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Otolaryngology -- Head and Neck Surgery 2011 145: 586 originally published online 25 June 2011
DOI: 10.1177/0194599811408778

The online version of this article can be found at:
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Otolaryngology–
Head and Neck Surgery
145(4) 586–593
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Surgery Foundation 2011
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DOI: 10.1177/0194599811408778
http://otojournal.org

Genetic Polymorphisms of *ADH1C* and *CYP2E1* and Risk of Oral Squamous Cell Carcinoma

Miroslav Brocic, MD[1,*], Gordana Supic, PhD[2,*], Katarina Zeljic, MS[2], Nebojsa Jovic, MD, PhD[1], Ruzica Kozomara, MD, PhD[1], Sladjana Zagorac, MS[2], Milica Zlatkovic, MS[3], and Zvonko Magic, MD, PhD[2]

No sponsorships or competing interests have been disclosed for this article.

Received February 10, 2011; revised April 6, 2011; accepted April 8, 2011.

Abstract

Objective. Several studies have suggested that the metabolism of alcohol is modulated by the polymorphisms in genes encoding ethanol-metabolizing enzymes, including alcohol dehydrogenase 1C, *ADH1C*, and cytochrome P450-dependent monooxygenase, *CYP2E1*. Genetic polymorphisms of ethanol-metabolizing enzymes may affect individual susceptibility to oral cancer. The purpose of this study was to investigate the associations between *ADH1C* and *CYP2E1* gene polymorphisms with oral squamous cell carcinoma in an ethnically homogeneous Caucasian population.

Design. Case-control study.

Setting. Serbian national general hospital.

Subjects and Methods. The study was conducted on 123 oral cancer patients and a control group of 177 individuals of the Caucasian race and the same ethnicity, matched in age and gender, without previous cancer history. The control group consisted of 120 population-based and 57 hospital-based controls of heavy-drinking individuals. Genetic polymorphisms of *ADH1C SspI*, *ADH1C HaeIII*, *CYP2E1 RsaI*, and *CYP2E1 Ins* were determined by the polymerase chain reaction and restriction fragment length polymorphisms.

Results. After adjustment by potential confounders, the significant increase of oral cancer risk, independent of alcohol drinking, was observed in individuals with the variant *ADH1C SspI*2/*2* genotype (odds ratio, 3.029; *P* = .014) and in combined *ADH1C SspI*1/*2* and *ADH1C SspI*2/*2* genotypes (odds ratio, 2.605; *P* = .002), compared to the *ADH1C*1/1** wild type. The association of other polymorphisms under study was not observed.

Conclusion. This study suggested that the *ADH1C SspI* polymorphism could play a significant role in the etiology of oral cancer, whereas *ADH1C HaeIII*, *CYP2E1 RsaI*, and *CYP2E1 Ins* could have minor influence.

Keywords

oral cancer risk, genetic polymorphisms, *ADH1C*, *CYP2E1*, alcohol

A number of studies have reported head and neck squamous cell carcinoma (HNSCC) and oral squamous cell carcinoma (OSCC) association with etiological factors such as smoking and quantity and type of alcohol beverages.[1-3] Although alcohol and smoking are well-known risk factors, only a fraction of smokers and alcohol consumers develop OSCC, suggesting that genetic susceptibility and interactions between genetic, epigenetic, and environmental factors could play an important role in the etiology of OSCC.[3]

The effect of alcohol could be modulated by the polymorphisms in the genes encoding ethanol metabolism enzymes, including alcohol dehydrogenase (*ADH*) and cytochrome P450-dependent monooxygenase (*CYP2E1*). Alcohol dehydrogenases catalyze the conversion of alcohols to acetaldehyde via oxidation, and aldehyde is subsequently detoxified into acetate by aldehyde dehydrogenases (*ALDH*).[4] The *ADH1C* gene has 2 alleles, *ADH1C*1* and *ADH1C*2* coding the $\gamma 1$ and $\gamma 2$ enzyme subunits that have different in vitro kinetic properties. Functional single nucleotide polymorphism (SNP) *ADH1C SspI* located in exon 8 is characterized by Ile349Val substitution, whereas *ADH1C HaeIII* is a site silent SNP, located in exon 5.[4] The isoenzymes encoded by the *ADH1C*1* (Ile349) allele metabolize ethanol into acetaldehyde 2.5 times faster than those encoded by the *ADH1C*2* allele,[5] which could lead to the profound generation and accumulation of acetaldehyde.

Another enzyme involved in ethanol metabolism *CYP2E1* is the main enzyme component of microsomal ethanol oxidation system, which oxidizes alcohol to acetaldehyde, metabolizes

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tobacco-associated procarcinogens to reactive metabolites, and generates reactive oxygen intermediaries. The single nucleotide polymorphism at the position G–1259C, located in the 5′-flanking region of the gene, is associated with a 10 times higher transcriptional activity, elevated protein levels, and increased enzyme activity of the *c2* mutant variant, compared to the wild-type *c1* allele.[6] The 96-bp insertion polymorphism in the 5′-flanking region was also found to be associated with increased transcriptional activity[7] and the increased induction of the enzyme by alcohol consumption.[8]

Findings of *ADH1C* and *CYP2E1* association with head and neck and oral cancer incidence are inconsistent. Although several studies have shown increased head and neck or oral cancer risk in individuals with the *ADH1C*1/*1* genotype,[9-13] other studies have shown opposite findings of the association of the *ADH1C*2/*2* genotype with increased head and neck cancer risk.[14-17] Although in the majority of studies *CYP2E1* was not associated with oral cancer risk,[18] in other studies, the *CYP2E1 c2* mutated allele was associated with OSCC.[19,20]

The aim of this study was to evaluate the associations between *ADH1C* and *CYP2E1* gene polymorphisms with oral cancer in a single Caucasian population, given that these genes are highly polymorphic in Caucasians as opposite of rare *ALDH* and *ADH1B* polymorphisms.[4] To our knowledge, *ADH1C* and *CYP2E1* polymorphisms in the Serbian population in general and in patients with OSCC have not been previously determined. Therefore, we hypothesized that polymorphisms of the *ADH1C* gene, identified by *SspI* and *HaeIII* digestion; *CYP2E1* polymorphisms, identified by *RsaI* digestion; and insertion of 96 bp could play an important role in the etiology of oral cancer, and we evaluated whether the effect of these polymorphisms could be modified by alcohol drinking and/or smoking.

Materials and Methods

Study Group

A case-control study was conducted on the Serbian population. Written informed consent was obtained from all study subjects, and the ethical committee of the Military Medical Academy, Belgrade, Serbia, reviewed this research and approved the investigations on human samples, according to human subjects protection regulations. The study cohort consisted of 123 OSCC patients and 177 healthy individuals, without previous cancer history. Cases and control group participants consisted of Caucasians of the same ethnicity, matched in age and gender.

Incident cases between 2002 and 2007 with histologically confirmed OSCC of the tongue, gums, and floor of the mouth were included in the study. All patients underwent operation at the Clinic for Maxillofacial Surgery, Military Medical Academy, Belgrade, Serbia. Primary OSCC was obtained at the time of surgery, and the tissue samples were snap frozen and stored at –20°C. Blood samples from the control group were stored at –20°C until the DNA extraction.

Face-to-face interviews by an experienced interviewer were conducted to obtain the information on demographic factors, smoking, and alcohol drinking in both cases and the control group. Given that the numbers of nonsmokers and nondrinkers were limited among cases, subjects were classified as current smokers and never/former smokers and nonheavy and heavy alcohol drinkers. Smokers were defined as individuals who had ever smoked cigarettes daily for at least 1 year, whereas former smokers were defined as those who quit smoking 1 year or more before having a diagnosis.

Subjects who consumed more than 21 alcohol drinks per week were classified as heavy drinkers, categorized by the distributions among cases, and according to previous studies.[21,22]

Because population-based controls included only 5 heavy drinkers, we have included 57 hospital-based controls of heavy-drinking individuals of the same race and ethnicity, matched in age and gender, without cancer history.

ADH1C *and* CYP2E1 *Genotyping*

Genomic DNA from OSCC samples was isolated using a TRIZOL reagent (Invitrogen, Darmstadt, Germany). Tumor as a source of OSCC patients' DNA was used in this retrospective study according to previous studies[23-25] because tumor specimens have been routinely archived. However, for 27 patients, paired blood samples and tumor samples were available for the comparison of SNPs in DNA isolated from blood and tumor tissue. Extraction of DNA from the blood from the control group of healthy blood donors was performed with the Blood Prep Isolation Kit (Qiagen, Hilden, Germany). All genotype determinations were performed in duplicates, blinded of the case or control status of isolated DNA and to the alcohol drinking status.

ADH1C SspI, ADH1C HaeIII, CYP2E1 RsaI, and *CYP2E1 Ins* polymorphism analysis was assessed by the polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLPs), according to the previously described genotyping protocols and primer sequences.[4,7,26,27] After initial amplification, fragments were digested with *HaeIII, SspI,* or *RsaI* enzyme (Fermentas, Sankt Leon-Rot, Germany) according to the manufacturers' protocols, whereas the insertion polymorphism, characterized by the insertion of 96 bp in the *CYP2E1* gene, was detected by PCR amplification, with subsequent gel electrophoresis. The digested fragments were resolved after electrophoresis in 2% Et-Br stained agarose gel. Details of the individual polymorphisms and genotyping protocols are given in **Table 1.**

Statistical Analysis

Obtained data were analyzed using SPSS 16.0 software (SPSS Inc, an IBM Company, Chicago, Illinois). Contingency tables were analyzed using the χ^2 test or Fisher's exact test when appropriate. Unconditional logistic regression analysis was performed in case-control analysis to estimate the odds ratio (OR), with a confidence interval (CI) of 95%. Adjustments were made to eliminate or reduce the effects of confounding factors, such as age, gender, alcohol use, and smoking status. All reported *P* values were 2-sided, and all associations were considered significant when *P* values were less than .05.

Results

The main characteristics of the studied OSCC cases and control group of this Serbian population are presented in **Table 2**. All participants were of the same race and ethnicity, and age and gender distributions were similar in cases and controls.

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Table 1. Details of the Individual Polymorphisms and Genotyping Protocols

Gene	Location	Polymorphism (RefSNP)	Method/Enzyme	Allele and Size, bp	Reference
ADH1C	Exon 8	(rs698) Ile349Val	PCR-RFLP/*SspI*	Wild type, 131; mutant variant, 68, 63	Groppi et al[27] (1990)
ADH1C	Exon 5	A to G trans (rs1693425); silent SNP	PCR-RFLP/*HaeIII*	Wild type, 435; mutant variant, 193, 242	Osier et al[4] (2002)
CYP2E1[a]	5′-flanking region	C to T transition (rs2031920)	PCR-RFLP/*RsaI*	Wild type, 360, 50; mutant variant, 410	Le Marchand et al[26] (1999)
CYP2E1	5′-flanking region	Insertion of 96 bp	PCR	Wild type, 633; mutant variant, 729	Fritsche et al[7] (2000)

[a]Single nucleotide polymorphism (SNP) is in linkage disequilibrium with 2 other SNPs: 1293G>C (RFLP *PstI*) and 7632T>A (RFLP *DraI*). PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Table 2. Demographic Characteristics and Polymorphism Prevalences in Oral Squamous Cell Carcinoma Patients and Control Group Participants

Variable	Cases	Controls	*P*
Sex			.752
Male, n	91	135	
Female, n	32	42	
Age,[a] y			.200
<58, n	54	92	
≥58, n	69	85	
Smoking			.730
Never/former, n	42	65	
Current, n	81	112	
Alcohol drinking			.000
Nonheavy, n	31	115	
Heavy, n	92	62	
ADH1C SspI			.000[b]
**1/*1 (wt)*, n	24	72	
**1/*2 (het)*, n	79	80	
**2/*2 (mut)*, n	20	25	
ADH1C HaeIII			.815[b]
**1/*1 (wt)*, n	48	69	
**1/*2 (ht)*, n	56	85	
**2/*2 (mut)*, n	19	23	
CYP2E1 RsaI			.089[b]
c1/c1 (wt), n	105	160	
c1/c2 (ht), n	13	16	
c2/c2 (mut), n	5	1	
CYP2E1 Ins			.720[b]
wt, n	117	165	
ht, n	4	6	
mut, n	2	6	

Abbreviation: *wt/ht/mut*, number of wild-type, heterozygotes, and mutant genotypes, respectively, in studied oral squamous cell carcinoma cohort.
[a]Age according to median value of 58 years.
[b]*P* values calculated by χ^2 test for mutant allele versus wild type.

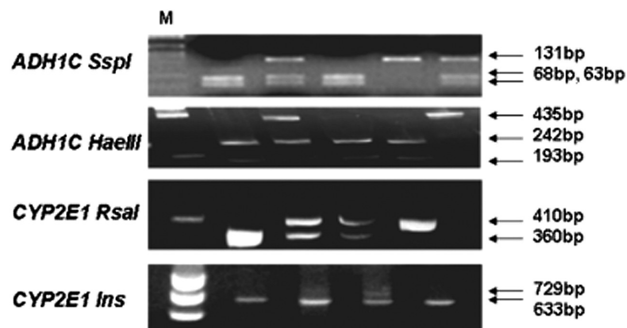

Figure 1. Representative examples of patient genotype analysis. *ADH1C SspI*, *ADH1C HaeIII,* and *CYP2E1 RsaI* polymorphism analysis was performed by PCR/RFLP. After initial amplification, fragments were subsequently digested with appropriate enzyme and analyzed by Et-Br stained agarose. For *ADH1C SspI*, *ADH1C HaeIII,* and *CYP2E1 RsaI,* individual lanes represent digestion fragments, whereas for *CYP2E1 Ins,* individual lanes represent PCR products. Detailed genotyping protocols and fragment sizes are presented in **Table 1**. To prevent incomplete digestions, equal amounts of DNA were subjected to the same digestion conditions, with the sufficient amount of the enzymes and prolonged incubation time, according to manufacturer protocols (Fermentas, Sankt Leon-Rot, Germany). In addition, multiple control heterozygote and mutant homozygote samples were tested to eliminate incomplete digestion bias. Lane 1: M, DNA marker. For *ADH1C SspI* polymorphism: lanes 2 and 4, homozygote mutant (*mut*); lanes 3 and 6, heterozygote (*het*); lane 5, homozygote wild type (*wt*). For *ADH1C HaeIII* polymorphism: lanes 2, 4, and 5, *mut*; lane 3, *het*; lane 6, *wt*. For *CYP2E1 RsaI* polymorphism: lane 2, *wt*; lanes 3 and 4, *het*; lane 5, *mut*. For *CYP2E1 Ins* polymorphism: lanes 2 and 5, *wt*; lanes 3 and 4, *het*. PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

For 27 patients, a complete concordance (100%) of the peripheral blood genotypes and the genotypes determined in corresponding tumor tissue was obtained for each of the individual polymorphisms under study, in accordance with previous studies.[23-25]

The significant differences in the frequencies of the *ADH1C SspI* genotypes were observed among the OSCC group and the control group, *P* = .000, χ^2 test (**Table 2**). The *ADH1C**2 variant allele showed a trend of higher incidence in healthy high-alcohol consumers compared to nondrinking controls (*P* = .110). For the other polymorphisms under study, the differences among the frequencies determined in the studied OSCC cohort and control group were not observed. Detailed genotyping protocols are presented in **Table 1**, and representative examples of genotyping analysis are presented in **Figure 1**.

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Table 3. Association between *ADH1C SspI*, *ADH1C HaeIII*, *CYP2E1 RsaI,* and *CYP2E1 Ins* Polymorphism and Clinical and Pathological Variables in the Studied Oral Squamous Cell Carcinoma Group

	wt/ht/mut			
Variable	*ADH1C SspI*	*ADH1C HaeIII*	*CYP2E1 RsaI*	*CYP2E1 Ins*
Gender				
Male	20/56/15	34/40/17	78/10/3	88/2/1
Female	4/23/5	14/16/2	27/3/2	29/2/1
[a]*P*	NS	NS	NS	NS
Age,[b] y				
<58	10/36/8	25/21/8	51/3/0	52/2/0
≥58	14/43/12	23/35/11	54/10/5	65/2/2
[a]*P*	NS	NS	NS	NS
Smoking				
Never/former	7/20/4	12/16/3	26/3/2	28/2/1
Current	17/59/16	36/40/16	79/10/3	89/2/1
[a]*P*	NS	NS	NS	NS
Alcohol drinking				
Nonheavy	15/52/14	33/38/10	69/8/4	76/4/1
Heavy	9/27/6	15/18/9	36/5/1	41/0/1
[a]*P*	NS	NS	NS	NS
Oral hygiene				
Good	15/32/7	22/23/7	59/9/1	68/1/0
Poor	9/47/13	26/31/12	46/4/4	49/3/2
[a]*P*	.041	NS	NS	.096
Stage				
II	9/17/3	14/12/3	26/1/2	27/1/1
III	15/62/17	34/44/16	79/12/3	90/3/1
[a]*P*	.073	NS	NS	NS
Tumor size				
T1/2	20/57/12	34/42/13	76/9/4	89/3/2
T3/4	4/22/8	14/14/6	29/4/1	33/1/0
[a]*P*	.099	NS	NS	NS
Nodal status				
N0	9/15/13	13/10/4	26/0/1	25/1/1
N+	15/64/17	35/46/15	79/13/4	92/3/1
[a]*P*	.040	NS	NS	NS

Data represent the total number of patients in the wild-type/heterozygotes/mutant (*wt/ht/mut*) genotypes in studied oral squamous cell carcinoma cohort. *P* < .100 presented as exact values. NS, not significant. *P* values for mutant allele versus wild type are not significant and thus not presented.
[a]*P* values for variant allele carriers (combined heterozygotes and variant homozygotes) versus wild type.
[b]Age according to median value of 58 years (range, 36-80 years).

The association between *ADH1C SspI*, *ADH1C HaeIII*, *CYP2E1 RsaI,* and *CYP2E1 Ins* polymorphism and clinical and pathological variables in the studied OSCC patient cohort is presented in **Table 3**. Homozygote mutant genotypes for any of the studied gene polymorphisms were not associated with clinicopathological characteristics and therefore are not presented. The *ADH1C*1/*1* and *ADH1C*1/*2* genotypes were combined in subsequent analyses as allele *ADH1C*2* carriers and were associated with poor oral hygiene, compared to the wild-type *ADH1C SspI *1/1** genotype, *P* = .041 (**Table 3**). In allele *ADH1C SspI *2* carriers, the presence of nodal metastases was more frequent, *P* = .040. An association of other polymorphisms under study with clinicopathological characteristics was not observed (**Table 3**).

The multivariate adjusted ORs are shown by *ADH1C* and *CYP2E1* genotypes in **Tables 4** and **5**. After adjustment for age, gender, alcohol use, and smoking status, the OR was significantly higher among individuals with the *ADH1C SspI *2/*2* genotype (OR, 3.029; 95% CI, 1.256-7.304; *P* = .014) and with the heterozygote *ADH1C SspI *1/*2* genotype (OR, 2.605; 95% CI, 1.496-4.536; *P* = .001) compared to the wild-type *ADH1C SspI *1/*1* genotype (**Table 4**). Allele *ADH1C*2* carriers, combined *ADH1C*1/*1* and *ADH1C*1/*2* genotypes, compared to the *wt ADH1C**1/1* genotype revealed significant increase of adjusted OR (OR, 2.605; 95% CI, 1.496-4.53; *P* = .002). An association of other polymorphisms under study with oral cancer risk was not observed (**Tables 4** and **5**).

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Table 4. *ADH1C* Polymorphisms and the Risk of Oral Squamous Cell Carcinoma

Gene/Genotype	Cases n	Cases %	Controls n	Controls %	Adjusted OR (95% CI)[a]	P
ADH1C SspI						
**1/*1 (wt)*	24	19.5	72	40.7	1.00	Reference
**1/*2 (ht)*	79	64.2	80	45.2	2.814 (1.450-5.461)	.002
**2/*2 (mut)*	20	16.3	25	14.1	3.029 (1.256-7.304)	.014
**1/*2 or *2/*2[b] (*2 carrier)*	99	80.5	105	59.3	2.605 (1.496-4.536)	.001
ADH1C HaeIII						
**1/*1 (wt)*	48	39	69	39	1.00	Reference
**1/*2 (ht)*	56	45.5	85	48	0.848 (0.462-1.557)	.594
**2/*2 (mut)*	19	15.4	23	13	1.581 (0.681-3.671)	.287
**1/*2 or*2/*2[b] (*2 carrier)*	75	61	108	61	1.016 (0.621-1.661)	.950

Abbreviations: CI, confidence interval; OR, odds ratio; *wt/ht/mut,* number of wild-type, heterozygotes, and mutant genotypes, respectively, in studied oral squamous cell carcinoma cohort.
[a]Adjusted for age, gender, smoking status, and alcohol intake.
[b]Heterozygotes *1/*2 and variant homozygotes *2/*2 combined as allele *2 carriers.

Table 5. *CYP2E1* Polymorphisms and the Risk of Oral Squamous Cell Carcinoma

Gene/Genotype	Cases n	Cases %	Controls n	Controls %	Adjusted OR (95% CI)[a]	P
CYP2E1 RsaI						
c1/c1 (wt)	105	85.4	160	90.4	1.00	Reference
c1/c2 (ht)	13	10.6	16	9	1.007 (0.393-2.581)	.988
c2/c2 (mut)	5	4.1	1	0.6	2.623 (0.258-26.639)	.415
c1/c2 or c2/c2 (c2 carrier)	18	14.6	17	9.6	1.597 (0.762-3.348)	.215
CYP2E1 Ins						
0 (wt)	117	95.1	165	93.2	1.00	Reference
1 (ht)	4	3.3	6	3.4	0.734 (0.165-3.264)	.684
2 (mut)	2	1.6	6	3.4	0.213 (0.029-1.586)	.131
1 or 2 (Ins carrier)	6	4.9	12	6.8	0.699 (0.242-2.024)	.509

Abbreviations: CI, confidence interval; OR, odds ratio; *wt/ht/mut,* number of wild-type, heterozygotes, and mutant genotypes, respectively, in studied oral squamous cell carcinoma cohort.
[a]Adjusted for age, gender, smoking status, and alcohol intake.
[b]Heterozygotes and variant homozygotes combined as variant allele 2 carriers.

In subsequent analyses, we examined the interaction of the *ADH1C SspI* genotype and alcohol use and smoking status. Although smokers with the *ADH1C*2/*2* genotype showed a trend toward elevated cancer risk (OR, 2.412; 95% CI, 0.979-5.941; $P = .056$) and heavy drinkers with the *ADH1C*2/*2* genotype had a nonsignificant tendency toward elevated cancer risk (OR, 1.650; 95% CI, 0.860-3.171; $P = .132$), interaction was not observed because the oral cancer risk tended to be decreased with the inclusion of smoking or drinking.

Discussion

In the present study, case-to-control analysis revealed that the variant *ADH1C*2/*2 SspI* genotype is associated with increased oral cancer risk, independently of alcohol drinking. After adjustment for potential confounders, our findings showed a 3-fold increase in the OR for oral cancer in the slow metabolizing variant *ADH1C SspI *2/2** genotype compared to the *ADH1C*1/1** genotype and a 2.6-fold increase in the risk of allele *ADH1C*2/*2* and *ADH1C*1/*2* genotypes compared to the wild-type *ADH1C*1/1** genotype. Silent SNP *ADH1C HaeIII* and functional SNPs *CYP2E1 RsaI* and *CYP2E1 Ins* were not associated with the increased risk for oral cancer in our study.

Although several studies have shown increased head and neck or oral cancer risk in individuals with the variant *ADH1C*2/*2* genotype, in accordance with our findings,[14-17] other studies have shown opposite findings of association of the wild-type *ADH1C*1/*1* genotype with increased head and neck cancer risk.[9-13]

The mechanism by which alcohol exerts its harmful effect in cancer etiology has not been fully defined. The hypothesis that the *ADH1C* genotype could play an important role in

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susceptibility to oral cancer is interesting because this enzyme is involved in a metabolic transformation of ethanol to carcinogen acetaldehyde.[28,29] The presence of the rapid metabolizing *ADH1C**1 allele could lead to a rapid metabolism of ethanol and an accumulation of acetaldehyde in the tissues, thus increasing head and neck cancer risk.[9-13] An alternative explanation in favor of our findings is that alcohol could exert its damaging effect directly, either acting as a solvent of carcinogens from tobacco smoke or damaging the oral mucosa, therefore enhancing the penetration of carcinogens from tobacco smoke. The *ADH1C*2/*2* genotype, associated with a 2.5-fold slower alcohol metabolism, could enhance that direct damaging effect and/or increase the effective dose of alcohol, thus increasing oral cancer risk.

Another mechanism of the role of ADH in the etiology of oral cancer includes oxidation of vitamin A to retinoic acid, which has anti-oncogenic and proapoptotic effects in oral cancer.[30] A recent study revealed the association of a slow ethanol-metabolizing *ADH1C* genotype and increased breast cancer risk by alcohol intake, possibly via aromatase (*CYP19*), an enzyme involved in estrogen metabolism.[31] Aromatase is expressed in oral cell lines and in primary oral carcinoma, and it could be involved in aberrant estrogen metabolism, observed in head and neck cancer patients.[32,33]

In head and neck and oral cancer, alcohol impairs folate metabolism.[34] Previously, we have observed gene-environment interactions between high alcohol intake and the C677T polymorphism of the methylenetetrahydrofolate reductase gene, a key enzyme involved in folate metabolism, with the significant impact on multiple DNA methylation of cancer-related genes.[3] Significant association of gene hypermethylation with alcohol use was observed in HNSCC[35]; thus, one of the major effects of ethanol could be an increase of epigenetic changes due to the methylation misbalance.

Contradictory findings of *ADH1C* and *CYP2E1* polymorphism association with head and neck and oral cancer in previous studies, including ours, may be attributed to the ethnic or racial variability among populations, which could likely affect the results of polymorphism association with head and neck and oral cancer risk. Although the *ADH1C*1/*1* genotype was associated with an increased oral cancer risk in Caucasians with high alcohol consumption,[20] in Asians who were moderate to heavy drinkers, this genotype was associated with decreased risk for oral and pharyngeal cancer.[36] Our study was conducted on Caucasians of the same ethnicity, thus eliminating the potential racial and/or ethnicity confounders.

In addition, several studies have shown a gene-environmental interaction between *ADH1C* and *CYP2E1* genotypes with alcohol use and/or smoking, although these findings were not observed in our study. The variant genotype *ADH1C*2/*2* was associated with a significantly increased oral cancer risk in heavy drinkers.[2,15] Head and neck cancer patients with the *ADH1C*2/*2* genotype who consumed high quantities of alcohol (>30 drinks per week) had a 7-fold increased cancer risk, whereas heavy smokers had a 4-fold elevated cancer risk compared to *ADH*1/*1* wild-type homozygotes.[14] The variant *ADH1C*2/2** genotype was associated with the increased head and neck cancer risk in a Turkish population[16] and in the Central European Slavic population, with a high level of smoking and alcohol intake.[17] In contrast, in other studies, the *ADH1C*1/*1* genotype was associated with a significantly increased oral and head and neck cancer risk in heavy drinkers.[10,12] Among the head and neck cancer patients with the *ADH1C*1/*1* genotype who consumed high quantities of alcohol, the risk was increased almost 4 times,[9] suggesting the significant interaction with high alcohol intake.

Contradictory findings of *ADH1C* and *CYP2E1* polymorphism association with head and neck and oral cancer may also be attributed to the regional differences in quantities and type of alcoholic beverages. A study conducted on US, Japan, Europe, and Balkan region populations revealed regional differences in predominant type of alcoholic beverages used and an association of spirits consumption with oral cancer mortality.[1]

The substantial differences in a definition of heavy alcohol drinkers among studies and the variation among the occurrence of heavy drinkers could be observed. In many studies of HNSCC,[21,22] including ours, heavy drinking is defined as the consumption of more than 21 drinks per week. In a previous study, 27% of high drinkers consumed more than 30 drinks per week[14]; in another study, 17% of cases consumed more than 43 drinks per week.[15] The highest reported proportion of heavy consumers was 46% of cases that consumed more than 57 alcoholic drinks per week.[10] In our study, 47% cases consumed more than 21 alcohol drinks per week, and such a high percentage of heavy drinkers among cases can be attributed to a relatively low borderline for heavy drinking. The genetic susceptibility to carcinogens may have a more significant effect at lower levels of alcohol exposure. At high-level exposure, saturation of enzyme activity can occur in both "rapid" (*ADH1C *1/*1*) and "slow" metabolizing genotypes (*ADH1C *2/*2*), whereas this effect could not occur at lower doses.

A possible limitation to our study is that self-reported alcohol status could not be validated with an objective method. Thus, subjects could have misreported their true alcohol consumption. Underestimation of actual alcohol consumption could diminish the association between alcohol, *ADH1C,* and oral cancer. To reduce this type of bias, we have included hospital-based controls of heavy-drinking individuals without cancer history because in the population-based healthy control group very few heavy drinkers were recorded. However, this may have influenced our results because the *ADH1C*2* variant allele showed a trend of higher incidence in healthy high-alcohol consumers compared to nondrinking controls ($P = .110$). Previously, it has been observed that the variant allele of the *ADH1C* gene was significantly higher in alcoholics,[37] suggesting that this polymorphism may be associated with high alcohol consumption and addiction.

Although oral mucosal *ADH* contributes to local acetaldehyde production, the oral bacteria, especially among tobacco and alcohol users, is responsible for higher levels of acetaldehyde production from ethanol in saliva.[38] In our study, poor oral hygiene was associated with the *ADH1C SspI* polymorphism; thus, the role of the *ADH1C* genetic polymorphism might be obscured by *ADH* production or acetaldehyde production by oral microflora.

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In conclusion, our study suggested that *SspI* polymorphism of the *ADH1C* gene could play a significant role in the etiology of oral cancer. The mechanism by which alcohol exerts its harmful effect has not been fully elucidated, and genetically determined interindividual differences in the metabolic ability to activate or eliminate alcohol- or tobacco-associated carcinogens may determine personal susceptibility to oral cancer. Further analysis of other ethanol- and tobacco-related polymorphisms, alcohol intake, smoking, and diet may provide important information on the etiology of oral cancer.

Author Contributions

Miroslav Brocic, conception and design, acquisition and interpretation of data, drafting and revising the article, final approval; **Gordana Supic**, conception and design, analysis and interpretation of data, drafting and revising the article, final approval; **Katarina Zeljic**, analysis and interpretation of data, drafting the article; **Nebojsa Jovic**, acquisition of data, revising the article; **Ruzica Kozomara**, acquisition of data, revising the article; **Sladjana Zagorac**, analysis and interpretation of data, drafting the article; **Milica Zlatkovic**, acquisition of data, drafting the article; **Zvonko Magic**, conception and design, interpretation of data, drafting and revising the article, final approval.

Disclosures

Competing interests: None.

Sponsorships: None.

Funding source: None.

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