doi:10.4149/neo_2012_010

Microsomal epoxide hydrolase polymorphisms, cigarette smoking and prostate cancer risk in the Slovak population

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Received July 25, 2011 / Accepted August 25, 2011

Polymorphisms in tobacco carcinogen metabolizing enzymes may generate interindividual variations towards the risk of developing prostate cancer. One of these enzymes is microsomal epoxide hydrolase (EPHX1) which metabolizes polycyclic aromatic hydrocarbons, or PAH, carcinogens found in cigarette smoke. The activity of this enzyme is affected by two polymorphisms, a substitution of Tyr113 by His in exon 3 and a substitution of His139 by Arg in exon 4. The aim of this study was to use a population-based case-control study to investigate whether or not such genetic polymorphisms in *EPHX1* gene can modify the relationship between smoking status and the risk of developing prostate cancer. We used restriction fragment length polymorphism, or PCR-RFLP to determine *EPHX1* genotypes in subjects comprising 194 patients with histologically verified prostate cancer and 305 healthy individuals as control. We found no overall association between prostate cancer risk and functional polymorphisms of *EPHX1* gene in exon 3 and exon 4. We further analysed the association between the *EPHX1* genotypes and smoking. Smokers carrying the exon 3 *Tyr/Tyr* and *Tyr/His* genotypes were at no significant risk compared to non-smokers with the "rapid" *Tyr/Tyr* genotype. By contrast, a significant interaction of smoking and the exon 4 polymorphism was present ($p < 0.05$). Excess statistically significant risk was apparent among smokers with imputed normal phenotype in comparison to non-smokers with normal phenotype ($p < 0.01$). Based on these results we conclude that in the Slovak population studied, *EPHX1* gene polymorphism in exon 4 would alter the risk of prostate cancer, particularly among smokers. Further analysis of other polymorphic variants in biotransformation enzymes and evaluation of gene-gene interactions may provide a more complete picture of how *EPHX1* might influence the risk of developing prostate cancer.

Key words: Microsomal epoxide hydrolase (EPHX1), genetic polymorphism, prostate cancer, Slovak population, cigarette smoking

Prostate cancer remains the most common male specific malignancy diagnosed in those aged over 50. The highest incidence of prostate cancer is found in the United States and Scandinavian countries and the lowest rates are found in China and Japan. In European countries, the rates are very high in the north and west and lower in the south [1]. In the Slovak Republic, the age-adjusted incidence of prostate cancer was 36.2/100,000 and the mortality rate, 14.9/100,000 in 2005 [2]. However, its aetiology remains poorly understood. It is a multifactorial disease, which means that age, ethnicity, geography, family history, genetics, socioeconomic and environmental factors, such as diet and lifestyle all play a possible predominant role in its development [3].

Genes coding for enzymes involved in the biotransformation of carcinogens can be used as markers of individual susceptibility to cancer. Many of these biotransformation enzymes are polymorphic, with the alleles presenting different enzymatic activities [4]. Different activity of these enzymes may increase or decrease the conversion of xenobiotics into its reactive metabolites and may confer an increased susceptibility to cancer mainly in the presence of environmental stresses such as smoking and UV light exposure [5].

One of these biotransformation enzymes is microsomal epoxide hydrolase (EPHX1), a member of the α/β-hydrolase enzyme family [6]. The *EPHX1* gene is localized on the long arm of chromosome 1q42.1 [7, 8]. It is approximately 20 kb in size, comprises eight coding exons and one non-coding exon, and encodes for a single monomeric protein of 455 amino acids [9]. This enzyme appears to be expressed in all tissues and cell types but mainly in the liver, gonads, kidneys, lungs, and bronchial epithelial cells [10]. It metabolizes a broad array of epoxide substrates, including PAHs (e.g. benzo(*a*)pyrene, benzo(*a*)anthracene, dibenzo(*a, h*)anthracene); carcinogens found in cigarette smoke and charred red meat [11]. PAHs are oxidized initially by cytochrome P4501A1/1B1 (CYP1A1/CYP1B1) to benzo(*a*)pyrene-derived benzo(*a*)pyrene 7,8-epoxide, which undergoes EPHX1-mediated hydrolysis to the less toxic transdihydrodiol derivative, benzo(*a*)pyrene 7,8-diol [11, 12]. On the other hand, less reactive dihydrodiols from PAHs can be substrates for further transformation into dihydrodiol-epoxides such as the carcinogen benzo(*a*)pyrene-7,8-diol-9,10 epoxide that intercalates into DNA by binding to the exocyclic 2-amino position of guanine and is active in DNA adduct formation [13]. Other xenobiotic compounds metabolized by EPHX1 are the epoxide derivatives of 1,3-butadiene, benzene, aflatoxin B1, chrysene, nitropyrene, naphthalene, and anthracene [9, 14].

EPHX1 gene is highly polymorphic and in its coding region are present two prominent gene polymorphisms [15, 16]. The first, within exon 3, thymine (T) to cytosine (C) substitution changes tyrosine residue 113 to histidine, and enzyme activity is reduced by 50% (slow allele). Another, within exon 4, an adenine (A) to guanine (G) changes histidine residue 139 to arginine, and produces an enzyme with an activity increase of 25% (fast allele) [17, 18]. The genotypic combinations of these *EPHX1* polymorphisms lead to the formation of EPHX1 metabolic phenotypes.

To our knowledge only two studies have been published with regards to the possible association of these coding region polymorphisms with altered xenobiotic disposition and prostate cancer incidence [19, 20]. The goal of the present study therefore was to determine whether or not *EPHX1* polymorphisms in exon 3 and exon 4 and their interaction with smoking may play a role in the risk of developing prostate cancer within the Slovak population.

Patients and methods

Study population. In this study, 194 patients with prostate cancer (median age of 67; lower and upper quartile limits 51-84 years) who had previously undergone surgery in the Department of Urology between May 2005 and December 2009 were selected. Further confirmation of their status was confirmed by histopathological evaluation with each tumor graded using the Gleason scoring system. Approximately, 36% patients had a Gleason score < 7 and the remaining 64% a Gleason score ≥ 7. For comparison, 305 healthy control subjects (median age of 62; lower and upper quartile limits 51-85 years) were included to determine the nominal distribution of polymorphisms in the population. Both groups were tested for serum total PSA levels. Controls with abnormal total PSA levels (higher than 4.0 ng/ml) were excluded from this study in order to preclude overlapping controls and cases. Cases and controls were interviewed regarding age, smoking status (i.e. habitual smokers and those who have never smoked, or never smokers), previous and/or current prostate diseased, and history of incidence of cancer and other chronic diseases. The study was approved by the Ethical Board of Jessenius Faculty of Medicine, Comenius University and informed written consent was obtained from all individuals prior to its start.

DNA extraction. Five millilitres of venous blood were collected in EDTA tubes as a source of peripheral blood leukocytes. Genomic DNA was extracted and purified using a standard phenol/chloroform methodology and stored at -20°C until genotype analysis.

Genotyping. Restriction fragment length polymorphism was used for genotyping *EPHX1* gene polymorphisms in exon 3 and exon 4 according to the protocol described by Zidzik et al. [21]. The PCR fragment was amplified using primer pairs for exon 3 variants *EPO1* (5′-GATCGATAAGTTCCGTTTCACC-3′), *EPO2* (5′-ATCCTTAGTCTTGAAGTGAGGAT-3′) and for exon 4 variants *EPO3* (5′-ACATCCACTTCATCCACGT-3′), *EPO4* (5′-ATGCCTCTGA GAAGCCAT-3′). Genotyping was performed using restriction digestion with *EcoRV* (exon 3) and *RsaI* (exon 4). The *EcoRV* digests were incubated at 37°C for 16 hours with expected fragment sizes of 162 bp for *His/His*; 162 and 140 bp for *Tyr/His*; and 140 bp for *Tyr/Tyr*. After *RsaI* digestion (37°C, 16 hours) products were classified into homozygotes for the *His/His* alleles (210 bp fragments), homozygotes for the *Arg/Arg* alleles (164 bp fragment) and heterozygotes for the *His/Arg* alleles (210 and 164 bp fragments). The resultant fragments were electrophoresed on a 3% agarose gel containing ethidium bromide and then visualized by UV transillumination.

Statistical analysis. Genotype distributions in the controls were tested for Hardy-Weinberg equilibrium to evaluate possible selection bias and genotyping errors. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated using an unconditional logistic regression method, all OR values were adjusted for age, which is considered a confounder. Chi^2 test was performed in order to assess differences in genotype prevalence and association between case and control groups. The ORs with their 95% CI were used as a measure of the strength of association since they are more illustrative than the Chi^2 test statistics. Statistical analysis was performed using StatsDirect statistical package version 2.7.8 (StatsDirect Ltd. http://www.statsdirect.com). Quantitative variables such as age and smoking status were compared by Student's t-test. All *P* values cited were two-sided and *P* values < 0.05 were judged as statistically significant.

Results and Discussion

In this case-control study we evaluated a possible association between *EPHX1* gene polymorphisms and susceptibility to prostate cancer in 194 prostate cancer patients and 305 healthy controls. To our knowledge, this is also the first report to examine this association in the Slovak population. A statistically significant difference in age between cases and controls were observed ($p < 0.001$). The alleles frequencies for *His113*, and *Arg139* of the *EPHX1* polymorphisms in our control group were estimated at 0.35 and 0.16, respectively. These frequencies were similar to that reported for the Caucasian populations, where the frequency of the *His113* allele ranged from 0.28 to 0.40; of the *Arg139* allele from 0.15 to 0.18 [22-24]. In our study, the frequency of the *His113* allele was lower and frequency of the *Arg139* allele more frequent in the cancer population than in healthy population (0.32 and 0.20, respectively).

Our primary aim was to investigate whether the observed individual variations could be related to polymorphic differences in exon 3 and exon 4 of the *EPHX1* gene, either alone, or in combination. Genotype frequencies for polymorphic sites in exon 3 and exon 4 were in Hardy-Weinberg equilibrium among the controls. Prevalence and ORs of the *EPHX1* gene polymorphisms in exon 3 and exon 4 in prostate cancer patients and controls are given in Table 1. All ORs were calculated relative to subjects with the *Tyr/Tyr* and *His/His* genotypes. We found no association between prostate cancer risk and functional polymorphism of *EPHX1* gene in exon 3. The "slow" *His/His* genotype was lower in the prostate cancer patients group (7%) compared to the control group (12%). For the exon 4 polymorphism of *EPHX1*, we examined that individuals with *Arg* allele, corresponding to increased enzyme activity, had no significantly increased risk for prostate cancer (OR=1.58, 95% CI 0.59-4.19).

An analysis based on combined genotypes and imputed phenotypes were also performed (Table 1). We observed that individuals with rapid phenotype had higher prostate cancer risk in comparison to individuals with normal phenotype (OR=1.44, 95% CI 0.81-2.55). The ORs calculated in cases with slow and very slow phenotype was lower than in cases with normal phenotype (0.92 and 0.77, respectively).

Although after reviewing the literature only two reports have been published to date regarding *EPHX1* gene polymorphisms in exon 3 and exon 4 and prostate cancer risk [19, 20], other reports have associated *EPHX1* genotypes with increased cancer susceptibility to lung, breast, colorectal, and esophageal squamous cell carcinoma [11, 13, 25, 26]. Nock et al. [19] for the first time investigated the effect of functional variants in PAHs metabolism and conjugation genes (*CYP1A1*, *EXPH1* in exon 4 and glutathione-S-transferases – *GST*) in 439 prostate cancer patients and 479 healthy controls, both from different ethnics groups (90% Caucasians, 9% African-Americans, and 1% Asian or Latino). In contrast with our findings they have found that the *Arg* allele occurs in the same frequency in the prostate cancer patients group and in the controls. They reported no correlation between prostate cancer risk and gene polymorphism of *EPHX1* in exon 4.

As previous studies have suggested that a remarkable number of human cancers are known to be related to exposure to PAHs, which are produced during cigarette smoking and can induce mutations in human cells [27, 28], we also performed an analysis based on smoking habits (Table 2). The

Table 1. Association between *EPHX1* genotypes and prostate cancer risk

Genotype	Cases No. (%)	Controls No. (%)	OR	95% CI	p
No.	194	305			
Exon 3					
Tyr/Tyr (rapid)	86 (44.3)	129 (42.3)	1.00 (ref.)	–	–
Tyr/His	94 (48.5)	140 (45.9)	1.01	0.69-1.47	NS
His/His (slow)	14 (7.2)	36 (11.8)	0.58	0.29-1.15	NS
Tyr/His+His/His	108 (55.7)	176 (57.7)	0.92	0.64-1.32	NS
Exon 4					
His/His (slow)	124 (63.9)	220 (72.1)	1.00 (ref.)	–	–
His/Arg	62 (32.0)	76 (24.9)	1.44	0.97-2.16	NS
Arg/Arg (rapid)	8 (4.1)	9 (3)	1.58	0.59-4.19	NS
His/Arg+ Arg/Arg	70 (36.1)	85 (27.9)	1.46	0.99-2.15	NS
Imputed phenotypes[#]					
Rapid	29 (16.0)	31 (11.4)	1.44	0.81-2.55	NS
Normal	91 (50.3)	140 (51.3)	1.00 (ref.)	–	–
Slow	60 (33.2)	100 (36.6)	0.92	0.61-1.40	NS
Very slow	1 (0.5)	2 (0.7)	0.77	0.07-8.61	NS

Note: OR adjusted for age. NS – not significant.

[#]Imputed phenotypes as classified by Tranah et al. [29]: **rapid**, exon 3 Tyr/Tyr and exon 4 Arg/Arg or His/Arg; **normal**, exon 3 Tyr/Tyr and exon 4 His/His or exon 3 Tyr/His and exon 4 His/Arg; **slow**, exon 3 Tyr/His and exon 4 His/His; **very slow**, exon 3 His/His and exon 4 His/His.

Table 2. Risk of prostate cancer associated with *EPHX1* genotypes and cigarette smoking in cases and controls.

Genotype	Non-smokers*	Smokers*
Exon 3		
Tyr/Tyr	59/98	27/31
OR (95% CI)	1.00 (ref.)	1.44 (0.78-2.66)
Tyr/His	72/113	22/27
OR (95% CI)	1.06 (0.68-1.64)	1.35 (0.71-2.59)
His/His	10/29	4/7
OR (95% CI)	0.57 (0.26-1.26)	0.95 (0.26-3.38)
Exon 4		
His/His	92/174	32/46
OR (95% CI)	1.00 (ref.)	1.32 (0.78-2.21)
His/Arg	43/59	19/17
OR (95% CI)	1.38 (0.86-2.20)	**2.11 (1.05-4.26)**
Arg/Arg	6/7	2/2
OR (95% CI)	1.62 (0.53-4.97)	1.89 (0.26-13.65)
Imputed phenotypes#		
Rapid	23/20	6/11
OR (95% CI)	**2.22 (1.13-4.37)**	1.05 (0.37-2.99)
Normal	58/112	33/28
OR (95% CI)	1.00 (ref.)	**2.27 (1.25-4.13)**
Slow	50/81	10/19
OR (95% CI)	1.19 (0.74-1.92)	1.02 (0.44-2.33)
Very slow	1/2	0/0
OR (95% CI)	0.96 (0.08-10.87)	–

NOTE: OR adjusted for age. Data in boldface are statistical significant.
*Reported as number of cases/number of controls.
#Imputed phenotypes as classified by Tranah et al. [29]: **rapid**, exon 3 Tyr/Tyr and exon 4 Arg/Arg or His/Arg; **normal**, exon 3 Tyr/Tyr and exon 4 His/His or exon 3 Tyr/His and exon 4 His/Arg; **slow**, exon 3 Tyr/His and exon 4 His/His; **very slow**, exon 3 His/His and exon 4 His/His.

distributions of smoking status (smokers and never smoked) among our cases and controls were approximately similar, 27% and 21% of smokers in prostate cancer patients group and controls, respectively.

In this study, we noticed that smokers carrying the exon 3 *Tyr/Tyr* and *Tyr/His* genotype were at increased prostate cancer risk (OR=1.44, 95% CI 0.78-2.66 and OR=1.35, 95% CI 0.71-2.59, respectively) relative to non-smokers with the "rapid" *Tyr/Tyr* genotype. Among smokers with "slow" *His/His* genotype, risk of prostate cancer was decreased (OR=0.95, 95% CI 0.26-3.38). By contrast, a significant interaction of smoking and the exon 4 polymorphism was present ($p < 0.05$). Smoking individuals were at significantly increased prostate cancer risk if they had *His/Arg* genotype (OR=2.11, 95% CI 1.05-4.26) relative to non-smokers with the "slow" *His/His* genotype. For the "rapid" *Arg/Arg* genotype, there was no significant increased risk for prostate cancer among smokers (OR=1.89, 95% CI 0.26-13.65) in comparison to "slow" *His/His* genotype in non-smokers.

Table 2 also presents risk of prostate cancer for the imputed phenotypes and smoking status. Excess statistical significant risk associated with the high predicted EPHX1 enzymatic activity was apparent among smokers with normal phenotype (OR=2.27, 95% CI 1.25-4.13, $p < 0.01$). By contrast, statistically significant elevated risk was associated with the rapid phenotype in non-smokers (OR=2.22, 95% CI 1.13-4.37, $p < 0.05$) in comparison to normal phenotype in non-smokers.

In the second study, Nock et al. [20] continued not only in the evaluation of the potential association between smoking and polymorphisms in genes that metabolize/detoxify PAHs (*CYP1A1, CYP1B1, CYP3A4, EPHX1* in exon 3 and exon 4, *GST*) but they also for the first time determined levels of PAH-DNA adducts in tumor and adjacent nontumor prostate cells in 400 men with prostate cancer (52.2% Caucasians and 44.3% African-Americans) by using immunohistochemical assay. They have found that Caucasian ever smokers had significantly higher PAH-DNA adducts than nonsmokers in tumor cells. Moreover, Caucasians carrying two copies of the *EPHX1 Arg* allele had decreased PAH-DNA adduct levels in both, tumor and nontumor cells. Contrary to our present study and the other studies [16, 29, 30], Nock et al. [20] marked *His/His* as a "rapid" and *Arg/Arg* as a "slow" genotypes at exon 4 polymorphism.

We hypothesize that the association between activity of EPHX1 and prostate cancer risk would be modified by PAHs (and/or other different carcinogenic substances) because they are lipophilic, hence easily absorbed and distributed in the whole human body [31]. It has been shown that the coding region substitutions appear to influence enzymatic activity of EPHX1 through alteration of protein stability rather than enzyme kinetics [11, 17, 32]. Additional genetic variants, possibly in regulatory regions of the *EPHX1* gene [11], variation in transcriptional/posttranscriptional modification may also play a role and have both, protective or promotional effect on developing of prostate cancer.

The results of our study are part of a continuing trend that demonstrates the importance of considering both gene-gene and/or gene-environment interactions in the context of cancer risk. Effects of variation of *EPHX1* on prostate cancer risk may also be enhanced when considering other important genes of activation/detoxification pathway for PAHs, such as *CYP1A1*, *CYP1B1*, *GST*, *NAT* (N-acetyltransferases), *UGT* (UDP-glucuronosyl-transferase), etc [33, 34]. Single nucleotide polymorphisms in these enzymes could also affect the balance of metabolic activation and detoxification in a given smoker, thus altering prostate cancer risk upon exposure to PAHs in cigarette smoke.

In summary therefore, in spite of a few limitations in the design and other minor considerations, we still observed no statistically significant overall associations of *EPHX1* polymorphisms with prostate cancer risk, although moderate associations of the *His/Arg* and *Arg/Arg* genotypes at exon 4 were suggested. We have found that smokers were at significantly increased risk if they have *His/Arg* genotype in compare with non-smokers with "slow" *His/His* genotype at exon 4. Significant interaction was found between normal phenotype

and smoking. Among non-smokers, risk was significantly increased for rapid genotype in comparison with non-smokers with normal phenotype. In order to further analyze the relationship between *EPHX1* polymorphisms and prostate cancer risk, large population studies are required that would take into account all the variables: association with other polymorphisms, exposure to environmental factors, ethnic and demographic particular features.

Acknowledgments: This work was supported by Ministry of Health of the Slovak Republic under the projects 2007/45-UK-10 "Genetic polymorphism of xenobiotic metabolising enzymes and susceptibility to prostate cancer in the Slovak population", MH SR 2007/57-UK-17 and by VEGA grant 1/426207. This work was supported by project „CENTER OF EXCELLENCE FOR RESEARCH ON PERSONALIZED THERAPY (CEVYPET)", code 2622012053, co-financed from EU sources and European Regional Development Fund. The authors are grateful to Dr N. A. Yeboah for critical reading of the manuscript and wish to thank Mrs M. Martinčeková for her technical assistance.

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