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EVALUATION OF RENIN-ANGIOTENSIN SYSTEM GENES POLYMORPHISMS AND ITS CORELATION WITH PARAOXONASE 1 ACTIVITY IN A HETEROGENOUS GROUP OF HYPERTENSIVE PACIENTS

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ABSTRACT. **Introduction**: The association of polymorphisms of the RAS genes on the incidence of hypertension its still under debate and more over it seems to be population-dependent. **Aim:** We studied correlations between the angiotensinogen T174M and M235T and angiotensin II receptor 1 (AT1R) A1166C and angiotensin II receptor 2 (AT2R2) C3123A gene polymorphisms and human paraoxonase 1 (PON 1) activity in hypertensive group of patients. **Method:** 100 patients of a medium and old age have been investigated clinically and subjected to a stress questionnaire. Familial inquiry up to 3rd generation has been recorded. The RFLP-PCR was used to detect the target mutations in RAS genes. Paraoxonase activity was recorded from plasma serum samples. **Conclusions:** Our results showed strong association of RAS polymorphism with PON1 in a specific study group. We stress out the importance of genetic analysis for essential hypertension and that the possibility of inter-population differences in genetic factors should be kept in mind.

Key words: hypertension, RAS polymorphism, human paraoxonase 1, cardiovascular risk

INTRODUCTION

Hypertension (HTA) is a major form of cardiovascular disease and a risk factor for stroke, heart disease and end-stage renal disease (*Cowley, 2006*). Despite of intensive research efforts no clear options of diagnosis, preventive measures or treatment of essential hypertension are available. It is estimated that about 40% of variation in human blood pressure is determined by genetic variants. Most studies searching for genetic causes of essential hypertension have been conducted with association analysis of candidate genes including renin gene, angiotensin-converting enzyme (ACE) gene, angiotensinogen gene (AGT), angiotensin II type 1 receptor (AGT1R) gene, ENaC genes, 11bHSD gene, sympathetic a-receptor genes, b-receptor genes, endothelial nitric oxide synthase (eNOS) gene, atrial natriuretic peptide (ANP) gene, adducin gene, cytochrome P450, family 11, subfamily B, polypeptide 2 (CYP11B2); nuclear receptor subfamily 3, group C, member 2 (NR3C2); renin (REN); adrenoceptor beta 2 (ADRB2); adrenoceptor alpha 2A (ADRA2A); adrenoceptor beta 1 (ADRB1); phenylethanolamine N-methyltransferase (PNMT) secretogranin II (SCG2) and others (*Agarwal et al., 2005*; *Delles et al., 2009*). AGT is the first gene to be linked to essential hypertension (EHT). Known and novel variants of AGT gene are found to contribute to the development of hypertension (*Padma et al., 2014 a,b*). The Millennium Genome Project for Hypertension identified ATP2B1 as a gene responsible for hypertension and certain alleles of ATP2B1 have been widely replicated in various populations (*Tbara et al., 2012*). The association of polymorphisms of the RAS genes on the incidence of hypertension its still under debate and more over it seems to be population-dependent (*Bautista et al., 2013*). The latest genetic researches has shown that from the paraoxonase family (PON1, PON2 and PON3) the human paraoxonase 1 (PON1) plays an important role in the protection of LDL cholesterol and HDL cholesterol molecules to be oxidized by the free radicals (*Bin et al., 2003*). A low serum activity of PON1 is linked with an increased risk of coronary heart disease, myocardial infarction, and carotid atherosclerosis (*Mackness et al., 1991*). We have previously reported low levels of PON1 in hypertensive patients that correlated with increased risk of cardiovascular diseases (*Popa et al., 2013*).

AIM: Here we compare the possible genetic profile among heterogeneous group of hypertensive patients by analyzing the common angiotensinogen (AGT) T174M and M235T polymorphism, and angiotensin II receptor 1 (AGT1R) A1166C and AGT2R2 - C3123A gene polymorphisms on the risk of hypertension and the correlation with PON1 activity.

METHODOLOGY

Study group: We included in our research 100 patients (45 males, 55 females) diagnosed with hypertension. Inclusion criteria for the study were

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hypertensive subjects over 35 years and less than 70 years of age, without major cardiovascular events (history of bypass, stenting, cardiac arrest, stroke or heart failure) and without recent consumption of tobacco, alcohol or lowering lipids drugs. After collecting the anamnestic data from the patients, we split our study group in four different study groups as follows: first group (H1) included 20 patients (9 males, 11 females) with hypertension 1st degree without treatment, age between 35-45 years; the second group (H2) with 20 patients (10 males, 10 females) with metabolic syndrome (hypertension grade I/II, decreased glucose tolerance, obesity, sedentary), age between 45-55 years; third group (H3) also with 20 patients (11 males, 9 females) included hypertensive patients with diabetes mellitus type 2, age between 35-60 years; the fourth group (H4) with 20 hypertensive patients (13 males, 7 females) associated with type 2 diabetes and chronic kidney disease at different stages, age between 45-70 years. The fifth group, control group (C) had 20 healthy individuals (15 males, 10 females), age between 35-45 years. Clinical data of hypertensive patients and control group were selected from anamnestic and clinical exams made to each patient at the moment of inclusion in our study. Blood pressure measurements were performed according to the guidelines of the European Society of hypertension (ESH). Familial inquiry up to 3rd generation has been recorded for all patients and pedigrees were contracted using CeGaT, GmbH – Center of Genomics and Transcriptomics (http://www.cegat.de/Pedigree-chartdesigner_l=1_155.html). Written consent was obtained from each patient included in the study.

PON1 quantification: Blood was taken from vein into vacutainers and after centrifugation (10 min at 1500×g) serum was immediately separated and stored in aliquots at –80°C until use. Specific ELISA PON1 kit was used for the quantitative determination of human serum paraoxonase 1 concentration (*Popa et al., 2014*).

DNA extraction and PCR_RFLP: Genomic DNA was isolated from peripheral blood using DNeasy Blood Isolation kit Qiagen. RAS polymorphisms were tested as described by Rupert *et al., 2003*, Russ *et al., 1993*, Caufield *et al., 1999*, Katsuya *et al., 1995* and *Ramachandran et al., 2009*. 50ng genomic DNA was amplified in a total volume of 20µl, using 12,5µl PCR Master Mix Fermentas, 10µM each primers (IDT). The PCR program was used as follows: 10 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s annealing at 64°C (Met235Thr), 57°C (Thr174Met), 57°C (A1166C), 52°C (C3123A), 1.30 min. elongation at 72°C, followed by a final extension of 2 min at 72°C. The amplicons were verified on agarose gel. 10µl of the PCR product was used for enzymatic digestion with *Tth111I* (Met235Thr), *NcoI* (Thr174Met), *DdeI* (A1166C) and *AluI* (C3123A). Homozygotes and heterozygotes were discriminated by separation of the digested fragments in 3% agarose gel electrophoresis.

Statistical analysis:

All results were expressed as mean and standard deviation (± SD). The comparison between the groups was performed using the Student *t-test.* The associations of the four genetic variants were estimated as odds ratio (OR) and 95% confidence interval (CI) were calculated. The genotypes and alleles frequencies were compared in patients and controls by using the Fisher exact test. Statistical significance means were considered at a *p-value* < 0.05.

RESULTS

The renin-angiotensin system or RAS regulates blood pressure and fluid balance in the body. If the renin-angiotensin system becomes overactive, consistently high blood pressure results. Among candidate genes of RAS, AGT gene is considered as an important player in the pathogenesis of essential hypertension. The frequency of the AGT-235T and 174M alleles, AGT1R-A1166C and AGT2R2 - C3123A of the angiotensinogen gene, previously reported to be associated with hypertension was compared between a heterogeneous group of hypertensive patients with additional associated chronic diseases from Arad county. We included in the study group subjects that are relatives. The individuals related accounted for 10% from the whole group. The patients selected for molecular variants of ATG and AGTR genes were part of a larger group study (*Popa et al., 2014*).

Our results obtained from the history and clinical examination specific for each patient from the study groups are shown in Table 1. The blood pressure (BP), body mass index (BMI) were very different for the study groups compared with the control group, so we can say that these parameters along with the age represent important risk factors for each hypertensive patient. Higher values for BP were found in the H4 group of study (hypertensive patients with diabetes and kidney disease), also higher values had the H2 group (hypertensive patients with cardio-metabolic syndrome) for the BMI parameter, in this group were the most numerous patients with obesity. The lipid fractions for all the study groups were modified, higher values were observed for the study group H2 (hypertensive patients with metabolic syndrome) also the values for the AIP (atherogenic index of plasma) were very high for this group compared with the control group (p=0.0025 for the male subjects in study group H2) or even with all other study groups.

Different frequency of the 4 mutated genotypes was observed among the study groups (Table 2). The frequency of AGT-MT heterozygous genotype was seen only in the H4 group (OR=0,85 96%CI (0,14-10,9 p=0,80). Similar frecqency of TT genotype was observed in same group (Fig.1).

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Table 1.

Anamnestic data distributed by gender in the study groups

	Age[1]		BMI[1]		BP[1]	
	♂	♀	♂	♀	♂	♀
Control	38.5±2.3	37±4.2	24.3±3.2	20.4±1.6	124±2.1 / 78±1.7	118±1.2 /74±1.7
H1	42±5.7	39±4.5	25.9±2.3	25.1±1.2	148±1.7 / 96±1.8	146±1.9 / 94±2.4
H2	46.5±3.6	44.5±4.8	31.5±4.6	29.5±5.3	150±3.2 / 92±2.4	148±2.8 / 98±2.3
H3	47±5.1	46±7.2	25.8±1.7	24.9±1.4	168±2.2 /106±2.4	156±2.6 /104±2.8
H4	49±4.2	52±3.1	25.3±3.8	25.5±4.7	176±2.8 /104±2.9	172±3.5 /108±4.1

For the T235T molecular variant of the ATG gene we detected high frecvency of homozygous individuals in H3 group (OR=0,37 95%CI (0,03-3,99), p=0,41) but also in the control group. The frecquency of the T174M variant the groups H3 (OR=0,25 95%CI (0,01-3,98), p=0,32), H4 (OR=0,25 95%CI (0,02-2,43), p=0,23) and the control group showed similar results.

Table 2.

Observed RAS genotypes in the study groups

	% of homozygous (T235/T235) / heterozygous (M2335/T235)	% of homozygous (T174/T174)/ heterozygous (T174/M174)	% of homozygous (C1166/C1166) heterozygous (A1166/ C1166)	% of homozygous (A3123/A3123)/ heterozygous (C3123/ A3123)
Control group	20/0	0/14	0/10	0/0
Group H1	0/0	0/0	0/0	20/0
Group H2	0/0	0/0	0/0	0/0
Group H3	60/0	0/20	0/10	0/0
Group H4	**20/20**	**0/18**	**0/20**	**0/30**

No homozygous T174T individuals were detected in the study groups (Fig.2). Heterozygous A1166C variants were detected with similar frecquency in the H3 and H4 but also in the control group (Fig.3). No homozygous individuals were detected. The C3123A variant was detected only in the H4 group compared to homozygous individuals that were detected only in the H1 group.

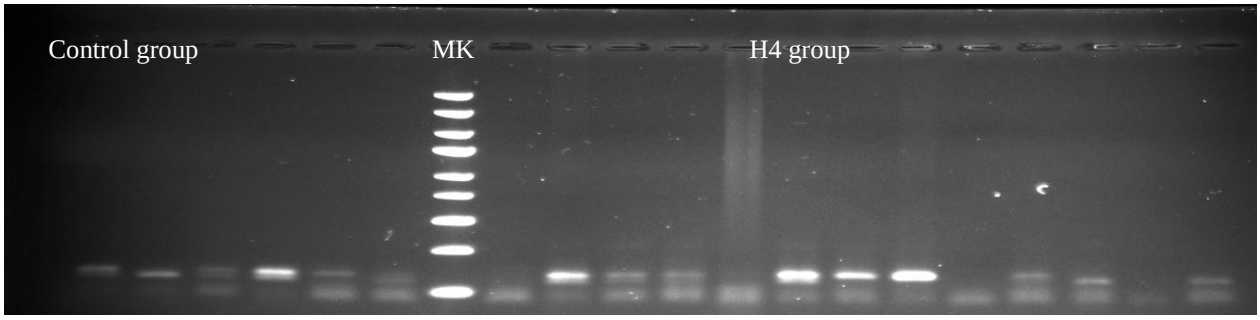

Fig.1. Agarose gel electrophoresis of the AGT M235T variant PCR-RFLP products. The mutated T235 allele: 141 and 21 bp. Mk- 100 bp DNA ladder SM1551.

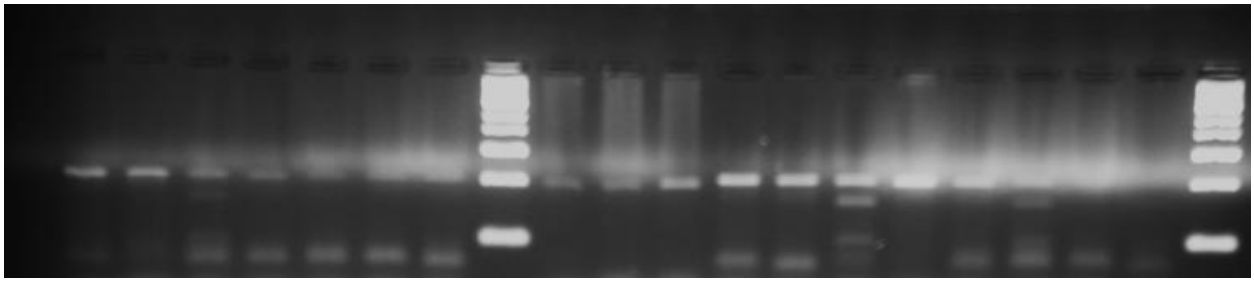

Fig.2. Agarose gel electrophoresis of the AGT T174M variant PCR-RFLP products. The mutated M174 allele: 211 and 92 bp. Mk- 100 bp DNA ladder SM1551

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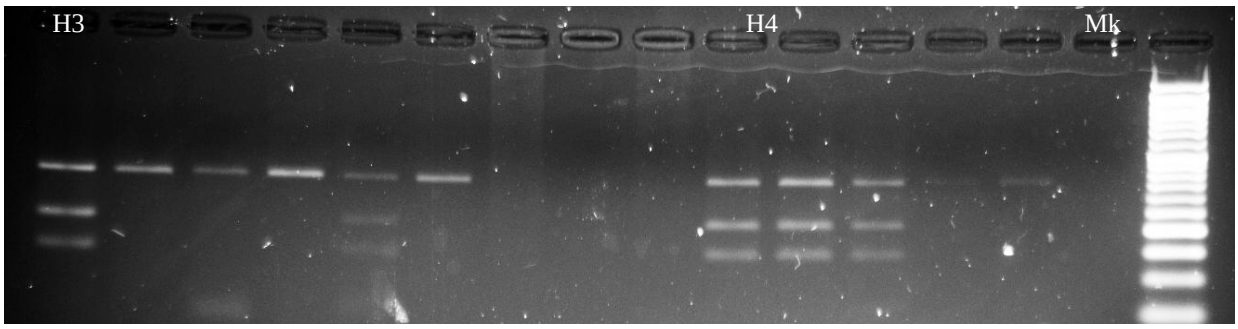

Fig.3. Agarose gel electrophoresis of the A1166C variant PCR-RFLP products. The mutated mutated C1166 allele: 211 and 139 bp fragments. Mk- 50 bp DNA ladder

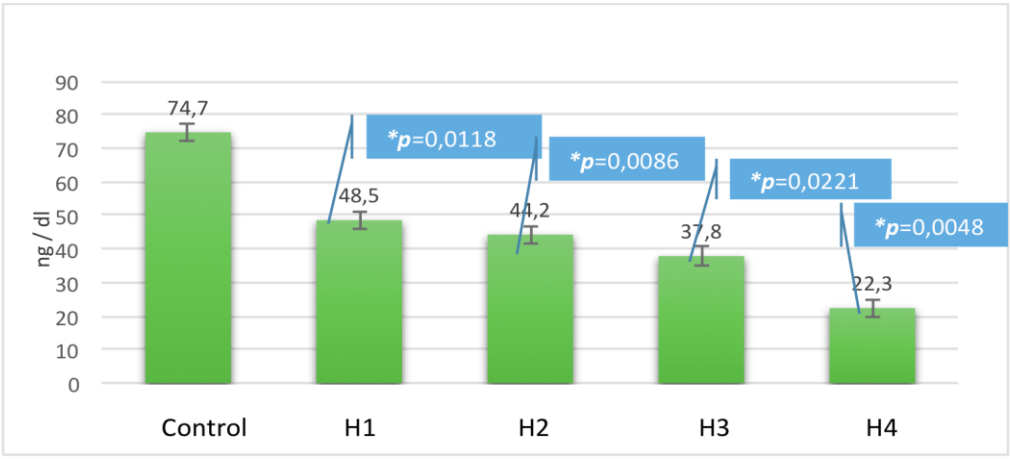

Fig.4. Human paraoxonase 1 values for the female patients

- *values expressed as mean (±standard deviation); the value of $p<0.05$ was considered significant, values of $p>0.05$ considered non-significant;*

The lowest serum levels for PON1 was for H4 group (patients with hypertension, type II diabetes and renal disease) that was on average 25,7ng/dl compared to the control group. As particular results, some subjects from the control group (3 females and 6 males) were having low serum levels of PON1 ranged between 55-68 ng/dl without having known risk factor from the anamnesis.

DISCUSSIONS

Our study is retrospective and thus provides only a small insight into causal processes. We failed to find significant association between analyzed RAS polymorphism and hypertension. From the study groups only H3 and H4 patients showed an overall high frequency of AGT and AGTR known polymorphism. We think that lack of significance is partly due to the low statistical power since the size of our groups is quite small compared to other studies. Still, we found strong correlation between PON 1 activity and genetic polymorphism of RAS genes in H3 patients characterized by hypertension and type II diabetes and H4 group, patients with hypertension, type II diabetes and renal disease. The lowest values of PON1 were found in H4 female patients (22,3 ng/dl compared to male patients (25,7 ng/dl). Clearly, our failure to identify a statistically significant association in a sample of 80 hypertensive and 20 control subjects cannot be taken as proof that no such association exists.

The family inquiry and anamnestic data collection allowed us to construct the pedigree charts for each patient in our study (examples are shown in the fig.5). Having in mind the % of family relatives in the study group we were able to analyze the genetic predisposition for hypertension and other associated chronic diseases in those families. We found same type of molecular variants in close relatives and interestingly few patients from group H3 and H4 expressed all 4 analyzed RAS polymorphisms and the lowest values for PON1. This data suggest a strong association of these polymorphism and susceptibility for HTA and the fact that PON 1which is a relative cheap test can provide a starting platform for further genetic testing. We are currently analyzing other members in the families for RAS polymorphism and

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PON1 levels.

Association between the genetic polymorphisms and HTA remains controversial. However, it is an efficient method to evaluate the associations of genotype frequencies of candidate genes with diseases to understand the genetic etiology of complex human traits. Taking this into an account, in this present study we determined the possible genetic profile and biochemical features of subjects with HTA and other chronic diseases. To our knowledge, few papers have been published correlations of RAS gene polymorphisms and PON 1 levels.

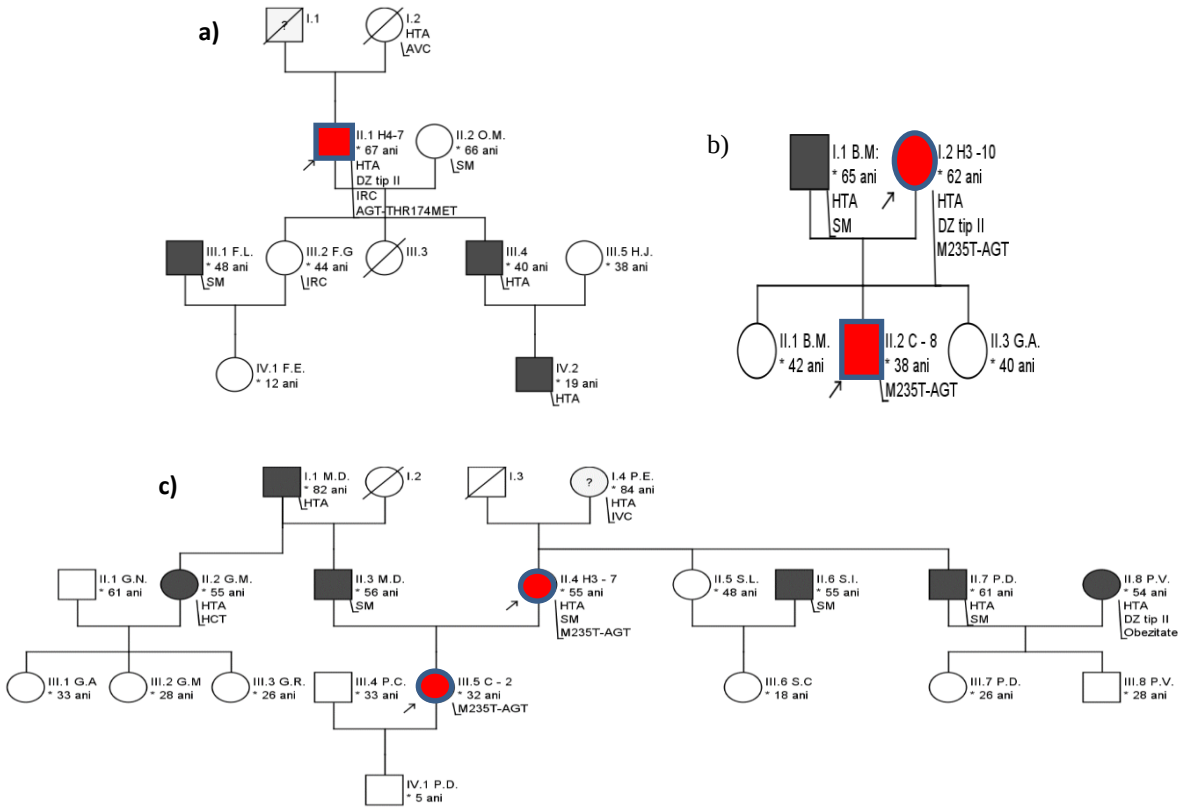

Fig.5. Pedigree charts constructed with CeGat based on anamnestic data collected from the subjects included in the study groups. a) pedigree of patient 4 from H4 group; b) pedigree of patient 10 from H3 group and C8; c) pedigree of patient 2 from control group and H3-7 patient. The red mark highlights the presence of the analyzed polymorphisms.

We can conclude that the results of this study are in agreement with previous reports, and reinforce the notion that due to the complex and heterogeneous aspects of HTA finding the responsible genetic component that will lead to a full understanding of the genetic contribution to the pathophysiology of essential hypertension is still controversial. More and intense genome analysis with accurate phenotype and personalized data may identify a panel of genes that will fish out the contribution of genetic component HTA.

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