Original Article

Mistyping of Angiotensinogen M235T Alleles

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Conflicting results are to be found in the literature on the relationship between the M235T polymorphism of the angiotensinogen (AGT) gene and hypertension. The controversy may be due to insufficient numbers of subjects, the variability of the inclusion criteria and the different genotype analysis methods used. We have experienced that the most frequently used, original polymerase chain reaction (PCR)–restriction fragment length polymorphism (RFLP) method involves significant uncertainties when the TT genotype is determined, independently of the restriction digestion. To make the determination more accurate, we improved the PCR by designing a new antisense primer containing only one mismatch instead of the two in the original protocol and also by adding DMSO to the PCR reaction mixture. The original and our improved methods were compared by using DNA from 123 patients: parallel determinations resulted in values of 33 MM, 90 MT and 0 TT with the original method and of 33 MM, 56 MT and 34 TT with the improved RFLP protocol. In summary, a plausible explanation for some of the conflicting data published on AGT M235T polymorphism may be that inaccuracies arose during the determination of the genotype. (*Hypertens Res* 2006; 29: 197–201)

***Key Words*: angiotensinogen, M235T polymorphism, polymerase chain reaction, restriction fragment length polymorphism, hypertension**

Introduction

Hypertension is a complex disease in which genetic and environmental factors contribute interactively. Molecular genetic studies have achieved noteworthy success in the identification of single nucleotide polymorphisms (SNPs). Among the SNPs affecting blood pressure, the renin-angiotensin system (RAS) is the best characterized. The RAS is involved in the control of blood pressure and in fluid and electrolyte homeostasis. The most important elements of the RAS are renin, angiotensinogen (AGT), angiotensin I (Ang I), angiotensin converting enzyme (ACE), angiotensin II (Ang II) and the angiotensin II receptors (type 1 [AT1R] and type 2 [AT2R]). Numerous SNPs have been identified in these components of the RAS, *e.g.*, AGT M235T, ACE I/D and AT1R A1166C. These polymorphisms identified in the RAS genes may be involved in the genetic predisposition to hypertension, nephropathy, myocardial infarction and left ventricular hypertrophy (*1*).

As regards the AGT gene, more than 20 molecular variants have been identified, with the replacement of methionine by threonine at position 235 in the amino acid sequence being of particular interest. The functional consequences of this polymorphism have been investigated in several ethnic groups and the results have given rise to considerable controversy. In short, about one-third of the published papers suggest a significant correlation between the 235T allele and hypertension, whereas the remainder indicate no correlation. The evaluation of 69 case-control reports pointed to a publication bias for hypertension based on a gap in the funnel plot (*2*).

One reason for the uncertainty may be that at least four different methods have been used to determine the M235T genotype, *i.e.*, 1) allele-specific oligonucleotide hybridization (PCR-ASO), 2) mutagenically separated polymerase chain reaction (MS-PCR) applying three primers to amplify all pos-

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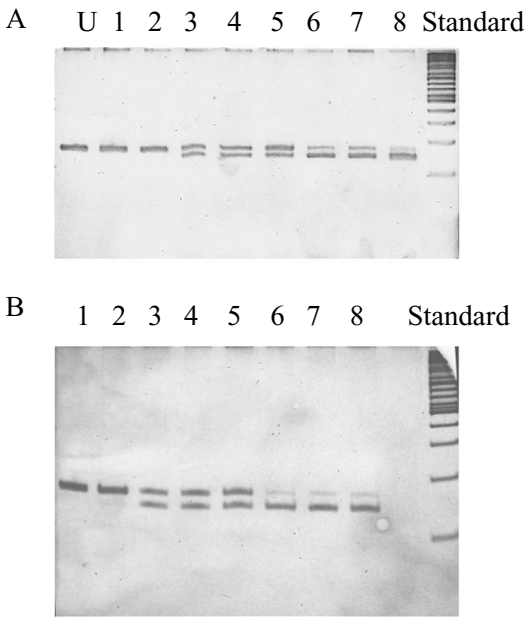

Fig. 1. *Optimization of PCR reaction. PCR was performed at 59°C (A) and 68°C (B), using DNA samples from the patients (U: uncleaved PCR control; 1–8: patient numbers; Standard: 100 bp DNA ladder).*

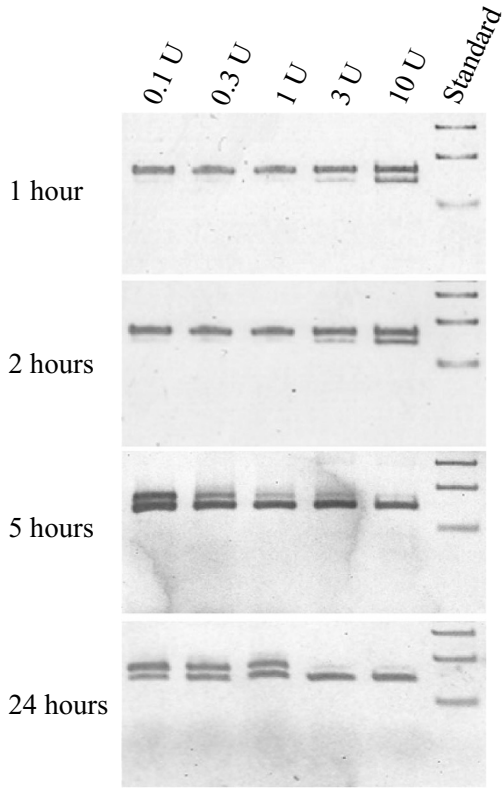

Fig. 2. *Optimization of restriction digestion. The PCR product of a patient with the TT genotype (patient No. 8) was digested under different conditions (incubation time, 1–24 h; restriction enzyme activity, 0.1–10 U).*

sible alleles in the polymerase chain reaction (PCR), 3) single strand conformation polymorphism analysis and 4) PCR–restriction fragment length polymorphism (PCR-RFLP).

In the present report we provide evidence that the most frequently used PCR-RFLP method can lead to an overestimation of the M allele. To achieve more reliable genotyping, we have developed an improved PCR-RFLP method which could be performed with the same equipment and by the same personnel.

Methods

Genomic DNA was extracted from the peripheral blood leukocytes of patients with clinically suspected ischemic heart disease by using a commercially available kit (FlexiGene DNA Kit; Qiagen, Hilden, Germany). The AGT gene M235T polymorphism was subjected to genotyping by utilizing a previously described (*3*) PCR-RFLP–based technique.

First, the original protocol (*3*) was applied to our assay conditions. The sense primer was 5′-CCGTTTGTGCAGGGC CTGGCTCTCT-3′ and the antisense primer was 5′-CA GGGTGCTGTCCACACTGGACCCC-3′ (Sigma-Genosys, Steinheim, Germany). Template DNA (50 ng) was amplified in a final volume of 25 µl containing 1 µmol/l of each primer, 200 µmol/l dNTP, 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3) and 1 U Taq DNA polymerase (Sigma-Aldrich, Schnelldorf, Germany). After initial denaturation at 94°C for 5 min, amplification was performed for 30 cycles of 94°C for 20 s, 59°C for 20 s and 72°C for 40 s with a final extension of 72°C for 5 min in a Rapid Cycler (Idaho Technology, Salt Lake City, UT; Figs. 1A and 2). Alternatively, PCR was performed under the same conditions except that 68°C was used as the annealing temperature (Fig. 1B). The PCR fragments were cleaved with either the restriction enzyme Tth 111I (New England Biolabs, Beverly, USA) or Psy I (Fermentas, Vilnius, Lithuania).

Secondly, an improved PCR-RFLP protocol was used (Fig. 3). In these experiments, the reaction conditions were: 50 ng genomic DNA, 1 µmol/l of each primer, 200 µmol/l dNTP, 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 10% DMSO and 1 U Taq DNA polymerase in a final volume of 25 µl. The sense primer was 5′-CCGTTTGTG CAGGGCCTGGCTCTCT-3′ (similarly to the original protocol detailed above) and the antisense primer was 5′-GCCA GGGTGCTGTCCACACTGACTCCC-3′ (Sigma-Genosys, Steinheim, Germany). After initial denaturation at 94°C for 5 min, amplification was performed for 30 cycles of 94°C for 20 s, 64°C for 20 s, and 72°C for 40 s with a final extension at 72°C for 5 min. The PCR products were subjected to Box I (Fermentas) digestions with 1 U enzyme at 37°C for 2 h.

The end-products of PCR-RFLP analysis were separated on

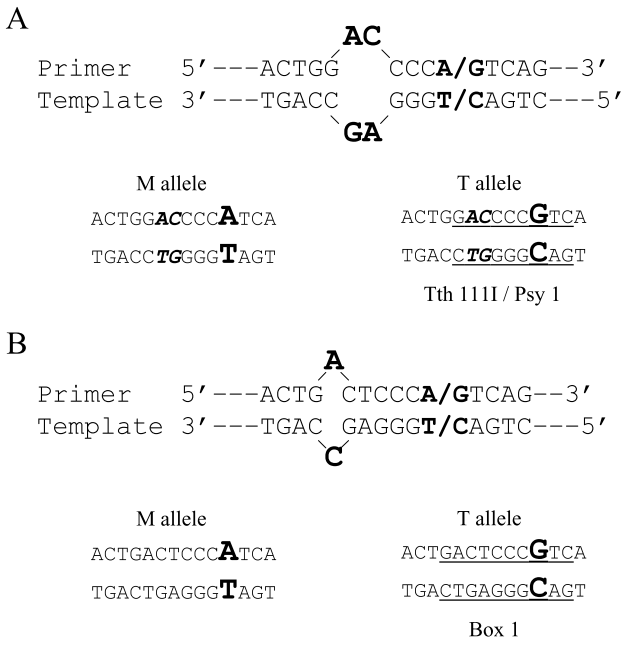

Fig. 3. *Schematic representation of the procedures. The original (A) and the modified protocol (B) are shown. Differences, including the sequence of the primer template and the generated cleavage sites are also shown.*

Table 1. Summary of Genotypization*

	MM	MT	TT
Method 1	33	90	0
Method 2	33	56	34

*One hundred and twenty-three DNA samples were analyzed with the original protocol (method 1) or with the modified protocol (method 2). The numbers of cases with MM, MT and TT genotypes are shown.

10% polyacrylamide gels and visualized by ethidium bromide staining. The undigested fragment represented the M allele, while for the T allele cleavage should occur with disappearance of the undigested fragment.

This study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association and was approved by the Institutional Ethical Committee. All patients gave their informed consent to the laboratory procedures.

Results

We sought to set up a method to determine AGT M235T polymorphism in our laboratory. On the basis of the method developed by Russ *et al*. (*3*), we first optimized the PCR reaction in our system and found that 59°C is optimal for hybridization. The PCR reaction was followed by restriction

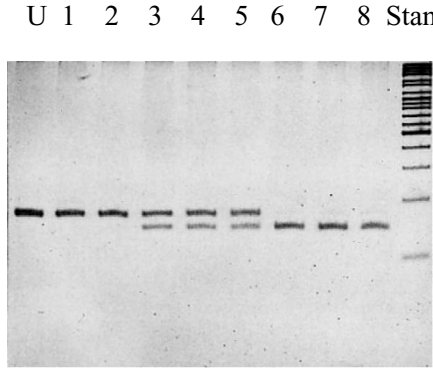

Fig. 4. *Results with the new method. PCR-RFLP analysis was performed with the developed procedure using DNA samples from the patients (U: uncleaved PCR control; 1–8: patient numbers; Standard: 100 bp DNA ladder).*

digestion with the enzyme Tth 111I to determine AGT M235T polymorphism (Fig. 1A).

Surprisingly, we found no TT alleles in the 123 patients evaluated with this method (Table 1, method 1). The allele frequencies of M and T were calculated to be 0.634 and 0.366, respectively. Therefore, the genotype of TT should be $0.366 \times 0.366 \times 123 = 16.48$, if the population remained in Hardy-Weinberg equilibrium. The statistical evaluation of the data revealed that the determined allele frequencies do not represent a population in Hardy-Weinberg equilibrium (significant deviation from the expected values, $p < 0.01$, as determined by χ^2 test). This suggested possible technical problems with the PCR amplification or with the restriction digestion, in view of the allele frequency previously reported (*3*). Indeed, we determined the genotype of the patient samples shown in Fig. 1A and found that patients 6–8 represented the TT genotype and were therefore mistyped. To eliminate various problems that may have led to the mistyping, we first used a higher hybridization temperature to ensure the specificity of the PCR reaction (Fig. 1B). This change resulted in a better efficiency of restriction digestion, but complete cleavage of the AGT fragments was still not obtained under these conditions. We next replaced the restriction enzyme Tth 111I with Psy 1 to investigate a postulated restriction enzyme–dependent effect on the digestion; again, we did not observe complete digestion (data not shown). To investigate the kinetics and the efficiency of the digestion we tested enzyme activities 0.1–10 U for 1–24 h (Fig. 2), using the PCR product of a patient (patient number 8) with the TT genotype (determined by direct sequencing). Here we expected complete digestion of the full length fragment. In fact, a clear tendency toward more complete digestion was observed, in parallel with longer time and higher enzyme activities. However, complete digestion was not achieved.

These results suggested uncertainties in the detection of the AGT M235T genotype. Hence, we made an effort to improve

the accuracy of the PCR-RFLP method. Unfortunately, no restriction enzyme is known that is specific for the AGT sequence in the region responsible for the M235T polymorphism, but the restriction site for the Box 1 enzyme required the insertion of only one base during the PCR reaction. We therefore designed a new primer in which only one mismatch is sufficient to generate a restriction site for Box 1 (Fig. 3).

After optimization of the PCR reaction with the new primer set, we tested the sensitivity of the fragments to Box 1 digestion and found complete cleavage for the TT genotype (Fig. 4). To confirm the identity of the products, direct sequencing was performed and revealed that indeed, patients 1 and 2 had the MM, patients 3–5 had the MT and patients 6–8 had the TT genotype. Re-testing of DNA of the 123 patients with the improved method revealed 33 MM, 56 MT and 34 TT genotypes, in contrast with the original protocol, where no TT genotype was detected (Table 1, method 1 [original] and method 2 [improved]). The statistical analysis of the data obtained by the improved protocol (Table 1, method 2) suggested that the allele frequencies for M and T alleles are 0.4959 and 0.5040, respectively, and confirmed that these alleles represent a population in Hardy-Weinberg equilibrium (no significant deviation from the expected values, $p>0.5$ as determined by χ^2 test), in contrast with the data of the original protocol.

Discussion

The various forms of treatment with antihypertensive drugs that have been developed to date have generally resulted in satisfactory therapeutic effects, but not in all of the patients. This has fueled efforts to characterize the molecular genetics of hypertension.

The very first gene that was found to be associated with human hypertension is the AGT gene. In their seminal paper, Jeunemaitre *et al*. (*4*) suggested that threonine at position 235 in the amino acid sequence (methionine to threonine polymorphism, M235T) is associated with human essential hypertension. To date their work has received more than 1,000 citations, indicating the interest in and debate on this original finding. The association of the AGT M235T polymorphism was reviewed in three large meta-analyses (*2*, *5*, *6*). These and other analyses demonstrated that the AGT 235T polymorphism is indeed, albeit weakly, associated with an increased risk of hypertension in Caucasians and possibly in Japanese, but probably not in Africans and Asians. However, certain of the data published remain controversial. Our MEDLINE search for the AGT M235T polymorphism in association with cardiovascular disorders revealed 3 articles that reported an association with the M235 genotype, 12 articles that found an association with the T235 genotype and 23 articles that concluded that there was no association. One of the reasons for these controversial data could be uncertainties in the methods applied to determine the genotype. Indeed, as pointed out by Kawada *et al*. (*7*), the application of different methods to determine the AGT M235T polymorphism resulted in different allele frequencies in Japanese studies.

In our work the PCR-RFLP method was chosen, mainly because it is readily available for most clinical laboratories. During the optimization of the reaction described by Russ *et al*. (*3*), we were not able to attain a satisfactory identification with our equipment, similarly to others (*7*). We also strived to optimize both the PCR and the restriction digestion, but we still encountered uncertainties. These could lead to overestimation of the M235 allele, since the MT and TT genotypes could not be differentiated unambiguously. Accordingly, we designed a new antisense primer and consequently employed a new restriction enzyme in order to identify the AGT M235T polymorphism.

There are alternatives to the use of PCR-RFLP for determining the AGT M235T polymorphism, such as direct sequencing, PCR-ASO (*8*), MS-PCR (*9*) and single strand conformation polymorphism (SSCP) (*10*). However, these methods require special instruments and personnel, which may well not be available in most clinics. They also have other drawbacks. In the case of PCR-ASO, the signal-to-noise ratio of the hybridization of the nucleotides to the PCR product might be a limiting factor. For SSCP, strict control of the temperature of the gel during separation is of extreme importance (*10*). Together, these facts suggest that the improved PCR-RFLP protocol presented here could serve as a widely available method with enhanced accuracy for AGT M235T polymorphism determination.

Finally, it should also be noted that several clinical features of hypertension were associated with genetic variants of the angiotensinogen gene, including not only the T235M, but also several other polymorphisms, such as the T31C (*11*).

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