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SERGEJ NADALIN[1], VELJKO FLEGO[2], SANJA DEVIĆ PAVLIĆ[1], DARIAN VOLARIĆ[1], ANĐELKA RADOJČIĆ BADOVINAC[1,3], MILJENKO KAPOVIĆ[1] and SMILJANA RISTIĆ[1]

[1]Department of Medical Biology and Genetics, Faculty of Medicine, University of Rijeka; [2]Department of Pulmonology, Clinical Hospital Center Rijeka; [3]Department of Biotechnology, University of Rijeka, 51000 Rijeka, Croatia

Abstract. The biologically active peptide angiotensin II is cleaved from angiotensinogen by the renin and the angiotensin-converting enzyme (ACE), an enzymatic cascade known as the renin-angiotensin system (RAS). RAS may be important in the etiology of nicotine dependence by influencing dopaminergic signaling. In the present study, the association between an insertion/deletion (I/D) polymorphism of ACE and nicotine dependence amongst patients with lung cancer was assessed. To date, several studies have shown the relevance of this polymorphic variant in both nicotine dependence and lung cancer. However, the present study is the first to address the potential role of the ACE-I/D polymorphism in nicotine dependence among patients with lung cancer. Genotyping was performed in 305 patients with lung cancer (males/females, 214/91). Significantly more male smokers had the ACE-I allele compared with male non-smokers (44.9 vs. 20.0%; P<0.05). The risk of smoking was ~5-fold higher for males with the ACE-I allele (ACE-II homozygous and ACE-ID heterozygous) vs. ACE-DD homozygous (odds ratio, 5.47; 95% confidence interval, 1.4-21.9; P=0.016). The pack-year smoking history in a subgroup of females with squamous cell carcinoma carrying the ACE-I allele was significantly lower compared with ACE-DD (37.1±14.1 vs. 57.0±29.1; F=4.5; P=0.046). The ACE-I/D polymorphism accounted for 17.6% of the smoking severity in this patient group (β, -0.42; multiple R^2 change, 0.176; P=0.046). These results suggest that the ACE-I/D polymorphism contributes to the risk of nicotine dependence and smoking severity in lung cancer patients in a sex-specific manner.

Correspondence to: Dr Sergej Nadalin, Department of Medical Biology and Genetics, Faculty of Medicine, University of Rijeka, Braće Branchetta 20, 51000 Rijeka, Croatia
E-mail: sergej.nadalin@medri.uniri.hr

Introduction

Lung cancer is a multifactorial disease with a complex interplay of genetic and environmental factors contributing to its progression. Environmental exposure, primarily to smoking, is the most well-established environmental risk factor for this disease (1,2). A previous meta-analysis, which included a large number of participants of European ancestry (n, 29,179), revealed that current male smokers with an average daily dose of >30 cigarettes had an odds ratios (ORs) of 103.5 for squamous cell carcinoma, 111.3 for small cell lung cancer and 21.9 for adenocarcinoma. In women, the corresponding ORs were 62.7, 108.6 and 16.8, respectively (3).

Although cigarette smoke contains >4,000 ingredients, the pharmacological effects of tobacco addiction are known to be primarily mediated by nicotine (4-7). Binding of nicotine to nicotinic cholinergic receptors results in stimulation of brain mesolimbic dopaminergic neurons, and these are hypothesized to underlie the addiction to tobacco/nicotine (8-12). Nicotine itself is not carcinogenic; however, nicotine dependence may be indirectly associated with an elevated risk of lung cancer through increasing smoking and consequently increasing tobacco carcinogen intake (1,13). Genome wide association studies of lung cancer have identified a locus on chromosome 15q25.1 that encodes three (CHRNA3, CHRNA5 and CHRNB4) of the eleven nicotinic acetylcholine receptor subunits in the brain mesolimbic system, and this is the most significant genome-wide location for lung cancer and nicotine dependence (14-16).

The activity of the renin-angiotensin system (RAS) is dependent on angiotensin II, which is a biologically active peptide generated from angiotensinogen via cleavage by renin and angiotensin-converting enzyme (ACE) (17-19). Although the classical function of the RAS is regulation of blood pressure and cardiovascular homeostasis (20), several studies have suggested that the RAS may also be important in the etiology of nicotine dependence by influencing dopaminergic signaling (21-23). Angiotensin II interacts with dopamine in mesocorticolimbic areas as a neurotransmitter, and ACE regulates turnover of dopamine in the rat striatum (21,22). Furthermore, dopamine release in the rat striatum exhibits a decreased response to captopril and enalaprilat, both antihypertensive drugs that inhibit the activity of ACE (23).

A functional insertion/deletion (I/D) polymorphism (rs1799752) in intron 16 of the ACE gene (17q23), which is characterized by the presence or absence of a 287 bp Alu repetitive sequence, accounts for ~50% of the ACE levels (24) and is the most widely-studied RAS-related polymorphic variant, and the only RAS-related polymorphism investigated in the etiology of nicotine dependence (25-29). To date, several studies have addressed the potential relevance of the ACE-I/D polymorphism in the etiology of nicotine dependence, but the results have been inconsistent (25-29). For example, there is a significantly greater risk of being a smoker observed amongst ACE-DD homozygous individuals suffering from depression in the German population (27) and amongst heterozygous (ID) females with schizophrenia in the Croatian population (29). Conversely, studies consisting of healthy subjects from the Czech Republic and German populations (26,27) and multiple sclerosis patients from the Croatian population (28) found no evidence of an association between the ACE-I/D polymorphism and the risk of smoking. The ACE-I/D polymorphic variant may also serve a role in smoking severity; the ACE-DD homozygous genotype was shown to contribute to an increase in the number of cigarettes smoked per day amongst patients suffering from depression, as well as an increase in the pack-years of smoking history amongst healthy individuals in the German population (27).

Numerous studies have investigated the influence of the ACE-I/D polymorphism in the etiology of lung cancer in European and Asian populations, but the conclusions of the studies are often contradictory (30-37). A meta-analysis showed no significant associations between the ACE-I/D polymorphic variant and the risk of lung cancer (38,39). In contrast, in our previous study in lung cancer patients from a Croatian population, the ACE-I/D polymorphism was shown to be associated with a higher risk of developing squamous cell carcinoma (35), which is strongly associated with smoking (3,40). In these patients, there was a significantly higher frequency of the ACE-DD genotype and ACE-D allele compared with the control group. Based on this finding and the hypothesis that the RAS may be involved in brain dopaminergic signaling (20-22), in the present study, whether the ACE-I/D polymorphism influenced smoking behavior amongst patients with lung cancer was investigated.

Patients and methods

Study participants. The study group recruited in the present consisted of 305 lung cancer patients who were treated at the Department of Pulmonology in the Clinical Hospital Center in Rijeka, Croatia. The patients' demographic and clinical characteristics are shown in Table I. Histology was graded according to the World Health Organization classification method (41) and the classification of tumor stages according to the Tumor-Node-Metastasis staging system (42). Patients were divided into smokers and non-smokers using data from questionnaires, with smokers defined as individuals who smoked >1 cigarette per day for >1 year and non-smokers defined as those who had smoked <100 cigarettes during their lifetime (43,44). The numbers of quitters, occasional smokers, ex-smokers as well as those who had been smoking for <1 year were too small for statistical analysis and were

thus excluded from the present study. To assess the severity of nicotine dependence, patients were also asked for their pack-year smoking history, which was calculated as follows: Number of cigarettes smoked per day x the number of years smoked. The Ethics Committee of the School of Medicine, University of Rijeka, Croatia approved the present study, and written informed consent was obtained from each subject after the study's purpose and methods were described. The study was performed in accordance with the ethical standards stated in the latest version of the Declaration of Helsinki (45).

Genotyping. Peripheral blood leukocytes were obtained as described previously (46), and genomic DNA was extracted using a FlexiGene DNA kit 250 (QIAGEN GmbH) according to the manufacturer's protocol. Genotyping was performed using allele-specific PCR as described previously (23). PCR amplification was performed on a Mastercycler personal (Eppendorf) and 2720 Thermal Cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). The polymorphism detected by PCR was characterized by an ~490 bp fragment in the presence of the ACE-I allele and as an ~190 bp fragment in the presence of the ACE-D allele. The PCR products were separated on 2% agarose gels and DNA was visualized using ethidium bromide staining under ultraviolet light. Each DNA sample appeared in one of three possible forms: A 490 bp band (ACE-II genotype), a 190 bp band (ACE-DD genotype), or both 490 and 190 bp bands (ACE-ID genotype). To avoid mistyping of the ACE-ID heterozygotes as ACE-DD homozygotes, all of the ACE-DD genotype samples were additionally confirmed using insertion-specific PCR as described previously (47).

Statistical analysis. Statistica for Windows version 12 was used to perform the statistical analysis. Data are presented as the mean ± standard deviation. The ACE genotype and allele distributions amongst smokers and non-smokers, as well as the observed and expected genotype proportions according to Hardy-Weinberg equilibrium, were compared using a χ^2 test with 2x2 and 3x2 contingency tables. Furthermore, odds ratios (ORs) and 95% confidence intervals (CIs) were used to examine the association between the ACE-II homozygous and ACE-ID heterozygous genotypes, and the risk of being a smoker amongst the male patients. The relationship between pack-year smoking history and the ACE genotype was assessed using a one-way ANOVA. Multiple regression analysis was used to examine the extent the ACE-I/D polymorphic variant contributed to pack-year smoking history amongst females with squamous cell carcinoma. P<0.05 was considered to indicate a statistically significant difference.

Results

Sex-specific differences in the effects of the ACE-I/D polymorphism in various diseases and/or conditions (48-52), including cancer (53) and nicotine dependence (29), have been shown previously. Thus, all analyses for male and female patients were performed separately.

The smoking rate was higher for both male and female patients compared with the rate in the general Croatian population (54), with ~95% of males and two-thirds of the females classified as smokers. A significantly greater prevalence

Table I. Characteristics of patients with lung cancer.

Clinicopathological characteristics	Males, n=214 (%)	Females, n=91 (%)	F or χ^2	P-value
Age at blood sampling	65.9±10.4	66.8±10.6	0.39	0.532
Tumor type (%)				
Adenocarcinoma	48 (22.4)	39 (42.9)	13.07	<0.001[c]
Squamous cell carcinoma	119 (55.6)	25 (27.5)	20.28	<0.0001[d]
Other NSCLC	26 (12.1)	15 (16.5)	1.03	0.310
Total NSCLC	193 (90.1)	79 (86.9)	0.75	0.385
SCLC	21 (9.9)	12 (13.1)	0.75	0.385
Tumor stage (%)				
I + II	43 (20.1)	28 (30.8)	4.07	0.043[a]
III + IV	171 (79.9)	63 (69.2)	4.07	0.043[a]
Smoking behavior				
Smokers/non-smokers	204/10	55/36	60.68	<0.0001[d]
Pack-year smoking history	55.6±32.2	40.3±24.3	10.78	<0.01[b]

[a]P<0.05, [b]P<0.01, [c]P<0.001, [d]P<0.0001. NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

Table II. Frequency of angiotensin converting enzyme genotypes and alleles according to smoking status.

A, Males

Allele	Smokers, n=204 (%)	Non-smokers, n=10 (%)	χ^2	df	P-value
DD	61 (29.9)	7 (70.0)	7.09	2	0.029[a]
ID	103 (50.5)	2 (20.0)			
II	40 (19.6)	1 (10.0)			
D allele	225 (55.1)	16 (80.0)	4.79	1	0.029[a]
I allele	183 (44.9)	4 (20.0)			

B, Females

Allele	Smokers, n=55 (%)	Non-smokers, n=36 (%)	χ^2	df	P-value
DD	18 (32.8)	10 (27.8)	0.25	2	0.881
ID	24 (43.6)	17 (47.2)			
II	13 (23.6)	9 (25.0)			
D allele	60 (54.5)	37 (51.4)	0.17	1	0.676
I allele	50 (45.5)	35 (48.6)			

[a]P<0.05.

of nicotine dependence and significantly higher pack-year smoking histories were observed amongst males compared with females (P<0.0001 and P<0.01, respectively; Table I). The allele and genotype frequencies for the ACE-I/D polymorphism are presented in Table II, according to the smoking status. The statistical power of the present study was 99% for detection of a 1.5-fold increase in ACE-D allele frequency and 80% for detection of a 1.5-fold increase in ACE-I allele frequency. Distribution of the ACE genotype amongst male or female patients, as well as in the male smokers and non-smokers, and female smokers and non-smokers, were consistent with the Hardy-Weinberg equilibrium (P>0.05). The ACE allele and genotype frequencies did not deviate significantly from those observed in the European population (55). No significant differences were observed in the genotype and allele distributions of ACE between the total male and female patients (P>0.05). Analysis of smokers and non-smokers separately showed a greater prevalence of the ACE-I allele amongst female non-smokers compared with male non-smokers (48.6% vs. 20.0%; P<0.05). Thus, the ACE-I/D polymorphic variant contributed significantly to the risk of nicotine dependence only for male patients (Table II). There was a significantly

Table III. ACE-I/D polymorphism and severity of nicotine dependence.

Sex	ACE genotype	Pack-year smoking history	F	P-value
Males	DD	57.7±35.3	0.51	0.600
	ID	56.0±32.2		
	II	51.2±27.1		
Females	DD	47.1±26.0	1.14	0.326
	ID	35.7±19.5		
	II	39.2±29.2		

ACE, angiotensin converting enzyme.

different ACE genotype distribution between male smokers and male non-smokers, with an increase in the ACE-I allele amongst male smokers vs. male non-smokers (44.9 vs. 20.0%; P<0.05). Furthermore, males harboring the ACE-I allele (ACE-II homozygous and ACE-ID heterozygous) had a ~5-fold higher risk of becoming smokers than the ACE-DD homozygous carriers (OR=5.47, 95% CI=1.4-21.9; P=0.016).

Whether the ACE-I/D polymorphism contributed to smoking severity was next determined. No significant association was found between the ACE-I/D polymorphism and pack-year smoking history in all lung cancer patients (P>0.05; Table III). There was no significant association between the ACE-I/D polymorphism and pack-year smoking history in subgroups of males with different types of lung cancer (P>0.05; Table SI). In subgroups of females with different types of lung cancer, significant associations between the ACE-I/D polymorphism and pack-year smoking history was observed only for females with squamous cell carcinoma (P<0.05; Table SII). Specifically, females with squamous cell carcinoma that harbored an ACE-I allele (ACE-II homozygous and ACE-ID heterozygous) had significantly lower pack-year smoking histories compared with those who were ACE-DD homozygous (37.1±14.1 vs. 57.0±29.1; F=4.5; P=0.046). The ACE-I/D polymorphism accounted for ~17.6% of the smoking severity in this patient group (β=-0.42, multiple R^2 change=0.176; P=0.046).

Discussion

Molecular epidemiological studies suggest that a large number of genes and several environmental factors contribute to the etiology of nicotine dependence. This complexity represents a considerable challenge, but also underlies the potential benefits of genetics studies on nicotine dependence (56,57). In the present study, whether smoking behavior amongst patients with lung cancer was influenced by a functional I/D polymorphism of the ACE gene was investigated. To date, several studies have shown the relevance of this polymorphic variant in both nicotine dependence and lung cancer (27,29,34-36). However, to the best of our knowledge, the present study is the first to address the potential role of the ACE-I/D polymorphism in nicotine dependence amongst patients with lung cancer.

The results of the present study suggested there was a sex-dependent influence of the ACE-I/D polymorphism on smoking behavior amongst patients with lung cancer. In addition, the ACE-I/D polymorphic variant had the opposite effects on the risk of nicotine dependence and smoking severity. The ACE-I/D polymorphism had a significant impact on the risk of nicotine dependence in male patients in this patient group, where the presence of the ACE-I allele contributed to a greater risk of being a smoker. These findings are not consistent with any of the previous reports on the potential relevance of the ACE-I/D polymorphism in the etiology of nicotine dependence (26-29), highlighting the presence of a mechanism by which the ACE-I/D polymorphism contributed to the risk of nicotine dependence in lung cancer which differs from that than in other diseases and/or conditions.

A protective effect of the ACE-I allele on pack-year smoking history amongst a subgroup of females with squamous cell carcinoma was identified. The ACE genotype satisfactorily described the severity of nicotine dependence in this patient group. These data are supportive of previous studies on the influence of the ACE-I/D polymorphism on smoking severity in a study on a German cohort (27), which suggested that the absence of the ACE-I allele (ACE-DD homozygosity) contributes to greater pack-year smoking history amongst healthy individuals as well as to a higher number of cigarettes smoked per day amongst patients suffering from depression. The protective effects of the ACE-I allele on the severity of nicotine dependence may be attributed to the functional properties of the ACE-I/D polymorphism. It has been established that subjects homozygous for the D allele have the highest ACE plasma levels, those homozygous for the ACE-I allele have the lowest levels and those who are ID heterozygous exhibit intermediate levels (24). Thus, it is possible that ACE-II homozygous and ACE-ID heterozygous individuals, due to lower ACE activity, release dopamine at lower concentrations in the mesolimbic dopaminergic reward pathway in their brains.

Several findings may account for the sex-specific differences of the ACE-I/D polymorphism observed in the present study (58-60). Postmenopausal women receiving estrogen replacement therapy may result in a genotype-associated decrease in ACE activity. A significant decrease in plasma ACE activity was observed either in women with the ACE-ID and ACE-II genotypes (59), and in those with the ACE-ID and ACE-DD genotypes (60). Estrogen may influence dopaminergic neurotransmission, as estrogen treatment has been shown to reduce dopamine receptor D2 levels in several regions of the rat brain (61). Dopaminergic response to cigarette smoking differs between men and women. In particular, males show a consistent and more rapid increase in activity of the ventral striatum; whereas females respond show a faster response in a subregion of the dorsal putamen (62).

The present study has several limitations. The recruited cohort consisted of a relatively small number of participants; thus, there is a possibility minor effects were not detected. In addition, there was an unbalanced number of male and female patients as well as smoking and non-smoking males, which may have led to statistical biases in the analyses. Thus, although the observed OR indicates a strong association between the increased risk of becoming a smoker and

the presence of the ACE-I allele amongst male patients, these results should be interpreted with caution. Moreover, the sample group also lacked a group of healthy control participants and data regarding smoking behavior were based exclusively on self-reporting, which itself is prone to unintended bias. Finally, only one RAS-related polymorphism with a distribution that differed significantly only between a subgroup of patients with squamous cell carcinoma and control participants in our previous study was investigated in the present study (35).

In conclusion, our results highlight that the ACE-I/D polymorphism contributes to both the risk and the severity of nicotine dependence amongst patients with lung cancer. A significantly increased risk of being a smoker was identified for male patients carrying the ACE-I allele (ACE-II homozygous and ACE-ID heterozygous) compared with those who were ACE-DD homozygous; whereas, a subgroup of female smokers with squamous cell carcinoma carrying the ACE-I allele had significantly lower pack-year smoking histories compared with the ACE-DD homozygous carriers. In line with the results of our previous study showing that the ACE-DD genotype and the ACE-D allele significantly increased the risk of developing squamous cell carcinoma (35), the latter findings suggest that the ACE-I/D polymorphism may be indirectly associated with an increased risk of squamous cell carcinoma by influencing the severity of nicotine dependence. Further studies are required, which include patients with other diseases and/or conditions, to assess nicotine dependence with more specific methods, such as the Fagerstrom test, and to investigate the relevance of other RAS-related polymorphic variants in smoking behavior (such as those of angiotensinogen and angiotensin II receptors), to validate and expand upon the results of the present study. Another issue that warrants consideration is that ACE, in addition to angiotensin II, has other potential substrates, some of which influence dopaminergic neurotransmission and are proposed to be associated with lung cancer, such as substance P and neurotensin (63,64). Finally, it has been shown that cigarette smoking may also influence ACE expression/activity. It has been observed that nicotine and its metabolites increased the expression and activity of ACE in human endothelial cells (65), enhanced RAS activation and increased the levels of circulating ACE in chronic cigarette smokers (65,66).

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SN designed the study, performed the data analysis and wrote the manuscript. VF recruited the patients, performed the medical examinations and drafted the manuscript. SDP performed the experiments and the data analysis. DV recruited the patients. ARB and MK interpreted the data, and drafted and revised the manuscript. SR designed and supervised the study as well as drafted the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of the School of Medicine, University of Rijeka, Croatia approved the present study, and written informed consent was obtained from each subject after the study's purpose and methods were described. The study was performed in accordance with the ethical standards stated in the latest version of the Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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