

The Angiotensinogen *AGT* p.M235T Gene Polymorphism May Be Responsible for the Development of Severe Anaphylactic Reactions to Insect Venom Allergens

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Key Words

Genetics • I/D *ACE* • Insect venom allergy • M235T *AGT*

Abstract

Background: Insect venom allergy (IVA) is present in 1–3% of the population. A group of patients with high specific IgE do not react to stings. In contrast, a proportion of patients with IVA have low specific IgE levels. These findings indicate that factors other than specific IgE may also be involved in IVA. Dysfunction of the renin-angiotensin system (RAS) has been described as a potential factor in IVA. The objective of this study was to determine the prevalence of angiotensin *AGT* p.M235T and angiotensin-converting enzyme *ACE* I/D, I/I, D/D gene polymorphisms in patients with IVA and to relate the presence of these gene variants to the course of IVA and the safety of treatment. **Methods:** A total of 107 patients with IVA and 113 controls were studied. *AGT* p.M235T and *ACE* (I/D, I/I, D/D) gene polymorphisms were examined, and angiotensin I levels were measured by immunoassay. **Results:** The frequency of the *AGT* MM M235T variant was significantly higher in IVA patients (29.9%) than in controls (17%, $p = 0.02$). The presence of the MM M235T genotype increased the risk of grade IV reactions (odds ratio = 2.5 and 95% confidence interval 1.04–6.08). There were no differences in the

prevalence of the *ACE* I/D polymorphism and angiotensin I levels between control groups and patients with different grades of anaphylactic reactions or patients with side effects of venom immunotherapy. **Conclusion:** The *AGT* M235T MM variant may be responsible for severe anaphylactic reactions to insect venom allergens in some patients.

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Introduction

Insect venom allergy (IVA), defined as at least one systemic IgE-mediated reaction (during an entire lifetime) following an insect sting, is present in approximately 1–3% of the population [1]. It is estimated that approximately 2.4% of the patients with IVA have experienced at least one life-threatening reaction graded as III or IV on the Mueller scale during their lifetime [1–5]. The treatment of choice for IVA patients is venom immunotherapy (VIT) [2, 3].

A large group of patients with high concentrations of specific IgE do not react to stings, whereas a proportion of patients with severe reactions have low specific IgE levels [1–4]. Consequently, factors other than the serum IgE level may also contribute to the allergic reaction to insect

venom. In previous studies, dysfunction of the renin-angiotensin system (RAS), for example, resulted in stronger allergic responses to Hymenoptera venom [6–11].

Angiotensin II belongs to a family of agents exerting strong vasoconstrictive effects [12]. The level of angiotensin II is regulated by the level of its precursors (angiotensinogen and angiotensin I), the activity of the enzyme converting angiotensin I into angiotensin II, and angiotensin II receptor activity [12].

Angiotensinogen is an inactive plasma protein produced by hepatocytes. Renin, an enzyme released by granular juxtaglomerular cells of the kidney, cleaves angiotensinogen into angiotensin I, while the angiotensin-converting enzyme (ACE) cleaves angiotensin I into angiotensin II, the main RAS effector [12].

In most cases, a Hymenoptera sting leads to a local reaction characterized by redness, swelling and local edema [1–4]. Constriction of the vessels mediated by angiotensin may prevent a generalized reaction. Furthermore, many patients show symptoms of arrhythmia and/or hypotension, which may be related to RAS activation [6–11]. Results of the study by Hermann and Ring [6–10] and Hermann et al. [11] showed that patients who experienced anaphylaxis to insect venom had significantly lower levels of angiotensin I, angiotensin II, renin and angiotensinogen, i.e. molecules involved in RAS, in comparison to non-allergic controls. Patients who repeatedly experienced anaphylactic reactions during VIT had significantly lower levels of these molecules compared with patients without side effects of VIT [6–11]. Interestingly, patients with good VIT tolerance had RAS molecules within normal ranges [6–11]. The low levels of these molecules also correlated with the severity of clinical symptoms, whereas no association with serum concentrations of aldosterone was observed [7]. Significant differences in RAS molecule levels were found in patients that reacted positively in a provocation test to a living insect. They had lower levels of angiotensinogen and angiotensin I/II, whereas in patients with a negative reaction, who may be regarded as successfully treated, the levels of angiotensinogen were even lower than in healthy subjects [6].

The *AGT* gene, encoding for angiotensinogen, is located on the long arm of chromosome 1 (1q42). Enzymatic activity is related to polymorphic variants (methionine → threonine) at codon 235, where a thymine is substituted by a cytosine [12–14]. The MM genotype of *AGT* is related to low levels of angiotensinogen [12–15].

The *ACE* gene is located on the long arm of chromosome 17, and several polymorphisms of this gene have been found so far. An insertion (I)/deletion (D) polymor-

phism of a 287-bp fragment within intron 16 was recently identified. The presence of the D allele is related to a higher plasma ACE concentration and cardiovascular diseases [12–18].

Both *AGT* and *ACE* gene polymorphisms are associated with cardiovascular disorders [12–18]. However, little is known about the association of the *ACE* and *AGT* gene variants to allergic diseases. Studies by Benessiano et al. [19] and Holla et al. [20] demonstrated a relationship between the DD genotype of the *ACE* gene and the prevalence of asthma in a Caucasian population, whereas the M235 allele of the *AGT* gene was more frequent among patients with asthma, allergic rhinitis and atopic dermatitis [19, 20]. Interestingly, such a relationship was absent in Korean populations [21–23]. ACE also inactivates kinins, substance P, bradykinin and prostaglandins (molecules involved in the pathogenesis of asthma) [23]. Decreased ACE activity was noted in patients with aspirin-intolerant asthma, which leads to bronchial hyperactivity and eosinophilic inflammation [23]. Although the results might explain differences in the concentration of molecules involved in RAS, no studies have defined an association between gene polymorphisms and IVA patients. It is likely that a pharmacogenetic approach to the diagnosis of IVA might predict the incidence of anaphylactic reactions during VIT and the efficacy of treatment.

The aim of this study was to determine the prevalence rates of *AGT* (p.M235T) and *ACE* (I/D, I/I and D/D) gene polymorphisms in patients with IVA and to relate the presence of particular gene variants to the course of the disease (severity of anaphylactic reactions prior to treatment) and the safety of treatment.

Patients and Methods

Patients

A total of 107 consecutive patients with Hymenoptera allergy treated at the Department of Allergology, Medical University of Gdansk, were studied. Their mean age was 41 years (range 18–75). There were 59 (55%) women (mean age 41 years; range 18–75) and 48 (45%) men (mean age 40 years; range 18–73). IVA was diagnosed according to the guidelines of the European Academy of Allergy and Clinical Immunology [1].

The control group consisted of 113 healthy blood donors recruited from the Regional Center for Blood Donation in Gdansk. There were 65 (58%) men and 48 (42%) women (mean age 41 years; range 21–74). The age and gender differences were not significant, and the genes in the current study were not dependent on the X chromosome.

Clinical data on age, sex, personal and family history of IVA, previous hospitalizations, home and work characteristics, environmental exposures and physical examination were obtained af-

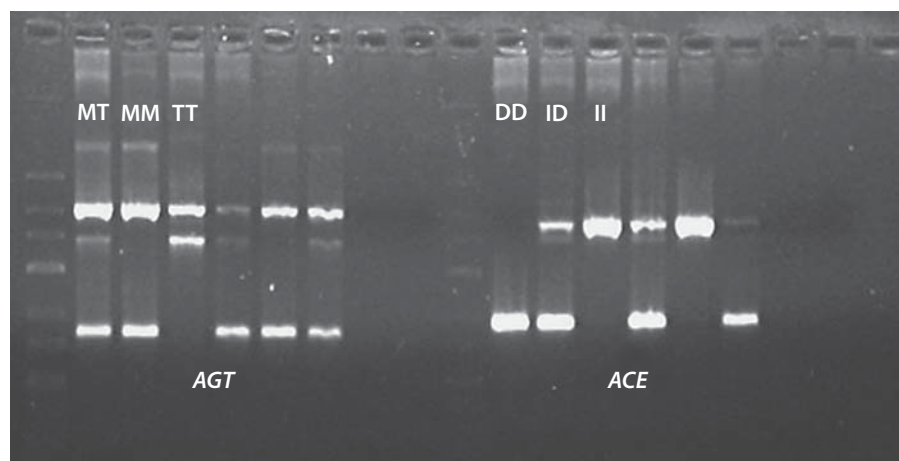


Fig. 1. AGT 235 MT and ACE I/D gene polymorphism analysis in IVA patients.

ter written informed consent. Exclusion criteria included malignancy, tuberculosis, pregnancy, and heart, renal or liver failure. None of the patients reported treatment with ACE inhibitors or β -blockers.

The study was approved by the Ethical Committee of the Medical University of Gdansk (NKEBN/811/2004).

Skin Test

Skin prick tests (SPTs; concentrations of 10 and 100 $\mu\text{g/ml}$) and intracutaneous tests (concentrations of 0.01 and 0.1 $\mu\text{g/ml}$) with allergens of yellow jacket and bee (HAL Allergy, Haarlem, The Netherlands) were carried out according to the recommendations of the European Academy of Allergy and Clinical Immunology [1]. Positive SPTs were defined as a mean wheal diameter 3 mm larger than that of the negative control. Intracutaneous tests were regarded positive when the mean wheal diameter was 5 mm larger than that of the negative control. Glycerol-buffered saline was the negative control, and histamine (1 mg/ml) was the positive control. Allergopharma lancets and 0.7-mm needles were used for SPT and intracutaneous tests, respectively.

Specific IgE

Specific IgE levels were measured with UniCAP (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. Results $\geq$ class I (IgE level of 0.35 kU/l) were considered positive.

Immunotherapy

All patients with IVA (either grade III or IV reactions according to the Mueller [5] scale) were treated with insect venom allergens. Bee allergens (Venomenhal Biene, HAL Allergy) were used in 26 (24%) cases, and yellow jacket allergens (Venomenhal Wespe, HAL Allergy) were used in 81 cases (76%). The initial phase was performed with the rush procedure according to the manufacturer's instructions. Maintenance doses (100 μg) were administered every 4–6 weeks.

Collection of Blood Samples

For genetic analysis, peripheral blood samples (10 ml) were collected into EDTA-containing tubes and stored at -80°C . Genomic DNA was isolated from peripheral blood leukocytes using Blood DNA Prep Plus according to the manufacturer's instruc-

tions (A&A Biotechnology, Gdynia, Poland). DNA concentration was assessed using the Beckman DU-600 spectrophotometer. Patient sera were collected and stored at -20°C . All samples were taken when patients were on the maintenance doses of VIT.

AGT M235T (rs699) Gene Polymorphism Analysis

To detect AGT (p.M235T) gene polymorphisms, allele-specific oligonucleotide polymerase chain reaction (PCR) was used [20–21]. In order to confirm the results of the analysis, every 10th sample was sequenced using the ABI PRISM 310 genetic analyzer. More information on the protocols used, including the choice of primers, can be obtained from the corresponding author upon request.

ACE I/D, I/I, D/D (rs1799752) Gene Polymorphism Analysis

To detect ACE (ID, I/I and D/D) gene polymorphisms, PCR was applied [13]. Due to the possibility of misclassification of ID as DD, all DD homozygotes were reanalyzed with primers specific for the insert allele (fig. 1). In order to confirm the results of the analysis, every 10th sample was sequenced using the ABI PRISM 310 genetic analyzer. More information on the protocols used, including the choice of primers, can be obtained from the corresponding author upon request.

Measurement of Serum Angiotensin I Levels

Serum angiotensin I levels were measured using a commercial ELISA (Phoenix Pharmaceuticals, Burlingame, Calif., USA). The samples were taken during the maintenance phase of VIT, and the 3rd or 4th year of the treatment. The assay was performed according to the manufacturer's instructions. The detection rate of the kit ranges from 0 to 25 ng/ml.

Statistical Analysis

Results were expressed as a percentage of the patients, while differences in percentages between groups were assessed by χ^2 test. Data on continuous variables (angiotensinogen level) were presented as medians and standard deviations. The differences in angiotensinogen levels between groups were analyzed using the Mann-Whitney U test. Hardy-Weinberg equilibrium was tested by χ^2 analysis using Tufts University calculator. Statistica 8.0 PL (StatSoft, Tulsa, Okla., USA) software was applied.

Table 1. Clinical data of the patients and controls

	IVA patients (n = 107)	Controls (n = 106)
Age, years (range)	41 (18–75)	41 (21–74)
Males/females, %	45/55	58/42
Yellow jacket/bee allergy, %	75/25	
Mueller class III/IV, %	47/53	
Hypertension, n (%)	34 (36)	
Hypercholesterolemia, n (%)	6 (6)	
Diabetes, n (%)	7 (6)	
Thyroid diseases, n (%)	13 (12)	
Coronary artery disease, n (%)	5 (5)	
Asthma, n (%)	18 (16)	
Allergic rhinitis, n (%)	22 (20)	
Chronic obstructive pulmonary disease, n (%)	3 (3)	
Mastocytosis, n (%)	4 (4)	

Results

Insect Venom Allergy

VIT was administered to 50 (47%) patients who experienced grade III anaphylactic reactions to insect venom and to 57 (53%) patients with grade IV reactions. During the rush phase of VIT, systemic reaction occurred in 11 (10%) cases. In 8 cases, the adverse reaction was graded as life-threatening (III or IV according to the Mueller [5] scale). Coexisting diseases diagnosed are described in table 1. All patients with mastocytosis suffered from grade IV anaphylactic reactions before treatment, other comorbidities did not relate to the severity of the preceding reaction. No relationship was found between co-morbidities present in the study group and the prevalence of side effects. Re-stings were reported in ~25% of the patients during VIT, but did not result in a systemic reaction.

High Frequency of the AGT M235T MM Polymorphism in Patients with Grade IV Anaphylactic Reactions

The AGT gene polymorphism, in both the study and the control groups, did not exhibit significant deviation from Hardy-Weinberg expectations. The frequency of the MM 235 AGT variant was significantly higher ($p = 0.02$) in patients with IVA ($n = 32$; 29.9%) in comparison with controls ($n = 19$; 17%). This genotype was significantly more prevalent ($p = 0.03$) in patients with grade IV anaphylactic reactions prior to treatment ($n = 22$; 39%) compared with subjects with III grade reactions ($n = 10$; 20%). The difference was significant for patients with grade IV

Table 2. AGT p.M235T and ACE I/D polymorphisms in the IVA patients and in the control group

	MM II	MT ID	TT DD	HWE p value
<i>Control group</i>				
MT235 AGT	19 (17%)*	57 (51%)	37 (32%)	0.70
I/D ACE	29 (25%)	64 (57%)	20 (18%)	0.13
<i>IVA patients</i>				
<i>All</i>				
MT235 AGT	32 (30%)*	52 (48%)	23 (22%)	0.82
I/D ACE	23 (21%)	51 (48%)	33 (31%)	0.69
<i>Grade III anaphylactic reactions</i>				
MT235 AGT	10 (20%)**	25 (50%)	15 (30%)	0.94
I/D ACE	11 (22%)	26 (52%)	13 (26%)	0.76
<i>Grade IV anaphylactic reactions</i>				
MT235 AGT	22 (39%)**	27 (47%)	8 (14%)	0.95
I/D ACE	12 (21%)	25 (44%)	20 (35%)	0.42

The frequency of the MM235 AGT genotype was significantly higher in the patients (30%) than in the controls (30%; * $p = 0.02$) and in the IVA patients with grade IV (39%) vs. grade III anaphylactic reactions (20%; ** $p = 0.03$). The presence of the MM genotype increased the risk of grade IV reaction (odds ratio: 2.5; 95% confidence interval: 1.04–6.08). HWE = Hardy-Weinberg equilibrium.

reactions vs. controls ($p = 0.001$) but not for patients with grade III reactions. Presence of the MM variant increased the risk of grade IV reactions (odds ratio: 2.5; 95% confidence interval: 1.04–6.08; table 2). No differences were found in the presence of the MM variant between patients with and without side effects during the rush phase of immunotherapy.

ACE I/D, D/D and I/I Polymorphisms

In the study and control groups, the ACE gene polymorphism did not exhibit significant deviation from Hardy-Weinberg expectations. The prevalence of the genotypes studied did not differ between the control group and patients with different grades of anaphylactic reactions or with patients that experienced side effects during immunotherapy (table 2).

Angiotensin I Level

The angiotensin I level was measured in 37 patients with IVA (35% of the study group) without coexisting diseases influencing the level of angiotensin I. The serum level of angiotensin in patients possessing the MM genotype of the AGT (1.04 ± 0.7 ng/ml) gene was not signifi-

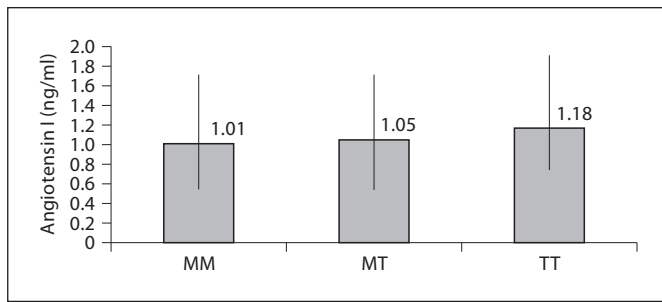


Fig. 2. Angiotensin I levels (means $\pm$ SD) in patients with different genotypes of the M235T *AGT* gene. The differences were not significant.

cantly lower than in patients having either the MT or TT genotype (1.16 ± 0.61 ng/ml; fig. 2). Patients with grade IV reactions also had a lower level of angiotensin (1.0 ± 0.6 ng/ml) in comparison to patients with grade III (1.12 ± 0.6 ng/ml), although not significantly different ($p = 0.1$). The *ACE* I/D polymorphism was also not related to the level of angiotensin I. An association between the level of angiotensin I and co-morbidities, including mastocytosis, was not detected.

Discussion

The results of this study confirmed the relevant role of RAS in IVA. The presence of the *AGT* p.M235T MM variant was found to be more prevalent in patients with IVA, particularly in those who suffered from the most severe (grade IV) reaction. No differences were found in the prevalence of the *ACE* I/D polymorphism and IVA. Significant differences between the serum level of angiotensin I and either polymorphic variants of the *ACE* gene or the grade of anaphylactic reactions were absent.

In the present study, the frequency of the *AGT* gene MM235 variant, which is related to lower angiotensinogen levels, was significantly higher in IVA patients (30%) compared with control subjects (17%). Levels of angiotensinogen and its metabolites, angiotensin I and angiotensin II, were reduced in IVA and in IVA patients with repeated anaphylactic reactions during Hymenoptera venom hyposensitization and sting challenge [6–11]. These findings might be partially explained by a trend to decreased angiotensin levels in carriers of the MM variant. Indeed, an inverse correlation of angiotensinogen and angiotensin I and II with the severity of anaphylaxis was reported [6–11].

The presence of the MM235 variant (39% of patients with grade IV reactions) was highly associated with an increased risk of grade IV reactions (odds ratio = 2.5; 95% confidence interval 1.04–6.08) in this study. Additionally, angiotensin I was decreased in patients with grade IV reactions. Our results suggest that the observed differences may have a genetic background. In the present study, plasma levels of angiotensin I were measured during the maintenance phase of VIT, thus this may explain why differences between the subgroups of patients with grades III and IV anaphylactic reactions were not significant. However, the lack of significance may also be due to the low number of subjects included in the analysis. Previous studies showed that successful VIT induced a 9-fold increase in angiotensin I and II levels [6, 10].

The association of the angiotensinogen gene p.M235T polymorphism was found in patients with asthma and atopic diseases (allergic rhinitis and atopic dermatitis) [20]. Similar to our study, the MM variant in association with the lowest angiotensin levels was more prevalent in patients with atopic disease ($p = 0.02$) [20]. This finding was not confirmed in Korean patients with allergic rhinitis [21, 22]. The differences in the frequencies of gene polymorphisms found in the above studies [20–22] may be related to different patient groups. Angiotensinogen is produced by hepatocytes and other cells, providing a constant level of the substrate for RAS [24]. Transcription of angiotensinogen might also be induced by acute-phase mediators, such as IL-1 α and TNF- α [20, 24]. Systemic inflammation, which affects acute-phase proteins, plays a more important role in asthma and atopic dermatitis than in IVA. This may explain the relevance of the *AGT* M235T gene variant in those patient groups.

The frequency of the *ACE* I/D polymorphism did not differ in the groups studied. ACE is a membrane-bound enzyme found in epithelial and endothelial cells of blood vessels, lungs and other organs [13, 15, 25]. The functions of the ACE enzyme (the cleavage of angiotensin I and inactivation of active substances like kinins, substance P and prostaglandins) are important in the pathology of allergic diseases. The role of the *ACE* I/D polymorphism in atopic diseases [20] and aspirin-intolerant asthma [23] was discussed previously. In contrast, no difference in the frequency of this polymorphism was found in the IVA patients studied, confirming the observation that there is no difference in plasma ACE activity between patients with IVA allergy and controls [6–8]. These results suggest that a low level of angiotensinogen is the most important factor responsible for the diminished function of the RAS pathway and severe insect venom anaphylaxis [6–11].

In IVA patients, treatment of hypertension with ACE inhibitors has been the subject of previous discussions [25–27]. A review of papers published from 1966 to 2006 suggested that ACE inhibitors may exacerbate the response to insect venom, resulting in potentially life-threatening allergic reactions to insect stings or VIT [25]. Consequently, avoidance of ACE inhibitors has been advocated in IVA patients [25, 27]. On the other hand, this group of drugs has proven protective effects in cardiovascular diseases and diabetes [12–14]. In a recently published retrospective analysis, these drugs were not found to be associated with an increased frequency of side effects to IVA [26]. The results of the present study indicate that 39% of the IVA patients possess the M235T polymorphism, which is related to the low level of angiotensinogen. No difference was evidenced between the prevalence of side effects of VIT and the polymorphisms studied. However, such a correlation cannot be excluded. Further studies are required to determine the risk-benefit ratio of ACE inhibitors in IVA patients.

IVA is classified as an IgE-mediated non-atopic reaction. The most important pathway involved is IgE-mediated degranulation of mast cells associated with previous sensitization and a shift in the T-regulatory/T-helper 2 cell balance [28]. Alternative mechanisms of systemic reaction were studied, including complement activation, calcitonin-gene-related peptide overproduction, osteopontine pathway involvement and diminished RAS function [6–11, 29–32]. In the present study, coexisting atopic diseases were found in 31% of patients. This result is similar to the prevalence of allergic diseases in the general

population [4]. This clinical observation may lead to the hypothesis that genes involved in insect venom anaphylaxis may be responsible for factors different from those involved in allergic reactions graded according to the Gell-Coombs classification.

In summary, pharmacogenetics is one of the most promising fields of allergy management. There is hope that, in the future, diagnosis based on genetic assessment may facilitate tailoring of therapy for particular patients.

Conclusion

The AGT MM235 gene polymorphism may be responsible for severe anaphylactic reactions to insect venom allergens and should probably be considered in the diagnosis of IVA. It is likely that a pharmacogenetic approach to the diagnosis of IVA may lead to safer and more effective treatment options.

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