Article

The association of ACE I/D gene polymorphism with severe carotid atherosclerosis in patients undergoing carotid endarterectomy

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Abstract

Introduction: The ACE I/D polymorphism was mostly investigated in association with intima-media thickness, rarely with severe atherosclerotic phenotype.

Materials and methods: We investigated the association of I/D polymorphism with severe carotid atherosclerosis (CA) (stenosis > 70%) in asymptomatic and symptomatic patients undergoing carotid endarterectomy. The 504 patients subjected to endarterectomy and 492 healthy controls from a population in Serbia were investigated as a case-control study.

Results: The univariate logistic regression analysis revealed ACE DD as a significant risk factor for severe CA (odds ratio [OR] = 1.3, 95% confidence interval [CI] 1.0–1.7, p = 0.04). After adjustment for the common risk factors (age, hypertension, smoking, and HDL) ACE was no longer significant. However, we found a significant independent influence of DD genotype on plaque presence in a normotensive subgroup of patients (OR 1.8, CI 1.2–3.0, p = 0.01, corrected for multiple testing). In symptomatic patients *D* allele carriers were significantly more frequent compared with asymptomatic patients (OR 1.6 CI 1.0–2.6, p = 0.05).

Conclusions: Our data suggests that ACE I/D is not an independent risk factor for severe CA. On the other hand, a significant independent genetic influence of ACE I/D appeared in normotensive and symptomatic patients with severe CA. This should be considered in further research toward resolving the complex genetic background of severe CA phenotype.

Keywords

ACE I/D, carotid plaque, endarterectomy, gene, polymorphism

Introduction

Atherosclerosis, with its major endpoint events such as myocardial infarction (MI) and stroke, is still the leading cause of death in both men and women in the Western world. The cardiovascular disease was the leading cause of death in Serbia and Montenegro in 2002, accounting for about 40% of all death cases.[1] During the year 2006 more than 57% of all deaths in Serbia were caused by cardiovascular diseases (in males 52%; in females 62.8%), according to data from the Ministry of Health of the Republic of Serbia.[2]

Angiotensin II (Ang II) has been implicated in the pathobiology of atherosclerosis and the arterial response to injury and restenosis, through mechanisms that include vascular hypertrophy, extracellular matrix production, and induction of cytokines.[3–7] ACE is the key enzyme of the RAS system, which converts angiotensin I (Ang I) to Ang II, the main

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effector molecule. It is expressed in endothelial cells of normal vessels,[8] as well as in functionally different vessel types.[9] ACE also promotes inflammatory cell production of Ang II,[10] and induction of premature senescence of smooth muscle cells.[4] Through its actions, ACE first induces endothelial dysfunction, than activates monocytes/macrophages, augments vascular inflammation, and, in doing so, enhances the atherogenic process.[5] The chronic exposure to high levels of circulating and tissue ACE may predispose to vascular wall remodelling and changes in its diameter and thickness.[6] The marked accumulation of tissue ACE and Ang II were shown in inflamed regions of plaques prone to rupture.[7]

The ACE gene has functional insertion/deletion (I/D) polymorphism of a 287 bp *Alu* sequence within intron 16.[11] The ACE DD carriers have higher local levels of ACE and that genotype may have a more pronounced effect on atherosclerosis due to higher Ang II levels.[11,12] The ACE inhibitors could reduce atherogenesis.[13] The polymorphisms within the RAS genes can affect response to therapy.[14] So, it was reasonable to investigate functional I/D polymorphism in atherosclerotic cardiovascular disorders. Investigation of the effect of ACE I/D polymorphism in previous studies has been mainly focused on an increased risk of MI and ischaemic heart disease[15,16] or hypertension.[17]

Considering carotid atherosclerosis (CA), most of the studies investigated ACE I/D polymorphism in association with subclinical, intermediate atherosclerotic phenotype, intima-media thickness (IMT) with conflicting results. Recent meta-analysis based on data from more than 20 studies, the majority of which were case-control studies, revealed moderate positive association of ACE *D* allele with common carotid IMT.[18] The association of ACE I/D gene polymorphism with severe CA and atherosclerotic plaques, as an end-stage atherosclerotic phenotype, is rare. Thus, we deliberately included a homogenous group of patients with clinically evident severe atherosclerosis, who had high grade carotid stenosis (> 70%) and were undergoing carotid endarterectomy. The aim of our study was to find whether there is an association of ACE I/D gene polymorphism with severe carotid atherosclerosis in asymptomatic and symptomatic patients undergoing carotid endarterectomy among the population of Serbia.

Materials and methods

Study population

In this case-control study, the 504 patients were recruited among subjects consecutively admitted for carotid endarterectomy to the Vascular Surgery Clinic, Dedinje Cardiovascular Institute, and Clinic for the Vascular and Endovascular Surgery Diseases, Clinical Centre of Serbia, all of them in Belgrade, Serbia, with evidence of carotid plaque presence in the internal or common carotid artery. The 492 controls, who underwent clinical, ultrasound and ECG examination and did not show any evidence of CA, cerebrovascular or cardiovascular disease, chronic inflammatory disease, renal failure or diabetes mellitus, were recruited from the individuals undergoing annual medical check-up at Occupational Medical Centres, Belgrade, Serbia.

Ultrasound assessment of bilateral carotid atherosclerosis was performed by high-resolution B-mode ultrasound applying the same protocol for all participants (Toshiba, PowerVision 6000, 7.5 MHz, Riverside, CA, USA or Acuson Antares™ system, Siemens, Munich, Germany). The ultrasonographers were blinded to genotyping results. Division into asymptomatic (never having ischaemic symptoms) or symptomatic patients were based on prior clinical symptoms: if a patient had suffered an ipsilateral stroke or transient ischaemic attack of the carotid territory. Classification was based on the clinical history obtained from the patients and confirmed by medical records. CT scans were obtained from all patients, and imaging studies were used to rule out silent infarcts in the asymptomatic population. Exclusion criteria for all patients were history of previous carotid endarterectomy (possible restenosis), carotid kinking, carotid aneurysm, tumours, chronic inflammatory diseases, autoimmune disease, or renal failure. Patients with atrial fibrillation or highly suspected cardiac sources of emboli were excluded from the study to avoid confusion between cardiac and carotid sources of ischaemic events. For all individuals enrolled in the study a medical history was completed, including information on smoking and drinking habits, presence of diabetes, coronary artery disease, peripheral arterial occlusive disease, and drug treatment. Subjects with fasting glucose level of ≥ 7.0 mmol/L, or taking insulin or oral hypoglycaemic drugs were characterized to have diabetes mellitus; those with previous myocardial infarction or with stable angina pectoris evaluated by selective coronarography that confirmed or revealed coronary artery disease were characterized to have coronary heart disease. Peripheral artery disease was diagnosed as ankle brachial index lower than 0.75. Hypertension was defined as a systolic blood pressure ≥ 140 mm Hg, a diastolic blood pressure ≥ 90 mm Hg, or current treatment with an antihypertensive drug.

The study was approved by the local research ethics committee and each participant gave their written informed consent to participate in the study.

Ultrasound assessment of the carotid arteries

After the subject had been placed at rest in a supine position, the common carotid artery, bulb, internal carotid artery, and external carotid artery on both sides were examined from several directions using B mode ultrasonography. Ultrasound assessment of the bilateral carotid arteries was performed by high-resolution B-mode ultrasound Acuson Antares™ system (Siemens, Munich, Germany) and HDL 3500 linear transducer (5 to 12 MHz; Philips ATL, Bothell, WA, USA).

The degree of the carotid stenosis was calculated using the North American Symptomatic Carotid Endarterectomy Trial (NASCET) method[19] or the European Carotid Surgery Trialists Collaborative Group (ECST) method,[20] depending on the clinic it was performed in. The values for the degree of stenosis measured according to NASCET were recalculated according to a formula which defines the relation between the two measurements: % stenosis by ECST = 0·6* % stenosis by NASCET + 40%.[21] All of the patients included in the study had stenosis > 70% according to NASCET, which is equivalent to 85% according to ECST.

The highest peak systolic (PSV) and end-diastolic (EDV) velocities as well as ICA/ CCA carotid artery ratio were recorded. Atherosclerotic plaques were defined as focal widening relative to adjacent segments as evidenced by protrusion into the lumen and/or localized roughness with increased echogenicity. For ultrasound carotid measurements the intraclass correlation coefficient for inter-rater and intra-rater reliability was 0.916 and 0.968, respectively. CA was defined as the presence of atherosclerotic plaques in the internal or the common carotid artery (ICA/CCA).

Laboratory measurements

Lipid concentrations and glucose levels were determined from fresh blood samples after overnight fasting. LDL-cholesterol (LDLC) levels were calculated using Friedwald's formula.[22] All biochemical analyses were routinely performed at the hospital laboratories.

Genetic analysis

Genomic DNA was isolated from whole blood samples collected with EDTA and purified by the proteinase K/phenol extraction method,[23] or by standardized BloodPrep® DNA Chemistry isolation kit (Applied Biosystems, Forester City, CA, USA) on the ABI PRISM™ 6100 Nucleic Acid PrepStation. The I/D polymorphism was typed by polymerase chain reaction (PCR) on ABI 9700 (Applied Biosystems, USA) with the three primer method, as previously described.[24] The 200 ng of genomic DNA was mixed with 20 pmol of sense primer 5′ctggagaccactcccatcctttct-3′, 7 pmol of anti-sense primer 5′gatgtggccatcacattcgtcagat-3′, and 1.5 pmol of the third, I allele sense specific primer 5′tgggaccacagcgcccgccactac-3′. Thermal cycling was performed under the following conditions: initial denaturation at 93°C for 60 s, 30 cycles at 93°C for 60 s, 65°C for 90 s, and 72°C for 90 s, and final extension at 72°C for 5 min. Sense and antisense primers flank 478 and 191 bp-long fragments for I and D allele respectively, whereas third primer and antisense primer flank the 292 bp-long fragment specific for the I allele. Amplification products were separated by electrophoresis, in 1.8% (w/v) agarose gels with ethidium bromide. The applied electric field was 7.5 V cm⁻¹. Bands were visualized by GDS8000 gel documentation

system (Ultra Violet Products Inc., Upland, USA). At the time of genotyping the researchers were completely blinded to clinical characterization of samples.

Statistical analysis

Statistical analysis was performed using the Statistica software package (Version 5, StatSoft, 1997). Results were evaluated by chi-square (χ^2), unpaired t-test, and non-parametric Mann–Whitney U-test. As a measure of strength of association between ACE genotypes and carotid stenosis, univariate and multivariate logistic regression analysis was used and expressed in terms of adjusted OR and 95% confidence interval (CI). In multivariate regression analysis we included the factors that were significantly different in patients compared with controls (age, BMI, smoking status, hypertension, serum total cholesterol [TC], HDL-cholesterol [HDLC], and triglycerides [TG]) and kept covariates in the model if the p value of the model was < 0.001. In multiple logistic regression analysis carotid plaque presence or presence of clinical symptoms were entered as the dichotomous dependent variable and genotype classes were coded on a ratio scale (0 and 1), according to three models of inheritance: model 1 – assumes dominant effect of D allele, model 2 – recessive effect of D allele, and model 3 – additive effect of D allele. In model 1 codes were: 1 for ID + DD and 0 for II; in model 2: 1 for DD and 0 for II + ID; in model 3: 0 for II, 1 for ID and 2 for DD. Differences with two-tailed alpha-probability $p \leq 0.05$ were considered significant in all tests. We used Bonferroni correction in multiple comparisons of ACE genotypes between patients and controls.

Results

Description of population

The main characteristics of the patients with severe CA, > 70%, and undergoing carotid endarterectomy and controls are shown in Table 1. The patients had significantly higher BMI, TC, LDLC, and TG values and lower HDLC levels compared with the controls. The patients were also significantly older and had a significantly higher proportion of male individuals, smokers, and individuals who suffered from hypertension.

Prevalence of allele and genotype frequencies

The ACE I/D genotype and allele frequencies and odds ratio for severe CA, > 70%, in the patients undergoing carotid endarterectomy are shown in Table 2. The expected genotype frequencies are provided by the control group. The genotype frequencies between the patients and the controls were significantly different (χ^2 = 8.8, df = 2, p = 0.012). The DD genotype was significantly more frequent among the patients (χ^2 = 5.76, df = 1, p = 0.016).

Table 1. Main characteristics of patients with severe CA, > 70%, undergoing carotid endarterectomy, and controls

Parameter	Patients	Controls	p^*
N	504	492	
Age, years	62.54 ± 12.58	50.44 ± 13.37	< 0.05
Gender F/M, n (%)	205/299 (40.6/59.3)	245/238 (49.8/50.2)	< 0.05**
BMI, kg/m^2	26.20 ± 3.16	25.39 ± 3.46	< 0.05
TC, mmol/L	5.89 ± 1.30	5.47 ± 1.26	< 0.01
LDLC, mmol/L	3.78 ± 1.17	3.34 ± 1.10	< 0.01
HDLC, mmol/L	1.15 ± 0.41	1.43 ± 0.39	< 0.01
TG, mmol/L	1.94 ± 1.32	1.56 ± 1.25	< 0.01***
Hypertension, %	83.2	7	< 0.05**
Smokers, %	64.2	52.0	< 0.05**

Values are mean ± SD, for age, Body mass index (BMI), total cholesterol (TC), LDL cholesterol (LDLC), HDL cholesterol (HDLC), and triglycerides (TG). *Student's *t*-test. **χ^2 test. ***Mann–Whitney *U*-test.

Association of ACE genotypes with severe CA in patients undergoing carotid endarterectomy

We performed both univariate and multivariate analysis of association of I/D polymorphism with severe CA, according to dominant, recessive, and additive models (see *Materials and methods*). Odd ratios (ORs) for the dose dependent effect of *D* allele according to the additive model were significant (Table 2). Significant crude OR for severe CA was 1.2 (CI 1.0–1.4) for one *D* allele and 1.5 (CI 1.1–2.1) for two *D* alleles. According to the recessive model OR was 1.3 (CI 1.00–1.7) for DD genotype (Table 2). The adjusted OR for severe CA was 1.3 (CI 0.8–1.6) for one *D* allele and 1.3 (CI 0.7–2.4) for two *D* alleles (both $p = 0.4$). Thus, multivariate logistic regression analysis, which included common risk factors (age, hypertension, smoking, and HDLC) showed that ACE I/D is not an independent risk factor for severe CA.

Table 2. The ACE I/D genotype and allele frequencies and odds ratio for severe CA, > 70%, in patients undergoing carotid endarterectomy

ACE	Patients n (%)	Controls n (%)	OR	p
Genotype	504	492		
II	83 (16.5)	96 (19.5)	Referent	
ID	246 (48.8)	254 (51.6)	1.2 (1.0–1.4)	0.04
DD	175 (34.7)	142 (28.9)	1.5 (1.1–2.1)	0.04
Recessive model				
DD *vs*. II + ID			1.3 (1.0–1.7)	0.04
Alelle				
I	0.41	0.45	Referent	
D	0.59	0.55	1.2 (1.0–1.4)	0.05

p: univariate logistic regression. OR for genotypes were calculated according to additive model (II *vs*. ID *vs*. DD).

Association of ACE genotypes with biochemical parameters that carry risk for atherosclerosis

We looked in the patients group for the association of ACE I/D genotypes with biochemical parameters which are commonly associated with higher risk for atherosclerosis. In patients with severe CA undergoing endarterectomy we did not find the association of I/D polymorphism genotypes with: lipid status (TC, HDLC, TG, ApoAI, ApoB, Lp(a)) or inflammatory (CRP, IL-6, WBC, fibrinogen) parameters (data not shown).

Association of ACE genotypes with severe CA in the subgroups of patients according to hypertension

We divided the tested group of patients according to their blood pressure status: normotensive or hypertensive. In the normotensive patients, the association between DD genotype and severe CA was more pronounced than in the tested group as a whole (OR 1.8, CI 1.2–3.0, $p = 0.01$, corrected for multiple testing). The OR remained significant after the adjustment according to their age and HDL (OR 2.6, CI 1.0–6.7, $p = 0.04$). The frequencies of ACE I/D genotypes in normotensive patients and controls are presented in Table 3.

Association of ACE genotypes with clinical symptoms in patients with severe CA undergoing carotid endarterectomy

We analysed ACE I/D as a risk factor for symptomatic CA (ipsilateral stroke or transient ischaemic attack) in patients with severe CA subjected to endarterectomy. Genotype frequencies in symptomatic patients were II = 15.38%, ID = 51.40%, DD = 33.22% and in asymptomatic patients II = 22.53%, ID = 46.15, DD = 31.32%. We found that in

Table 3. The ACE I/D genotype frequencies in normotensive patients with severe CA, >70%, undergoing carotid endarterectomy, and controls

ACE	Normotensive patients *n* (%)	Normotensive controls *n* (%)	*p* (χ^2)
Genotype	85	458	
II	16 (18.82)	90 (19.65)	0.03
ID	33 (38.82)	238 (51.97)	
DD	36 (42.35)	130 (28.38)	

symptomatic patients ACE *D* allele carriers were significantly more present compared with asymptomatic patients (OR 1.6, CI 1.0–2.6, *p* = 0.05, according to the dominant model of inheritance, corrected for age, smoking, and HDLC).

Discussion

Few studies in various populations have focused on the relationship between ACE and early atherosclerotic changes, such as carotid IMT, in case/control design and large population based design.[18,25–27] In those studies, however, the results are conflicting. In two large population-based studies in Caucasians[25] and Japanese[26] ACE *D* allele was not associated with common carotid IMT, while in a part of the Rotterdam Study, the association was revealed only among the smokers.[27] In this study, we have investigated association of ACE I/D polymorphism with severe carotid atherosclerosis in the Serbian population.

In this population we found significant crude OR for *D* allele and DD genotype carriage for carotid stenosis over 70%. We showed that the patients undergoing carotid endarterectomy had higher *D* allele frequency compared with the controls. Previous to this study, the ACE gene polymorphism was investigated only in small study groups, with carotid plaques as the phenotype of interest. The power of our study (999 subjects) to detect the association of DD genotype with an OR 1.3 at the significance level of 0.05 was 70%. It was shown that ACE *D* allele/DD genotype correlated with internal carotid artery stenosis > 50%, or plaque presence in a relatively small group of patients (43 patients in a Japanese study[28] and 68 patients in a Swedish study[29]). Nevertheless, the frequency of *D* allele in our patients group was similar to the patient group in the Swedish study[29] as well as to the group in an Italian study.[30] The *D* allele was also shown to promote the severity of coronary atherosclerotic lesions.[31] These results, as well as ours, suggest the certain influence of ACE polymorphism on severe atherosclerotic phenotype. Still, in the whole patient group this influence was not independent from common risk factors for atherosclerosis such as age,

hypertension, smoking, and HDLC, similarly to the other study.[32] However, we found statistically a significant independent influence of DD genotype on plaque presence in the normotensive subgroup of patients. We previously showed that *D* allele was associated with hypertension in males in the Serb population.[17] The analysis of the normotensive patients as a separate group revealed an association that was not influenced by the possible association of ACE with hypertension. The previous clinical study, which used multivariate regression analysis, revealed that age was a significant risk factor for thicker IMT in normotensive patients with moderate to severe CA.[33] We corrected the OR for age and HDLC and it remained significant. The association of DD genotype with plaque presence in patients without hypertension could be linked to Ang II proinflammatory actions, beside its effects on blood pressure regulation. It is known that many researchers have argued that the benefits of ACE inhibitors in stable coronary artery disease are largely independent of their blood pressure lowering effects. Carotid artery ACE transcription and translation was increased within regions of plaque inflammation.[3] The ACE DD genotype may have a more pronounced effect due to higher local levels of ACE and Ang II.[11,12] Among our symptomatic patients (ipsilateral stroke or transient ischaemic attack) *D* allele was significantly more frequent. Previously it was shown that in subjects who died from coronary heart disease, the frequency of *D* allele was significantly increased compared with controls.[34]

It was of importance to investigate the potential role of ACE I/D in severe carotid atherosclerotic phenotype, since there is a dualism in human CA defined as the difference between early non-stenotic atherosclerosis and advanced stenotic atherosclerosis. It is well known that the frequency of ACE I/D gene polymorphism is different among worldwide populations' origins. Studies on a larger sample should be performed to evaluate ACE gene in advanced stenotic atherosclerosis in different populations, as had previously been done for intermediate phenotypes, i.e. IMT. The IMT concerns only part of the athero-pathogenic process and could represent the remodelling changes in response to common vascular risk factors, such as hypertension. The strength of our study lies with the fact that it was undertaken on a homogenous group of patients, all of them with high grade stenosis > 70%. Still, a relatively small number of normotensive patients with severe CA represent one of the limitations of this study which should be overcome in the future by pooling samples or using a meta-analysis approach. The RAS and its main molecules, such as ACE are still in the focus of the research since the ACE inhibitors represent one of the great therapeutic success stories of cardiovascular pharmacotherapy.[35]

In conclusion, our present data suggests a significant and an independent genetic influence of ACE I/D polymorphism

in normotensive and symptomatic patients with severe CA. Further studies are needed to confirm our findings in larger sample sizes in different populations. The ACE as well as other components of RAS should not be excluded from further research toward resolving the complex genetic background of severe CA and towards a more personalized use of preventive therapies.

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Conflict of interest

None declared.

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