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Title: Gene polymorphisms of the renin-angiotensin system and early development of hypertension

Running title: Renin-angiotensin gene polymorphisms and hypertension

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Abstract

Background: A case-control association study was conducted to investigate a possible involvement of polymorphisms of three renin-angiotensin system genes (*ACE - I/D* and *T-3892C*, *AGT - M235T* and *T174M, AT1R - A1166C*) in the early development of hypertension.

Methods: One hundred and twenty five hypertensive and 119 normotensive participants aged 18-40 were selected from a broader sample representative of the general population of Croatia. The selection criteria for hypertensive cases were systolic blood pressure higher than 140 mmHg and/or diastolic blood pressure higher than 90 mmHg and a history of hypertension according to patient interview.

Results: Among the polymorphisms investigated, only those located on the *ACE* gene were associated with hypertension. For *ACE I/D*, the odds ratio for hypertension of *DD* versus *II* homozygote individuals was 2.50 (95% CI 1.19 to 5.25) and for *ACE T-3892C*, the odds ratio of *CC* versus *TT* individuals was 2.32 (95% CI 1.05 to 5.10). Both polymorphisms of the *ACE* gene were in tight linkage disequilibrium. Out of the investigated risk factors for hypertension, only the body mass index showed an influence on the early development of hypertension, acting independently of the *ACE* polymorphism. Their additive effect gives rise to 86 % of hypertensives in subjects having both the *DD* genotype and BMI $\geq$ 30 kg/m^2.

Conclusions: The present study provides evidence of the association of the *ACE* gene polymorphisms and premature hypertension. Additionally, BMI proved to be another important predictor of the disorder acting independently of the *ACE* gene.

Keywords: *ACE*, *AGT* and *AT1R* polymorphisms, premature hypertension, association, BMI

Introduction

Hypertension is a complex disorder that represents one of the most important risk factors for myocardial infarction, stroke, end-stage renal disease and peripheral vascular disease [1]. It is influenced by both environmental as well as by genetic factors. The genetic contribution is estimated to be between 30% and 40% of blood pressure variation [2,3]. Several susceptibility genes for hypertension showing Mendelian inheritance have been identified to date [1], but their contribution to blood pressure variation in the general population is very small. Searching for genes that contribute to the complex form of hypertension has been much less successful. In recent years, a series of genes have been proposed to influence blood pressure and the evidence of the association with hypertension has been shown for some of them [4-6]. However, these associations were not always confirmed [7-9].

Genes encoding components of the renin-angiotensin system (RAS) have been proposed as candidate genes for hypertension since RAS is a well-known pathophysiological pathway involved in blood pressure regulation. RAS genes encoding angiotensinogen (*AGT*), angiotensin-1-converting enzyme (*ACE*) and angiotensin II type 1 receptor (*AT1R*) have been extensively studied and their involvement in the development of hypertension has been analyzed by linkage and association studies in various populations [4-11].

The most extensively studied polymorphism in the *ACE* gene is the *Alu* insertion polymorphism (*ACE I/D*) in intron 16 of the gene, which is known to be associated with plasma ACE level [12,13]. The *ACE I/D* polymorphism is presumed to be in linkage disequilibrium with functional variant that determines ACE plasma level [14] . However, in spite of a great number of polymorphisms detected in *ACE* gene – including the *T*-

3892C polymorphism in the DNA upstream of the gene [15] – the functional variant has not yet been identified. Although there is strong evidence that there is a variant in the *ACE* gene that influences ACE plasma level, the available data on *ACE* gene influence on the development of hypertension are conflicting [5,8,16]. However, two large population-based studies found linkage evidence and significant association of *I/D* with hypertension which was restricted to males [5,17]. Moreover, strong evidence for a quantitative-trait locus for blood pressure on chromosome 17, which was close to the *ACE* gene, was found by a genome-scan analysis [18].

The potential role of *AGT* gene in hypertension was originally explored by Jeunemaitre et al. [4]. Among the 15 molecular variants of *AGT* that had been identified, significant association with hypertension was observed with 2 amino acid substitutions – *M235T* and *T174M*. The results of subsequent studies that tried to detect an association between these two variants and hypertension have been contradictory [e.g. 7,10].

The polymorphism *A1166C* in the 3' untranslated region of the *AT1R* gene was detected by Bonnardeaux et al. [6] who also identified its association with hypertension. Subsequent studies yielded conflicting results [e.g. 9].

In the present case-control study we examined possible associations between polymorphisms of the *AGT*, *ACE* and *AT1R* genes and hypertension in the Croatian population under 40 years of age. Being aware of the composite nature of hypertension with the cumulative effects of environmental risk factors in the course of life we chose a sample of relatively young adults assuming that the genetic influence on hypertension might be more clearly detected. We studied *I/D* and *T-3892C* polymorphisms in *ACE* gene, *M235T* and *T174M* in *AGT* gene, and *A1166C* in *AT1R* gene.

Methods

Study population

The study sample was extracted from a broader sample of 10,074 participants that was collected from 1995 to 1996 within the subproject "Health Promotion" included in the First Croatian Health Project [19]. The participants were adult volunteers aged 18-80 from 30 randomly selected settlements belonging to all four major geographical regions of Croatia. A stratified multistage sampling design was used that provided the sample with a known probability distribution for variables age and sex. The age group of 18-40 numbered 4115 participants, which was 2.6 % of the population of Croatia falling in that age range (according to the 1991 census). From all hypertensive participants aged 18-40 yrs, 119 cases were selected randomly for the purpose of this study. The control group (N = 125) was chosen by matching cases with respect to age, sex and place of residence.

Our criteria for selection of hypertensive participants were the following: 1. blood pressure greater than 140 mmHg for systolic pressure and/or 90 mmHg for diastolic pressure and 2. history of hypertension as assessed by patient interview. The inclusion of persons suffering from secondary hypertension was minimized using detailed health questionnaire.

Arterial blood pressure (systolic and diastolic) was measured twice and the lower value was taken into account. The measurement was performed on the left upper arm by auscultation method after the subject had been seated for at least 15 minutes. Mercurial sphygmomanometers were used and the appropriate adult cuff size (medium or large) was applied.

Body height and weight were measured according to the IBP protocols [20] and used to calculate body mass index (BMI = weight (kg) / height^2 (m^2)). Data on blood triglycerides and cholesterol levels were also available [19]. Basic characteristics of the sample are shown in Table 1. All the participants gave their informed consent.

DNA analysis

Samples of venous blood were collected in EDTA tubes for extraction of DNA from whole blood by using salting out procedure.

Genotyping of the *ACE I/D* polymorphism was performed after polymerase chain reaction amplification of the region encompassing the polymorphism with 3 primers as described by Harrap [21] and detection of fragments on 2 % agarose gel. The genotypes of *T-3892C* in *ACE* gene, *M235T* and *T714M* polymorphisms in *AGT* gene and *A1166C* polymorphism in the *AT1R* gene were detected by allele-specific oligonucleotide hybridization as described previously [15,22,6].

Statistical analysis

For each polymorphism, allele frequencies were calculated from genotype frequencies in cases and controls. Deviation from Hardy-Weinberg equilibrium was assessed by χ^2-test with 1 df. In the control group, χ^2-test was also used to test the differences in genotype distributions among four geographical regions of Croatia. Since no statistically significant differences were found, we assumed the homogeneity of Croatian population with respect to polymorphisms studied, and further analyses were carried out on total samples of cases and controls. Differences in genotype and allele

distributions between cases and controls were tested by χ^2-test with 2 df and 1 df respectively.

In order to compare the prevalence of hypertension among the genotypes of each polymorphism, genotypic odds ratios for hypertension were estimated using logistic regression analysis. The same analysis was used in order to calculate odds ratios for combined effects of sex, age, body mass index (BMI), triglycerides and cholesterol blood concentrations and genotypes on hypertension. BMI was compared across genotypes by univariate analysis of variance.

Pairwise linkage disequilibrium (LD) was estimated as $D = P_{11} - p_1 q_1$, where P_{11} is the frequency of haplotype A_1B_1, and p_1 and q_2 are the frequencies of alleles A_1 and B_1 at loci A and B, respectively. The value of LD was expressed as Lewontin's coefficient [23]. Haplotype frequencies were estimated by expectation-maximization algorithm [24].

A value of $p \leq 0.05$ was considered statistically significant for all statistical tests.

Results

Genotype distributions of all five studied polymorphisms were compatible with Hardy-Weinberg expectation in cases as well as in controls. Differences between cases and controls in genotype distributions were observed for both *ACE* gene polymorphisms (Table 2) with *DD* genotype of *I/D* polymorphism and *CC* genotype of *T-3892C* polymorphism being more prevalent in cases than in controls. Genotype distributions of other polymorphisms investigated in *AGT* and *AT1R* genes did not differ between these two groups. The difference in allele distributions was only observed for *ACE ID* with the *D* allele being more frequent in cases (56.7 % versus 45.5 % in controls).

To test the association of *ACE* gene polymorphisms with hypertension, genotypic odds ratios were calculated. Taking the *II* genotype of the *ACE ID* polymorphism as a reference, the odds ratio for hypertension associated with the *ID* genotype was 0.97 (95 % confidence interval (CI) 0.51 – 1.83) and the odds ratio associated with the *DD* genotype was 2.50 (95 % CI 1.19 – 5.25), indicating a recessive effect of the *D* allele on risk (Table 3). A similar situation was observed for the *T-3892C* polymorphism in the *ACE* gene. Taking *TT* genotype as a reference, the odds ratio for hypertension associated with the *CT* genotype was 0.82 (95 % CI 0.47 – 1.45) and that associated with the *CC* genotype was 2.32 (95 % CI 1.05-5.10).

The linkage disequilibrium (LD) between the two studied polymorphisms in the *ACE* gene was expressed as Lewontin's coefficient *D*' was –0.85. Such a high LD between two polymorphisms having similar allele frequencies suggests that their association with hypertension may have the same source that might possibly be a third polymorphism in LD with the ones investigated.

The odds ratio associated with the combination of *DD* and *CC* genotypes versus all other combinations was 2.57 (95 % CI 1.20 – 5.52) i.e. very similar to the one observed in the separate analyses of these two polymorphisms (Table 3).

In order to unravel the possible influence of several risk factors for hypertension - sex, age, body mass index and triglyceride and cholesterol concentrations – on the development of hypertension in the sample, these factors were incorporated as independent variables in the multivariate logistic regression together with *ACE ID* genotypes (Table 4). Beside the *DD* genotype that had in the univariate analysis already been shown as significant predictor for the presence of hypertension, the multivariate analysis revealed BMI as another significant predictor (OR 1.15 with 95 % CI 1.07 –

1.23). In order to check for independent influence of BMI and *ACE* on hypertension, the distribution of BMI was tested across the *ACE* genotypes. The univariate F of 0.274 for controls (d.f. = 2 and 118; $p = 0.761$) and 0.001 for cases (d.f. = 2 and 115; $p = 0.999$) indicated there were no differences in BMI distributions among the genotypes within each group.

The relationship between BMI and hypertension across *ACE ID* genotypes is illustrated in Figure 1 where BMI is shown as a categorical variable with the cut-off point of 30 kg/m^2 that divides non-obese and obese subjects [25]. The proportions of hypertensives across BMI and genotype clearly indicate the additive effect of obesity and *ACE DD* genotype. In non-obese *ACE II* and *ID* groups, the proportions of hypertensive cases are at the 40 % level. In the obese with these genotypes, the proportions increase up to the level found in the non-obese *DD* group (60 %). The proportion of hypertensives is as high as 86% in the obese *DD* group.

Discussion

In this study, we performed an association analysis of hypertension with polymorphisms in genes encoding components of the renin-angiotensin system in a general population sample from Croatia younger than 40 years of age. We chose to sample younger persons in order to enhance the detection of the genetic influence on hypertension assuming a stronger genetic component if the affected individuals are of a younger age. Since case-control studies may suffer from several biases that may lead to false-positive and false-negative results, we have matched our case and control groups for age, sex and place of residence, thus minimizing the biases.

Among the three RAS genes, we found that only the polymorhisms in *ACE* gene showed an association with hypertension. The association of *DD* genotype of *ACE I/D* polymorphism suggested the recessive effect of this susceptibility allele on the risk. A similar association was observed for the *-3892 CC* genotype. As these two polymorphisms are in strong LD, the association observed for each genotype separately probably reflects the same mechanism.

Despite the strong evidence from both association and linkage studies that the *ACE D* allele influences ACE plasma levels [12,13], the data on its association with hypertension as well as on increased angiotensin II (potent vasoconstrictor molecule) levels are contradictory [5,8,16,17,21,26]. However, *ACE* may also influence blood pressure regulation through the degradation of bradikinin, a strong vasodilatator and suppressor of smooth muscle cell growth. Some studies have shown that bradikinin concentration is decreased in hypertension [27] and Brown et al., 1998 [28] have reported an association of the *ACE D* allele with increased degradation of bradykinin.

The lack of association between *ACE* polymorphisms and hypertension in initial studies [8,29] led to the opinion that *ACE* polymorphisms are not involved in the pathogenesis of hypertension. Recently, however, two large population-based studies found the evidence of both linkage and significant association of the *I/D* polymorphism with hypertension, which were restricted to male subjects only [5,17]. Furthermore, a genome-scan analysis revealed strong evidence of a quantitative-trait locus for blood pressure on chromosome 17 in the proximity of *ACE* gene [18].

Our negative findings on the association of the polymorphisms in other two studied genes with hypertension is in accordance with some of the recently published papers [e.g.7,9,10]. Jeunemaitre [30] states that such findings may result from the small sample sizes

that lack the statistical power to detect differences. The same remark may generally be applied to this study. The lack of association of the polymorphisms in *AGT* and *AGT1R* genes with hypertension may likewise be the consequence of the small sample size.

Among the several known risk factors for hypertension (sex, age, body mass index, triglyceride and cholesterol blood concentrations), BMI was the only significant predictor for the presence of hypertension. The *DD* genotype and BMI seem to act independently on the development of hypertension since BMI distribution is the same regardless of *ACE ID* genotype.

Finding of 86 % of hypertensives among the subjects having both *DD* genotype and BMI $\geq$ 30 kg/m^2 – compared to 60 % of those having only one of these two risk factors and 40 % of those having neither of them – is consistent with an additive effect of *DD* genotype and obesity. Moreover, similar frequencies of obese and non-obese cases in both *II* and *ID* genotypes confirm the recessive nature of *ACE ID* polymorphism i.e. the influence of *ACE ID* on hypertension only in the case of the presence of two *D* alleles (Table 3, Figure 1).

In conclusion, the present study detected the association of *ACE* gene polymorphsims with premature hypertension while no association with *AGT* and *AT1R* gene polymorphisms was obtained. Among several known risk factors for hypertension, BMI was the only one found to be associated with its development acting independently from the *ACE* gene.

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Table 1. Basic characteristics of study sample

	Controls	Cases
N	125	119
Male/Female	66/59	61/58
Age (yrs)	34.82 ± 5.34	35.34 ± 5.28
SBP (mm Hg)	124.82 ±10.95	150.22 ± 11.68
DBP (mm Hg)	82.22 ± 9.15	96.24 ± 9.36
BMI (kg/m^2)	25.20 ± 3.85	27.53 ± 4.69
Triglycerides (mmol/l)	1.67 ± 1.42	1.77 ±1.19
Cholesterol (mmol/l)	5.57 ± 1.27	5.59 ± 1.27

Table 2. Distributions of genotype and allele frequencies in cases and controls

	Genotypes			Alleles
ACE ID[a]	*II* (%)	*ID* (%)	*DD* (%)	*D* (%)
Controls	32 (26.0)	70 (56.9)	21 (17.1)	112 (45.5)
Cases	25 (21.0)	53 (44.5)	41 (34.5)	135 (56.7)
ACE T-3892C[b]	*TT* (%)	*TC* (%)	*CC* (%)	*C* (%)
Controls	42 (34.1)	68 (55.3)	13 (10.6)	94 (38.2)
Cases	39 (32.8)	52 (43.7)	28 (23.5)	108 (45.4)
AGT T174M	*CC* (%)	CT (%)	*TT* (%)	*T* (%)
Controls	82 (68.9)	34 (28.6)	3 (2.5)	40 (16.8)
Cases	75 (68.8)	27 (24.8)	7 (6.4)	41 (18.8)
AGT M235T	*TT* (%)	*TC* (%)	*CC* (%)	*C* (%)
Controls	36 (29.5)	56 (45.9)	30 (24.6)	116 (47.5)
Cases	34 (31.8)	52 (48.6)	21 (19.6)	94 (43.9)
AGTR A1166C	*AA* (%)	*AC* (%)	*CC* (%)	*C* (%)
Controls	55 (47.8)	50 (43.5)	10 (8.7)	70 (30.4)
Cases	48 (50.6)	33 (34.7)	14 (14.7)	61 (32.1)

[a]Significant difference in genotype and allele frequencies between cases and controls ($p = 0.01$; $p = 0.02$ respectively)

[b]Significant difference in genotype frequencies between cases and controls ($p = 0.02$)

Table 3. Odds ratios for the presence of hypertension by *ACE ID* and *ACE T-3892C* genotypes

		OR	95 % CI
ACE ID	*ID versus II*	0.97	0.51 – 1.83
	DD versus II	2.50	1.19 – 5.25[a]
ACE T-3892C	*TC versus TT*	0.82	0.47 – 1.45
	CC versus TT	2.32	1.05 – 5.10[b]
ACE ID and *ACE T-3892C*	*DD + CC versus all other combinations of genotypes on two loci*	2.57	1.20 – 5.52[c]

Significance: [a] $p = 0.02$; [b] $p = 0.03$; [c] $p = 0.02$

Table 4. Odds ratios for the presence of hypertension by *ACE ID* genotypes, sex, age, body mass index (BMI) and triglycerides and cholesterol blood concentrations

		OR	95 % CI
ACE ID	*ID versus II*	0.94	0.48 – 1.85
	DD versus II	2.26	1.03 – 4.99[a]
Sex		1.34	0.74 – 2.42
Age (yrs)		0.99	0.94 – 1.05
BMI (kg/m^2)		1.15	1.07 – 1.23[b]
Triglycerides (mmol/l)		1.04	0.81 – 1.34
Cholesterol (mmol/l)		0.89	0.69 – 1.16

Significance: [a] $p = 0.04$; [b] $p = 0.001$

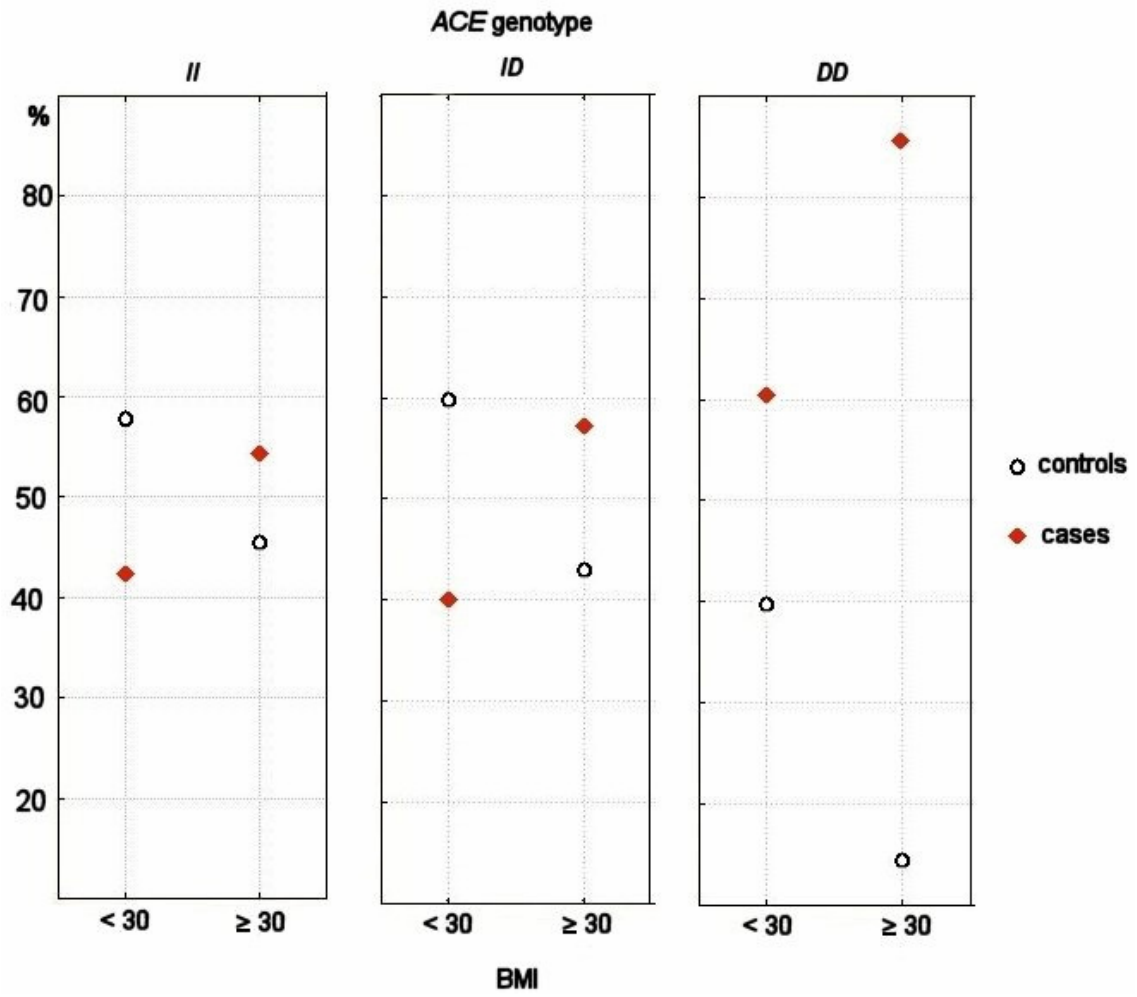

Figure 1. The frequency of cases and controls according to *ACE ID* genotype and BMI category.