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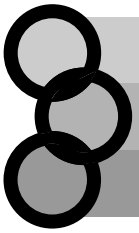

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Polymorphisms of xenobiotic metabolizing genes in oropharyngeal carcinoma

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Objective. The objective was to determine the prevalence of the polymorphisms of the microsomal epoxide hydrolase (*Ephx1*), glutathione S-transferase μ1 (*GSTM1*), θ1 (*GSTT1*), and π1 (*GSTP1*) genes in patients with oropharyngeal carcinoma.
Study design. Gene polymorphisms in 137 patients with oropharyngeal carcinoma were determined by polymerase chain reaction and restriction enzyme digestion for xenobiotic metabolizing enzymes that have been implicated in the carcinogenesis of tobacco-related neoplasias and compared with a population sample of 99 persons.
Results. At *Ephx1* (microsomal epoxide hydrolase) codon 113, an overrepresentation of the greater activity genotype (*Tyr/Tyr*) was observed for male ever-smokers alone, both male and female ever-smokers, female never-smokers alone, and in both male and female never-smokers, compared with a control population sample. At codon 139, *Ephx1* showed no differences. There was an overrepresentation of homozygosity for the *GSTT1* (glutathione S-transferase θ1) null allele [but not for the *GSTM1* (glutathione S-transferase μ1) null allele] in ever-smokers, when compared with controls. Polymorphisms at the *GSTP1* (glutathione S-transferase π1) locus did not show differences versus controls, although in the never-smoker cancer sample there was a higher prevalence of the *B/B* genotype compared with ever-smokers.
Conclusion. The *Ephx1* codon 113 *Tyr/Tyr* variant, as well as homozygosity for the *GSTT1* null allele, is associated with oropharyngeal carcinogenesis.
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Smoke from cigarettes contains polycyclic aromatic hydrocarbons (PAH) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), as well as other carcinogens, cocarcinogens, and tumor promoters.[1] The effects of these molecules are generally manifested through formation of DNA adducts, which when not repaired result in permanent mutations of developmental genes and/or tumor suppressor genes that may

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Table I. Characteristics of the cancer patients and of the control population sample (investigators were masked to any characteristics of the controls except for sex and race)

	Controls	*Ever-smokers*	*Never-smokers*
Age (y)	Unknown	32-82	33-78
Sex			
Male	49	103	9
Female	50	19	6
Ethnicity			
White	90	114	14
Black	8	8	1
Native American	1	0	0
Type of cancer			
Squamous cell cancer	Unknown	114	13
Other cancer	Unknown	8	2
Tobacco use*	Unknown		None
Cigarettes		100	
Cigars		11	
Pipe		11	
Chew		6	
Snuff		7	
Alcohol use	Unknown		
Beer		44	4
Wine		3	0
Liquor		21	1
Beer + wine		0	0
Beer + liquor		27	0
Wine + liquor		2	0
Beer + wine + liquor		3	0
None		20	10
Not known		2	
Relatives with cancer	Unknown		
Mouth and throat cancer		11	1
Other cancer		51	9
None		56	5
Not known		4	
Site of primary†	Unknown		
Lip		4	0
Mouth		59	9
Pharynx		17	6
Parotid		1	1
Larynx		34	0
Unknown		18	0

*Some patients used more than one form of tobacco; therefore, the number totals are greater than the number of patients.
†Some patients had more than one primary.

initiate carcinogenesis. Certain combinations of xenobiotic enzyme coding gene polymorphisms have been shown to promote the formation of higher levels of PAH-induced DNA adducts.[2]

Cytochrome P_{450} enzymes initially metabolize many carcinogens in tobacco smoke. Enzymes like microsomal epoxide hydrolase (mEH) and glutathione S-transferases (GSTs) may subsequently metabolize the resulting intermediate metabolites. Recent studies indicate that specific alleles of the genes coding for these types of enzymes might be correlated with higher incidences of tobacco-related neoplasias, including oral cancer.[3]

Some studies suggest that the combined lack of active *GSTM1* and *GSTT1* (glutathione S-transferases μ1 and θ1) products is related to an increased susceptibility to neoplasias of the head and neck, as well as to the occurrence of preneoplastic lesions.[4-5] We studied the prevalence of the polymorphisms of the microsomal epoxide hydrolase (*Ephx1*), glutathione S-transferase μ1 (*GSTM1*), θ1 (*GSTT1*), and π1 (*GSTP1*) genes in patients with oropharyngeal carcinoma.

MATERIAL AND METHODS

Human subjects

Patients with a diagnosis of oropharyngeal carcinoma were recruited at the Head and Neck Tumor clinics of the Indiana University Cancer Pavilion and the Roudebush Veterans Administration Medical Center

Table II. Characteristics of the polymerase chain reaction assays and the restriction enzyme digestions

A	
Microsomal epoxide hydrolase (*Ephx1*)-113	
Primers	
EH1	GAC TTA CAC CAG AGG ATC GAT AAG
EH2	GCC CTT CAA TCT TAG TCT TGA AGT GAC GGT
PCR	5 cycles (3´ @ 95°C, 1´ @ 55°C, 1´ @ 72°C)
	35 cycles (3´ @ 94°C, 1´ @ 55°C, 1´ @ 72°C)
Digestion with *Tth111*I	20 U for 3 h @ 65°C
Detection	2.5% agarose gel
B	
Microsomal epoxide hydrolase (*Ephx1*)-139	
Primers	
mEH4prime1	CAG AGC CTG ACC GTG CAG
mEH4prime2	GCT CAC CCC GCC GGA
PCR	Same as above
Digestion with *Rsa*I	20U for 3h @ 37°C
Detection	2.0% agarose gel
C	
Glutathione S-transferases μ1 and θ1 (*GSTM1* and *GSTT1*)	
Primers	
G5	GAA CTC CCT GAA AAG CTA AAG C
G6	GTT GGG CTC AAA TAT ACG GTG G
T1	TTC CTT ACT GGT CCT CAC ATC TC
T2	TCA CCG GAT CAT GGC CAG CA
PC04	CAA CTT CAT CCA CGT TCA CC
GH20	GAA GAG CCA AGG ACA GGT AC
PCR	1 cycle (4´ @ 94°C)
	35 cycles (60´´ @ 94°C, 45´´ @ 55°C, 60´´ @ 72°C)
Detection	2.5% agarose gel
D	
Glutathione S-transferases π1 (*GSTP1*)	
Primers	
P1	TCC TTC CAC GCA CAT CCT CT
P2	AGC CCC TTT CTT TGT TCA GC
PCR	Same as above
Digestion with *Alw26*I	20 U for 3 h @ 37°C
Detection	2.5% agarose gel

(Indianapolis, Ind). Subsequent to Institutional Review Board–approved informed consent, a 15-mL venous blood sample was obtained. One hundred twenty-two ever-smokers and 15 never-smokers agreed to participate. A *never-smoker* was defined as a patient who had never smoked more than two cigarettes, cigars, or pipe loads per day for longer than 6 months during a lifetime. Thus, the total lifetime tobacco consumption of these patients was ≤1 pipe-year. If the patient had smoked a greater quantity or had smoked for a longer time, she or he was considered an *ever-smoker*.

A sample from the Indiana population consisting of 99 persons who had previously participated in paternity testing and had agreed to have their DNA anonymously banked was used as the control population. All participants in the control group signed a consent for testing and release that included the statement: "I also understand that a sample of my blood may be stored for future additional testing and/or for research/teaching purposes." Only sex was specifically recorded for control patients, and their smoking and cancer status were unknown. The investigators were totally masked to any other information concerning the control subjects. Whereas ideal control matching would have given a greater power to detect further differences between patient groups and controls, the differences detected are valid because they are detected despite the decrease in statistical power. The control population sample was 91% white, 8% black, and 1% Native American. The characteristics of patients and controls are summarized in Table I.

DNA isolation

Leukocytes were isolated from peripheral blood to prepare genomic DNA. The DNA was extracted by a high-salt method followed by ethanol precipitation and was stored frozen until assayed by primer-directed

Table III. Genotyping of the various polymorphisms in patients with mouth or throat cancer (data presented as percent of the total number of patients in each specific group; wild-type genotypes are shown in **bold)**

	Controls			*Ever-smokers*			*Never-smokers*
	Male	*Female*	*All*	*Male*	*Female*	*All*	*All*
a) Ephx1-113				*P* = .012*	N.S.	*P* = .001	*P* = .006
Tyr/Tyr	22.0	20.8	21.4	42.2	38.9	41.7	60.0
Tyr/His	46.0	47.9	46.9	45.1	44.4	45.0	33.3
His/His	32.0	31.3	31.6	12.8	16.7	13.3	6.7
b) Ephx1-139				N.S.	N.S.	N.S.	N.S.
His/His	74	73.5	73.7	67.7	88.9	70.8	86.7
His/Arg	20.0	22.5	21.2	25.5	5.6	22.5	0.0
Arg/Arg	6.0	4.1	5.1	6.9	5.6	6.7	13.3
c) GSTM1 & GSTT1				N.S.	N.S.	N.S.	N.S.
+/? & +/?	34.0	40.8	37.4	30.7	38.9	31.9	33.3
+/? & null/null	4.0	4.1	4.0	8.9	5.6	8.4	6.7
null/null & +/?	56.0	49.0	52.5	49.5	27.8	46.2	53.3
null/null & null/null	6.0	6.1	6.1	10.9	27.8	13.5	6.7
d) GSTP1				N.S.	N.S.	N.S.	N.S.
A/A	24.4	29.6	27.1	29.3	14.3	27.1	33.3
A/B	63.4	63.6	63.5	65.9	64.3	65.6	40.0
B/B	12.2	6.8	4.9	4.9	21.4	7.3	26.7

*Statistical differences are all versus the Indiana control sample. Comparisons were not significantly different if *P* > .05.

polymerase chain reaction (PCR),[6] with or without subsequent restriction enzyme digestions.

Polymorphism analysis

Polymorphism within the *Ephx1* locus was determined for codons 113 and 139 by using PCR followed by restriction enzyme digestions (Table II).[7] Gene polymorphism within the *GSTM1* and *GSTT1* loci was determined by using multiplex PCR, with the β-globin gene as a control reaction.[7] To analyze polymorphism within the *GSTP1* locus, PCR was used, followed by a restriction enzyme digestion.[8]

Statistical analysis

Comparisons between the Indiana control population sample, ever-smokers with oropharyngeal carcinoma and never-smokers with oropharyngeal carcinoma, were made by using the Fisher exact test.[9] No adjustments were made for multiple comparisons or multiple hypothesis testing. A *P* value of less than .05 was considered to be statistically significant. The SAS statistical software package[10] was used to perform all analyses.

RESULTS

There is a significant difference within the genotypic pattern for *Ephx1* at codon 113 in those patients with oropharyngeal carcinoma. A significant overrepresentation of the genotype (*Tyr/Tyr*) with greater in vitro microsomal epoxide hydrolase specific activity than the *His/His* variant[7] was observed when compared with the Indiana control population sample (Table III). This was observed for male ever-smokers (*P* = .012), for all ever-smokers (*P* = .001), and all never-smokers (*P* = .006). An overrepresentation of the *Tyr/Tyr* genotype was observed in female never-smokers (*P* = .042). In contrast, analysis of polymorphism at codon 139 of *Ephx1* did not show differences in the patterns among the various groups (Table III).

The prevalence of the homozygous null *GSTM1* or *GSTT1* genotypes suggests that ever-smokers, and in particular female ever-smokers, tend to have overrepresentation of the combination of null alleles at both of these loci, although this was not statistically significant (Table III).

Although an analysis of these combined GST loci data did not yield any significant differences between the study and control groups, there is an increase in the occurrence of homozygous *GSTT1* null allele in ever-smokers versus the Indiana population sample (21.85% vs 10.10%; *P* = .027). There also was an increase in the occurrence of homozygous *GSTT1* null allele for female ever-smokers over that of the Indiana population female sample, although the difference was not significant (33.34% vs 10.20%; *P* = .06).

Polymorphism at the *GSTP1* locus did not reveal significant differences between patients with oropharyngeal carcinoma and the Indiana population sample (Table III). However, there was a significant overrepresentation of the low-activity *B* allele in male never-smokers when compared with that of male ever-smokers (*P* = .043).

DISCUSSION

The *Ephx1* 113 *Tyr/Tyr* coding genotype is overrepresented in oropharyngeal carcinoma. These data indi-

cate that the presence of the *Tyr/Tyr* genotype at this position plays a role in carcinogenesis of the mouth and throat. In nonsmokers with cancer, the *Ephx1* 113 *Tyr/Tyr* genotype was the most common one. Because we do not know the tobacco use or cancer status of persons in the Indiana population sample, comparisons with this group are conservative. Cancer patients with a history of tobacco use had a higher prevalence of the *Ephx1* 113 *Tyr/Tyr* genotype than the Indiana population, although not to the degree seen in cancer patients with no history of tobacco use.

It is interesting that the *Ephx1* 113 *Tyr/Tyr* genotype is increased in oropharyngeal carcinoma regardless of smoking history, as the metabolic model of activation involves constituents of tobacco smoke, suggesting that either the never-smokers who developed cancer had some other similar exposure, such as passive smoking or occupational exposure, or that this genotype is predisposing or a marker regardless of tobacco use. These differences could be explained by taking into account that tobacco users are subjected to mutagenic insults from smoking-related chemicals and physical agents. Thus, the *Tyr/Tyr* genotype would be one of the etiopathogenic factors, whereas smoking-related factors in nonsmokers would be absent. In the absence of tobacco use, the *Tyr/Tyr* genotype may be of greater importance. The lack of differences in polymorphism at codon 139 of *Ephx1* could indicate that an increase in specific activity, which has been found to be associated in vitro with the *Arg/Arg* genotype,[7] does not affect susceptibility to carcinogens.

Although combined absence of *GSTM1* and *GSTT1* is increased, but not to statistically significant levels in ever-smoker patients with cancer of the mouth and throat, the absence of the θ1 isozyme may play a role within the combination. Whereas the Indiana population sample has only a *GSTT1* homozygous null genotype incidence of 10.1%, cancer patients with a history of tobacco use have an incidence of 21.5%. Cancer patients who never used tobacco had a *GSTT1* null allele incidence of 13.3%. Thus, these enzymes may play a greater role in tobacco-related carcinogenesis than in other kinds of carcinogenesis. In never-smokers, this role may be that of *GSTP1*, because the low-activity *B* allele is overrepresented in those who developed oropharyngeal or laryngeal malignant neoplasms as compared with ever-smokers with cancer.

Recent experimental and clinical evidence appears to support the role of mEH in some carcinogenic events.[11-13] When *Ephx1*-null mice lacking mEH activity were produced, these mice were resistant to induction of skin malignancies when exposed to 7,12-dimethylbenz[*a*]anthracene.[11] A recent study found that patients with *Ephx1* high-activity–associated polymorphism had a high risk of developing a neoplasia in the region of the mouth and throat. When this polymorphism was associated with certain *GSTM3* genotypes, the risk of developing these neoplasms increased further.[12] In addition, mEH has been identified as a brain tumor antigen associated with a group of gliomas, illustrating the need for further study of this protein in cancer.[13]

Under typical circumstances, many tobacco carcinogens are metabolized first by P_{450}-1A1, P_{450}-3A, or other P_{450} enzymes, then by a number of enzymes including GSTs and mEH, ultimately allowing for inactivation, detoxification, and excretion of the final metabolite. However, in our patients at least, there could be decreased or absent GST activity that overloads the preceding pathways. As a consequence, in the presence of relatively greater mEH activity, the metabolic flow would shift toward the formation of the dihydrodiol epoxides and its subsequent metabolites, "bay region" dihydrodiol epoxides. The benzo rings of arene oxides containing certain benzylic carbons in a benzo ring that has previously undergone an epoxidation-hydration reaction may form a bay region. Bay region dihydrodiol epoxides are potent carcinogens capable of forming DNA adducts that induce point mutations (mainly transitions) at the site of these adducts. As a result, the cellular repair/replication machinery recognizes the new structural conformations differently, and the machinery's fidelity is decreased. Thus, it would appear that in the presence of a deficiency in overall GST activity, those patients that have reduced mEH activity would be at a lesser risk of developing neoplasms in the presence of certain xenobiotics (substances whose origin is extrinsic to the organism), especially those present in tobacco. In this case, the slower metabolism may be "balanced," with less accumulation of dangerous metabolites such as the bay region dihydrodiol epoxide.

Our use of an anonymous control group is not ideal because it most likely includes some smokers and does not specifically match any particular group characteristics, aside from representing the same geographic region as the study group. However, the anonymous control group was available, and using this group we wished to determine if future study on a greater scale was warranted. Adequate resources would facilitate recruiting a group of matched controls. Also, these appropriate controls would have to be matched not only by sex, smoker status, and ethnicity, but also by year and month of birth. Recent data indicate that genomic composition and seasonality of birth individually play important roles in susceptibility to the development of cancer.[14-16] We believe the results of our study warrant the undertaking of a more comprehensive investigation.

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