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Angiotensinogen gene T174M polymorphism is related to Hashimoto's thyroiditis

Katarzyna Wojciechowska-Durczynska [1,2], Marta Pacholczyk [3], Arkadiusz Zygmunt [1,2], Kinga Krawczyk-Rusiecka [1,2], Tomasz Ferenc [3], Andrzej Lewinski [1,2]

1 Department of Endocrinology and Metabolic Disease, Medical University of Lodz, Poland
2 Polish Mother's Memorial Hospital – Research Institute, Lodz, Poland
3 Department of Biology and Medical Genetics, Medical University of Lodz, Poland

Correspondence to: Prof. Andrzej Lewinski
Department of Endocrinology and Metabolic Diseases, Medical University of Lodz, Polish Mother's Memorial Hospital – Research Institute, 281/289 Rzgowska Street, 93-338 Lodz, Poland
tel.: +48 (42) 271-11-41; fax: +48 (42) 271-11-40;
e-mail: andrzej.lewinski@umed.lodz.pl

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Abstract

OBJECTIVES: The Hashimoto thyroiditis is found to be Th1-related autoimmunity. Recently, it has been proved that the renin-angiotensin-aldosterone system (RAAS) may be involved in promoting Th1-mediated autoimmune diseases. However, the role of RAAS in HT pathogenesis remains still unknown. The aim of this study was to determine whether the polymorphisms of *ACE*, *AGTR1* and *AGT* genes are associated with HT.
MATERIAL AND METHODS: Polymerase Chain Reaction (PCR) was performed to determine *ACE* I/D, *AGTR1* A1166C and *AGT* T174M polymorphisms and next chi-square test was used to compare allele frequencies of genes between HT patients (n=53) and the control group (n=31).
RESULTS: TM genotype of *AGT* gene has been more often presented in HT patients (p <0.05). No others statistically significant differences were found in the distribution of I/D *ACE* and A1166C, *AGTR1* genes polymorphisms between studied groups.
CONCLUSION: Our study has examined for the first time the association of genes related to RAAS with autoimmune thyroid disease and results suggest that *AGT* TM genotype individuals might be at higher risk of HT. Although in the present study we have not found any association between increased activation of RAAS and the risk of HT, still this issue seems to be interesting and worthy further research, considering patients with thyroid cancers.

INTRODUCTION

Hashimoto's thyroiditis (HT) is the most common autoimmune thyroid disease (AITD). HT is characterized by the presence of circulating thyroid antibodies and infiltration by autoreactive lymphocytes of the thyroid gland. In HT, a Th1 response predominates, with a strong inflammatory cell infiltration, leading to the destruction of the thyroid.

The renin-angiotensin-aldosterone system (RAAS) is a major regulator of blood pressure. Angiotensinogen (AGT) undergoes two enzymatic cleavages by renin and angiotensin converting enzyme (*ACE*) to form angiotensin I (Ang I) and angiotensin II (Ang II), respectively.

ORIGINAL ARTICLE

Previous studies have demonstrated that the thyreometabolic status affects the enzymatic activity of RAAS components (Stasiolek & Lewinski, 2011). Recently, it has been also showed that the RAAS plays a role in autoimmunity. Angiotensin I has been induced in immune cells during autoimmune neuroinflammation (Platten *et al.* 2009). Moreover, the *ACE* inhibitors suppressed autoreactive Th1 and Th17 cells and promoted antigen-specific regulatory T cells (Platten *et al.* 2009). The results of studies imply that the RAAS may be involved in promoting Th1-mediated autoimmune diseases.

Genetic polymorphisms involving RAAS have been studied extensively by several investigators. The insertion/deletion (I/D) polymorphism of *ACE* gene is characterized by insertion (I) or deletion (D) of 287-bp Alu repeat sequence in intron 16 – non-coding polymorphism (Sayed-Tabatabaei *et al.* 2006). Furthermore, the angiotensin II type I receptor gene (*AGTR1*) A1166C polymorphism consists of A/C nucleotide transversion and has been located at the 1166 position in the 3'untranslated region of the *AGTR1* gene (non-coding polymorphism). In the human, three possible genotypes exist: homozygotes – AA, CC and heterozygotes - AC. Finally, the gene for angiotensinogen, AGT, is characterized by common single-nucleotide polymorphisms (SNPs) T174M – substitution of threonine (T) to methionine (M) at position 174 of the mature protein and is located in exon 2 – coding polymorphism (Mohana *et al.* 2011). T174M polymorphism has been found to be associated with angiotensinogen levels (Miller & Scholey 2004).

Specific polymorphic variants of *ACE*, *AGTR1* and *AGT* genes affect the activity of protein products of these genes and enhance RAAS activation. Nevertheless, the exact role of these polymorphisms in the thyroid autoimmune diseases pathophysiology is unknown.

The aim of this study was to analyze the polymorphism of *ACE*, *AGTR1* and *AGT* genes in patients with HT. To the best of our knowledge, no reports have been published related to the role of RAAS gene polymorphism and their interaction in AITD, thus our study is pioneering in this field.

METHODS

Peripheral blood samples from patients with HT hospitalized in Department of Endocrinology and Metabolic Disease, Polish Mother's Memorial Hospital were obtained. Inclusion criteria for HT were the presence of positive autoantibodies against thyroid antigens (anti-thyroperoxidase, anti-thyroglobulin) and negative antibodies against thyrotropin receptor. Blood samples from the patients without thyroid autoimmunity served as a control. A group of 53 patients with HT (aged 8-77 years; mean age 34.0) and controls (aged 9-86 years; mean age 50.2) were enrolled into the study (Table 1, Table 2). The study procedures were approved by the Local Ethical Committee of Medical University of Lodz and written informed consent was obtained from all participating individuals.

Genomic DNA was isolated from 200 uL of peripheral blood leucocytes with the use of DNA extraction kit (Gene JET^{TM} genomic DNA Purification Kit, Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol.

Polymerase Chain Reaction (PCR) was performed to determine *ACE* I/D polymorphism of the *ACE* gene according to the method described by Rigat et al (1990) with modification. PCR was performed with thermal cycler and thermostable Taq polymerase (Fermentas) using oligonucleotide primers that sequences were as follows: forward (sense) 5' – CTG GAG ACC ACT CCC ATC CTT TCT – 3' and reverse (antisense) 5'- GAT GTG GCC ATC ACA TTC GTC AGAT – 3'. Each DD sample was subjected to the second independent amplification with a primer pair that recognizes an insertion-specific sequence (F5' – TGG GAC CAC AGC GCC CGC CAC TAC – 3'; R5' – TCG CCA GCC CTC CCA TGC CCA TAA – 3'). PCR was performed under the same conditions except the annealing temperature.

The *AGTR1* A1166C polymorphism was determined using polymerase chain reaction restriction fragment length polymorphism method with primers: forward [sense] F 5'- GCA GCA CTT CAC TAC CAA ATG GGC-3' and reverse [antisense] R 5'-CAG GAC AAA AGC AGG CTA GGG AGA-3' (Bonnardeaux *et al.* 1994). The 255 bp PCR products were digested with the restriction enzyme BsuRI (Fermentas).

The *AGT* T174M (521*C/T*) polymorphism was investigated by PCR amplification of genomic DNA followed by restriction endonuclease digestion. The primers for PCR amplification *AGT* T174M polymorphism were: forward [sense] F 5' – TAC AGG CAA TCC TGG GTG TTC CTTG – 3' and reverse [antisense] R 5' – AGC AGA GAG GTT TGC CTT ACC TTG – 3'. PCR was performed with thermal cycler and thermostable *Taq* polymerase (Fermentas, Vilnius, Lithuania), in a final volume of 25 µl reaction mixture containing 100 ng of genomic DNA as template, 50 pM of each primer, 0.5 µM of each dNTP, 1 U of *Taq* polymerase, buffer PCR 10x and distilled water. DNA fragments were amplified for 35 cycles, cycling conditions were: denaturation at 94°C for 60 s, annealing 58°C for 40 s and extension at 72°C for 40 s after initial denaturation at 94°C for 5 min and with final 10-minute elongation. The 405 bp PCR products were digested with endonuclease *Nco*I (Fermentas, Vilnius, Lithuania) with 1 U enzyme at 37°C for 10 to 30 min (Fast Digest).

To certify genotyping quality, all polymorphisms were regenotyped in 10% randomly selected samples. The check confirmed the previous genotyping results by 100%.

Statistical analysis

Chi-square test was used to compare variables between the groups and to evaluate statistical differences of

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Tab. 1. Distribution of *ACE*, *AGTR1* and *AGT* genes genotypes in patients with HT.

Genotype distribution in patients with HT				
Age	**Gender**	***ACE* I/D**	***AGTR1* A1166C**	***AGT* T174M**
8	F	DI	AA	TT
9	F	II	AA	TM
9	F	DI	AA	TT
10	M	DD	AA	TM
12	F	DD	CC	TT
13	F	DI	AC	TM
13	F	DI	AC	TM
13	F	II	AC	TT
14	F	DD	AA	TM
14	F	DD	AC	TT
15	F	II	AA	TM
16	F	II	AA	TT
16	F	II	AC	TM
16	F	II	AA	TT
17	F	DI	AC	TT
17	F	DD	AC	TT
18	F	DI	AA	TM
18	F	DD	AC	TT
18	F	II	AA	TT
19	F	DD	AC	TT
21	F	DI	CC	TT
21	F	II	AC	TT
25	F	II	AA	TT
26	F	II	AC	TT
26	F	DI	AA	TT
28	F	DI	AC	TT
28	F	DI	AC	TT
31	F	DD	AA	TM
32	F	II	AC	TM
34	F	DI	AC	TM
34	F	II	AC	TT
36	F	II	AA	TT
37	F	DI	AA	TT
42	M	DI	AA	TT
43	F	DD	CC	TT
43	F	DD	AC	TM
49	F	II	AA	MM
53	F	DI	AC	TM
59	F	II	CC	TT
61	F	DI	AA	TT
65	F	DI	AA	TT
65	F	DD	AA	TT
66	F	II	AA	TT
66	F	DI	AC	TM
67	F	II	AA	TT
67	F	II	AA	TT
68	F	DI	AA	TT
69	F	DD	AA	TT
70	F	DI	AA	TM
75	F	II	AA	TT
77	F	DD	AC	TT
14	F	II	AC	TM
20	F	DI	AA	TM

Tab. 2. Distribution of *ACE*, *AGTR1* and *AGT* genes genotypes in controls

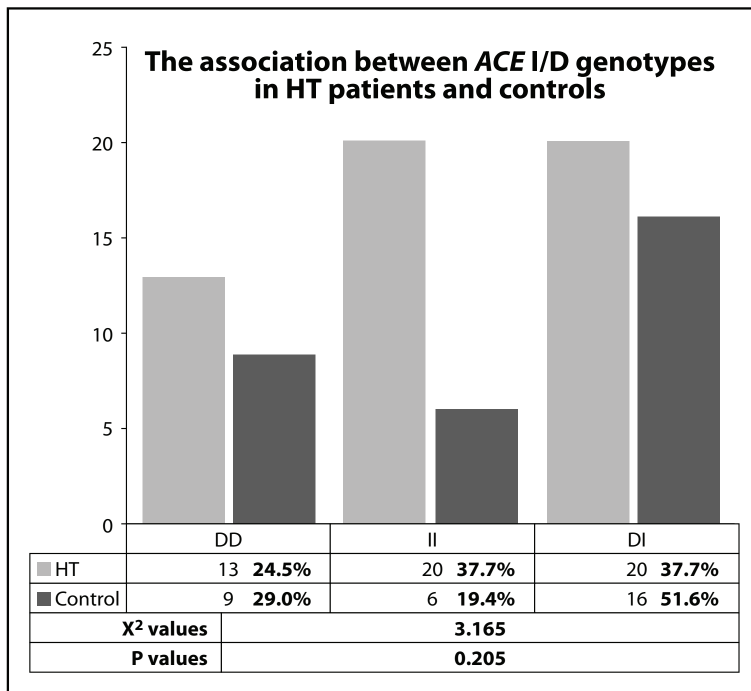
Genotype distribution in controls				
Age	**Gender**	***ACE* I/D**	***AGTR1* A1166C**	***AGT* T174M**
9	M	DI	AA	TT
9	K	DD	AC	TT
18	M	DI	AA	TT
27	K	DD	AA	TT
28	K	DI	AA	TT
30	K	DI	AA	TT
33	M	DI	AC	TT
36	K	DI	AA	TT
36	K	DI	AC	TT
45	K	DI	AA	TT
49	M	DI	AA	TM
49	K	DD	AA	TT
50	M	DD	AC	TT
51	K	II	AA	TT
51	K	II	AA	TT
52	K	DI	AA	TT
52	K	DD	AA	TT
52	K	II	AC	TT
53	K	DI	AC	TT
55	K	DD	AC	TT
58	K	II	AC	TT
58	K	DI	AA	TM
58	M	DD	AA	TT
69	K	DD	AA	TT
69	M	II	AA	TT
71	M	DD	AA	TT
71	M	II	AC	TT
74	K	DI	AC	TT
78	K	DI	AC	TT
79	K	DI	AA	TM
86	K	DI	CC	TT

genotype distributions and allele frequencies of the *ACE*, *AGTR1* and *AGT* genotypes between patients with HT and the control group; p value of 0.05 or less was considered significant.

RESULTS

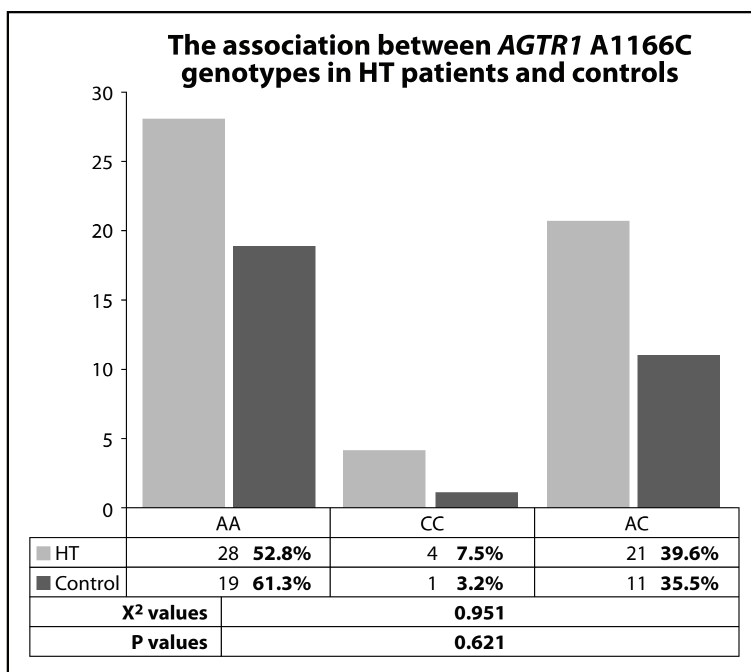
The genotype and allele frequencies of examined *ACE*, *AGTR1* and *AGT* genes polymorphisms in HT patients and healthy subjects are depicted in Table 1 and Table 2.

The frequencies of three genotypes (II, DD and ID) of *ACE* gene in patients with HT did not differ significantly from those in controls ($\chi 2$ =3.165; p =0.205) (Fig 1). The I/D polymorphism of the amplified *ACE* gene was demonstrated in agarose gels by the presence of a 490-bp fragment (insertion polymorphism – I allele) or/and of a 190-bp fragment (deletion polymorphism – D allele).



	DD		II		DI	
HT	13	**24.5%**	20	**37.7%**	20	**37.7%**
Control	9	**29.0%**	6	**19.4%**	16	**51.6%**
X^2 values	**3.165**					
P values	**0.205**					

Fig. 1. The association between *ACE* I/D genotypes in HT patients and controls.



	AA		CC		AC	
HT	28	**52.8%**	4	**7.5%**	21	**39.6%**
Control	19	**61.3%**	1	**3.2%**	11	**35.5%**
X^2 values	**0.951**					
P values	**0.621**					

Fig. 2. The association between *AGTR1* A1166C genotypes in HT patients and controls.

Furthermore, no statistically significant differences were found in the distribution of AA, AC and CC genotypes of *AGTR1* gene polymorphism between the control group and the study group of patients with HT ($\chi2$ =0.951; p =0.621) (Fig 2). The A/C polymorphism of the amplified *AGTR1* gene was demonstrated in agarose gels by the presence of a 255-bp fragment or/and of a 230-bp fragment.

Finally, our analysis has showed that the TM genotype of *AGT* gene has been more often present in HT patients in comparison to controls ($\chi2$ =6.244; p =0.04). The TT, MM genotypes frequencies were no statistically different in controls and in subjects with HT (Fig 3). The TM polymorphism of the amplified *AGT* gene was demonstrated in agarose gels by the presence of a 405-bp or/and of a 295-bp, 146-bp fragments (Fig 4).

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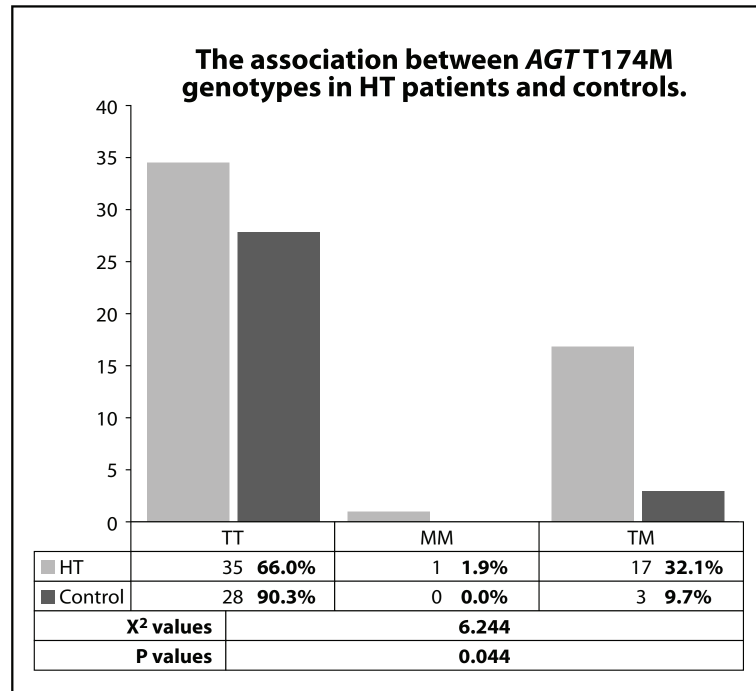

Fig. 3. The association between *AGT* T174M genotypes in HT patients and controls.

DISCUSSION

Hashimoto thyroiditis (HT) is found to be Th1-related autoimmune disease. The Th1 cells synthesize cytokines and activate cell mediated immune responses, mainly through cooperation with macrophages and lymphocytes. Furthermore, Th1 lymphocytes trigger a strong lymphocyte infiltration of the thyroid which results in subsequent thyroid destruction. Early clinical data has indicated that *ACE* inhibitor therapy is associated with decreased Th1/Th2 cytokine rations and inflammatory cytokine production (Gage *et al.* 2004). These observations have been supported by another studies, showing that AngII stimulates IFN and TNF in peripheral T lymphocytes (Guzik *et al.* 2007) and that AngII elicits a Th1 response (Mazzolai *et al.* 2004).

Moreover, epidemiological evidence supports the hypothesis that RAAS is involved in neoplasia. Suppression of the RAAS for hypertension treatment reduces cancer risk (Lever *et al.* 1998). Recent animal studies have also demonstrated that telmisartan, an *AGTR1* antagonist, significantly reduces tumour growth (Koyama *et al.* 2014). Until now, there is no evidence for increased risk of thyroid carcinoma in patients with HT, however studies in this field are still ongoing. Both diseases are inseparably associated with immune response and generally autoimmune processes and inflammation are believed to contribute to cancer development. Since immune cells also express angiotensin receptors (Gomez *et al.* 1993), angiotensin II inhibitors may also directly affect immune functions (Odaka & Mizuochi 2000) and thus regulate immune-mediated diseases. The question whether individuals with the autoimmune diseases including HT benefit more from therapeutic blockade of the renin-angiotensin system than other individuals, remains without answer.

Results of our study have demonstrated that *AGT* T174M polymorphism is related to HT. For the first time the association of RAAS genes polymorphisms with the occurrence of HT has been proved. Considering T174M polymorphism, firstly it is worth mentioning that T allel of *AGT* gene is associated with greater stimulation of *AGT* secretion in plasma (Azizi *et al.* 2000) and the *AGT* T174M polymorphism may be a susceptible predictor of the risk of ischemic stroke (Ou *et al.* 2015). We have demonstrated an increased incidence of TM genotype in HT in comparison to the controls. These results may suggest that *AGT* TM genotype individuals might be at higher risk of HT. It is difficult to compare our results with findings of other studies on the role of *AGT* T174M polymorphism in the thyroid diseases since there have been published no reports related to this topic. Nevertheless, it should be noted that earlier study conducted by Pacholczyk *et al.* (2013) also confirmed higher proportion of TM genotype among extremely obese patients and in obese patients with diabetes mellitus type 2 as compared to lean subjects (Pacholczyk *et al.* 2013). The findings of both studies may also indicate that obesity and HT could have similar genetic background.

More than 100 polymorphisms in *ACE* gene have been reported and considering I/D *ACE* gene polymorphism, DD genotype is associated with higher tissue and plasma levels of *ACE*, whereas the *ACE* II genotype

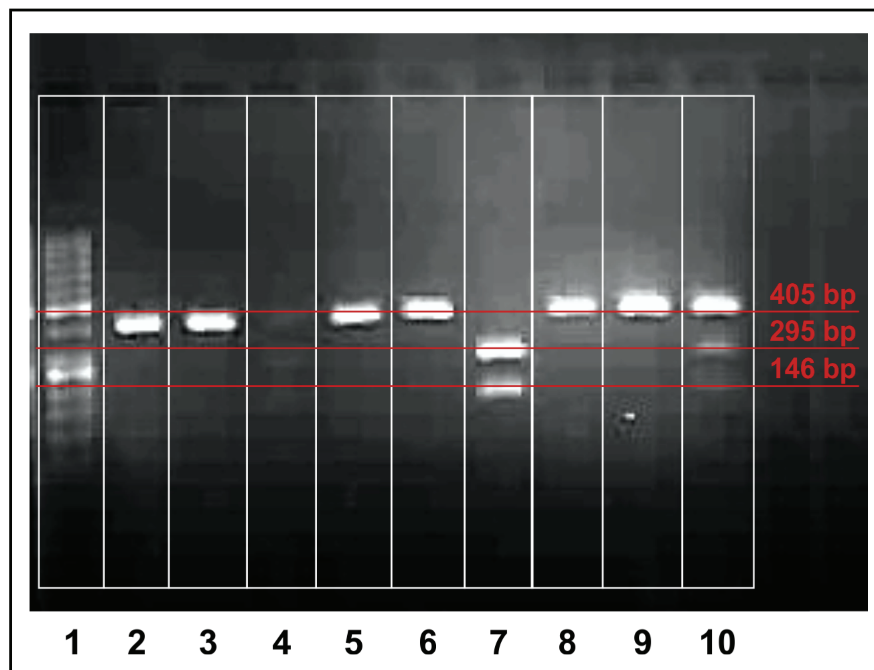

Fig. 4. PCR products from amplification of the polymorphic region of exon 2 the *AGT* gene. Lane 1 contains DNA marker; lanes 2, 3, 4 (small amount of product), 5, 6, 8, 9 contain the 405-bp product from a TT homozygote; lane 7 contains the products from MM homozygote with both 295-bp and 146-bp fragments; lane 10 contain the 405-bp, 295-bp and 146-bp products from TM heterozygote.

is associated with lower *ACE* levels and ID genotype with intermediate levels (Rigat *et al.* 1990). An association between *ACE* polymorphisms and left ventricular hypertrophy (Saeed *et al.* 2005) and resistant hypertension (Abouelfath *et al.* 2018) were suggested, however, there are hardly studies investigating influence of I/D *ACE* gene polymorphisms on immune response including autoimmunity. In one of the few studies, it has been proved that in multiple sclerosis (MS), *ACE* DD genotype and D allel is associated with susceptibility to disease, and increased *ACE* activity in the serum of patients with MS (Lovrecic *et al.* 2006). Additionally, it has been demonstrated that *ACE* DD genotype is associated with negative response to immunosuppressive therapy in MS (Ristic *et al.* 2017). It is also known that HT is frequently associated with other autoimmune disease like for example alopecia areata, which is also Th1 cytokine profile disease. In the study by Namazi *et al.* (2014), the increased serum *ACE* activity has been found in patients with alopecia areata, although the results did not reach the border of statistical significance (Namazi *et al.* 2014). On the basis of these findings, it can be assumed that RAAS activity may be increased in Th-1 related thyroiditis, by a higher incidence of D allele. Although, results of our study did not confirm that hypothesis.

The angiotensin II receptor includes two subtypes: type 1 receptor (*AGTR1*) and type 2. The *AGTR1* is a member of the G-protein-coupled receptor superfamily expressed in most tissues. *AGTR1* A1166C is implicated in etiology of various diseases including myocardial infraction. Zhang *et al.* (2013) have found the association of the presence of *AGTR1* gene C allel with myocardial infraction risk. Due to presented results, it can be presumed that *AGTR1* gene C allele probably leads to increased RAAS activity and this may be also associated with pathogenesis of HT. However present results have not confirmed the differences in incidence of CC, AC and AA genotypes of *AGTR1* gene in HT patients comparing to controls. This indicates that the *AGTR1* gene polymorphism in HT development is negligible. In accordance with our findings are the results of Beniamerian *et al.* (2017), in which the association between *AGTR1* A1166C polymorphism and the risk of systemic lupus erythematosus has not been confirmed.

To conclude, our study has examined for the first time the association of genes related to RAAS with autoimmune thyroid disease and results suggest that *AGT* TM genotype may be linked with increased risk of HT. Although in the present study we have not found any association between increased activation of RAAS and the risk of HT, still this issue seems to be interesting and worthy further research, considering patients with thyroid cancers.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the study.

AUTHORS' CONTRIBUTIONS

KWD designed and coordinated the study, writing a manuscript.
MP carried out the molecular genetic studies.
AZ performed statistics.
KKR participated in coordination of the study.
TF participated in coordination of the study.
AL senior supervision, writing a manuscript.
All authors have read and approved the final manuscript.

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