Polymorphisms in Metabolic Genes Related to Tobacco Smoke and the Risk of Gastric Cancer in the European Prospective Investigation into Cancer and Nutrition

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Abstract

Metabolizing enzymes, which often display genetic polymorphisms, are involved in the activation of compounds present in tobacco smoke that may be relevant to gastric carcinogenesis. We report the results of a study looking at the association between risk of gastric adenocarcinoma and polymorphisms in genes *CYP1A1, CYP1A2, EPHX1*, and *GSTT1*. A nested case-control study was carried out within the European Prospective Investigation into Cancer and Nutrition, developed in 10 European countries. The study includes 243 newly diagnosed cases of histologically confirmed gastric adenocarcinoma and 946 controls matched by center, age, sex, and date of blood collection. Genotypes were determined in nuclear DNA from WBCs. We found an increased risk of gastric cancer for homozygotes for C (histidine) variant in *Y113H* of EPHX1 (odds ratio, 1.91; 95% confidence interval, 1.19-3.07) compared with subjects with TC/TT. There was also a significant increased risk for smokers carrying at least one variant allele A in Ex7+129C>A (*m4*) of CYP1A1 and never smokers with null GSTT1 and allele A in the locus −3859G>A of CYP1A2. Most of these genes are involved in the activation and detoxification of polycyclic aromatic hydrocarbons, suggesting a potential role of these compounds in gastric carcinogenesis. (Cancer Epidemiol Biomarkers Prev 2006;15(12):2427–34)

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Introduction

Although the relationship between gastric cancer and tobacco smoking had been controversial, a recent review concluded that there is sufficient evidence that tobacco smoking causes cancer of the stomach in humans (1). Tobacco smoke is a complex mixture of chemical substances, including >60 known or potential carcinogens, such as polycyclic aromatic hydrocarbons (PAH), *N*-nitrosamines, and heterocyclic amines (2). Several components of these groups have been determined as carcinogenic in experimental animals, but the available evidence about gastric cancer risk in humans is scarce and rather indirect.

As many chemicals, PAHs do not react directly with cellular constituents and require enzymatic conversion into the ultimate carcinogenic form; the process of activation of PAH involves the formation of bay-region diol epoxides (3). The transformation into the ultimate carcinogen (PAH-DE) is achieved through oxidation by cytochrome *P*450 enzymes, mainly CYP1A1, and hydrolysis by microsomal epoxide hydrolase (EPHX1); the PAH-DE can be detoxified through conjugation with glutathione by phase II enzymes glutathione *S*-transferases (GST). Another cytochrome *P*450 enzyme, such as CYP1A2 and CYP2E1, as well as *N*-acetyltransferases activate tobacco procarcinogens, notably heterocyclic amines, *N*-nitrosamines, and aromatic amines (3).

We have previously reported (4) the association between tobacco smoking and increased risk of cancer of the stomach in the large cohort European Prospective Investigation into Cancer and Nutrition (EPIC). A nested case-control study within the cohort was set up to examine, among other purposes, the contribution of genetic susceptibility to gastric carcinogenesis: most metabolizing enzymes involved in the process of carcinogenic activation often display genetic polymorphisms and can thereby modulate the risk of cancer (5). We explored the effect of polymorphisms in genes involved in the metabolism of tobacco smoking–related carcinogens that could also be associated with gastric cancer (6). In this article, we present the results on the association between risk of gastric adenocarcinoma and genetic polymorphisms in genes *CYP1A1, CYP1A2, EPHX1*, and *GSTT1* and their possible differential effect according to tobacco smoking. Other polymorphisms encoding for enzymes involved in the metabolism of potential carcinogens (CYP2E1, GSTM1, NAT1, and NAT2) were analyzed; because no association with gastric cancer was found for any of them, detailed results about these genes have been reported separately (7).

Materials and Methods

Subjects. The study subjects were selected from the EPIC cohort according to a nested case-control design. The methods and rationale of the EPIC study have been presented elsewhere (8). Briefly, the EPIC cohort includes about half million individuals recruited between 1992 and 1998 in 23 centers in 10 European countries: Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom. At enrollment, each subject provided information about usual diet and lifestyle factors; anthropometric data and blood samples were also collected for most participants. Follow-up is based on population cancer registries in most countries, except in France, Germany, and Greece where it is mainly achieved by active contact with study subjects, and review of health insurance and pathology reports. In this study, the follow-up was complete until December 2000 or December 2001 for countries using cancer registry data and December 2002 for the remaining three countries. The follow-up in Norway was too short for what the Norwegian cohort was excluded. Only subjects having blood collected were considered for inclusion in the case-control study.

Cases were subjects newly diagnosed during the follow-up of cancer of the stomach, defined by code C16 of the International Classification of Diseases, 10th Revision. An independent panel of pathologists reviewed original slides and/or cuts from paraffin blocks as well as pathology reports provided by each EPIC center to confirm and validate the diagnosis, tumor site, and morphology. Initially, 290 gastric cancer cases were identified; 4 cases of cancer located in gastric stump as well as 31 tumors other than adenocarcinoma were excluded. For each case, up to four control subjects were randomly selected among cohort members alive and free of cancer at the time of diagnosis of the case with blood samples available, matched by center, gender, age (±2.5 years), and date of blood collection (±45 days). There were no controls available for three cases, and thus, these cases were not included in the study; on the other hand, nine cases had no information on tobacco smoking and/or genotypes of genes of interest. The final group of analysis in this study consisted of 243 cases and 946 matched controls.

DNA Extraction. Genomic DNA from patients and controls was extracted from a 0.5 mL aliquot of buffy coat, which had been kept deep frozen since blood extraction and processing. With the exception of Malmö samples, all other DNAs were extracted at the IARC by use of the Puregene DNA Purification System (Gentra Systems, Minneapolis, MN) adapted to the Gentra Autopure LS DNA preparation platform (Gentra Systems). A Tecan Genesis pipetting robot was used to distribute DNA samples to 96-well plates for DNA concentration measurement with PicoGreen dsDNA quantitation assay and kit (Molecular Probes, Inc., Leiden, the Netherlands), drying, and further distribution. DNA from the frozen buffy coat straws from Malmö samples was extracted by the phenol-chloroform method and also distributed dried in 96-well plates. Before use, dried DNAs were reconstituted with water to a final concentration of 20 ng/μL for the IARC samples and 2 ng/μL for the Malmö samples and kept frozen.

Genotyping Analysis. Single-nucleotide polymorphism (SNP) genotyping was done in a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) by melting curve analysis of a fluorescently labeled sensor probe specific for each PCR-amplified variant.

Specific primers and hybridization probes for SNP analyses were obtained from published literature (9) or they were designed by TIB-MOLBIOL (Berlin, Germany) or by ourselves by use of the LightTyper Probe Design software from Roche Diagnostics according to the gene or cDNA sequences published in the Genbank or European Molecular Biology Laboratory databases. All PCR primers as well as the 3′-fluorescein and the 5′-LC-Red640 or 5′-LC-Red705 probes were synthesized by TIB-MOLBIOL and can be provided by the authors on request. A minimum of 10 test DNAs, different from the EURGAST ones, was used to standardize all the LightCycler genotyping protocols. The results obtained were confirmed by a second genotyping method, such as restriction analysis, single-strand conformational polymorphism analysis, or direct DNA sequencing, of a new PCR product. The CYP1A1 rs1048943 and rs1799814 (*m2* and *m4*) variants were detected with the same sensor probes, and therefore, they were analyzed simultaneously in the same LightCycler runs.

PCR mixtures contained 2 to 4 mmol/L $MgCl_2$, 0.4 or 0.5 μmol/L of each primer, 0.12 to 0.2 μmol/L of each hybridization probe, 1 μL of LightCycler FastStart DNA Master Hybridization mix, and 2 ng (Malmö samples) or 5 to 10 ng (IARC samples) of DNA in a final volume of 10 μL. General PCR conditions were as follows: initial denaturation at 95°C for 5 to 10 min and 45 cycles of 95°C for 2 to 5 s, 55°C to 62°C for 12 to 20 s, and 72°C for 15 to 25 s at transition rates of

20°C/s. The program for the melting curve analysis was 95°C for 5 s, 37°C to 65°C for 10 to 30 s with 20°C/s transition rate, and then ramping to 75°C to 98°C at transitions rates between 0.1°C/s and 0.25°C/s.

Analysis of the GSTT1-null allele was also done in the LightCycler instrument by melting curve analysis of the fragments amplified in a capillary PCR that contained specific primers for the amplification of *GSTT1* and *Bcl2* (used as an amplification control) fragments and the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics). The primers used to amplify the *GSTT1* (257 bp) and *Bcl2* (154 bp) fragments had been previously described (10), but the reverse primer for the *GSTT1* fragment was modified to obtain a shorter amplification product of 257 bp.

Gene and Polymorphism Nomenclature. Genes have been named according to the HUGO Gene Nomenclature committee.[34] Polymorphisms have been identified according to the SNP500Cancer database[35] and to the ID numbering of the dbSNP database of the National Center for Biotechnology Information.[36] Metabolic gene allele nomenclature according to that recommended by Garte et al. (11) is also provided.[37]

Other Factors. Subjects were classified according to smoking habit at recruitment as current, former, or never smokers of any type of tobacco products, although the majority of them smoked cigarettes exclusively or in addition to cigars and/or pipe. In the stratified analysis according to smoking status, current and former smokers were grouped together within the category of ever smokers. Antecedent of *Helicobacter pylori* (Hp) infection was determined by anti-Hp antibodies measured in plasma using ELISA.

Statistical Analysis. For each polymorphism, Hardy-Weinberg equilibrium (HWE) was tested separately for cases and controls. Pair-wise linkage disequilibrium was also tested for polymorphisms within the same gene (12). Association was assessed by the odds ratio (OR), with corresponding 95% confidence intervals (95% CI) estimated by logistic regression (13). ORs were calculated for each genotype compared with the homozygous group of the most frequent allele as well as for the dominant and/or recessive model depending on the frequency of each genotype. Effect modification of polymorphisms by smoking status was assessed by testing the homogeneity of the ORs in smokers and nonsmokers (12), whereas interaction between the two genes was assessed by the likelihood ratio test.

Given the matched design, the ORs for the main effect of each polymorphism with gastric cancer were calculated by conditional logistic regression (13). However, matched analysis when stratifying according to smoking status may produce loss of power because of exclusion of some risk sets due to stratification. For this reason, stratified analysis by smoking was carried out using unconditional logistic regression, including the matching variables in the model. We verified that both approaches, conditional and unconditional, with adjustment for matching variables gave approximately the same results in the whole data set.

We have previously shown that meat intake (14) as well as the consumption of vegetables and citrus fruits (15) are associated, respectively, with an increased risk or protection of gastric cancer; furthermore, it is well known that Hp infection is a major determinant of gastric cancer and that gastric cancer risk is associated with low educational level. All the estimates reported here may be considered adjusted by age, sex, and center given the matched design, but no further adjustment was initially done. Although the above-mentioned factors may be causally related to the disease, the subject's genotype is determined by an apparently random process, and thus, the distribution of genetic variants is meant to be independent of behavioral or environmental factors and its potential relationship with disease should not be confounded by such factors (16). Nevertheless, the inclusion of such factors in the logistic model for the main effects of SNPs did not modify the results, and in the remaining analyses, they were not adjusted for.

Results

In our study, 56% of cases (and matched controls) were males, with average age at recruitment of 59 years (Table 1). A lower proportion of cases attained the highest educational level and they had higher prevalence of Hp infection; furthermore, cases tended to consume regularly higher amounts of meat but less citrus fruits than controls. Of the 243 cases, 68 (28%) were located in the cardia (including 16 in the gastroesophageal junction) and 128 (53%) in noncardia regions; in 19% of cases, the tumor site was unknown. Histologic types intestinal and diffuse had about the same proportion of 38% (93 and 94 cases, respectively); the remainder 56 cases were classified as mixed or the histologic subtype was unknown. Compared with never smokers, current smokers had an increased risk of gastric cancer (adjusted by education, Hp infection, and the intake of vegetables, citrus fruit, and meat), with OR (95% CI) of 2.21 (1.46-3.33), whereas the corresponding figures for former smokers were 1.53 (1.06-2.21) and 1.76 (1.26-2.45) for ever smokers (current and former combined). The ORs (95% CI) for ever smokers were 2.03 (1.01-4.07) and 1.86 (1.17-2.96) for tumors located in the cardia and noncardia regions, respectively, 1.71 (0.95-3.05) for intestinal tumors, and 1.66 (1.00-2.76) for diffuse tumors. These results are in good agreement with those previously reported for the whole cohort (4).

The genotype distribution for all the polymorphisms analyzed is shown in Table 2. The polymorphism *Y113H* of EPHX1 was the only one showing no HWE in controls (P = 0.01): the expected proportions according to allelic frequencies were 50.2% (TT), 41.3% (TC), and 8.5% (CC) compared with the observed 48.3%, 45%, and 6.6%, respectively. Polymorphisms *m1* and *m2* of CYP1A1 showed linkage disequilibrium in controls: 57 of 62 (92%) subjects with the allele G in *m2* of CYP1A1 had also the allele C in *m1*.

Only the homozygous variant CC of *Y113H* in EPHX1 was significantly associated with increased gastric cancer risk (Table 3). There is some suggestion that the allele A in the CYP1A1 *m4* polymorphism and in CYP1A2 −3859G>A has an increased risk, whereas a decreased risk was suggested for allele C in CYP1A1 *m1*; none of these associations reached statistical significance. The point estimates and corresponding 95% CIs for most polymorphisms are quite similar with or without taking into account Hp infection, education, and the intake of meat, vegetables, and citrus fruits, confirming that these factors are not related to the genotype distribution and thus do not confound the association with gastric cancer. In the remaining analyses, only the raw estimates (adjusted only by age, sex, and center) are shown.

To avoid loss of power, the stratified analyses according to smoking status (Table 4) were carried out only for dominant models, except for the two SNPs of EPHX1, where the effect was rather associated with recessive model. The significant association for *Y113H* in EPHX1 observed in the overall analysis seems to be limited to ever smokers. Statistically significant associations were also observed in two SNPs of CYP1A1, *m1* (decreased risk) and *m4* (increased risk), whereas

[34] http://www.genomic.unimelb.edu.au.
[35] http://snp500cancer.nci.nih.gov/home.cfm.
[36] http://www.ncbi.nlm.nih.gov/SNP.
[37] http://www.gsec.net.

Table 1. Main characteristics of cases and controls

		Cases (n = 243), n (%)	Controls (n = 946), n (%)
Sex	Male	136 (56.0)	520 (55.0)
	Female	107 (44.0)	426 (45.0)
Country	France	3 (1.2)	11 (1.2)
	Italy	44 (18.1)	174 (18.4)
	Spain	29 (11.9)	115 (12.2)
	United Kingdom	28 (11.5)	105 (11.1)
	The Netherlands	18 (7.4)	80 (8.5)
	Greece	11 (4.5)	44 (4.7)
	Germany	30 (12.3)	124 (13.1)
	Sweden	58 (23.9)	225 (23.8)
	Denmark	22 (9.1)	68 (7.2)
Smoking status	Never smoker	83 (34.2)	425 (44.9)
	Former smoker	87 (35.8)	328 (34.7)
	Current smoker	73 (30.0)	193 (20.4)
Education	None	12 (4.9)	65 (6.9)
	Primary	103 (42.4)	360 (38.1)
	Technical	57 (23.5)	211 (22.3)
	Secondary	37 (15.2)	146 (15.4)
	University	32 (13.2)	151 (16.0)
	Not specified	2 (0.8)	13 (1.4)
Hp infection*	Not infected	39 (16.2)	299 (31.7)
	Infected	202 (83.8)	644 (68.3)
Age (y) at recruitment	Mean (SD)	59.1 (0.51)	59.5 (0.25)
Total vegetables (g/d)	Mean (SD)	184.5 (141.1)	180.4 (131.0)
Citrus fruit (g/d)	Mean (SD)	51.3 (68.8)	59.0 (75.6)
Total meat (g/d)	Mean (SD)	115.0 (62.3)	107.4 (57.6)

*Two cases and three controls without information on Hp serology.

the significantly higher risks for −3859G>A of CYP1A2 and deletion of GSTT1 were restricted to never smokers; deletion of GSTT1 showed statistically significant effect modification by tobacco smoking status. Only polymorphisms with significant associations, either in the overall or smoking-stratified analyses, were further analyzed by tumor site or histologic type (Table 5). Associations tended to be more evident and significant for tumors located in the noncardia regions and for those of intestinal type. In both cases, there were particularly consistent associations for CYP1A1 *m4* and *Y113H* in EPHX1, mainly among smokers, whereas for GSTT1 the effect was observed only among never smokers.

Table 2. Frequency distribution of genotypes for polymorphisms in metabolic genes in gastric adenocarcinoma cases and controls

Gene	Polymorphism	Genotype	Cases, n (%)	Controls, n (%)
*CYP1A1**	1,188 bp 3′ of STP T>C (*m1*) rs4646903	TT	210 (86.4)	765 (81.4)
		TC	33 (13.6)	167 (17.8)
		CC	—	8 (0.9)
		Total	243	940
	Ex7+131A>G (*I462V*, *m2*) rs1048943	AA	229 (94.2)	874 (93.4)
		AG	13 (5.3)	62 (6.6)
		GG	1 (0.4)	0 (0.0)
		Total	243	936
	Ex7+129C>A (*T461N*, *m4*) rs1799814	CC	216 (88.9)	864 (92.3)
		CA	27 (11.1)	69 (7.4)
		AA	—	3 (0.3)
		Total	243	936
CYP1A2	IVS1-154A>C rs762551	AA	111 (45.9)	474 (50.3)
		AC	115 (47.5)	399 (42.3)
		CC	16 (6.6)	70 (7.4)
		Total	242	943
	−3859G>A rs2069514	GG	235 (96.7)	926 (98.0)
		GA	8 (3.3)	19 (2.0)
		AA	—	—
		Total	243	945
EPHX1	Ex3-28T>C (*Y113H*) rs1051740	TT	105 (43.2)	453 (48.3)
		TC	110 (45.3)	422 (45.0)
		CC	28 (11.5)	62 (6.6)
		Total	243	937
	Ex4+52A>G (*H139R*) rs2234922	AA	156 (64.5)	615 (65.1)
		AG	76 (31.4)	289 (30.6)
		GG	10 (4.1)	40 (4.2)
		Total	242	944
GSTT1	del{GSTT1}	Present	205 (84.7)	801 (85.9)
		Null	37 (15.3)	131 (14.1)
		Total	242	932

*SNP *m1* and *m2* in linkage disequilibrium: $r^2 = 0.23$, D′ = 0.86 ($P < 0.001$).

Table 3. OR and 95% CI of gastric adenocarcinoma for SNPs in metabolic genes

Gene	Polymorphism	Model	Effect	Reference	OR (95% CI)*	OR (95% CI)†
CYP1A1	1,188 bp 3′ of STP T>C (*m1*)	Genotype	TC	TT	0.74 (0.49-1.10)	0.74 (0.49-1.11)
			CC	TT	—	—
		Dominant	CC/TC	TT	0.70 (0.47-1.04)	0.70 (0.47-1.06)
	Ex7+131A>G (*I462V*, *m2*)	Genotype	AG	AA	0.82 (0.44-1.51)	0.90 (0.48-1.68)
			GG	AA	—	—
		Dominant	GG/AG	AA	0.88 (0.49-1.60)	0.98 (0.53-1.80)
	Ex7+129C>A (*T461N*, *m4*)	Genotype	CA	CC	1.57 (0.99-2.50)	1.55 (0.96-2.50)
			AA	CC	—	—
		Dominant	AA/CA	CC	1.53 (0.96-2.43)	1.51 (0.94-2.43)
CYP1A2	IVS1-154A>C	Genotype	AC	AA	1.27 (0.94-1.71)	1.29 (0.95-1.76)
			CC	AA	1.01 (0.56-1.82)	1.02 (0.56-1.88)
		Dominant	CC/AC	AA	1.23 (0.92-1.65)	1.26 (0.93-1.70)
		Recessive	CC	AC/AA	0.89 (0.51-1.56)	0.89 (0.50-1.59)
	−3859G>A	Genotype	GA	GG	1.92 (0.79-4.65)	2.12 (0.85-5.31)
			AA	GG	—	—
		Dominant	AA/GA	GG	1.92 (0.79-4.65)	2.12 (0.85-5.31)
EPHX1	Ex3-28T>C (*Y113H*)	Genotype	TC	TT	1.15 (0.85-1.56)	1.11 (0.81-1.51)
			CC	TT	2.05 (1.25-3.37)	1.96 (1.18-3.26)
		Dominant	CC/TC	TT	1.27 (0.95-1.68)	1.22 (0.91-1.63)
		Recessive	CC	TC/TT	1.91 (1.19-3.07)	1.87 (1.14-3.04)
	Ex4+52A>G (*H139R*)	Genotype	AG	AA	1.07 (0.78-1.46)	1.07 (0.78-1.48)
			GG	AA	0.90 (0.45-1.82)	0.94 (0.46-1.92)
		Dominant	GG/AG	AA	1.04 (0.78-1.40)	1.06 (0.78-1.43)
		Recessive	GG	AG/AA	0.88 (0.44-1.77)	0.92 (0.45-1.86)
GSTT1	del{GSTT1}		Null	Present	1.13 (0.76-1.67)	1.05 (0.70-1.57)

*OR (95% CI) by conditional logistic regression; matching variables: sex, age, center, and date of blood extraction.
†OR as (*) and further adjusted for Hp infection, total meat, total vegetables and citrus fruit intake, and educational level.

Finally, we considered gene-gene interactions of EPHX1 (*Y113H*) with CYP1A1 (*m4*) and with GSTT1 deletion (Table 6) because they are supposed to be involved in a common metabolic pathways. Combination of allele A in CYP1A1 *m4* with homozygous CC in *Y133H* of EPHX1 among smokers had an OR close to the multiplicative effect, whereas combination of CC in *Y133H* of EPHX1 with null GSTT1 among nonsmokers had an estimate far higher than expected by multiplicative effect of both SNP, although interaction was not statistically significant.

Discussion

We have observed an increased risk of gastric adenocarcinoma for subjects with homozygous variant in *Y113H* of EPHX1; there was also an increased risk for smokers carrying at least one variant allele A (coding for asparagine) in CYP1A1 *m4* (*T461N*), whereas null GSTT1 and allele A in CYP1A2 −3859G>A were associated with increased risk of developing gastric cancer among nonsmokers. Overall, the effects reported are more evident for tumors located in the noncardia region of the stomach and especially for those of intestinal type.

Genes from the cytochrome *P*450 family encode enzymes involved in oxidation of a variety of compounds; CYP1A1 is involved in the activation of PAH and aromatic amines and is present in many epithelial tissues. SNPs of CYP1A1 termed as *m1* (at 1,188 bp 3′ of the stop codon) and *m2* (*I462V*) have been often found associated with risk in several smoking-related tumors (3). Only two case control studies have reported results for gastric cancer in relation with *m2* mutation: one in Korea observed a weak but significant 34% increase in risk (17), whereas no association was found in a study in Japan (18). We observed a tendency to decreased risk for both *m1* (significant among smokers) and *m2* mutations. On the other hand, we found an increased risk for the relatively uncommon SNP *m4* involving the substitution of asparagine for threonine in the amino acid position 461 of the protein. This mutation was found not to be associated with lung or breast cancer in Caucasian populations (3); thus far, we are not aware of any association reported for CYP1A1 *m4* about gastric cancer. The two SNP *m1* and *m2* were in linkage disequilibrium; in the haplotype analysis (results not shown), it was found that a

Table 4. OR and 95% CI of gastric adenocarcinoma for SNPs in metabolic genes according to smoking status

Gene	Polymorphism	Model	Never smokers (83 cases, 425 controls)		Ever smokers (160 cases, 521 controls)		*P* for homogeneity
			Cases/controls*	OR† (95% CI)	Cases/controls*	OR† (95% CI)	
CYP1A1	1,188 bp 3′ of STP T>C (*m1*)	CC/TC vs TT	15/73	1.09 (0.58-2.05)	18/102	0.50 (0.29-0.87)	0.06
	Ex7+131A>G (*I462V*, *m2*)	GG/AG vs AA	7/31	1.26 (0.52-3.08)	7/31	0.75 (0.32-1.76)	0.42
	Ex7+129C>A (*T461N*, *m4*)	AA/CA vs CC	8/42	0.87 (0.38-1.99)	19/30	2.27 (1.21-4.27)	0.12
CYP1A2	IVS1-154A>C	CC/AC vs AA	48/213	1.56 (0.95-2.55)	83/256	1.15 (0.80-1.66)	0.52
	−3859G>A	AA/GA vs GG	5/8	3.44 (1.01-11.7)	3/11	1.01 (0.27-3.80)	0.22
EPHX1	Ex3-28T>C (*Y113H*)	CC vs TC/TT	7/26	1.45 (0.60-3.55)	21/36	2.10 (1.16-3.79)	0.46
	Ex4+52A>G (*H139R*)	GG vs AG/AA	5/23	1.23 (0.44-3.41)	5/17	0.84 (0.28-2.52)	0.68
GSTT1	del{GSTT1}	Null vs present	19/57	2.10 (1.13-3.90)	18/74	0.80 (0.46-1.41)	0.02

*Number of cases and controls in the comparison category (dominant, recessive, and null).
†OR (95% CI) estimated by unconditional logistic regression, adjusted by sex, age, center, and date of blood extraction. Ever smokers further adjustment by cigarettes/day and duration of smoking (years). OR (95% CI) for smoking status (compared with never smokers, adjusted by education, Hp infection, and the intake of vegetables, citrus fruit, and meat): ever smokers, 1.76 (1.26-2.45); current smokers, 2.21 (1.46-3.33); and former smokers, 1.53 (1.06-2.21).

Table 5. OR and 95% CI of gastric adenocarcinoma for SNPs in metabolic genes according to smoking status, by localization, and by histologic type

Gene	Polymorphism	Model	Overall, OR* (95% CI)	Never smokers, OR* (95% CI)	Ever smokers, OR* (95% CI)	*P* for homogeneity
Cardia (68 cases, 250 controls)						
CYP1A1	*m1*	CC/TC vs TT	0.94 (0.47-1.86)	1.81 (0.39-8.32)	0.65 (0.26-1.60)	0.17
CYP1A1	*m4* (*T461N*)	AA/CA vs CC	1.71 (0.67-4.36)	1.89 (0.35-10.1)	2.31 (0.59-9.12)	0.95
CYP1A2	−3859G>A	AA/GA vs GG	1.47 (0.25-8.50)	6.90 (0.16-299)	1.29 (0.12-14.3)	0.56
EPHX1	*Y113H*	CC vs TC/TT	1.84 (0.79-4.32)	—	1.91 (0.72-5.07)	
GSTT1	del{GSTT1}	Null vs present	0.82 (0.38-1.79)	0.37 (0.06-2.38)	0.97 (0.34-2.78)	0.70
Noncardia (128 cases, 507 controls)						
CYP1A1	*m1*	CC/TC vs TT	0.79 (0.46-1.36)	1.31 (0.58-2.98)	0.48 (0.20-1.14)	0.11
CYP1A1	*m4* (*T461N*)	AA/CA vs CC	1.44 (0.76-2.71)	0.97 (0.34-2.80)	1.91 (0.74-4.91)	0.36
CYP1A2	−3859G>A	AA/GA vs GG	2.13 (0.69-6.64)	3.08 (0.62-15.2)	2.32 (0.37-14.4)	0.87
EPHX1	*Y113H*	CC vs TC/TT	2.64 (1.41-4.95)	2.94 (0.99-8.78)	3.07 (1.29-7.33)	0.90
GSTT1	del{GSTT1}	Null vs present	1.15 (0.68-1.92)	2.94 (1.30-6.67)	0.70 (0.31-1.56)	0.04
Diffuse (93 cases, 366 controls)						
CYP1A1	*m1*	CC/TC vs TT	0.71 (0.37-1.35)	1.19 (0.44-3.27)	0.46 (0.17-1.27)	0.12
CYP1A1	*m4* (*T461N*)	AA/CA vs CC	0.43 (0.15-1.25)	0.39 (0.08-1.84)	0.44 (0.09-2.13)	0.95
CYP1A2	−3859G>A	AA/GA vs GG	2.41 (0.67-8.66)	9.59 (1.39-66.1)	—	
EPHX1	*Y113H*	CC vs TC/TT	1.43 (0.63-3.29)	2.33 (0.72-7.49)	1.14 (0.28-4.69)	0.34
GSTT1	del{GSTT1}	Null vs present	0.87 (0.44-1.72)	1.75 (0.60-5.26)	0.59 (0.20-1.72)	0.22
Intestinal (94 cases, 365 controls)						
CYP1A1	*m1*	CC/TC vs TT	0.57 (0.29-1.12)	1.34 (0.38-4.78)	0.39 (0.15-1.04)	0.14
CYP1A1	*m4* (*T461N*)	AA/CA vs CC	3.03 (1.60-5.73)	3.48 (0.95-12.8)	4.35 (1.67-11.3)	0.66
CYP1A2	−3859G>A	AA/GA vs GG	1.60 (0.31-8.25)	—	3.27 (0.46-23.1)	
EPHX1	*Y113H*	CC vs TC/TT	2.38 (1.24-4.56)	2.12 (0.34-13.2)	2.68 (1.18-6.11)	0.54
GSTT1	del{GSTT1}	Null vs present	1.20 (0.65-2.22)	3.85 (1.15-12.5)	0.63 (0.25-1.56)	0.03

*OR (95% CI) by unconditional logistic regression, adjusted by sex, age, center, and date of blood extraction plus cigarettes/day and duration of smoking (ever smokers). OR (95% CI) for ever smokers (adjusted by education, Hp infection, and the intake of vegetables, citrus fruit, and meat): cardia, 2.03 (1.01-4.07); noncardia, 1.86 (1.17-2.96); diffuse, 1.66 (1.00-2.76); and intestinal, 1.71 (0.95-3.05).

clear inverse relationship with gastric cancer was associated with *m1* but no *m2*, whereas a positive weak association remained for *m4*. The latter is a relatively new finding needing replication, whereas the inverse association with *m1* is difficult to interpret given the role as activating enzyme of CYP1A1 and a faster catalytic activity of this SNP (3). No results have been reported on gastric cancer for two promoter polymorphisms of CYP1A2; we found a marginally significant increased risk for allele A in −3859G>A confined to never smokers. CYP1A2 seems to be mainly expressed in the liver and is involved in the activation of heterocyclic amines and aromatic amines; its activity can be determined by measuring the excretion of caffeine metabolites. The allele C of IVS1-154A>C seems to be associated with decreased caffeine metabolism among smokers, whereas the effect on enzymatic activity of −3859G>A is not clear (19).

Deletion of GSTT1 was found not to be associated with gastric cancer risk in several studies in a variety of populations (17, 20-27). One study in Poland found a significant increase in risk for null GSTT1 limited to cases with age <50 years (28); another study in China reported a significant OR of 2.5 for GSTT1 deletion (29). A recent study in Italy (30), in a population similar to ours, found a significant increase in gastric cancer risk of 1.53, with a positive interaction between GSTT1- and GSTM1-null genotypes. We also observed an increased risk of gastric cancer for deletion of GSTT1 restricted to never smokers; actually, there is a significant heterogeneity between GSTT1 estimates for smokers and nonsmokers. It could be that the detoxifying effect of GSTT1 is more evident at lower doses of carcinogen exposure experienced by nonsmokers. Furthermore, GSTT1 is but one of the large family GST; whereas conjugation with PAH seems to be the main

Table 6. Gene-gene interaction of polymorphism *Y133H* in EPHX1 with CY1A1 *m4* and deletion of GSTT1

Gene and polymorphism		EPHX1 *Y113H*		*P* for interaction*
		TT/TC, OR† (95% CI)	CC, OR† (95% CI)	
Overall				
CYP1A1	CC	1.00 (reference)	2.13 (1.30-3.48)	
m4 (*T461N*)	AA/CA	1.60 (0.98-2.62)	0.98 (0.11-9.02)	0.25
GSTT1	Present	1.00 (reference)	1.90 (1.14-3.18)	
del{GSTT1}	Null	1.13 (0.74-1.73)	2.38 (0.67-8.42)	0.89
Never smokers				
CYP1A1	CC	1.00 (reference)	1.61 (0.65-4.01)	
m4 (*T461N*)	AA/CA	0.96 (0.42-2.22)	—	
GSTT1	Present	1.00 (reference)	1.20 (0.43-3.34)	
del{GSTT1}	Null	1.97 (1.03-3.75)	8.49 (1.01-71.4)	0.30
Ever smokers				
CYP1A1	CC	1.00 (reference)	2.42 (1.31-4.47)	0.92
m4 (*T461N*)	AA/CA	2.37 (1.24-4.54)	4.86 (0.25-95.7)	
GSTT1	Present	1.00 (reference)	2.33 (1.24-4.38)	
del{GSTT1}	Null	0.85 (0.46-1.54)	1.23 (0.22-6.82)	0.62

*By likelihood ratio test.
†OR (and 95% CI) estimated by unconditional logistic regression, adjusted by sex, age, center, and date of blood extraction. Ever smokers further adjustment by cigarettes/day and duration of smoking.

activity of other classes, such as GSTM1, GSTT1 may play a role in the conjugation of other epoxides, such as butadiene or ethylene oxide (31). On the other hand, although the predominant role of GST is believed to involve detoxification, glutathione conjugates are, in some instances, more reactive than the parent compounds; examples of this phenomenon are conjugates of dichloromethane, dihaloethanes, and several haloalkenes (31).

One of the most remarkable findings in our study is the increased risk of stomach cancer associated with the homozygous variant CC in Ex3-28T>C of EPHX1 (polymorphism *Y113H*). The amino acid histidine instead of tyrosine at the position 113 of the protein is known to produce decreased enzymatic activity (32). The role of EPHX1 in PAH metabolism is complex: CYP1A1-mediated epoxides are partly detoxified by EPHX1, which catalyzes their hydrolysis. However, some dihydrodiols are substrates for additional oxidation to more highly reactive diol epoxides, probably by the action of CYP3A4 (33, 34). Furthermore, inhibition (or slow activity) of EPHX1 may lead to an alternative pathway involving CYP1A1 and CYP2C9 (34). No results have been thus far reported for association between EPHX1 activity and gastric cancer. Interestingly, a marked decreased risk for slow EPHX1 activity has been often observed for respiratory tumors strongly related to smoking, such as lung (35) and larynx (36), whereas an increased risk for slow activity has been reported for digestive tumors, such as esophagus (37) and colorectal adenoma (38), the latter was significant only among smokers.

Several methodologic issues must be considered to get a proper interpretation of our results. Familial gastric cancer is a rare syndrome accounting for ~1% of all gastric cancer cases: on the other hand, there is a well-recognized predisposition to gastric cancer risk. Furthermore, some genetic or epigenetic traits are related to family history (39, 40). Thus, it would have been of interest to analyze the association with gastric cancer of metabolic SNPs among subjects with or without family history; unfortunately, in our study, information on the family history of gastric cancer was not collected. All subjects from our study were recruited in European countries. Although we did not have information on ethnicity, most of them are Caucasians, and for the genetic point of view, our population may be considered homogeneous; however, all our analyses were stratified by center. The mixture of individuals from heterogeneous genetic background (population stratification) can cause spurious allele-disease association but is likely to occur rarely: it has been pointed out that, even when ethnicity is ignored, the smaller the difference in allele frequency or baseline risk, the less likely it is that population stratification will lead to confounding (41). In our study, the prevalence of SNPs found to be associated with gastric cancer did not show remarkable heterogeneity across countries: without taking into account France (only 11 controls included), the proportion of subject with variant C in the SNP *m1* of CYP1A1 is 19%, with a range from 13% to 22%. The corresponding figures for variant A in *m4* were 8% (range, 5-16%) and 7% (range, 4-9%) for the homozygous AA genotype of *Y113H* in EPHX1. Only the frequency of deletion of GSTT1 showed geographic variation: overall, it was 14%, with two countries with prevalence above 20% (Spain and the Netherlands) and two with proportions below 10% (United Kingdom and Sweden), but these differences did not correspond to similar patterns of association with gastric cancer. Each polymorphism was tested for HWE in controls; only the distribution of genotypes for *Y113H* in EPHX1 was different from the expected according to allelic frequency. Because Hardy-Weinberg disequilibrium could result from genotyping errors, independent genotyping was carried out for this SNP in addition to those already done for standard quality control; overall, in a sample of 202 subjects, the proportion of agreement was 100%. Given these results and taking into account that deviation from equilibrium was rather modest, we decided to include this SNP in the analysis. Furthermore, the association seen could hardly be explained by lack the of HWE: the chance of a false-positive result is increased if homozygotes for the putative high-risk allele are more common in the population than predicted by HWE (42). In our study, the proportion of CC among controls was 6.6%, whereas the expected frequency under HWE was 8.5%, suggesting that the test is likely to give a conservative result.

Given the number of cases and the magnitude of association, our study has a relatively low statistical power to detect significant gene-environmental interaction (43). We simply explored the potential effect modification of the main genetic associations observed according to smoking status looking at the homogeneity between estimates among smokers and nonsmokers, although we also explored possible gene-gene interactions, but our main conclusions are based on the main effects of each SNP analyzed in the whole set. A related issue is the statistical model used to estimate relative risk: given the matched design, conditional logistic regression must be applied so that each case is compared with its matched controls, preserving the risk sets (13). However, when one stratifies by a factor different from those used as matching variables, this may produce loss of power because breaking the risk sets results in the exclusion of some of them from the analysis. To avoid this, we conducted an unconditional analysis, including the matching variables in the model, to approach the matched design. Because results in the whole sample were almost the same for both approaches, we used the unconditional-adjusted method in the stratified analysis by smoking status. The genes found to be associated with increased risk of gastric cancer in our study are involved in the metabolic pathway of activation and detoxification of PAH; CYP1A2 is involved in activation of heterocyclic amines as well. However, another limitation of our study is that not all the relevant genes encoding enzymes involved in a metabolic pathway were included, mainly because of scarcity of data about gastric carcinogenesis and metabolic genes when the study was designed. In summary, according to our results, gastric cancer risk is associated with an increased oxidizing activity of CYP1A1 due to the polymorphisms *m4*, a reduced activity of epoxy hydrolase induced by the homozygous variant in *Y113H*, and a decreased glutathione conjugation due to GSTT1 deletion. All these effects seem to point to a potential role of PAH in gastric carcinogenesis, a process that may be related to PAH formation during smoking or grilling of meat over open flames.

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