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Association of CYP1B1 Codon 432 Mutant Allele in Head and Neck Squamous Cell Cancer Is Reflected by Somatic Mutations of p53 in Tumor Tissue

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ABSTRACT

Tobacco use is causally associated with head and neck squamous cell cancer (HNSCC). Here, we present the results of a case-control study that investigated the effects that the genetic variants of the *cytochrome* (*CYP)1A1, CYP1B1, glutathione-S-transferase (GST)M1, GSTT1,* and *GSTP1* genes have on modifying the risk of smoking-related HNSCC. Allelisms of the *CYP1A1, GSTT1, GSTM1,* and *GSTT1* genes alone were not associated with an increased risk. *CYP1B1* codon 432 polymorphism was found to be a putative susceptibility factor in smoking-related HNSCC. The frequency of *CYP1B1* polymorphism was significantly higher ($P < 0.001$) in the group of smoking cases when compared with smoking controls. Additionally, an odds ratio (OR) of 4.53 (2.62–7.98) was discovered when investigating smoking and nonsmoking cases for the susceptible genotype *CYP1B1*2/*2,* when compared with the presence of the genotype wild type. In combination with polymorphic variants of the *GST* genes, a synergistic-effect OR was observed. The calculated OR for the combined genotype *CYP1B1*2/*2* and *GSTM1*2/*2* was 12.8 (4.09–49.7). The calculated OR for the combined genotype was 13.4 (2.92–97.7) for *CYP1B1*2/*2 and GSTT1*2/*2,* and 24.1 (9.36–70.5) for the combination of *CYP1B1*2/*2* and *GSTT1*-expressors. The impact of the polymorphic variants of the *CYP1B1* gene on HNSCC risk is reflected by the strong association with the frequency of somatic mutations of the *p53* gene. Smokers with susceptible genotype *CYP1B1*2/*2* were 20 times more likely to show evidence of *p53* mutations than were those with *CYP1B1* wild type. Combined genotype analysis of *CYP1B1* and *GSTM1* or *GSTT1* revealed interactive effects on the occurrence of *p53* gene mutations. The results of the present study indicate that polymorphic variants of *CYP1B1* relate significantly to the individual susceptibility of smokers to HNSCC.

INTRODUCTION

Tobacco smoke and alcohol abuse play a major role in the etiology of HNSCC[2] (1). Other factors discussed are occupational exposures to wool dust, wood dust, mineral fibers (2–6), and low intake of vegetables and fruits (7). In Europe, the highest incidence rates for HNSCC are observed in the southern countries. However, the geographic distribution of incidences was not strongly correlated with the distribution of tobacco consumption (8), suggesting that other, possibly genetic, factors might modulate the risk for HNSCC.

Molecular epidemiology has begun to have an increasing impact on analyzing the complexity of gene-environment interactions at the molecular level. Whereas germ line mutations in genes of high penetration, such as *APC*, resulted often in a high risk for cancer *per se* (9), polymorphisms of genes with low penetration (*i.e.*, genes involved in metabolisms of xenobiotics), are thought to predispose the risk of an individual if exposed to a chemical. Most environmental compounds require metabolic initiation to reactivate electrophilic intermediates before exerting their carcinogenic effects. This biotransformation is catalyzed by the cytochrome P450 mono-oxygenase system (CYP), consisting of several P450 isoenzymes in humans (10). CYP1A1 is involved in the activation of many compounds, including benzo(*a*)pyrene and other PAHs that are present in substances such as tobacco smoke. At least four polymorphic variants of the *CYP1A1* gene have been identified (11), two of which are thought to result in increased enzyme activity (12, 13): the first is an isoleucine/valine substitution in the heme-binding region in exon 7 at nucleotide A4889G; the second is a thymine/cytosine point mutation in the 3′-noncoding region at nucleotide T6235C. The homozygotes of the variant allele of each mutation are reported to correlate with an enhanced susceptibility to smoking-induced lung cancer in a Japanese population but not in Caucasians (14–16). The isoform CYP1B1 has been demonstrated to induce metabolic activation of a variety of chemical classes of carcinogens such as arylamines, nitroaromatics, and PAHs (17). CYP1B1 catalyzes both the formation of procarcinogenic dihydrodiols of certain PAHs and their additional oxidation to ultimate carcinogenic dihydrodiolepoxide. Several polymorphisms have been identified in the coding region of the *CYP1B1* gene. Most polymorphisms resulted in the formation of a truncated or nonfunctional protein. Four sense polymorphisms were found in the *CYP1B1* gene: at position 48 (Arg to Gly), at position 119 (Ala to Ser), at position 432 (Val to Leu), and at position 453 (Asn to Ser; Refs. 18, 19). The Val432Leu-polymorphism was suggested to have a profound impact on the catalytic activity of the enzyme (20). An association between susceptibility to colorectal cancer and polymorphisms at residue 432 of *CYP1B1* has been reported (21). In breast cancer patients, an association between CYP1B1 polymorphism and steroid receptor status has been observed (22). Similar to CYP1A1, the expression of CYP1B1 can be induced by several Ah receptor agonists, like dioxin and PAHs (23, 24).

Activated chemicals are subjected to detoxification, and the *GST* supergene family is an important part of the cellular defense system against chemicals with carcinogenic potential. The human GST family consists of four classes of GST isoenzymes: GSTA, GSTM, GSTT, and GSTP. Polymorphisms of the GSTM, GSTT, and GSTP classes have been shown to present an increased susceptibility to cancer. The *GSTM1* polymorphism is a gene deletion providing two alleles: the null allele *GSTM1*2* and the *GSTM1*1*. The homozygous *GSTM1*2/*2* genotype expresses no GSTM1 enzyme, and ~50% of Caucasians lack GSTM1 activity (25–27). The *GSTT1* locus also has functional (*GSTT1*1*) and nonfunctional (*GSTT1*2*) alleles, and ~20% of Caucasians lack the functional alleles (28, 29). In the GSTP1 family, four *GSTP1* alleles have been identified. The wild

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[2] The abbreviations used are: HNSCC, head and neck squamous cell cancer; CYP1A1, cytochrome P450 1A1; CYP1B1, cytochrome P450 1B1; PAH, polycyclic aromatic hydrocarbon; OR, odds ratio; CI, confidence interval; GST, glutathione-*S*-transferase.

type allele *GSTP1*A* differs by an A/G transition at nucleotide +313 (Val105-A114) from *GSTP1*B* and *GSTP1*C* by this transition and by a C/T transition at +341 (Val105-Val114). A *GSTP1*D* (Ile105-Val114) allele has been found. *GSTP1* polymorphisms are thought to be associated with altered substrate specificities (30, 31). The importance of polymorphisms of the *GST* genes on the risk of several cancers, including skin, lung, and bladder cancers, has been intensively reviewed (26, 27, 32).

Inherent mutations of genes coding for drug-metabolizing enzymes can result in chemically induced somatic mutations of genes, which are vital for cell growth and differentiation. The *p53* tumor suppressor gene controls cell cycle regulation and has been identified as a vulnerable target of critical DNA damage. Loss of p53 function is attributed to uncontrolled cell proliferation and neoplastic formation. Mutations that are caused by carcinogens in cigarette smoke occur in the *p53* gene early in carcinogenesis. In lung tumors, 40% of *p53* gene mutations are G to T transversions, which are thought to be putative fingerprints of certain types of PAHs. Benzo(*a*)pyrene metabolite adducts along the *p53* gene occur predominately at G positions in codons that have been found to be mutational hot spots (33, 34). An association between the frequency of tobacco-induced *p53* mutations and *CYP1A1* polymorphisms has been reported for patients with lung cancer. The probability of individuals homozygous for the susceptible *CYP1A1* genotypes was about nine times higher than for those individuals with *CYP1A1* wild type. The probability increased synergistically when susceptible *CYP1A1* genotypes were combined with *GSTM1*2/*2* genotypes (35).

In the current study, we investigated the interactive effects of the polymorphic variants of several phase 1 and phase 2 enzymes on the risk for HNSCC. Our main interest was focused on the putative role of *CYP1B1* codon 432 polymorphism in cancer susceptibility. The data indicates an association between *CYP1B1* polymorphism and HNSCC in smokers. The importance of *CYP1B1* polymorphism is reflected by the high probability of susceptible genotypes for smoking-induced *p53* gene mutations.

MATERIALS AND METHODS

Study Group. The patient group consisted of a total of 312 people, including 251 males with a mean age of 59.7 years and 61 females with a mean age of 59.7 years. All were histologically verified to have HNSCC. The control group consisted of 300 unrelated healthy individuals, including 176 males with a mean age of 46.7 years and 124 females with a mean age of 48.0 years, all without a history of cancer. The patients and controls were gathered between 1996 and 1998 at the Department of Oto-Rhino-Laryngology of the University of Bonn and the Medizinische Universitäts-Poliklinik, Bonn, Germany. Donors gave their informed consent, and the Ethics Committee of the Rheinische-Friedrich-Wilhelms University of Bonn approved the study. All study subjects completed a questionnaire covering medical, residential, occupational, and smoking history. For the purpose of this study, smokers were defined as individuals having smoked five or more cigarettes or pipes per day for at least 4 years during their lifetime. Nonsmokers were defined as individuals who had never smoked or who had smoked less than one pack per year.

Genotyping. Genomic DNA was isolated from whole blood using the QIAamp Blood Maxi Kit (Qiagen, Hilden, Germany). CYP1A1 *Msp*I and CYP1A1 IVA were genotyped by RFLP-analysis according to standard protocols (36, 37). Real-time PCR-analysis and melting-curve analysis were performed for genotyping *GSTT1, GSTM1, GSTP1* (38), and *CYP1B1* codon 432 (39).

Tissue Sample Preparation. A subset of 150 paraffin embedded biopsy specimens was available. For DNA extraction, 8-μm sections were taken from each paraffin block, mounted onto glass slides and air-dried overnight in an incubator at 48°C. Sections were deparaffinized with xylene and ethanol, transferred to microfuge tubes (1.5 ml; Eppendorf, Germany), and subjected to protein digestion overnight (proteinase K; Qiagen, Hilden, Germany). DNA was extracted according to Weirich *et al.* (40) and DNA concentration was determined photometrically.

***p53* Sequence Analysis.** Primer used for amplification of exon 5–8 of the *p53* gene are listed in Table 1. For specificity and sensitivity enhancement, the DNA was denaturated for 30 min at 95°C in 41.8 μl of reaction mixture containing 5 μl of 10× reaction buffer [10 mM Tris-HCl, (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin], 34.8 μl of sterile water, and 2 μl of diluted template DNA. Afterward, the amplification mixture, consisting of 4 μl dNTPs (0.2 mM dATP, dTTP, dCTP, and dGTP), 1 μl of each primer (20 pmol), and 0.5 μl Taq-polymerase (5 units/μl; Amersham Life Science, Cleveland, OH) was added. The PCR conditions were as follows: (*a*) initial denaturation for 7 min at 94°C; (*b*) 45 cycles of denaturing for 60 s at 94°C; (*c*) annealing for 60 s at 60°C; (*d*) extension for 90 s at 72°C; and (*e*) final extension for 20 min at 72°C. Fragment analysis was done by horizontal polyacrylamid-gelelectrophorese on CleanGel DNA-HP 15% 36S and the DNA-Delect buffer (ETC, Kirchentellinsfurt, Germany) according to the supplier's instructions. DNA fragments were visualized by silver staining. Sequencing of DNA-fragments was carried out according to the dye terminator method, using the oligonucleotides described in Table 1 (Qiagen, Hilden, Germany) on an automated DNA sequencer (ABI 377; Perkin-Elmer, Weiterstadt, Germany).

Nomenclature. The following nomenclature was used to describe the different polymorphic variants. For the CYP1A1 T6235C polymorphism the genotypes were *CYP1A1*1/*1* for wild type, *CYP1A1*1/*2* for heterozygous, and *CYP1A1*2/*2* for mutant genotypes. For the CYP1A1 Ile462Val polymorphism, the genotypes were *CYP1A1*1/*1*, *CYP1A1*1/*3*, and *CYP1A1*3/*3*. For the CYP1B1 Val432Leu polymorphism, the genotypes were *CYP1B1*1/*1*, *CYP1B1*1/*2*, and *CYP1B1*2/*2*. For the GSTP1 Ile104Val polymorphism, the genotypes were *GSTP1*1/*1*, *GSTP1*1/*2*, and *GSTP1*2/*2*. The *GSTM1*1/*1* and *GSTM1*1/*2* genotypes were referred to as GSTM1 expressors, with the *GSTM1*2/*2* genotype being used to indicate the deleted

Table 1 *Primer for amplification and sequencing of p53 exon 5–8 (sequence GenBank accession no. U94788)*

Exon	Name	Localization		Fragment size
			5′-PCR-primer	
			3′-PCR-primer	
			Forward-sequencing-primer	
			Reverse-sequencing-primer	
5	5′-1	12983–13006	5′-GCCGTGTTCCAGTTGCTTTATCTG-3′	298
	3′-B	13280–13260	5′-TGGGCAACCAGCCCTGTCGTC-3′	
	2428	13001–13020	5′-TATCTGTTCACTTGTGCCCT-3′	
6	5′-C	13301–13319	5′-CCTCACTGATTGCTCTTAG-3′	186
	3′-2	13486–13467	5′-CACTGACAACCACCCTTAAC-3′	
	2930	13302–13319	5′-CTCACTGATTGCTCTTAG-3′	
	2485	13468–13445	5′-ACCCCTCCTCCCAGAGACCCCAGT-3′	
7	5′-6	13974–13993	5′-CATCTTGGGCCTGTGTTATC-3′	292
	3′-6	14265–14243	5′-GAGTGGGAGCAGTAAGGAGATTC-3′	
	2931	13976–13993	5′-TCTTGGGCCTGTGTTATC-3′	
	2666	14199–14180	5′-GAAATCGGTAAGAGGTGGGC-3′	
8	5′-8	14373–14394	5′-GGAGCCTGGTTTTTTAAATGGG-3′	308
	3′-9	14680–14659	5′-CTAGGAAAGAGGCAAGGAAAGG-3′	
	2575	14650–14627	5′-GTGAATCTGAGGCATAACTGCACC-3′	

Table 2 *Genotype distribution of the described CYP1A1, CYP1B1, GSTP1, GSTM1, and GSTT1 polymorphisms in control individuals and HNSCC patients*

	Control individuals (n = 300)			HNSCC patients (n = 312)				
	wt/wt[a]	wt/mt	mt/mt	wt/wt	wt/mt	mt/mt	OR[b] (95% CI)	P
CYP1A1 T6235C	81.4	17.3	1.3	79.5	19.9	0.6	0.94 (0.60–1.48)[c]	0.78
CYP1A1 Ile462Val	91.7	8.3	0.0	90.4	9.6	0.0	1.01 (0.54–1.91)[c]	0.99
CYP1B1 Val432Leu	36.3	46.0	17.7	25.7	50.6	23.7	1.41 (0.94–2.11)[c]	0.10
GSTP1 Ile104Val	43.3	46.0	10.7	46.5	39.1	14.4	0.78 (0.53–1.13)[c]	0.19
	+[d]	−[e]		+[d]	−[e]			
GSTM1	51.7	48.3		46.8	53.2		1.03 (0.71–1.49)	0.89
GSTT1	79.7	20.3		79.5	20.5		1.00 (0.64–1.60)	0.99

[a] wt, wild type; mt, mutant.
[b] The associations between the genotype distributions and the patients' status were assessed by ORs and CIs that were calculated by unconditional logistic regression, adjusting for age and gender.
[c] Calculation for wt/wt genotype vs. wt/mt and mt/mt genotypes.
[d] GSTM1- and GSTT1-expressors.
[e] Deleted *GSTM1* and *GSTT1* genotypes.

variant. Similarly, the GSTT1 expressors included the genotypes *GSTT1*1/*1* and *GSTT1*1/*2*, with the *GSTT1*2/*2* genotype indicating the deleted phenotype.

Statistical Analysis. The associations between the genotype distributions and the patients' status were assessed by ORs and CIs that were calculated by unconditional logistic regression, adjusting for age and gender. Preliminary analyses suggested the inclusion of both a linear and a quadratic term for age when comparing HNSCC patients with the control patients; whereas a model with only a linear term for age was considered to be appropriate for the subgroup analyses of HNSCC patients. The goodness-of-fit was assessed by a likelihood ratio test that compared the likelihood of the applied variable with that of an expanded model, with additional explanatory variables. This consisted of all third-order terms (*i.e.,* cubic trend for age and the corresponding interaction terms), for the comparisons of cases with controls, and all second-order terms in the subgroup analyses (41). The results of the goodness-of-fit tests confirmed the appropriateness of the chosen model. Fisher's exact test was used to evaluate differences in the distribution of the p53 mutation spectra between subgroups of cancer patients. All computations were carried out using the statistical software SAS, Version 6.12 (41).

RESULTS

Genotype Distribution in Controls and HNSCC Patients. Inheritable traits of different phase 1 (CYP1A1 and CYP1B1) and phase 2 (GSTP1, GSTM1, and GSTT1) enzymes were analyzed in 312 patients with HNSCC and in 300 controls as host factors of cancer risk. The prevalence of different genotypes in the control population and in HNSCC patients is listed in Table 2. The observed frequencies in the control population were within the range described for Caucasians (11, 21, 26, 42). When comparing the genotype distribution between control individuals and HNSCC patients, we observed that the frequency of the mutated *CYP1B1* genotypes was slightly elevated in HNSCC patients, but the difference was not significant. The genotype distribution of *CYP1A1*, *GSTP1*, *GSTM1*, and *GSTT1* showed no significant differences between the control and cancer group (Table 2), indicating a unique distribution of the respective polymorphic alleles within the examined population.

Stratification by Smoking. One hundred and seventy-seven members of the control group and 195 members of the HNSCC group reported having smoked cigarettes (Table 3). Genotype distribution of polymorphic phase 1 enzymes revealed a significantly higher frequency of the mutated *CYP1B1* genotypes ($P < 0.001$) in the HNSCC group. In the HNSCC patients, 65 smokers carried the *CYP1B1*2/*2* genotype (33.3%), 101 patients carried the *CYP1B1*1/*2* genotype (51.8%), and only 29 patients carried the wild type *CYP1B1*1/*1* genotype (14.9%); whereas, in the control group, 18.6% were homozygous, 49.2% heterozygous, and 32.3% were wild type in the described *CYP1B1* polymorphism. An adjusted OR of 2.70 (1.53–4.86) was observed. Among the other polymorphic enzymes investigated, no significant difference of genotype distribution was found between smoking controls and smoking HNSCC patients (Table 3).

Analysis of Genotype Distribution in Smoking and Nonsmoking Patients and the Effect of Combined Genotypes of *CYP1B1* and *GSTM1, GSTT1,* or *GSTP1* on Risk of HNSCC. When subgrouping the cancer patients into smokers (195 patients) and nonsmokers (117 patients), the data revealed a significantly higher frequency of

Table 3 *Genotype distribution of xenobiotic metabolizing enzymes in smoking control individuals and HNSCC patients (stratification by smoking)*

	Control individuals (n = 177)			HNSCC patients (n = 195)				
	wt/wt	wt/mt	mt/mt	wt/wt	wt/mt	mt/mt	OR[a] (95% CI)	P
CYP1A1 T6235C	82.5	16.4	1.1	81.0	18.5	0.5	0.89 (0.49–1.60)[b]	0.68
CYP1A1 Ile462Val	93.2	6.8	0.0	91.8	8.2	0.0	0.93 (0.40–2.25)[b]	0.87
CYP1B1 Val432Leu	32.3	49.2	18.6	14.9	51.8	33.3	2.70 (1.53–4.86)[b]	<0.001
GSTP1 Ile104Val	44.1	45.7	10.2	43.6	38.5	17.9	0.80 (0.50–1.29)[b]	0.37
	+[c]	−[d]		+[c]	−[d]			
GSTM1	53.1	46.9		44.1	55.9		1.33 (0.83–2.13)	0.23
GSTT1	77.6	22.4		78.9	21.1		0.94 (0.54–1.67)	0.84

[a] The associations between the genotype distributions and the patients' status were assessed by ORs and CIs that were calculated by unconditional logistic regression, adjusting for age and gender.
[b] Calculation for wt/wt genotype vs. wt/mt and mt/mt genotypes.
[c] GSTM1- and GSTT1-expressors.
[d] Deleted *GSTM1* and *GSTT1* genotypes.

Table 4 *Genotype distribution of described XME polymorphisms in smoking and nonsmoking HNSCC patients*

	Nonsmokers (n = 117)			Smokers (n = 195)				
	wt/wt	wt/mt	mt/mt	wt/wt	wt/mt	mt/mt	OR[a] (95% CI)	P
CYP1A1 T6235C	76.9	22.2	0.9	81.0	18.5	0.5	0.80 (0.45–1.43)[b]	0.45
CYP1A1 Ile462Val	88.0	12.0	0.0	91.8	8.2	0.0	0.65 (0.30–1.42)[b]	0.27
CYP1B1 Val432Leu	43.6	48.7	7.6	14.9	51.8	33.3	4.53 (2.62–7.98)[b]	<0.001
GSTP1 Ile104Val	51.3	40.2	8.5	43.6	38.5	17.9	1.36 (0.86–2.16)[b]	0.19
	+[c]	−[d]		+[c]	−[d]			
GSTM1	51.3	48.7		44.1	55.9		1.33 (0.84–2.11)	0.22
GSTT1	80.3	19.7		78.9	21.1		1.09 (0.61–1.93)	0.77

[a] The associations between the genotype distributions and the patients' status were assessed by ORs and CIs that were calculated by unconditional logistic regression, adjusting for age and gender.
[b] Calculation for wt/wt genotype vs. wt/mt and mt/mt genotypes.
[c] GSTM1- and GSTT1-expressors.
[d] Deleted *GSTM1* and *GSTT1* genotypes.

CYP1B1 polymorphism in the group of smokers ($P < 0.001$; Table 4). The odds of smokers carrying the *CYP1B1*2/*2* allele were 4.5 times higher (95% CI, 2.62–7.98) than for nonsmokers. No significant differences were found in genotype distribution of *GSTM1*, *GSTT1*, *GSTP1*, or *CYP1A1* polymorphisms between smoking and nonsmoking patients (Table 4).

Because the polymorphism of the *CYP1B1* gene seems to have an impact on the risk for HNSCC in smokers, we investigated whether combined polymorphisms of *CYP1B1* and described *GST* genes act synergistically on the cancer risk. Tables 5, A and B, show the results of our combined genotype analyses. As shown in Table 5A, genotypes with *CYP1B1*2* and *GSTM1*2* alleles were more frequent in the group of smokers when compared with the group of nonsmokers, and the ORs increased synergistically with the exchange of Val to Leu in CYP1B1. The combined *CYP1B1*2/*2* and *GSTM1*2/*2* genotypes had an OR of 12.8 (95% CI, 4.09–49.7) compared with smokers with *CYP1B1* and *GSTM1* wild types (Table 5A). The combined genotype analysis of *CYP1B1* and *GSTP1* (Table 5B) showed a similar trend. The OR increased with the exchange of Val to Leu in the CYP1B1 and Ile to Val in the GSTP1 protein. In the group of smokers, eight patients were homozygous for the mutated *CYP1B1* and *GSTP1*, whereas this combination was not present in the group of nonsmoking patients (Table 5B). In contrast with the results obtained by combined genotype analyses of *CYP1B1* and *GSTM1* or *GSTP1*, the *GSTT1*2/*2* genotype, in combination with the polymorphic alleles of *CYP1B1*, seemed to attenuate the probability of smoking-induced HNSCC (Table 5A). The OR for smokers carrying the mutated *CYP1B1*2* and *GSTT1*2* alleles were nearly half of that which have been found for smokers having mutated *CYP1B1*2* alleles and functional GSTT1 protein (Table 5*A*). The value for combined *CYP1B1*2/*2* and *GSTT*2/*2* genotype was 13.4 (95% CI, 2.92–97.7), and the value for the genotype *CYP1B1*2/*2* and *GSTT1*-expressor was 24.1 (95% CI, 9.36–70.5; Table 5A).

Table 5

A. *Genotype combinations of xenobiotic metabolizing enzymes in cases (non smokers versus smokers)*

Genotypes		HNSCC patients		
CYP1B1	*GSTM1*	Nonsmokers (n = 117)	Smokers (n = 195)	OR[a] (95% CI)
**1/*1*	+[b]	25	17	1.00[c]
	−[d]	26	12	0.72 (0.27–1.84)
**1/*2*	+	30	40	1.93 (0.87–4.36)
	−	27	61	3.59 (1.64–8.08)[e]
**2/*2*	+	5	29	10.9 (3.57–39.3)[e]
	−	4	36	12.8 (4.09–49.7)[e]
	GSTT1			
**1/*1*	+[b]	44	16	1.00[c]
	−[d]	7	13	5.45 (1.82–17.7)[e]
**1/*2*	+	43	82	5.39 (2.72–11.1)[e]
	−	14	19	3.80 (1.53–9.81)[e]
**2/*2*	+	7	56	24.1 (9.36–70.5)[e]
	−	2	9	13.4 (2.92–97.7)[e]

B. *Genotype combinations of CYP1B1 and GSTP1 in cases (non-smokers versus smokers)*

CYP1B1	*GSTP1*	Nonsmokers (n = 117)	Smokers (n = 195)	
**1/*1*	Ile/Ile	26	13	1.00[c]
	Ile/Val	19	11	0.78 (0.29–2.04)
	Val/Val	6	5	0.86 (0.21–3.36)
**1/*2*	Ile/Ile	30	39	1.63 (0.76–3.54)
	Ile/Val	23	40	2.14 (0.98–4.79)
	Val/Val	4	22	7.01 (2.20–27.6)[e]
**2/*2*	Ile/Ile	4	33	11.2 (3.62–43.6)[e]
	Ile/Val	5	24	6.24 (2.09–21.8)[e]
	Val/Val	0	8	N.A.[f]

[a] The associations between the genotype distributions and the patients' status were assessed by ORs and CIs that were calculated by unconditional logistic regression, adjusting for age and gender.
[b] GSTM1- and GSTT1-expressors.
[c] Reference category.
[d] Deleted *GSTM1* and *GSTT1* genotypes.
[e] $P < 0.01$.
[f] N.A., not analyzed.

Aberrations of the *p53* Gene among Smokers with HNSCC Classified by *CYP1B1* Genotypes. We also investigated the possible interaction of *CYP1B1* polymorphism with an early end point of carcinogenesis, as indicated by somatic mutations of the *p53* gene. Tumor DNA from 140 HNSCC patients were screened for aberrations of the *p53* gene, and tumor-specific aberrations were detected in 66 cases (47.1%). The frequency of somatic mutations of the *p53* gene in smokers (46 of 76) was 1.9 times higher than in nonsmokers (20 of 64). A strong association (Table 6) between aberration frequencies of the *p53* gene and *CYP1B1* genotypes was found for smokers. Patients with the genotype *CYP1B1*1/*2* were ~7 times more likely (OR, 7.19; 95% CI, 1.56–52.4) to show smoking-induced somatic mutations of the *p53* gene than those with *CYP1B1* wild type (Table 6). The OR value increased to 20 for patients with genotype *CYP1B1*2/*2* (OR: 20.0; 95% CI, 3.95–160), indicating that the exchange of amino acid Val to Leu is strongly associated with the frequency of smoking-induced *p53* gene mutations. The strong gene-dose-dependent effect of mutant *CYP1B1* alleles on *p53* mutations indicates the importance of CYP1B1 in tobacco-induced HNSCC, because no such association was found in nonsmokers (Table 6).

Table 6 *Association between p53-mutations and CYP1B1-genotypes in smokers and nonsmokers*

	HNSCC Patients Nonsmokers (n = 64)			HNSCC Patients Smokers (n = 76)		
	Normal p53	Mutant p53	OR[a] (95% CI)	Normal p53	Mutant p53	OR[a] (95% CI)
*CYP1B1*1/*1*	13	16	1.00[b]	10	2	1.00[b]
*CYP1B1*1/*2*	24	4	0.10 (0.02–0.37)[c]	14	21	7.19 (1.56–52.4)
*CYP1B1*2/*2*	7	0	N.A.[d]	6	23	20.0 (3.95–160)[c]

[a] The associations between the genotype distributions and the patients' status were assessed by ORs and CIs that were calculated by unconditional logistic regression, adjusting for age and gender.
[b] Reference category.
[c] $P < 0.01$.
[d] N.A., not analyzed.

Effects of the Combined Genotypes of *CYP1B1* and *GSTM1* or *GSTT1* Genes on *p53* Gene Aberrations among Smokers with HNSCC. To determine whether different genotype combinations of C*YP1B1* and *GSTM1* or *GSTT1* affect the frequency of smoking-related *p53* gene mutation, each of three genotypes of *CYP1B1* gene were combined with each of the two genotypes of the *GSTM1* and *GSTT1* genes. The results showed that mutation frequencies of the *p53* gene were lower or higher in each of the combined genotypes of *CYP1B1* and *GSTM1* or *GSTT1* (Table 7). A mutation frequency of 80% (16 of 20) was observed for the combined *CYP1B1*2/*2* and *GSTM1*2/*2* genotypes. The odds of these patients having smoking-induced mutations of the *p53* gene was about 26 times higher (OR, 26.8; 95% CI, 3.41–590) than those having the *CYP1B1*1/*1* and *GSTM1*-expressor genotype. Similar results were obtained through the combined genotype analysis of *CYP1B1* and G*STT1* (Table 7). The data indicates a high probability (OR, 25.8; 95% CI, 2.42–700) of *p53* gene mutations for smokers with the combined *CYP1B1*2/*2 and *GSTT1*2/*2* genotypes. The results of this combined genotype analysis suggest a synergistic effect of *CYP1B1* and *GSTM1* or *GSTT1* polymorphisms on the occurrence of *p53* mutations. However, the low number of cases in this study must be considered.

p53 Mutational Spectrum in HNSCC. To confirm the mutation sites and types of the *p53* gene, PCR direct sequence analyses were performed. In smokers, G→T transversions were found more frequently (27%) than in nonsmokers (10%); whereas A→G mutations were more frequent in nonsmokers (25%) than in smokers (15%). The other mutations found in smokers with HNSCC were: G→A, 7%; C→T, 11%; T→C, 4%; A→T; 0%; T→A, 2%; G→C, 4%; C→A, 2%; Del, 24%; and Ins, 4%. The respective mutations in nonsmokers were: 0%, G→A; 15%, C→T; 5%, T→C; 10%, A→T; 5%, T→A; 5%, G→C; 0%, C→A; 20%, Del; and 5%, Ins. There was no correlation between the mutational spectrum of the *p53* gene and *CYP1B1* polymorphisms (data not shown).

DISCUSSION

This is the first study presenting data on the role of *CYP1B1* codon 432 polymorphism as a susceptibility factor in smoking-related HNSCC. Genotype analyses of *CYP1B1* and polymorphic *GST* genes revealed a significant influence of the combined polymorphisms on the vulnerability of smokers for HNSCC. Several groups have investigated the possible interference of polymorphic variants of phase 1 and phase 2 enzymes on HNSCC. Matthias *et al.* (43) found no differences in the distribution pattern of certain polymorphic *CYP1A1* alleles or *GSTT1* alleles between patients and control individuals. A moderate influence on the risk of HNSCC has been observed for *GSTM1* polymorphisms. The genotype *GSTM1*A/*B* was found to be protective, whereas the *GSTM3*A/*A* genotype seemed to confer an increased risk (43, 44). Deakin *et al.* (45) studied the influence of *GSTT1* and *GSTM1* polymorphisms on the susceptibility to oral cancer. They found no association between *GSTT1* or *GSTM1* genotypes and susceptibility to oral cancer. Similar results have been reported for *GSTM1* polymorphism (46) and for *GSTM1* or *GSTT1* polymorphisms (47), respectively. A small-sized study (48) revealed that the absence of GSTM1 and GSTT1 proteins confer an increased risk of HNSCC. An association between *GSTP1* polymorphism and susceptibility to oral squamous cell carcinoma was found in a Japanese population (49). However, the effect was moderate (OR, 1.93; 95% CI, 1.05–3.58), and no consistent difference in the smoking status between patients and the corresponding controls could be observed. The effect of *GSTP1* polymorphism on susceptibility to oral/pharyngeal and laryngeal carcinomas was also investigated previously (42). The frequency of *GSTP1*A/*A* was lower in the oral/pharyngeal cancer cases than in the controls. The effect was strongly correlated with the site-specific location of the carcinoma after adjustment for age and gender, but was independent of the smoking behavior of the patients. In the present study, we found no significant differences among the genotype distribution of polymorphic *GSTM1*,

Table 7 *Effect of combined genotypes of CYP1B1 and GSTM1 or CYP1B1 and GSTT1 on p53 mutation frequencies*

Genotypes		HNSCC Patients Smokers (n = 76)			Genotypes		HNSCC Patients Smokers (n = 76)		
CYP1B1	*GSTM1*	Normal p53	Mutant p53	OR[a] (95% CI)	*CYP1B1*	*GSTT1*	Normal p53	Mutant p53	OR[a] (95% CI)
**1/*1*	+[b]	7	1	1.00[c]	**1/*1*	+[b]	3	2	1.00[c]
	−[d]	3	1	1.59 (0.05–53.7)		−[d]	7	0	N.A.[e]
**1/*2*	+	4	8	12.5 (1.48–283)	**1/*2*	+	12	18	7.57 (1.60–56.5)
	−	10	13	7.23 (1.00–149)		−	2	3	5.19 (0.47–74.4)
**2/*2*	+	2	7	20.0 (1.91–536)	**2/*2*	+	5	18	19.1 (3.58–159)[f]
	−	4	16	26.8 (3.41–590)[f]		−	1	5	25.8 (2.42–700)

[a] The associations between the genotype distributions and the patients' status were assessed by ORs and CIs that were calculated by unconditional logistic regression, adjusting for age and gender.
[b] GSTM1- and GSTT1-expressors.
[c] Reference category.
[d] Deleted *GSTM1* and *GSTT1* genotypes.
[e] N.A., not analyzed.
[f] $P < 0.01$.

GSTT1, or *GSTP1* genes between HNSCC cases and controls, and no consistent association of different *GST* genotypes to patient smoking behavior could be observed. Although there is a body of evidence which suggests that polymorphisms of different *GST* genes influence the susceptibility to cancer (especially to lung cancer), the results of the present study suggest that polymorphisms of *GSTM1, GSTT1,* or *GSTP1* genes alone have only minor importance on the susceptibility of smokers to HNSCC.

CYP1A1 is also a candidate for susceptibility for smoking-related cancer, and several studies on a Japanese population have indicated that the mutant *CYP1A1* alleles, alone or in combination with *GSTM1*2/*2* genotype, contribute to an increased risk of lung cancer (35). However, the frequency of mutant *CYP1A1* alleles is rare in Caucasians, and it is therefore difficult to reproduce the same results. We could not identify any effect of *CYP1A1* alone or in combination with either *GSTM1* or *GSTT1* genotypes on the risk of HNSCC; confirming the findings of Matthias *et al.* (43). It is likely that the *CYP1A1* gene plays only a minor role in detoxification of tobacco-derived chemicals in Caucasians. Alternatively, the effect may also be masked by the involvement of other *CYP* genes in these metabolisms.

The data obtained from *CYP1B1* genotyping, clearly indicates an association between the susceptible genotypes of *CYP1B1* and HNSCC. Smoking cases were 4.5 times more likely to carry the susceptible *CYP1B1* genotype than nonsmoking cases; and the OR increased considerably when the susceptible genotypes of *CYP1B1* were combined with either polymorphic *GSTM1* or *GSTP1* genes. Similar results have been reported for the combination of *CYP1A1* polymorphisms and the *GSTM1*2/*2* genotype in lung cancer (50, 51), confirming the hypothesis that imbalances in the drug metabolism system attributable to inherent genetic variations can confer a risk to individuals when exposed to chemicals. Our observation that the combination of *CYP1B1* variants with the *GSTT1*2/*2* genotype was underrepresented in smoking HNSCC cases is remarkable, because most studies have reported that *GSTM1*- or *GSTT1*-deficient individuals are more susceptible to the genotoxic actions of chemicals. The conclusion that there is likely an increased susceptibility of the *CYP1B1*2/*2* and GSTT1-expressors is of particular biological significance because GSTT1 is involved in both the activation and inactivation of a variety of environmental and industrial chemicals, including methylene chloride, ethylene chloride, and 1,3-butadiene (52). For example, a bioactivation of haloalkanes catalyzed by GSTT1 has been described (52). Combining these haloalkanes with glutathione generates episulfonium ions, which bind to DNA. Another explanation for the lower risk of *CYP1B1*2/*2* and *GSTT1*2/*2*-genotypes could be that individuals lacking GSTM1 or GSTT1 enzymes are thought to possess higher tissue levels of glutathione than GSTM1- or GSTT1-expressors (27). Thus, increased tissue levels of glutathione might be protective against the accumulation of reactive intermediates, including oxygen radicals, which are thought to be involved in tobacco-induced carcinogenesis.

The role of *CYP1B1* in smoking-induced HNSCC carcinogenesis is reflected by the results of mutation analysis of the cancer target gene *p53*, which was affected significantly by the *CYP1B1* genotypes. Smokers with HNSCC who have the susceptible *CYP1B1*2/*2* genotype are 20 times more likely to show *p53* mutations than those with the nonsusceptible *CYP1B1*1/*1* genotype. This data indicates a strong and consistent association of the *CYP1B1* Val432Leu polymorphism and smoking-induced *p53* mutations along with a strong gene-dose-dependent effect. In addition, the findings of combined *CYP1B1* genotype with either the *GSTM1* or *GSTT1* genotype indicate an interactive effect on *p53* mutation frequency. However, because of the small number of cases and the wide range of CIs, the data has to be cautiously interpreted. In addition, the small number of cases in the *p53* analysis may be the cause of the obvious discrepancies of the data in Tables 5 and 7. Whereas, in Table 5, an under-representation of the combined *CYP1B1*2/*2* and *GSTT1*2/*2* genotypes in smokers with HNSCC was presented, the data in Table 7 possibly indicates an interactive effect of the combined genotypes on *p53* mutations. Although these problems have to be resolved by an extended study, our findings nevertheless support the hypothesis that polymorphic variants of *GSTM1* or *GSTT1* are moderately strong susceptibility factors for HNSCC, but may become a dominant factor in the presence of certain gene-gene combinations, as has been demonstrated for *CYP1B1*.

The phenotypic importance of *CYP1B1* codon 432 polymorphism for metabolic activation of xenobiotics, is still unknown. Recently, it was shown that this polymorphism of *CYP1B1* is associated with a 3–4-fold increase of the K_m values for 2- and 4-hydroxylation of estradiol (20), whereas Hanna *et al.* (53) reported that the four polymorphic variants of *CYP1B1* displayed 2.4–3.4-fold higher catalytic efficiencies in estrogen hydroxylation activity than the wild type enzyme. The polymorphisms at residue 432 in *CYP1B1,* however, had no significant influence on CYP1B1-mediated epoxidation of (−)-*trans*-(7R,8R)benzo(*a*)pyrene-7.8-dihydrodiol, deethylation of ethoxyresorufine and hydroxylation of bufuralol (20). Influence of different allelic variants of the *CYP1B1* gene on the catalytic activity toward 19 procarcinogenic compounds has been investigated. It was found that the Arg48, Ser119, Leu432, and Asn 453 variants were slightly more active in catalyzing activation of PAHs, *e.g.,* (+)- and (−)-benzo(*a*)pyrene-7–8-diols, 7,12-demethylbenz(*a*)anthracene-3,4-diol, benzo(*g*)chrysene-11–12-diols, and benzo(*b*)fluoreanthene-9–10-diol (54). In considering these data, and based on our findings, we speculate that the carcinogenic agents in cigarette smoke preferentially metabolized by polymorphic CYP1B1 seem unrelated to PAHs. It is clear that these compounds have to be identified. In addition, the phenotypic importance of *CYP1B1* codon 432 polymorphism in the metabolism of xenobiotics remains to be seen.

In conclusion, we have demonstrated that the *CYP1B1* Val432Leu polymorphism is an inheritable predisposing factor for smoking-induced HNSCC, and we have shown that *CYP1B1* polymorphism is associated with an increased frequency of smoking-induced *p53* mutations. Furthermore, we have provided preliminary data of an interactive effect of combined *CYP1B1* and *GST* genotypes on the risk of smokers for HNSCC and on the frequencies of *p53* mutations. To substantiate our findings, we are carrying out an extended molecular epidemiological study, which should provide additional clues to the importance of the combination of polymorphic variants of the *CYP1B1* and *GST* genes as genetic determinants for HNSCC.

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