^f	7 (9)	10 (8)
EH*1/EH*3	34 (24)	37 (17)	11 (14)	15 (12)
EH*2/EH*3	11 (8)	22 (10)	8 (10)	10 (8)
EH*2/EH*4	2 (1)	1 (0.5)	2 (2)	2 (2)
EH*3/EH*3	13 (9) ^g	38 (18) ^e	1 (1)	7 (6)
EH*3/EH*4	5 (4)	10 (5)	3 (4)	2 (6)
EH*4/EH*4	1 (1)	2 (1)	0	0
Total	142	213	81	122

^aNumbers indicate allelic frequencies.

^bAllelic frequencies were significantly different ($P<0.05$) between Caucasian cases and matched controls.

^cAllelic frequencies were significantly different ($P<0.001$) between African American and Caucasian controls.

^dNumbers in parentheses denote percentages.

^eGenotype prevalence was significantly different ($P<0.005$).

^f $P<0.01$, in African American versus Caucasian controls.

^gGenotype prevalence was significantly lower ($P<0.0025$) in Caucasian cases as compared to matched controls.

EH polymorphic variants and orolaryngeal cancer risk in Caucasians and African Americans

Table 3

EH polymorphisms	Caucasians		African Americans	
	Cases	Controls	OR (95%CI) ^{a,b}	Controls
113 his/his	19 (13) ^d	50 (23)	1.0 (referent)	4 (5)
113 tyr/his+tyr/tyr	123 (87)	163 (77)	2.1 (1.1–4.0)	77 (95)
139 his/his	86 (61)	144 (68)	1.0 (referent)	31 (38)
139 arg/his +arg/arg	56 (39)	69 (32)	1.3 (0.8–2.2)	50 (62)
				62 (51)
				1.3 (0.6–2.7)

^aORs were calculated by adjusting for sex, age, smoking (py), alcohol consumption (categorical variables), and region of subject recruitment.

^bNine subjects were excluded from OR calculations due to incomplete questionnaire data.

^cThree subjects were excluded from OR calculations due to incomplete questionnaire data.

^dNumbers in parentheses denote percentages.

Table 4

Oral cavity cancer risk for predicted EH activity genotypes stratified by smoking

Race	Predicted EH activity genotypes ^a	Total subjects	Smokers	
			<35 py	35 py
Caucasians	Low+intermediate	Cases 103 (73) ^b Controls 178 (84)	Cases 31 (82)	Controls 59 (80)
	High	Cases 39 (27) Controls 35 (16)	Cases 7 (18)	Controls 15 (20)
	OR (95%CI)	1.7 (0.9–3.1) ^{c,d}	1.0 (0.3–2.8) ^{e,f}	3.4 (1.2–9.6) ^{e,f}
African Americans	Low+intermediate	Cases 42 (51) Controls 72 (59)	Cases 12 (40)	Controls 31 (57)
	High	Cases 39 (49) Controls 50 (41)	Cases 18 (60)	Controls 23 (43)
	OR (95%CI)	1.2 (0.6–2.6) ^{c,h}	2.1 (0.7–6.0) ^{e,f}	1.1 (0.2–4.6) ^{e,g}

^a (High), subjects with either the EH*1/EH*2, EH*2/EH*2, or EH*2/EH*4 genotypes; (intermediate), subjects with either the EH*1/EH*1, EH*1/EH*4, EH*2/EH*3 or EH*4/EH*4 genotypes; (low), subjects with either the EH*1/EH*3, EH*3/EH*3, or EH*3/EH*4 genotypes.

^b Numbers in parentheses denote percentages.

^c ORs were calculated by adjusting for sex, age, smoking (py), alcohol consumption (categorical variables), and region of subject recruitment.

^d Nine subjects were excluded from OR calculations due to incomplete questionnaire data.

^e ORs were calculated by adjusting for sex, age, alcohol consumption (categorical variables), and region of subject recruitment.

^f Two subjects were excluded from OR calculations due to incomplete questionnaire data.

^g Four subjects were excluded from OR calculations due to incomplete questionnaire data.

^h Three subjects were excluded from OR calculations due to incomplete questionnaire data.

Table 5

Orolaryngeal cancer risk for combined EH and GSTM1 genotypes in Caucasians

Combined genotypes		Cases	Controls	OR (95%CI) ^{a,b}
EH (predicted activity)	GSTM1			
Low+intermediate	[+] ^c	53 (78) ^d	86 (80)	1.0 (referent)
High	[+]	15 (22)	22 (20)	0.9 (0.4–2.1)
Low+intermediate	(0/0)	44 (65)	86 (87)	1.0 (referent)
High	(0/0)	24 (35)	13 (13)	3.5 (1.3–9.3)
Low+intermediate	[+]	53 (39)	86 (42)	1.0 (referent) ^e
Low+intermediate	(0/0)	44 (32)	86 (42)	1.0 (0.6–1.9)
High	[+]	15 (11)	22 (10)	0.9 (0.4–2.1)
High	(0/0)	24 (18)	13 (6)	3.7 (1.4–9.8)

^aORs were calculated by adjusting for sex, age, smoking (py), alcohol consumption (categorical variables), and region of subject recruitment.

^bNine subjects were excluded from OR calculations due to incomplete questionnaire data.

^c[+]=homozygous (+/+) and heterozygous (+/0) genotypes.

^dNumbers in parentheses denote percentages.

^eChi-square trend test, $P<0.02$.