# Cardiovascular diseases and molecular variants of the renin-angiotensin system components in Slovak population

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**Abstract.** Cardiovascular diseases associated with molecular variants of individual components of renin-angiotensin system are reported to constitute inherited predisposition in humans. Molecular variant frequencies are race- and population-dependent. We examined frequencies of the M235T variant of angiotensinogen gene and I/D polymorphism of gene for angiotensin-converting enzyme in Slovak population: in hypertensive patients, coronary heart disease (CHD), dilated cardiomyopathy (DCM) and myocardial infarction (MI) patients compared to healthy subjects. Frequency of M235T was significantly increased in hypertensive, CHD and DCM patients compared to controls (0.48 and 0.50 *vs.* 0.40, $p < 0.001$). Significant increase in D allele frequency compared to controls was observed in the group of patients after MI (0.58 *vs.* 0.50, $p < 0.001$), CHD (0.59 *vs.* 0.50, $p < 0.001$) and DCM (0.60 *vs.* 0.50, $p < 0.001$). These results correlate with other Caucasian populations. In Slovak population, M235T is associated with increased blood pressure and D allele of ACE gene is associated with MI, chronic CHD and DCM, rather than with hypertension. Our results suggest that in Slovak population, D alelle and M235T variant represent a risk factor for several cardiovascular diseases and these polymorphisms might have a cumulative effect on development of cardiovascular diseases.

**Key words**: Polymorphisms — Hypertension — Coronary heart disease — Dilated cardiomyopathy — Slovak population

## Introduction

In population of modern countries, cardiovascular diseases (CVD) contribute to the highest morbidity and mortality. Among patients suffering from CVD, coronary heart disease (CHD) and hypertension are very frequent. Human hypertension has been recognized as one of the most important and primary risk factors for the development of CHD and myocardial infarction (MI) (Horan and Lenfant 1990; Jeunemaitre et al. 1992).

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Human hypertension is a multifactorial, polygenic disease, in which one or more genes control the level of blood pressure (Jeunemaitre et al. 1992; Caulfield et al. 1994). Hypertension is defined as a repeated blood pressure reading of 140/90 mm Hg or greater (Rouse et al. 1983). Two types of hypertension are known: primary (essential or idiopathic) and secondary, developed due to some definable cause, such as kidney failure or atherosclerosis. About 90% of hypertension cases are considered to be primary hypertension. In patients with hypertension, approximately half of the cases can arise from genetic susceptibility (Ward et al. 1990). Studies conducted in previously treated patients point to the relationship between renin-angiotensin system (RAS) genotype and the severity of hypertension. An association of the polymorphism of angiotensinogen (AGT) gene is highly race-dependent (Beige et al. 1997; Gharavi et al. 1997).

Dilated cardiomyopathy (DCM) is one of the serious cardiovascular diseases. In DCM, the heart muscle becomes weakened and loses the strength to pump blood throughout the body. As greater amounts of blood fill and remain in the heart's ventricles, the ventricles expand, dilate. DCM is a leading indication for heart transplantation. The etiology of DCM is idiopathic in about half patients and up to 25% of cases had a familial origin (Michels et al. 1992; Keeling et al. 1995). Indeed, several genetic loci responsible for rare autosomal dominant or X-linked forms of DCM have been subsequently localized by linkage analysis in large pedigrees (for review, see Olson and Keating (1997)), suggesting that genetic factors might contribute to the disease susceptibility.

In complex diseases (like CVD) that do not exhibit a clear pattern of familial aggregation, the candidate gene approach is a strategy widely used (Lander 1996; Cambien et al. 1997). In mentioned cardiovascular diseases (hypertension, CHD and DCM), all genes coding for proteins involved in biochemical or physiological abnormalities of cardiac function are potential candidates. Among those candidates, genes of components of RAS are expected to play the major role.

In the human gene encoding for AGT – the main substrate of RAS, fifteen molecular variants have been identified, but only three have so far been reported to have a possible genetic association with hypertension (Jeunemaitre et al. 1992, 1993, 1997; Chiang et al. 1997).

I/D polymorphism of the angiotensin-converting enzyme (ACE) gene has been implicated in the development of myocardial infarction. The D/D genotype is associated with increased tissue and circulating ACE levels and elevated angiotensin II. Presence of the D/D genotype is more frequent in patients after MI than in controls, indicating that this genotype could be a significant risk factor and a predictor for the disease development (Espinel et al. 2005). Candy and coworkers (1999) pointed that D allele is associated with both, severity of decrease in the left venricular systolic performance, as well as increase in the left ventricular cavity size that occurs in idiopathic DCM. These results are consistent with these of Raynolds et al. (1993) who demonstrated that greater percentage of patients with end-stage idiopathic DCM are D/D homozygots.

Presented study is built on our previous results (Krizanova et al. 1997, 1998), where we have shown that in Slovak population M235T molecular variant of AGT gene is associated with predisposition to hypertension and D allele of ACE gene is associated with myocardial infarction rather than hypertension. Here we analyze both molecular variants on larger complex of patients from Slovak population suffering from hypertension and myocardial infarction, and we provide new data on the association of molecular variants M235T of AGT gene and I/D polymorphism of ACE gene with predisposition to CHD and DCM. All these groups were compared with group of healthy controls from Slovak population.

## Material and Methods

### *Patients*

In our study, 1203 patients (674 males and 529 females) and 156 healthy control subjects matched to patients by gender and age were taken into the trial. Many patients taken into the trial suffered from multiple cardiovascular diseases. From all patients, 134 suffered from hypertension only (61 males, 57.7 ± 11.9 years). 465 patients (332 males, 59.3 ± 14.5 years) experienced MI. 701 patients (485 males, 58.8 ± 16.2 years) suffered from other forms of CHD and 110 patients (84 males, 54.6 ± 17.4 years) suffered for DCM.

All patients were from Caucasian population of Slovakia. Samples were obtained from Slovak Institute of Cardiovascular Diseases, Bratislava, Slovakia, and patient facility of Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovakia. The Ethic Committee of the Slovak Institute of Cardiovascular Diseases, Bratislava, Slovakia, and Ethic Committee of Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava, Slovakia, approved presented study and written agreement was obtained from the participants prior to blood sample collection.

### *DNA extraction and purification*

Blood samples were collected into EDTA and were stored at –20°C until DNA extraction. DNA was extracted using proteinase K and sodium-dodecyl-sulphate digestion at 37°C overnight and purified using phenol-chloroform extraction. Extracted DNA was precipitated using 1/10 volume of 3 mol/l sodium acetate (pH 5.2) and 2 volumes of cold 96% ethanol. Purity and concentration of DNA was measured spectrophotometrically (GeneQuant Pro, Amersham Biosciences). Purified DNA was solubilized in TE buffer (10 mmol/l Tris, pH 8.0, 1 mmol/l EDTA, pH 7.5) and stored at –20°C until used for DNA amplification.

### *Polymerase chain reaction (PCR)*

Polymorphism of the gene for AGT was determined by PCR with specifically designed primers (Russ et al. 1993) (upstream primer: 5' CCG TTT GTG CAG GGC CTG GCT CTC CT 3' and downstream primer: 5' CAG GGT GCT GTC CAC ACT GGA CCC C 3') that after the exchange of methionine (M) for threonin (T) at codone 235 created a restriction site for Tth 111 I restriction endonuclease. PCR program specific for AGT started with initial denaturation

at 94°C for 5 min, followed by 10 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 1 min and polymerization at 72°C for 1 min and continued with 30 cycles of denaturation at 94°C for 30 s, annealing at 68°C for 1 min and polymerization at 72°C for 30 s. PCR was terminated by final polymerization at 72°C for 10 min. AGT PCR products were digested with Tth 111 I endonuclease for 16 h at 37°C. For I/D polymorphism following primers were used: the upstream primer 5' CTG GAG ACC ACT CCC ATC CTT TCT 3' and the downstream primer was: 5' GAT GTG GCC ATC ACA TTC GTC AGA 3' (Rigat et al. 1992). PCR program started with initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and polymerization at 72°C for 2 min. Specific PCR was terminated by final polymerization at 72°C for 7 min. PCR and digestion products were analyzed on 2 and 3% agarose gels.

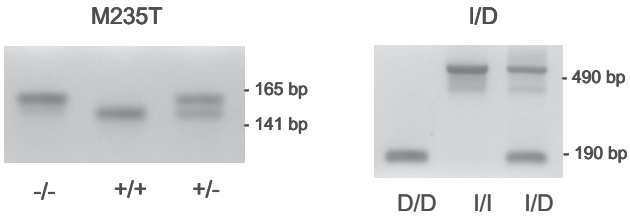


**Figure 1.** Representative gels of AGT and ACE gene polymorphisms PCR amplification. After PCR amplification of AGT gene and Tth 111 I restriction endonuclease digestion of PCR products we detected –/– homozygots (only 165 bp fragment; no mutation), +/+ homozygot (only 141 bp fragment; both alleles carrying mutation) and +/– heterozygots (165 and 141 bp fragments; 1 allele carrying mutation). After PCR amplification of ACE gene we identified D/D homozygots (only 190 bp fragment), I/I homozygots (only 490 bp fragment) and I/D heterozygots (490 and 190 bp fragments).

*Allele frequencies and statistical analysis*

Allele frequencies among patients with individual diagnosed cardiovascular diseases and the controls were determined by the gene evaluation method and by the chi-square analysis (STATISTICA 7, StatSoft, Czech Republic). Allele frequencies were compared with calculated Hardy–Weinberg distributions representing expected distributions of the alleles in population.

## Results

M235T molecular variant of AGT gene was identified after specific PCR followed by digestion with Tth 111 I restriction endonuclease. Heterozygots (+/–) were identified by appearance of two fragments of 165 bp and 141 bp, while mutant homozygots (+/+), where both alleles were cleaved by restriction enzyme, revealed only one band of size 141 bp. Individuals lacking this mutation (–/–) showed only one band sized 165 bp (Figure 1). The number of individual genotypes in tested groups of patients with different CVD was calculated and the frequency of M235T molecular variant was evaluated. Our data showed that M235T frequency is significantly higher in group of hypertensive patients (0.48 *vs.* 0.40; $p < 0.001$), CHD patients (0.48 *vs.* 0.40; $p < 0.001$) and also DCM patients (0.50 *vs.* 0.40; $p < 0.001$) compared to healthy controls. In a group of patients after MI, the frequency of mutant allele of AGT gene was 0.38 ($p < 0.001$), that was similar to frequency of controls (0.40). Described data are summarized in Table 1.

Patients were also examined for I/D polymorphism on ACE gene. Our results on group of all patients that had undergone MI show the frequency of 0.58 compared to healthy

**Table 1.** Genotypes distribution and frequency of molecular variant M235T of AGT gene in Slovak population

| Subject | *n* | –/– | –/+ | +/+ | q | *p* |
|---|---|---|---|---|---|---|
| Controls | 312 | 62 | 69 | 25 | 0.40 | |
| Control HW | | 56.2 | 74.9 | 24.9 | | |
| MI only | 64 | 10 | 17 | 5 | 0.38 | n.s. |
| MI only HW | | 12.3 | 15.1 | 4.6 | | |
| Hypertensive only | 268 | 31 | 72 | 31 | 0.48* | <0.001 |
| Hypertensive only HW | | 36.2 | 66.9 | 30.9 | | |
| CHD | 1402 | 181 | 360 | 160 | 0.48* | <0.001 |
| CHD HW | | 189.6 | 349.9 | 161.5 | | |
| DCM | 220 | 31 | 51 | 28 | 0.50* | <0.001 |
| DCM HW | | 27.5 | 55.0 | 27.5 | | |

*n*, number of alleles; q, frequency of M235T molecular variant of AGT gene; HW, Hardy–Weinberg calculation; +, M235T molecular variant of AGT gene present; n.s., non significant; * significant.

**Table 2.** Genotypes distribution and frequency of I/D polymorphism of ACE gene in Slovak population

| Subject | *n* | I/I | I/D | D/D | q | *p* |
|---|---|---|---|---|---|---|
| Controls | 312 | 38 | 78 | 40 | 0. 50 | |
| Control HW | | 39.0 | 78.0 | 39.0 | | |
| Hypertensive only | 268 | 31 | 65 | 38 | 0.53 | n.s. |
| Hypertensive only HW | | 29.6 | 66.8 | 37.6 | | |
| MI | 930 | 109 | 200 | 156 | 0.58* | <0.001 |
| MI HW | | 82.0 | 226.5 | 156.4 | | |
| CHD | 1402 | 150 | 308 | 243 | 0.59* | <0.001 |
| CHD HW | | 117.8 | 339.1 | 244.0 | | |
| DCM | 220 | 21 | 50 | 39 | 0.60* | <0.001 |
| DCM HW | | 17.6 | 52.8 | 39.6 | | |

*n*, number of alleles; I, insertion allele; D, deletion allele; q, frequency of D allele of ACE gene; HW, Hardy–Weinberg calculation; n.s., non significant; * significant.

controls frequency of 0.50 ($p < 0.001$). In two other CVD patients our results showed a significant increase in frequency of D allele compared to controls: in CHD patients (0.59 *vs.* 0.50; $p < 0.001$) and patients with dilated cardiomyopathy (0.60 *vs.* 0.50; $p < 0.001$). In a group of hypertensive patients, the frequency of D allele of ACE gene was 0.53 ($p < 0.001$), that was similar to frequency of controls (0.50). Results are summarized in Table 2.

## Discussion

Most of the cardiovascular diseases may have multifactorial etiologies resulting from interaction between genetic and environmental influences. Genes of the RAS are good candidates for the study of the genetics of CVD, because this system is well known to be involved in the control of blood pressure, plays an autocrine or paracrine role in cardiac remodeling and fibrosis (Dzau 1994; Kawaguchi and Kitabatake 1995) and contributes to the pathophysiology of CVD.

In the past few years, many studies confirming a positive association between M235T variant of AGT gene and hypertension were published (Jeunemaitre et al. 1992; Caulfield et al. 1994), but on the other hand there were many of those who did not detect any association at all (Beige et al. 1997; Gharavi et al. 1997). An extensive study of the potential role of the AGT gene in human essential hypertension was performed by Jeunemaitre et al. (1992). Genetic linkage between essential hypertension and AGT M235T allele frequency in Caucasian population was claimed by Caulfield and collegues (1995). In the study conducted in Japanese population no association was found (Hata et al. 1994; Ichihara et al. 1997). Similarly, Rotimi and coworkers (1994), who studied this association in the African–American population found no evidence for a linkage between T235 allele and essential hypertension. Our results presented here and also those published earlier (Krizanova et al. 1997) on Slovak population confirm the association of M235T molecular variant of AGT gene with higher incidence of diagnosed hypertension.

Hypertensive patients were also examined for I/D polymorphism of ACE gene. Consistently with our previous results (Krizanova et al. 1997, 1998), results of Fildes and coworkers (2005) and others done on Caucasian population, we found no association of ACE I/D polymorphism with hypertension in Slovak population.

In the group of patients that had undergone MI, consistently with our previous results (Krizanova et al. 1997, 1998) and results of others (Cambien et al. 1992), we confirmed the association of D alelle of ACE gene with the increased predisposition to MI.

AGT and ACE genes have been found to underlie cardiomyopathies and they either individually or in combination appear to contribute to genetic susceptibility to DCM. Molecular variant M235T of AGT gene seems to be a predisposing factor for cardiac hypertrophy in hypertrophic cardiomyopathies (Ishanov et al. 1997). Ludwig and coworkers (1997) showed positive association of M235T molecular variant with CHD complicated with the serious dysfunction of the left ventricle, which can precede the development of cardiomyopathies. In concordance with these authors, we found increased frequency of the M235T molecular variant of AGT gene in patients with CHD and DCM in Slovak population.

Although some authors failed to detect association between ACE genotype and either the diagnosis of the idiopathic DCM or progression of this disease in Caucasians (Montgomery et al. 1995), Raynolds et al. (1993) showed that

the ACE D/D genotype is a risk factor for development of end-stage heart failure caused by CHD or idiopathic DCM. Goncalvesova et al. (2005) also found increased frequency of the D alelle in patients with chronic heart failure due to DCM. Our results indicating that D allele is also related to the DCM in Slovak subjects are consistent with results of these authors.

The role of AGT and ACE genotypes in the pathogenesis of CHD and DCM remains still not clear and requires further investigation. Analysis of genetic variation has the potential to improve our understanding of the reason of development and progression of cardiovascular diseases and also of determinants of drug response. Using a candidate gene approach, interest is now focused on identifying genetic polymorphisms that influence the pharmacodynamic determinants of antihypertensive response.

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