EPHX1 polymorphisms do not modify esophageal carcinoma susceptibility in Dutch Caucasians

POLAT DURA[1], CARO V.V. BREGITHA[1], RENE H.M. TE MORSCHE[1], HENNIE M.J. ROELOFS[1], JON O. KRISTINSSON[1], THEO WOBBES[2], BEN J.M. WITTEMAN[3], ADRIAAN C.I.T.L. TAN[4], JOOST P.H. DRENTH[1] and WILBERT H.M. PETERS[1]

Departments of [1]Gastroenterology and [2]Surgery, Radboud University Medical Center, Nijmegen; [3]Department of Gastroenterology and Hepatology, Hospital Gelderse Vallei, Ede; [4]Department of Gastroenterology and Hepatology, Canisius-Wilhelmina Hospital, Nijmegen, The Netherlands

Received December 20, 2011; Accepted February 3, 2012

DOI: 10.3892/or.2012.1734

Abstract. Esophageal cancer (EC) has a globally increasing incidence with poor curative treatment options and survival rates. Crucial risk factors are exposure to toxins or carcinogens. Microsomal epoxide hydrolase (mEH) is a biotransformation enzyme essential for the detoxification of xenobiotics. Polymorphisms in exon 3 and exon 4 of the microsomal epoxide hydrolase gene (*EPHX1*) modify catalytic activity of this enzyme and subsequently may play a role in EC etiology. This case-control study investigated whether these polymorphisms in the *EPHX1* gene influence esophageal cancer susceptibility in a Dutch Caucasian population. A case-control study including 349 Caucasian EC patients and 581 Caucasian healthy controls was conducted and the polymorphisms Tyr113His (exon 3) and His139Arg (exon 4) in the *EPHX1* gene were determined, using polymerase chain reaction. The distribution of exon 3 and exon 4 genotypes were compared between cases and controls. Analyses included a stratification according to tumor histology; esophageal adenocarcinoma (EAC) or squamous cell carcinoma (ESCC). Furthermore, on the basis of allelic *in vitro* enzyme activity assays, exon 3 and 4 genotypes were combined and categorized according to their predicted high, medium or low enzyme activity. Homozygosity and heterozygosity for both exon 3 and 4 polymorphisms were correlated with a decreased esophageal squamous cell carcinoma risk. Heterozygosity and homozygosity for both polymorphisms correlated with an increased and a decreased esophageal adenocarcinoma risk, respectively. Predicted intermediate and high activity genotypes were risk and protective factors for esophageal squamous cell carcinoma and esophageal adenocarcinoma, respectively. However, none of these associations were statistically significant. In conclusion, the polymorphisms in exon 3 and exon 4 of the *EPHX1* gene do not seem to be modifiers of esophageal squamous cell carcinoma or esophageal adenocarcinoma risk in Dutch Caucasians.

Correspondence to: Dr Polat Dura, Department of Gastroenterology, 455, Radboud University Nijmegen Medical Center, P.O. Box 9101, 6500 HB, Nijmegen, The Netherlands
E-mail: p.dura@mdl.umcn.nl

Key words: microsomal epoxide hydrolase, genetic polymorphism, detoxification, esophageal adenocarcinoma, esophageal squamous cell carcinoma

Introduction

Esophageal cancer (EC) is globally one of the most lethal malignancies (1). In the past decennia, the incidence in the Netherlands has risen to 8.5 per 100,000 persons as a consequence of the increase in esophageal adenocarcinomas (EAC) (2). Gastroesophageal reflux disease (GERD) and obesity, among others, are risk factors for EAC. Dietary and lifestyle factors such as tobacco and alcohol consumption are major contributors to esophageal squamous cell carcinoma. The squamous cell subtype is primarily found in Asian countries (3). Lifestyle and dietary factors are involved in both subtypes of EC but the magnitude of attributing risk differs among persons, making a genetic element in EC etiology more credible. Consequently functional polymorphisms in biotransformation enzymes such as microsomal epoxide hydrolase (mEH), correlating with modified catalytic activity, may influence EC susceptibility via altered carcinogen detoxification (4).

Humans express five epoxide hydrolases: soluble EH (sEH), microsomal EH (mEH), cholesterol EH (ChEH), hepoxilin hydrolase and leukotriene A4 (LTA4) hydrolase (5). The most studied EHs are mEH and sEH. Soluble EH is well studied in cardiovascular diseases for its protective potential, while mEH has an important role in biotransformation of carcinogens (5). Along with glutathione S-transferases, mEH detoxifies electrophilic epoxides (EE) (5,6). These compounds are cytochrome P450 (CYP) derived mutagenic metabolites of tobacco-specific PAH, organic amines or fungeal aflatoxin (7). Microsomal EH hydrolizes the epoxides, generating water-soluble, less mutagenic, chemically less-active and excretable glycols. Conversely other hydrocarbons such as benzo(a)pyrene, are metabolized by

Table I. Characteristics of patients with esophageal cancer and controls.

Characteristics	Patients			Controls (%)
	ESCC (%)	EAC (%)	Total EC group (%)	
No. (% of total)	86[a] (24.6)	258[a] (73.9)	349[a]	581
Age (years; mean ± SD)	63.7±10.2	65.3±11.1	65.0±10.9	63.5±11.9
Gender				
Male	57[b] (66.3)	220 (85.3)	281 (80.5)	471 (81.1)
Female	28[b] (32.6)	38 (14.7)	67 (19.2)	110 (18.9)

[a]Note that for 5 patients data on the exact tumor type are missing, [b]whereas for 1 ESCC patient the gender is unknown. ESCC, esophageal squamous cell carcinoma; EAC, esophageal adenocarcinoma; EC, esophageal cancer.

mEH resulting in more carcinogenic diol epoxides with DNA alkylating abilities (8). This supports the concept that mEH, depending on the substrate, has carcinoprotective as well as toxicity-enhancing properties.

The mEH locus is located on chromosome 1q42 and it is expressed in nearly all human tissues (8). In the *EPHX1* gene, two frequent substitution polymorphisms are known; c.337T→C on exon 3 and c.416A→G on exon 4, respectively, resulting in the replacement of Tyrosine (Y) → Histidine (H) at codon 113 and Histidine (H) → Arginine (R) at codon 139 (9). The first polymorphism causes a decrease, while the latter is correlated with an increase in enzyme activity. The *EPHX1* polymorphisms have been associated with a diversity of neoplasms such as lung cancer (9), leukaemia (10), ovarian cancer (11), colorectal adenoma (12) and hepatocellular carcinoma (13). The issue is much more controversial for EC risk, most likely due to the substrate-dependent controversial function of mEH, relatively small patient groups and interracial differences in genetics (14-18).

Most studies originate from Asia and subsequently provide insights only on ESCC risk (15,17,19). Because of data regarding *EPHX1* polymorphisms and EC risk are inconsistent, we investigated the correlation between *EPHX1* exon 3 and 4 polymorphisms and ESCC and EAC susceptibility. Consequently we conducted a case-control study in a population of Dutch Caucasians with esophageal cancer.

Materials and methods

Patients and controls. The study was approved by the Medical Ethics Review Committee, region Arnhem-Nijmegen and written informed consent was received from all participants. Blood or tissue samples from 349 Caucasian patients with esophageal cancer were collected in the period October 2002 to July 2011 at 4 different hospitals, all located within 30 km distance in the South-East area of The Netherlands (20). Only patients with a diagnosis of esophageal carcinoma, as confirmed by a pathologist, were included in the study. As a source of DNA, in 91 cases tissue biopsies of normal esophagus or stomach from patients were collected after surgery, whereas in 258 cases EDTA blood was collected. Blood and tissue samples were frozen at -20 and -80˚C, respectively. Caucasian healthy controls (n=581) were recruited from the same geographical area of The Netherlands after advertisement in local papers as described earlier (20). Controls were matched with the EC patients for age, ethnicity and gender.

Genotyping. DNA isolation was performed by usage of the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the instructions of the manufacturer. Post-extraction DNA was stored at 4˚C. *EPHX1* exon 3 genotypes were detected with the iCycler iQ Multicolour Real-Time Detection System (Bio-Rad Laboratories, Veenendaal, The Netherlands), as described before (21). The c.416A→G polymorphism creating the exon 4 genotypes of *EPHX1* was analyzed using polymerase chain reaction (PCR) followed by polyacrylamide gel electrophoresis as described by Harrison *et al* (22).

Statistical analyses. For the comparison between cases and controls, the most common genotypes of exons 3 and 4 were set as reference. Earlier Benhamou *et al* correlated *EPHX1* genotypes with the corresponding predicted enzyme activity (9). In our study the predicted low activity genotype was used as reference in the comparison between cases and controls.

Haplotypes were generated using the PLEM program (23). The most common haplotype was taken as reference in the comparison between cases and controls. Only participants with complete genotypes were included in the haplotype analyses.

The independent samples t-test was applied for the differences in continues variables between characteristics of patients and controls. The χ^2 test was used for analyzing nominal variables of patient characteristics and to test for differences of frequencies in predicted enzyme activity genotypes between two groups. Odds ratios (OR) with 95% confidence interval (95% CI) were calculated. Stratified analyses were performed according to tumor histology. All P-values were two-sided and a probability level of $P<0.05$ was considered to be significant. The analyses were performed with the software SPSS for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA).

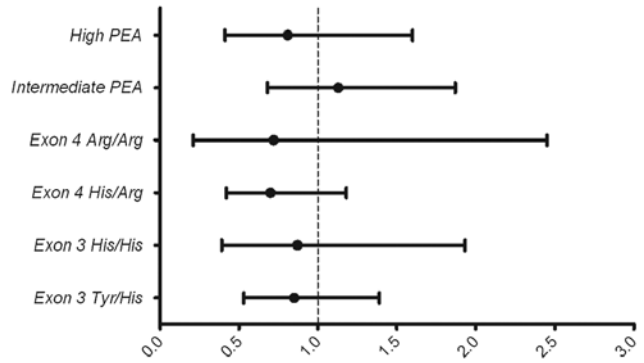

Figure 1. mEH polymorphisms and ESCC risk. Left y-axis, predicted enzyme activity mEH genotypes. x-axis, odds ratio with 95% confidence interval. PEA, predicted enzyme activity; mEH, micsrosomal epoxide hydrolase; ESCC, esophageal squamous cell carcinoma.

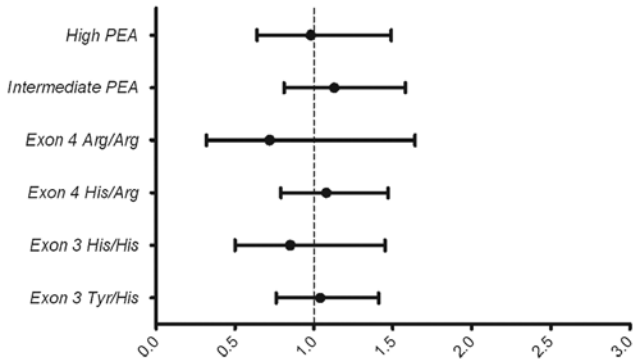

Figure 2. mEH polymorphisms and EAC risk. Left y-axis, predicted enzyme activity mEH genotypes. x-axis, odds ratio with 95% confidence interval. PEA, predicted enzyme activity; mEH, micsrosomal epoxide hydrolase; EAC, esophageal adenocarcinoma.

Table II. mEH genotypes with corresponding odds ratios for patients with ESCC or EAC compared to controls.

mEH genotypes	ESCC (n=86) (%)	OR (95% CI)	EAC (n=258) (%)	OR (95% CI)	Controls (n=581) (%)
Exon 3					
Tyr/Tyr	47 (54.7)	Ref.	131 (50.8)	Ref.	295 (50.8)
Tyr/His	31 (36.0)	0.85 (0.53-1.39)	105 (40.7)	1.04 (0.761.41)	228 (39.2)
His/His	8 (9.3)	0.87 (0.39-1.93)	22 (8.5)	0.85 (0.50-1.45)	58 (10.0)
Exon 4					
His/His	61 (70.9)	Ref.	161 (62.4)	Ref.	364 (62.7)
His/Arg	22 (25.6)	0.70 (0.42-1.18)	89 (34.5)	1.08 (0.79-1.47)	187 (32.2)
Arg/Arg	3 (3.5)	0.72 (0.21-2.45)	8 (3.1)	0.72 (0.32-1.64)	25 (4.3)
PEA[a]					
Low	29 (33.7)	Ref.	84 (32.6)	Ref.	198 (34.1)
Intermediate	43 (50.0)	1.13 (0.68-1.87)	125 (48.4)	1.13 (0.81-1.58)	260 (44.8)
High	14 (16.3)	0.81 (0.41-1.60)	49 (19.0)	0.98 (0.64-1.49)	118 (20.3)

Note that in 5 controls the exon 4 genotype is unknown. ESCC, esophageal squamous cell carcinoma; EAC, esophageal adenocarcinoma. OR, odds ratio; CI, confidence interval. [a]PEA, predicted enzyme activity.

Results

Demographics. This case-control study consisted of 349 patients with EC and 581 community-based healthy controls. Table I shows the demographics of the participants and includes a stratification of cases according to histology. The distribution of gender and the mean age did not differ between patients and controls (P=0.904 and 0.054, respectively). The ESCC subgroup consisted of 32.6% females, significantly more in comparison to patients with adenocarcinoma (14.7%; P=0.000), but there was no significant difference in age (P=0.216).

Genotype frequencies, haplotype distributions and esophageal cancer risk. Genotype frequencies in controls and patients with EC for exon 3 (P=0.16 and 0.43, respectively) and exon 4 (P=0.90 and 0.50, respectively) were according to the Hardy-Weinberg equilibrium, supporting random sampling and absence of population stratification.

Table II displays the genotype distribution in cases and controls whereby cases are stratified according to histology. The genotype distributions of the Tyr113Tyr and Tyr113His were similar in ESCC patients in comparison to controls. This was also true for the His139His and His139Arg exon 4 genotypes. Both exon 3 and 4 genotype frequencies were comparable between patients with EAC and controls. As shown in Table II and Figs. 1 and 2, the various exons 3 and 4 based genotypes did not significantly modify ESCC or EAC risk in our population.

For EC patients the absolute numbers of the genotype frequencies for the predicted mEH enzyme activity classification according to Benhamou *et al* (9) are given in Table III.

Table III. Absolute numbers of predicted low, intermediate and high enzyme activity genotypes[a] of patients with ESCC or EAC and Controls.

	Exon 4			
Exon 3	His/His	His/Arg	Arg/Arg	Total
ESCC				
Tyr/Tyr	35[b]	11[c]	1[c]	47
Tyr/His	21[d]	8[b]	2[c]	31
His/His	5[d]	3[d]	0[b]	8
Total	61	22	3	86
EAC				
Tyr/Tyr	86	41	4	131
Tyr/His	62	39	4	105
His/His	13	9	0	22
Total	161	89	8	258
Controls				
Tyr/Tyr	184	94	15	293
Tyr/His	143	75	9	227
His/His	37	18	1	56
Total	364	187	25	576

Note that for 5 patients data on the exact tumor type are missing. [a]Based on Benhamou *et al* (9). [b]Predicted intermediate activity. [c]Predicted high activity. [d]Predicted low activity. ESCC, esophageal squamous cell carcinoma; EAC, esophageal adenocarcinoma.

The predicted intermediate and high activity genotypes when set off against the low activity genotype were more and less frequent in patients with ESCC or EAC respectively, when compared to controls (Figs. 1 and 2). However, no association reached statistical significance.

Table IV shows the distribution of *EPHX1* gene haplotypes of all cases and controls. In descending order of presence, the following haplotypes 113Tyr-139His, 113His-139His, 113Tyr-139Arg and 113His-139Arg were observed for patients and controls. Setting the most frequent haplotype 113Tyr-139His as reference in the comparison between ESCC, EAC and controls, none of the other haplotypes were found to influence cancer risk.

Discussion

Our study concludes that the separate exon 3 and 4 polymorphisms do not modify ESCC or EAC risk in our Dutch Caucasian population. Also the combined effect of both polymorphisms, a classification of predicted enzyme activity, did not influence esophageal cancer susceptibility.

Recently, Ihsan *et al* showed that the His139Arg and Arg139Arg genotypes were associated with a higher esophageal squamous cell carcinoma (ESCC) risk in an Indian population, whereas the Tyr113His genotype was reported to be a protective factor in the same patient group (15). In contrast to these findings Jain *et al* found opposite results and reported that Tyr113His as well as His113His genotypes were independent risk factors for ESCC in a Northern Indian population (16). In two different Chinese studies exon 3 and 4 polymorphisms did not modify ESCC risk (19,24). Furthermore, a Taiwanese

Table IV. *EPHX1* haplotypes with corresponding odds ratios for patients with EC compared to controls.

EPHX1 haplotypes	ESCC (n=172) (%)	OR (95% CI)	EAC (n=516) (%)	OR (95% CI)	Controls (n=1162) (%)
113Tyr-139His[a]	110 (63.9)	Ref.	314 (60.8)	Ref.	685 (59.0)
113His-139His	34 (19.8)	0.88 (0.58-1.33)	97 (18.8)	0.88 (0.67-1.16)	240 (20.6)
113Tyr-139Arg	15 (8.7)	0.70 (0.40-1.24)	53 (10.3)	0.87 (0.62-1.23)	133 (11.4)
113His-139Arg	13 (7.6)	0.78 (0.42-1.43)	52 (10.1)	1.09 (0.76-1.56)	104 (9.0)

[a]The most common haplotype is taken as reference. ESCC, esophageal squamous cell carcinoma; EAC, esophageal adenocarcinoma; EC, esophageal cancer; OR, odds ratio; CI, confidence interval.

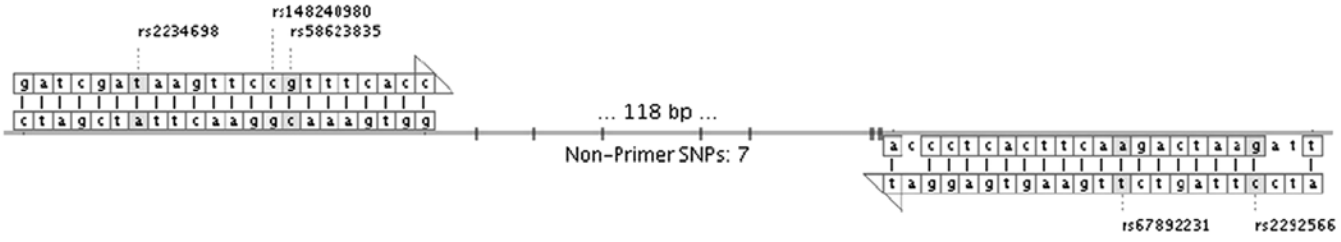

Figure 3. Polymorphisms in primer binding sites.

Table V. Prevalence of *EPHX1* genotypes observed in case-control studies of patients with esophageal cancer.

Study	Year	Country	N[b]	Exon 3 in Controls/ESCC/EAC[a] (%)			Exon 4 in Controls/ESCC/EAC[a] (%)		
				Tyr/Tyr	*Heterozygous*	*His/His*	*His/His*	*Heterozygous*	*Arg/Arg*
Present study	2011	Netherlands	349 (EAC/ESCC)	50.8/54.7/50.8	39.2/36.0/40.7	10.0/9.3/8.5	62.7/70.9/62.4	32.2/25.6/34.5	4.3/3.5/3.1
Ihsan, *et al* (15)	2010	India	142 (ESCC)	29.7/38.7/-	51.9/34.5/-	18.4/26.8/-	74.6/61.3/-	24.3/33.8/-	1.1/4.9/-
Jain, *et al* (16)	2008	India	107 (ESCC)	41.6/26.2/-	48.8/61.7/-	9.6/12.1/-	58.4/66.4/-	36.3/27.1/-	5.3/6.5/-
Wang, *et al* (19)	2006	China	107 (ESCC)	Not given	Not given	Not given	Not given	Not given	Not given
Casson, *et al* (14)	2006	Canada	56 (EAC)	44.0/-/61.0	36.0/-/28.0	20.0/-/11.0	58.0/-/62.0	35.0/-/36.0	7.0/-/2.0
Lin, *et al* (17)	2006	Taiwan	145 (ESCC)	30.4/35.9/-	39.8/35.2/-	29.8/29.0/-	81.5/80.7/-	18.5/19.3/-	-/-/-
Zhang, *et al* (23)	2003	China	257 (ESCC)	30.2/32.7/-	28.2/22.6/-	41.6/44.7/-	Not given	Not given	Not given
Wang, *et al* (18)	2003	China	62 (ESCC)	61.0/37.0/-	26.0/35.0/-	13.0/27.0/-	84.0/81.0/-	13.0/18.0/-	3.0/2.0/-

[a]ESCC, esophageal squamous cell carcinoma; EAC, esophageal adenocarcinoma. [b]Number of EC patients included in the study (histologic type is indicated between parentheses).

study suggested a protective role for His113His genotypes against ESCC in smokers and areca seed chewers (17). Lastly a Canadian study reported no significant association between the two *EPHX1* polymorphisms and EAC susceptibility in a Canadian population (14). Primary data of all studies cited above are summarized in Table V. It illustrates the diversity of the genotype frequencies and the highly variable degree of inheritance of the *EPHX1* gene in different populations. It also demonstrates that most studies deal with ESCC patients and that patient numbers are generally very low.

Although it is suggested that the *EPHX1* gene is expressed in many mammalian tissues (5,8) including those of the aero-digestive tract (15,25), to our knowledge no data on *EPHX1* mRNA expression in the cancerous esophagus is known. Consequently it is complicated to interpret the most recent results of Ihsan *et al* (15), since additionally the overall data report inconsistent findings regarding esophageal cancer risk. Moreover, three recently performed genome-wide association studies, using detection of SNPs or cDNA microarray techniques, did not identify the *EPHX1* gene as a susceptibility locus for ESCC or EAC (26-28).

The outcome of our study corresponds with the conclusion of Casson *et al* (14) that there is no association between *EPHX1* genotypes and EAC risk in Caucasian subjects. The enzyme activity classification comparisons showed a tendency that the intermediate activity genotypes, which is the major group, were correlated with an increased risk. One can dispute that this study lacked the power for this tendency to become statistically significant. Although no odds ratios were reported by Casson *et al*, the frequency distribution did not corroborate our results (14).

Mutant homozygosity and heterozygosity for exon 3 and exon 4 resulted mutually in a decreased ESCC risk while both polymorphisms have opposing influences on the enzyme activity. However, the correlation was not significant. This trend is not in line with the results regarding ESCC risk by Ihsan *et al* (15) and Jain *et al* (16). Both studies, however, did not include an analysis according to predicted enzyme activity. Moreover Lacko *et al* demonstrated the same absence of correlation in a larger group of Dutch Caucasians with head and neck cancer (25).

Reviewing all these incompatible data, a surfacing inevitable question is why the influence of mEH polymorphisms on EC susceptibility differs globally. The crux may be triple fold: global area-dependent differences in exposure to environmental (pro)carcinogens along with the ambiguous role of mEH, variations in genetic background and a genotyping flaw in earlier studies.

Firstly, detoxification of xenobiotics present in cigarette smoke and hot/spicy foods, both risk factors more frequent in Eastern continents, may play a vital role in ESCC prevention. This could explain the more prominent role of mEH in this subtype and a less crucial role in EAC. This hypothesis is confirmed by significant associations found in an Indian study on ESCC (15) and not in a Canadian study on EAC (14). Our study shows the same tendencies, although not significant, more frequently deviations are detected between ESCC and controls in comparison to EAC and controls. One can argue that our study lacked sufficient power to reach significance. Moreover, the Tyr113His polymorphism and the His139Arg are associ-

ated with, respectively, a decrease and an increase in enzyme activity. As a result, important esophageal carcinogens such as food- and tobacco smoke-derived nitrosamines (29) and high-cooking induced heterocyclic amines or polycyclic aromatic hydrocarbons (30) along with the mycotoxin aflatoxin B1 (19), might not be efficiently detoxified by the less active His113His genotype. Alternatively, the more active Arg139Arg genotype might form more procarcinogenic metabolites. These contradictions are visualized by the results of the two studies originating from India with analogous ethnic populations, one in a low-risk (16) and the other in a high risk region (15), showing increasing and decreasing risks for the heterozygous exon 3 genotype, respectively.

Secondly, inter-ethnic differences in genetics are a common observation in case-control studies concerning *EPHX1* genotypes and esophageal cancer risk (14-19,24). As shown in Table V, the genotype frequencies variate per race and nation. The deviation between nations may grossly be accounted for by a different ancestral gene pool and by natural selection, as some populations have a greater degree of exposure to environmental pollutants than others.

Lastly, the reported differences can partly be explained by the fact that earlier studies (Table V) may have a flaw in their genotyping. The used primers were verified using SNPCheck (National Genetics Reference Laboratory, Genetic Medicine, St. Mary's Hospital, Manchester, UK). Fig. 3 illustrates the four possible SNP's in the binding sites of the reverse and the forward primers for the exon 3 polymorphism used in former studies (14,16,17,19) and by Ihsan *et al* (15). The rs2234698 and the rs58623835 SNPs relevant for the forward primer and the rs67892231 SNP in the binding site of the reverse primer, are reported to have wild-type genotype frequencies >95%. Consequently genotyping errors based on these SNP's will most likely have insignificant effects on the results. The rs2292566 SNP, however, is a silent mutation in codon 119 and the key polymorphism on the binding site for the exon 3 reverse primer to influence study results by creating a possible mismatch at the 3-end. This can lead to overrepresentation of for example the exon 3 wild-type genotype, as the silent substitution polymorphism can interact with the binding of the primer, as described by Baxter *et al* (11) and Peters *et al* (21). Although the HAPMAP database does not have rs2292566 genotype frequencies of a population of Indian origine, it is described to be a common SNP in Europeans and Asians with heterozygous genotypes frequencies of 19.5 and 34.9%, respectively. Overclassification of the exon 3 wild-type genotype by Ihsan *et al* (15) and Jain *et al* (16) could lead to an alteration of the exon 3 genotype frequencies resulting in a significant difference between patients and controls and consequently may account for the inconsistent results between the two Indian studies. We used attuned primers excluding eventual negative effects of all 4 known mutations.

In summary, genetic *EPHX1* polymorphisms do not seem to be modifiers of EC risk in a cohort of Dutch Caucasians and are not likely to be involved in the etiology of EAC. Although we included a total of 349 patients and 581 controls, stratifying according to histology provided a relatively small group of 258 EAC patients. As a rule the statistical comparisons between categories are based upon smaller numbers. This increases the role of coincidence in our findings, so studies with larger groups of participants are desirable. Furthermore, this study did not examine risk or preventive factors such as tobacco, alcohol, red meat or fruits and vegetable consumption, which increases the chance of confounding.

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