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Polymorphisms in metabolizing enzymes and the risk of head and neck squamous cell carcinoma in the Slavic population of the central Europe

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The question of susceptibility to squamous cell carcinoma of head and neck (SCCHN) in the environmental context was addressed by analysis of functional polymorphisms in enzymes metabolizing smoke constituents and/or alcohol (CYP2A13, CYP1B1, EPHX1, NQO1, GSTM1, GSTP1, GSTT1, ADH1B and ADH1C). Case-control study of 122 age- and sex-matched pairs of subjects was performed using so far unexplored Central European Slavic population with high level of tobacco and alcohol abuse.

Age-, gender-, smoking- and alcohol-adjusted logistic regression failed to demonstrate any significant association of the analyzed polymorphisms with the SCCHN risk. When interactions between potential modifiers of effect, i.e. smoking and alcohol were tested, drinkers seemed to be at lower risk than nondrinkers when carrying the heterozygous genotype Ile/Val in codon 432 of *CYP1B1* (OR=0.42; 95% CI=0.21-0.83; p=0.013 vs. OR=1.02; 95% CI=0.34-2.94; p=0.977). Similarly, drinkers were at lower risk than nondrinkers when carrying the heterozygous genotype Pro/Ser in codon 187 of *NQO1* (OR=0.41; 95% CI=0.19-0.88; p=0.022 vs. OR=0.96; 95% CI=0.29-3.12; p=0.948). More interestingly, drinkers carrying the rare homozygous genotype Val/Val in codon 350 of *ADH1C* were at significantly higher risk than nondrinkers carrying this genotype (OR=4.01; 95% CI=1.61-10.01; p=0.003 vs. OR=0.93; 95% CI=0.25-3.57; p=0.919). This result confirmed findings of previously published studies. Smoking did not significantly modify the effect of genotypes.

Our data thus demonstrate that genetic susceptibility to SCCHN shall be further followed on populations with different genetic background and lifestyle.

Keywords: oral cancer; exposure; metabolism; polymorphism; risk

Squamous cell carcinoma of the head and neck (SCCHN, OMIM no.: 275355) represents a serious health problem. In 2002, SCCHN was ranked as the eighth leading cause of cancer death worldwide. Approximately 481,100 new cases developed, and 320,000 persons died of this disease [1]. Although the SCCHN etiology remains unknown, the majority of cases can be attributed to tobacco and alcohol use [2]. Certain subsets of SCCHN have been related to Human Papillomavirus [3].

It has been estimated that main-stream tobacco smoke contains over 60 pro-carcinogens or carcinogens [4]. Additionally, a number of studies have suggested that ethanol along with elements of cigarette smoke may act as co-carcinogen and enhance tumor formation [5, 6]. Regarding the ethanol itself, its main metabolite acetaldehyde is a known mutagen and carcinogen, but discrimination between the role of ethanol and tobacco smoke is usually difficult due to synergic effects [7].

Sporadic carcinogenesis is a complex, multistage process, putatively modulated by genetic polymorphisms (SNPs) in a number of genes. SNPs in the coding and regulatory sequences of genes encoding xenobiotic-metabolizing (XME), DNA repair and cell cycle enzymes may result in subtle structural alterations with an effect on the protein function [8]. Thus, sporadic cancer may develop in individuals with combinations of relatively common allelic variants with low penetrance and moderately altered functions, but with significantly increased susceptibility to disease in connection with various environmental exposures and lifestyle factors [9, 10].

In order to address the question of susceptibility to SCCHN in the environmental context, we have analyzed functional polymorphisms in XME metabolizing smoke constituents and/or alcohol (CYP2A13, CYP1B1, EPHX1, NQO1, GSTM1, GSTP1, GSTT1, ADH1B and ADH1C). Case-control study was performed using Central European Slavic population with high level of tobacco and alcohol abuse. Our study thus reflected conclusion of the recent review: "Cohort studies that simultaneously consider multiple genetic and environmental factors possibly involved in carcinogenesis of the head and neck are needed to ascertain not only the relative contribution of these factors to tumor development but also the contributions of their putative interactions" [11].

Patients and methods

Subjects. Studied cases included 122 Czech and Polish patients of Slavic Caucasian origin with histologically diagnosed squamous cell carcinoma of the upper-aerodigestive tract (i.e. pharynx, hypopharynx and larynx) and the oral cavity (SCCHN). Both the Czech (n=39) and Polish (n=83) cases were sampled in the period between September 2004 and February 2007 by Otorhinolaryngology and Oncology departments in Prague and Lodz. Clinical-pathological data on patients were collected from their medical records (date of diagnosis, localization of tumor, TNM stage according to UICC and histological type of cancer). Information concerning sex, age and tobacco and alcohol abuse was obtained from interviews or clinical records at the time of diagnosis. Randomly selected controls were 179 cancer-free subjects recruited during the 3 month period after the cases recruitment by general practitioners in Prague. Controls were included into the study under the condition that the difference in their age was not larger than 5 years from cases recruited in the same period. Basic epidemiological data on all participants were collected from face-to-face questionnaire survey (personal and family history, short occupational history, smoking and drinking history, history of physical activity, reproductive history and nutritional information). At the end of the recruitment period controls were age- and sex-matched to the cases and thus 122 controls were included into the genotyping stage. Blood samples of 122 cases and 122 controls were available in sufficient quality for genotyping. All participants were informed and gave their written consent to participate in the study. The design of the study was approved by the Ethical Committees of the 1st and 3rd Faculty of Medicine, Charles University in Prague, Czech Republic and Bioethical Committee of the Nofer Institute of Occupational Medicine in Lodz, Poland.

DNA isolation and genotyping. Blood was collected during diagnostic procedures using tubes with K_3EDTA anticoagulant. Genomic DNA was extracted from peripheral lymphocytes using a BioSprint 15 DNA Blood Kit (Qiagen, Valencia, CA) by KingFisher mL automated system (Thermo Electron Corporation, Vantaa, Finland) according to the procedure supplied by the manufacturer. Polymorphisms in *CYP1B1* (Leu432Val, dbSNP: rs1056836 and Asn453Ser, rs1800440), *GSTM1* (gene deletion) and *GSTT1* (gene deletion) were assayed by published polymerase chain reaction (PCR) restriction fragment length polymorphism-based methods and allele-specific multiplex PCR [12]. Oligonucleotide primers were synthesized by Generi Biotech (Hradec Kralove, Czech Republic). Polymorphisms in *GSTP1* (Ile105Val, rs1695), *NQO1* (Pro187Ser, rs1800566), *EPHX1* (His113Tyr, rs1051740 and Arg139His, rs2234922), *CYP2A13* (Arg101STOP, rs72552266), *ADH1B* (Arg48His, rs1229984), and *ADH1C* (Ile350Val, rs698) were assayed by allelic discrimination with TaqMan Drug Metabolism Genotyping Assays (Applied Biosystems, Foster City, CA) using real time PCR in RotorGene 6000 (Corbett Research, Brisbane, Australia). The TaqMan assays (*GSTP1,* Ile105Val, C__3237198_20; *NQO1,* Pro187Ser, C__2091255_30; *EPHX1,* His113Tyr, C___14938_30, Arg139His, C__11638783_30; *CYP2A13*, Arg101STOP, C_30634006_10; *ADH1B*, Arg48His, C_2688467_20 and *ADH1C*, Ile350Val, C_26457410_10,) were used according to instructions of manufacturer (Applied Biosystems). EPHX1 enzyme activity was deduced according to the results of His113Tyr and Arg139His genotyping [13]. The non-template control consisted of a reaction tube in which water was used in place of DNA sample. 10% of randomly selected samples were reanalyzed with 100% concordance of the results.

Statistical analysis. In the first round of analyses, Hardy-Weinberg equilibrium was assessed for the case and control group. Then the Pearson´s χ^2 was used to test differences in genotype and allele distribution between case and controls and unadjusted risk was estimated. Binary logistic regression was performed to estimate odds ratios (OR), 95% confidence interval for OR and corresponding p-values of different genotype frequencies among the case and control group, adjusting for the age at recruitment, sex, smoking and alcohol consumption. Furthermore, we tested for modifying effect of smoking and alcohol, on the associations of interest. The $p<0.05$ would be considered significant. Analyses were performed using Win SPSS v15.0 (SPSS, Chicago, IL).

Results

Characteristics of the studied population. Complete characteristics of the studied population are presented in Table 1. The gender distribution, age at diagnosis and alcohol consumption did not significantly differ between both groups. Analyses confirmed smoking as SCCHN risk predictor (OR = 15.49; $p<0.001$; Table 1).

Polymorphisms and SCCHN risk. Unadjusted analyses showed that carriers of the heterozygous genotype in codon 432 of *CYP1B1* (Ile/Val) were at significantly decreased risk of SCCHN in comparison with the common homozygous genotype (Ile/Ile) carriers (OR=0.54; 95% CI=0.31–0.96; p=0.035). However, inheritance of the rare homozygous Val/Val genotype did not significantly affect the risk (OR=1.27; 95% CI=0.57–2.78; p=0.565) and the risk was not modified by

TABLE 1: Characteristics of the studied population
Percentages in brackets

	Controls	Cases	OR	95% CI	p
Gender					
Females	28 (23.0)	26 (21.5)	1.00 (reference)		
Males	94 (77.0)	96 (78.5)	1.09	0.59 – 1.99	0.784*
Age at recruitment (years)					
Mean ± SD	60.5 ± 10.0	60.6 ± 10.1	-	-	0.916#
Range	31 - 84	31 - 86			
Smoking status					
Nonsmokers	77 (65.3)	12 (10.8)	1.00 (reference)		
Smokers	41 (34.7)	99 (89.2)	15.49	7.62 – 31.48	<0.001*
Unknown	4	11			
Drinking status					
Nondrinkers	33 (28.0)	34 (28.8)	1.00 (reference)		
Drinkers	85 (72.0)	84 (71.2)	0.96	0.54 – 1.69	0.885*
Unknown	4	4			

* Pearson Chi-Square Test; OR, crude odds ratio; 95% CI, 95% confidence interval, p-two sided
# ANOVA test

the second followed polymorphism in codon 453 of *CYP1B1* (results not shown). Rare homozygotes for polymorphism in codon 350 of *ADH1C* (Val/Val) were at significantly higher SCCHN risk than carriers of the common homozygous genotype Ile/Ile (OR=2.70; 95% CI=1.28–5.56; p=0.009). Neither polymorphisms in *EPHX1, GSTM1, GSTP1, GSTT1, NQO1, CYP2A13*, *ADH1B* nor the deduced EPHX1 activity showed significant association with the SCCHN risk in the unadjusted analyses (results not shown). Age-, gender-, smoking- and alcohol-adjusted logistic regression failed to demonstrate any significant association of the analyzed polymorphisms with the SCCHN risk (Table 2). The effect of heterozygous genotype in codon 432 of *CYP1B1* was at the border of significance (p=0.055, Table 2) and the effect of rare homozygous genotype of *ADH1C* was not significant (p=0.237). Combination of EPHX1-low activity with GSTM1-null genotype did not significantly affect the SCCHN risk (OR=0.73; 95% CI=0.34-1.59; p=0.426). When interactions between potential modifiers of effect, i.e. the followed exposure factors (smoking and alcohol) and the studied genotypes were tested, drinkers seemed to be at lower risk than nondrinkers when carrying the heterozygous genotype Ile/Val in codon 432 of *CYP1B1* (OR=0.42; 95% CI=0.21-0.83; p=0.013 vs. OR=1.02; 95% CI=0.34-2.94; p=0.977). Similarly, drinkers were at lower risk than nondrinkers when carrying the heterozygous genotype Pro/Ser in codon 187 of *NQO1* (OR=0.41; 95% CI=0.19-0.88; p=0.022 vs. OR=0.96; 95% CI=0.29-3.12; p=0.948). More interestingly, drinkers carrying the rare homozygous genotype Val/Val in codon 350 of *ADH1C* were at significantly higher risk than nondrinkers carrying this genotype (OR=4.01; 95% CI=1.61-10.01; p=0.003 vs. OR=0.93; 95% CI=0.25-3.57; p=0.919). Other interactions with alcohol were not significant (results not shown). Smoking did not significantly modify the effect of genotypes as well (results not shown).

Discussion

It is assumed that genetic variation in carcinogen metabolism modifies the risk of exposure-related cancer [14]. Tobacco carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are metabolized in the human body by cytochromes P450 (P450, EC 1.14.14.1). NNK induces lung tumors in all laboratory animals tested as well as the nasal cavity, pancreatic cancer, and liver tumors in rats [15]. P450 2A13 (OMIM: 608055) was suggested the most active enzyme in metabolic activation of NNK [16]. The nonsense polymorphism, *CYP2A13*1/*7* (Arg101Stop, rs72552266,) may be important modifier of tobacco-associated cancer risk because the truncated protein lacks enzymatic activity [17]. The role of CYP2A13 knock-out polymorphisms in SCCHN was so far not studied and our recent study suggested a possible role of this polymorphism in pancreatic cancer etiology [18]. However, due to the low numbers of studied SCCHN cases the power of our present study was not high enough to answer this question and larger multi-centric study should be used in order to clarify the role of rs72552266 in SCCHN.

Cytochrome P450 1B1 (OMIM: 601771) plays an important role in activating polycyclic aromatic hydrocarbons (PAH) or heterocyclic amines to reactive metabolites that cause DNA damage. Certain *CYP1B1* haplotypes have recently been associated with SCCHN [19]. Our results suggested possible association of the heterozygous genotype Ile/Val in codon 432 of *CYP1B1* with the increased SCCHN risk in unadjusted analyses (p=0.035). However, this observation was not confirmed by age-, gender-, smoking- and alcohol-

TABLE 2: Associations of polymorphisms in *CYP1B1, EPHX1, NQO1, GSTM1, GSTP1, GSTT1, CYP2A13, ADH1B* and *ADH1C* with SCCHN risk
Numbers of genotype carriers presented (percentages in brackets).

Gene	Genotype	Controls	Cases	OR†	95% CI†	p†
CYP1B1	*Leu/Leu*	37 (30.3)	46 (39.7)	1.00 (reference)		
codon 432	*Leu/Val*	71 (58.2)	48 (41.4)	0.49	0.24 – 1.01	0.055
	Val/Val	14 (11.5)	22 (19.0)	1.02	0.36 – 2.94	0.982
	Leu/Val+Val/Val	85	70	0.58	0.29 – 1.14	0.115
CYP1B1	*Asn/Asn*	83 (68.0)	79 (68.1)	1.00 (reference)		
codon 453	*Asn/Ser*	34 (27.9)	31 (26.7)	1.56	0.72 – 3.45	0.252
	Ser/Ser	5 (4.1)	6 (5.2)	1.37	0.30 – 6.25	0.684
	Asn/Ser+Ser/Ser	39	37	1.51	0.74 – 3.12	0.257
NQO1	*Pro/Pro*	83 (68.6)	92 (79.3)	1.00 (reference)		
codon 187	*Pro/Ser*	35 (28.9)	21 (18.1)	0.53	0.25 – 1.14	0.105
	Ser/Ser	3 (2.5)	3 (2.6)	1.10	0.12 – 10.0	0.935
	Pro/Ser+Ser/Ser	38	24	0.57	0.28 – 1.19	0.134
GSTM1	*plus*	52 (42.6)	52 (44.8)	1.00 (reference)		
(deletion)	*null*	70 (57.4)	64 (55.2)	0.99	0.71 – 1.37	0.946
GSTT1	*plus*	93 (85.3)	92 (79.3)	1.00 (reference)		
(deletion)	*null*	16 (14.7)	24 (20.7)	1.02	0.68 – 1.56	0.908
GSTP1	*Ile/Ile*	57 (46.7)	56 (48.3)	1.00 (reference)		
codon 105	*Ile/Val*	55 (45.1)	53 (45.7)	1.22	0.63 – 2.38	0.564
	Val/Val	10 (8.2)	7 (6.0)	1.06	0.33 – 3.45	0.920
	Ile/Val+Val/Val	65	60	1.20	0.64 – 2.27	0.557
EPHX1	*Tyr/Tyr*	53 (46.9)	60 (51.7)	1.00 (reference)		
codon 113	*Tyr/His*	48 (42.5)	42 (36.2)	0.71	0.34 – 1.45	0.348
	His/His	12 (10.6)	14 (12.1)	1.69	0.56 – 5.01	0.354
	Tyr/His+His/His	60	56	0.83	0.43 – 1.61	0.591
EPHX1	His/His	77 (63.1)	71 (61.2)	1.00 (reference)		
codon 139	His/Arg	41 (33.6)	40 (34.5)	0.96	0.49 – 1.89	0.910
	Arg/Arg	4 (3.3)	5 (4.3)	0.94	0.20 – 4.55	0.940
	His/Arg+Arg/Arg	45	45	0.94	0.49 – 1.82	0.861
EPHX1	low	44 (38.9)	38 (32.8)	1.05	0.53 – 2.08	0.870
activity	medium	49 (43.4)	55 (47.4)	1.00 (reference)		
	high	20 (17.7)	23 (19.8)	0.89	0.39 – 2.04	0.796
CYP2A13	*Asn/Asn*	119 (68.0)	120 (99.2)	1.00 (reference)		
codon 101	*Asn/STOP*	2 (1.7)	1 (0.8)	1.12	0.07 – 16.67	0.932
	STOP/STOP	0 (0)	0 (0)	–‡	–‡	–‡
ADH1B	*Arg/Arg*	111 (91.0)	101 (90.2)	1.00 (reference)		
codon 48	*Arg/His*	10 (8.2)	21 (9.0)	2.38	0.61 – 9.10	0.214
	His/His	1 (0.8)	0 (0)	–‡	–‡	–‡
	Asn/Ser+Ser/Ser	11	21	1.67	0.45 – 6.25	0.443
ADH1C	*Ile/Ile*	39 (32.2)	30 (24.8)	1.00 (reference)		
codon 350	*Ile/Val*	64 (52.9)	54 (44.6)	0.98	0.46 – 2.13	0.971
	Val/Val	18 (14.9)	37 (30.6)	1.75	0.69 – 4.55	0.237
	Ile/Val+Val/Val	82	91	1.12	0.54 – 2.33	0.756

† Odds ratios (OR) and 95% confidence intervals (95% CI) adjusted for age at diagnosis, gender, smoking and alcohol
‡ Statistics could not be performed due to the absence of individuals in one or more of analyzed groups

adjusted analyses (p=0.055) and the contribution of the rare homozygous genotype to the risk was also non-significant. On the contrary, the observed modification of SCCHN risk by interaction between alcohol and this polymorphism (p=0.013) warrants further study.

NAD(P)H:quinone oxidoreductase (NQO1, EC 1.6.99.2, OMIM: 125860) is an obligate two-electron reductase that can either bioactivate or detoxify quinones and has been proposed to play an important role in chemoprevention [20]. NQO1 polymorphism in codon 187 (Pro187Ser, rs1800566), encoding

an inactive enzyme was shown to influence the risk of breast cancer in Czech [21] and Austrian [22] populations. Moreover, recent study showed that *NQO1* Pro187Ser polymorphism may play role of a strong prognostic and predictive factor in breast cancer [23]. Begleiter et al [24] did not find significant association of two NQO1 polymorphisms (Pro187Ser and Trp465Arg) with SCCHN. Similarly, Harth et al. [25] also did not find association of Pro187Ser polymorphism in NQO1 with SCCHN. We observed, that drinkers had significantly lower SCCHN risk when carrying the NQO1 heterozygous genotype (p=0.022) in comparison with drinkers carrying the common genotype (Pro/Pro) but in nondrinkers no effect on the risk was evident (p=0.948). However, not much was published about interaction between NQO1 and alcohol. The association between alcohol and colorectal adenoma was modified by NQO1 Pro187Ser genotype in UKFSS Study. Higher risk was found among individuals with the common Pro/Pro genotype (OR=1.49; 95% CI=1.11-2.02; P-interaction=0.024; [ref. 26]). Therefore, larger studies should confirm or rule out our observation of interaction between *NQO1* knock-out polymorphism and alcohol consumption towards SCCHN risk.

Epoxide hydrolase (EPHX1, EC 3.3.2.3, OMIM: 132810) catalyzes the hydrolysis of epoxides (originating from e.g. P450 1B1-mediated metabolism of PAHs) to less reactive *trans*-dihydrodiols. Two common alleles of *EPHX1* that can be detected in codons 113 (Tyr113His, rs1051740) and 139 (His139Arg, rs2234922) change the enzyme activity [27]. Glutathione *S*-transferases (GST, EC 2.5.1.18) GSTM1 (OMIM: 138350), GSTP1 (OMIM: 134660) and GSTT1 (OMIM: 600436) belong to the most frequently studied XME in molecular epidemiology of cancer. Large genomic deletions (null genotype) of *GSTM1* and *GSTT1* produce complete lack of enzyme activities. *GSTP1* polymorphism in codon 105 (Ile105Val, rs1695) generates enzyme with different heat stability and substrate affinity [28]. We have found that combination of EPHX1-low deduced activity with either *GSTM1*-null or *GSTT1*-null genotypes significantly increased levels of DNA single strand breaks as an early genotoxicity marker in peripheral blood lymphocytes of 158 healthy volunteers [9]. SCCHN is cancer highly dependent at environmental factors (alcohol and smoking) and thus it seemed to be a suitable candidate for evaluation of the previously found genotoxicity relevance of metabolic polymorphisms. However, we did not find association of either single *EPHX1, GSTM1, GSTP1, GSTT1* polymorphisms, the deduced EPHX1 activity nor the combination of EPHX1 activity and *GSTM1* polymorphism with SCCHN risk. Thus, our results confirmed the lack of association of functional *EPHX1* polymorphisms and the deduced enzyme activity with SCCHN risk published previously [29, 30]. Our results also support the conclusion of recent meta-analysis that *GSTM1* null genotype significantly increases susceptibility to oral cancer in Asians but not Caucasians [31] and comply with negative results on *GSTP1* and *GSTT1* polymorphisms [32].

Ethanol metabolism is mediated by both the oxidative and the non-oxidative pathways [33]. The oxidative pathway is particularly catalyzed by alcohol dehydrogenases (ADH, EC 1.1.1.1), aldehyde dehydrogenases (ALDH, EC 1.2.1.5) and less by P450 2E1 [34]. Previous case-control studies showed the association between *ADH1B*1* (Arg) allele and an increased risk of SCCHN [35, 36]. Carriage of *ADH1C*2/*2* (Val/Val) genotype increased risk of SCCHN in heavy (OR=2.65; 95% CI=1.08-2.14) and moderate (OR=1.6; 95% CI=1.15-2.03) drinkers [37]. Similar results were published by Hashibe et al. [38] in a large study on Central European population (Czech, Polish, Romanian, Russian and Slovak) but this study followed more heterogeneous patient group including SCC esophageal cancers. Our study thus did not confirm the published associations of SCCHN with the *ADH1B* (OMIM: 103720) common homozygous genotype. However, in our study the *ADH1C* (OMIM: 103730) rare homozygous genotype (Val/Val) was associated with SCCHN risk in all participants (unadjusted OR=2.70, p=0.009) and especially in drinkers (OR=4.01, p=0.003). On the other hand, the common Ile/Ile genotype of *ADH1C* was identified as an independent risk factor for the development of alcohol-associated tumors among heavy drinkers, indicating a genetic predisposition of individuals carrying this genotype [39]. Another study on Caucasians, however reported an inversed association in heavy drinkers carrying the rare *ADH1C* Val/Val genotype (OR=7.1; 95% CI=2.3-22.0) suggesting its association with susceptibility to smoking and drinking-related SCCHN by modifying the biologically effective dose of alcohol [40]. It remains to be discovered e.g. by meta-analyses, whether these discrepancies were caused by different populations under study (Asians vs. Caucasians) or variation in study design. Reports on null or confirmatory results may play an important role in this effort.

In conclusion, our data demonstrate complex nature of interactions between genetic susceptibility, environmental factors and SCCHN risk. More studies in various populations with different genetic background and lifestyle habits are needed to understand the variations in SCCHN risks reported so far and to move towards reliable biomarker of susceptibility for targeting of preventive measures.

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