

Association of the -344T/C Aldosterone Synthase Gene Variant with Essential Hypertension

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Received November 19, 2007

Accepted October 24, 2008

On-line December 17, 2008

Summary

The aldosterone synthase gene (CYP11B2) is an important candidate gene region in essential hypertension. We therefore studied the association of -344T/C polymorphism of the CYP11B2 gene with the presence and severity of hypertension in a case-control study. We studied 369 individuals, of whom 213 were hypertensive patients (139 controlled hypertensive, 74 resistant hypertensive) and 156 were healthy normotensive subjects. The -344T/C polymorphism of the CYP11B2 gene was determined using polymerase chain reaction - restriction fragment length polymorphism analysis. The distribution of genotypes in normotensive controls and hypertensive subjects were: TT 25.6 vs. 31.9 %, TC 51.9 vs. 57.3 % and CC 22.4 vs. 10.8 %. The -344T/C variant was associated with hypertension. Subjects carrying the -344T allele had a greater risk of hypertension compared to those having C allele ($\chi^2=5.89$, $p<0.05$). The frequency of CC genotype was significantly lower in hypertensive patients than in normotensive controls ($\chi^2=9.44$, $p<0.01$). A stepwise logistic regression analysis confirmed these findings. We did not find an association of -344T/C variant with the resistance of hypertensive patients to combination therapy, but we observed an association of -344T/C polymorphism of aldosterone synthase gene with increased risk of hypertension. These results support a potential role of -344T/C CYP11B2 gene polymorphism in genetic predisposition to develop hypertension.

Key words

Aldosterone synthase • Hypertension • Polymorphism • Aldosterone

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Introduction

The renin-angiotensin-aldosterone system is an important regulator of blood pressure and molecular variants in genes that encode components of this system have been associated with several cardiovascular diseases, such as essential hypertension, myocardial infarction and hypertrophic cardiomyopathy.

Among them the CYP11B2 gene encodes a key enzyme of the aldosterone biosynthesis – aldosterone synthase. Aldosterone is an independent risk factor for cardiovascular diseases and development of cardiac hypertrophy and fibrosis. Aldosterone synthase includes the steroid 11 β -hydroxylase, 18-hydroxylase and 18-oxidase activities that are required for the final steps of aldosterone biosynthesis. A related enzyme, 11 β -hydroxylase (CYP11B1), is responsible mainly for cortisol biosynthesis, although changes in its activity can also influence biosynthesis of steroid metabolites with mineralocorticoid actions. The two relevant genes (CYP11B2, CYP11B1) are located in a close proximity on chromosome 8q22. It is now apparent from investigation of rare monogenic forms of hypertension

and animal models that changes in the expression and activity of these enzymes can affect sodium homeostasis and thereby blood pressure through defects in mineralocorticoid metabolism. CYP11B2 is thus an obvious gene to test for association with hypertension (Brand *et al.* 1998, Freel and Connell 2004).

To date, three common genetic variants of the aldosterone synthase gene (CYP11B2) have been identified as possible determinants of high blood pressure in patients with essential hypertension (White and Slutsker 1995, Mulatero *et al.* 2000, Zhu *et al.* 2003). One is a single nucleotide polymorphism in the 5' promoter region at -344T/C that alters a putative recognition site for steroidogenic transcription factor-1 (SF-1). The other polymorphism involves intron 2 of CYP11B2, which is partly replaced by the corresponding intron of CYP11B1 gene. These two polymorphisms are in close linkage disequilibrium. The third polymorphism is a point mutation K173R in exon 3.

Several studies of association between -344T/C polymorphism and essential hypertension have been published, with controversial results. Whereas some have found that the T-allele is more common in hypertension and subjects with increased urinary aldosterone excretion rate (Brand *et al.* 1998, Davies *et al.* 1999, Kumar *et al.* 2003, Casiglia *et al.* 2005), others have demonstrated no association of either allele with hypertension or other cardiovascular disease (Brand *et al.* 1999, Schunkert *et al.* 1999, Kato *et al.* 2000, Tsujita *et al.* 2001). It was also suggested that the C allele may be associated with genetic predisposition to hypertension in Hani and Yi minorities in China (Tang *et al.* 2006). Several factors such as ethnicity, gender and age could be involved in the phenotypic expression of this polymorphism.

Some studies evaluated the impact of -344T/C polymorphism of the CYP11B2 on the severity of hypertension and blood pressure-lowering response to antihypertensive drugs (Kurland *et al.* 2002, Tiago *et al.* 2003). Because the severity of hypertension determines cardiovascular risk and aldosterone is an important therapeutic target in hypertension, genotyping for the -344T/C polymorphism of the CYP11B2 gene may have prognostic as well as therapeutic potential in hypertensive patients.

The present study was designed to investigate the association of -344T/C polymorphism of the CYP11B2 gene with genetic predisposition to essential hypertension. Furthermore, we examined whether this polymorphism is associated with resistance of

hypertensive patient to combined antihypertensive therapy.

Materials and Methods

Study groups and clinical assessment

We examined a total of 369 individuals, of whom 213 were patients with essential hypertension and 156 were healthy normotensive subjects. All subjects were unrelated Caucasians from the Bohemia region of the Czech Republic. Hypertensive patients were sequentially enrolled from the outpatient clinic of Second Medical Department – Clinical Department of Cardiology and Angiology, First Faculty of Medicine and General University Hospital, Prague. Hypertension was defined according to the following criteria: (i) systolic blood pressure (BP) ≥ 140 mm Hg or diastolic BP ≥ 90 mm Hg, or both, measured on two consecutive visits for untreated subjects; (ii) patients receiving long-term antihypertensive treatment; (iii) no clinical or biochemical evidence of secondary hypertension. Normotensive subjects were selected from outpatients who participated in voluntary health checks and hospital staff undergoing annual medical examinations. The subjects were considered to be normotensive when their systolic blood pressure was lower than 140 mm Hg and diastolic blood pressure below 90 mm Hg. All subjects had normal renal function and no history of diabetes, heart disease or other serious illness. Informed consent was obtained from each subject and the study was approved by the local ethic committee.

To phenotype hypertensive subjects further they were subdivided according to the severity of hypertension into two groups: patients well-controlled after adjustment of lifestyle and medication [controlled hypertension group (CH), $n=139$] and subjects resistant to combined antihypertensive therapy [resistant hypertension group (RH), $n=74$]. Hypertensive patients were considered resistant to therapy if they had systolic BP ≥ 140 mm Hg and/or diastolic BP ≥ 90 mm Hg when on a triple antihypertensive therapy including diuretics.

Genotyping

Genomic DNA was isolated from peripheral leukocytes according to the standard method (Miller *et al.* 1988). The -344T/C polymorphism of the CYP11B2 gene was determined by the analysis of restriction fragment length polymorphism (RFLP) (Komiya *et al.* 2000) (Fig. 1). The DNA fragment containing -344T/C of the

CYP11B2 gene was amplified by polymerase chain reaction (PCR). The PCR was performed using Taq polymerase Boehringer Ingelheim and oligonucleotide primers CTCACCCAGGAACCTGCTCTGGAAACATA and CAGGAGGGATGAGCAGGCAGAGCACAG using hot start and touchdown PCR to increase specificity of the reaction. PCR was subjected to 94 °C for 30 s and from 62 °C to 58 °C, two cycles 1 °C lower temperature annealing, 72 °C for 30 s for amplification to the total amount of 35 cycles followed by final extension for 5min, producing a fragment of 639 bp. This fragment was subsequently cleaved by HaeIII, creating fragments for allele T 402, 138, 51 and 48 bp, and for allele C 334, 138, 68, 51 and 48 bp, which were subjected to electrophoresis on a metaphoragarose gel and visualized with ethidium bromide. The samples from hypertensive patients and healthy controls were also analyzed by DNA sequence analysis to confirm results of the PCR-RFLP method.

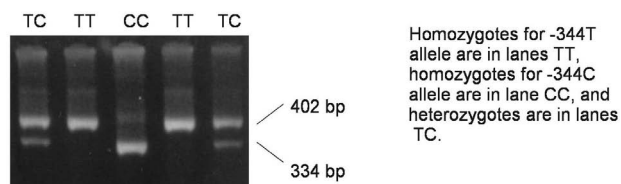


Fig. 1. Genotype determination of the -344T/C CYP11B2 gene polymorphism.

Statistical analysis

Statistical analysis was performed using SYSTAT 10 statistical software (SPSS Inc., 2000). Data are given as means \pm S.D. Differences in clinical variables between case and control groups were tested using one-way analysis of variance (ANOVA). The Hardy-Weinberg equilibrium and allele frequency and genotype distributions were tested by χ^2 statistic (Falconer and Mackay 1996). A stepwise logistic regression analysis was conducted to adjust for covariates including age, gender, body mass index (BMI), blood glucose, serum triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol plasma levels. Association of phenotypic parameters (BP levels, age, sex, BMI) with genotype was assessed by one-way ANOVA for continuous variables and χ^2 test for discrete variables. All statistical tests were two-tailed and values of $p < 0.05$ were considered to represent statistically significant differences.

Results

Clinical characteristics of the study groups

The overall characteristics of the study subjects genotyped for the -344T/C CYP11B2 gene polymorphism are listed in Table 1. Systolic and diastolic blood pressure was markedly higher in patients with essential hypertension (H group) than in normotensive

Table 1. Clinical characteristics of the study groups.

	Normotensive subjects	Hypertensive subjects n = 213		p (ANOVA)
		Controlled hypertensive	Resistant hypertensive	
n	156	139	74	
Age (years)	51.3 \pm 9.7	56.6 \pm 10.5*	61.4 \pm 8.74*	p<0.001
Systolic BP (mmHg)	122 \pm 10.4	144 \pm 16.9*	152 \pm 16.1*	p<0.001
Diastolic BP (mmHg)	79.6 \pm 6.45	90.1 \pm 8.61*	95.3 \pm 8.27*	p<0.001
BMI (kg/m ²)	26.1 \pm 3.41	28.5 \pm 4.37*	29.8 \pm 3.79*	p<0.001
Fasting glucose (mmol/l)	5.29 \pm 0.73	5.98 \pm 1.74*	6.36 \pm 2.27*	p<0.001
Serum-total cholesterol (mmol/l)	5.43 \pm 0.94	5.45 \pm 1.16	5.19 \pm 0.94	n.s.
Triglycerides (mmol/l)	1.43 \pm 0.94	1.87 \pm 1.22*	1.87 \pm 0.84*	p<0.01
HDL-cholesterol (mmol/l)	1.40 \pm 0.30	1.29 \pm 0.31*	1.23 \pm 0.30*	p<0.01
LDL-cholesterol (mmol/l)	3.39 \pm 0.77	3.36 \pm 0.91	3.11 \pm 0.81	n.s.

Data are expressed as means \pm S.D., * $p < 0.05$ for controlled hypertensives, resistant hypertensives versus normotensives by one-way ANOVA. BP = blood pressure, BMI = body mass index, HDL = high density lipoprotein, LDL = low density lipoprotein, n.s. = not significant, ANOVA = analysis of variance.

group (N group). In addition, as expected, body mass index (BMI) and fasting blood glucose were significantly higher in H group than in N group. No significant differences were detected between groups with respect to height, total cholesterol and LDL-cholesterol levels. Plasma levels of LDL-cholesterol revealed a non-significant tendency to lower concentrations in hypertensive than in normotensive subjects. This finding corresponds to a significant number of hypertensive patients treated by statin therapy. However, serum triglycerides were higher and serum HDL cholesterol was lower in hypertensive than in normotensive group.

Table 2. Association analysis of -344T/C CYP11B2 gene polymorphism in hypertensives versus normotensives.

Genotype frequencies (n, %)				
Group (n)	TT (n=108)	TC (n=203)	CC (n=58)	p
Normotensive subjects	40 (37.0 %)	81 (39.9 %)	35 (60.3 %)	p<0.01 $\chi^2=9.44$
Hypertensive patients	68 (63.0 %)	122 (60.1 %)	23 (39.7 %)	

Allele frequencies (n, %)			
Group (n)	T (n=419)	C (n=319)	p
Normotensive subjects	161(38.4 %)	151(47.3 %)	p<0.05 $\chi^2=5.89$
Hypertensive patients	258 (61.6 %)	168 (52.7 %)	

Genotypes and alleles indicated as percentage and number. Statistical analysis by χ^2 test.

Association between aldosterone synthase variants and essential hypertension

Table 2 shows the distribution of genotypes and alleles for the -344T/C CYP11B2 gene polymorphism in hypertensive subjects and normotensive controls.

Frequencies of genotypes were in accordance with the Hardy-Weinberg equilibrium in normotensive group. On the contrary the observed genotype frequencies deviated from that expected from the Hardy-Weinberg equilibrium in hypertensive group (p<0.01).

We found a significant association of the -344T/C CYP11B2 gene polymorphism with essential hypertension. Subjects carrying the -344T allele had

significantly greater risk of essential hypertension compared with those carrying C allele. The CC genotype was twice as prevalent in the normotensive as in hypertensive patients (Table 2). In the logistic regression analysis we observed significant association of -344T/C aldosterone synthase gene polymorphism and hypertension (TC vs. CC: odds ratio = 3.4, 95 % confidence interval 1.3-9.1; TT vs. CC: odds ratio = 5.4, 95 % confidence interval 1.8-16) after adjustment for the covariates of age, gender, BMI, blood glucose and blood lipid levels. We failed to observe any significant difference in the genotype distribution or allele frequencies between hypertensive subjects well-controlled with antihypertensive therapy (CH group) and resistant hypertensive group (RH group) either by χ^2 statistics or logistic regression analysis.

The associations between phenotypic characteristics and aldosterone synthase genotype were analyzed for the normotensive and hypertensive groups separately, as well as in the combined group of normotensive and hypertensive subjects. One-way ANOVA analysis did not show a significant influence of genotype on systolic or diastolic BP. Finally, no significant differences in terms of gender distribution, age, body mass index, glucose and cholesterol concentrations were detected among the genotypes.

Discussion

In the present study, we investigated the association between the -344T/C polymorphism in the promoter region of the human CYP11B2 gene and essential hypertension. Our results showed that the genotype and allele distribution differed significantly between hypertensive and normotensive groups. The -344T allele and TT genotype were associated with genetic predisposition to develop hypertension.

A number of studies have suggested the implication of CYP11B2 gene polymorphism in the pathogenesis of cardiovascular disease. However, previous data on the association between the -344T/C polymorphism and hypertension or with hypertensive intermediate phenotypes such as plasma renin activity and plasma aldosterone concentration gave controversial results. Our findings are in agreement with several studies previously conducted in Caucasians, also showing association of -344T allele with hypertension or blood pressure levels (Brand *et al.* 1998, Davies *et al.* 1999, Casiglia *et al.* 2005). At variance with these

reports, other studies conducted in Caucasian populations did not find any association (Brand *et al.* 1999, Schunkert *et al.* 1999). Kumar *et al.* (2003) reported that the C allele was associated with hypertension in Caucasian women. No association between the T allele and hypertension has also been reported in several Japanese studies (Kato *et al.* 2000, Tsujita *et al.* 2001, Isaji *et al.* 2005) and in one of them hypertension was associated with the C allele (Tamaki *et al.* 1999). Similar inconsistencies have characterized the reports linking this polymorphism to plasma or urinary aldosterone levels (Brand *et al.* 1998, Davies *et al.* 1999, Connell *et al.* 2004). Several reasons could account for these inconsistent results. First, the associations could be influenced by the different genetic background and environmental factors in geographically separated populations. Furthermore, the differences in study design and selection criteria, such as different age (Casiglia *et al.* 2005), gender (Tsujita *et al.* 2001) or different proportions of individuals with low renin hypertension (Zhu *et al.* 2003) might be responsible for these discrepancies. In a recent meta-analysis Sookoian *et al.* (2007) showed that subjects homozygous for the -344T allele of the CYP11B2 gene have, at least, a 17 % greater risk of essential hypertension than their -344CC counterparts.

The exact mechanism, whereby the -344T/C polymorphism of CYP11B2 gene variant may lead to higher blood pressure remains unknown. Recent studies suggest that the -344T/C variant by itself does not directly influence promoter activity despite its location within a SF-1 binding site. Rather, binding of SF-1 to this site downregulates activity of CYP11B2 promoter by making SF-1 less available to functionally affect other CYP11B2 promoter sites, which could alter expression of the gene (Clyne *et al.* 1997). Moreover, *in vitro* studies showed that C allele binds SF-1 fourfold more strongly than it does the T allele (White and Slutsker 1995). This may allow a change in CYP11B2 promoter activity and expression (Hautanen *et al.* 1998, Matsubara *et al.* 2004).

In a previous study conducted in normotensive healthy men we observed higher plasma renin activity (PRA) in TT homozygotes and higher values of left ventricular mass index in individuals with TT genotype and high PRA (Heller *et al.* 2004). In agreement with these findings a recent meta-analysis also revealed higher PRA and no difference in plasma aldosterone levels in homozygous TT individuals (Sookoian *et al.* 2007). We may speculate that higher PRA reported in TT homozygous individuals may be adaptive to a lack of

adequate response of the CYP11B2 promoter bearing the T allele to the angiotensin II-mediated stimulus. Therefore the elevated plasma angiotensin II may make TT homozygous subjects more prone to hypertension (Heller *et al.* 2004, Sookoian *et al.* 2007, Staessen *et al.* 2007).

The evaluation of hypertensive patients selected according to the severity of hypertension may increase the genetic component of hypertension and thus improve the likelihood of detecting any existing genetic association with hypertension. Studies conducted in hypertensive patients that examined the relationship between candidate gene polymorphisms and the severity of hypertension or presence of resistant hypertension has provided contradictory data. While some studies have reported an association of -344T/C polymorphism of aldosterone synthase gene, I/D polymorphism of ACE gene, A1166C polymorphism of AT1-receptor gene and G894T polymorphism of the endothelial nitric oxide synthase gene with severity of BP changes, resistant hypertension, malignant hypertension or hypertensive crisis, others did not confirm these results (Brand *et al.* 1998, O'Donnell *et al.* 1998, Stefansson *et al.* 2000, Jáchymová *et al.* 2001, Sunder-Plassmann *et al.* 2002, Tiago *et al.* 2003). In the present study we evaluated the impact of -344T/C polymorphism of CYP11B2 gene on the severity of hypertension by comparing hypertensive subjects well-controlled by conventional therapy and patients with resistant hypertension. We did not observe association between CYP11B2 genotype groups and presence of resistant hypertension. We found high frequency of resistant hypertension in our study. Although the prevalence of resistant hypertension is unknown, the data from recent hypertension outcome trials (VALUE, ALLHAT, CONVINCENCE) indicate that it is greater than previously thought (Cushman *et al.* 2002, Black *et al.* 2003, Julius *et al.* 2003). On the other hand, patients enrolled in hypertension clinic of our hospital are generally those with moderate to severe or uncontrolled hypertension. Thus, we think that the number of patients with resistant hypertension in our study overestimates the occurrence of resistant hypertension in hypertensive population.

Several studies investigating the role of -344T/C aldosterone synthase gene polymorphism on blood pressure described an association of BP levels with -344T allele (Brand *et al.* 1998, Brand *et al.* 1999, Matsubara *et al.* 2001, Tiago *et al.* 2003, Barbato *et al.* 2004). However, in the study of Brand *et al.* (1998) the

relationship was significant only in the subgroup of severely hypertensive patients of African ethnicity with BMI greater than 27 kg/m². In contrast, other cross-sectional and case-control studies as well as the meta-analysis by Sookoian *et al.* (2007) did not confirm the role of aldosterone synthase gene polymorphism in determining BP levels (Brand *et al.* 1998, Brand *et al.* 1999, Kupari *et al.* 1998, Tsujita *et al.* 2001, Tiago *et al.* 2003). In our study, we did not demonstrate the influence of genotype on systolic and diastolic BP levels. However, since we were unable to discontinue therapy in our outpatients, and the assessment of pretreatment blood pressure levels was not available, such analysis is inevitably biased. The genetic effects on BP levels in our study population may have been modified by the antihypertensive medication. Finally, we did not observe any significant differences among the genotypes in terms of gender distribution, age, BMI, glucose and cholesterol

concentrations.

In summary, we have observed an association of -344T/C polymorphism of aldosterone synthase gene with hypertension in case-control study in Caucasian subjects from Czech Republic. These results support a potential role of -344T/C CYP11B2 gene polymorphism in mechanisms affecting blood pressure regulation. Further studies of this locus should be performed in large-scale populations to confirm our results and to define the underlying physiological and clinical implications of observed association.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

Study supported by the research project from the Ministry of Health of the Czech Republic (MZO VFN2005).

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