Real time PCR for fast detection of the angiotensinogen polymorphisms

Abstract. Objective: to develop a rapid and reliable real-time PCR to detect polymorphisms of angiotensinogen (AGT), to compare the two methods of MS-PCR (Mutagenically Separated PCR) and real-time PCR to determine three polymorphisms of the angiotensinogen gene M235T, the A (-6) G and A (-20) C. Methods: the method of real-time PCR was developed on the PLC Roche LightCycler1 with SYBR Green I. We used two sense primers and a primer nonsense. Detection of polymorphisms of angiotensinogen gene was performed by comparing the melting curves. Results: the DNA samples were analyzed by two methods: real-time PCR and MS-PCR. In our study, no differences were found between the two techniques. Discussion: The real-time PCR is a rapid and reliable method for detecting gene polymorphisms on the AGT M235T, the A (-6) G and A (-20) C. Conclusion: this method of real-time PCR is a reliable genetic test, which is fast and cheap and can be used in practice to study particular polymorphisms of AGT gene associated with cardiovascular disease.

Key words: angiotensinogen (AGT), real-time PCR, polymorphisms, SYBR Green
symptoms differs not only between disease entities but also between individual patients [1]. With regards to disease risk, much effort has been directed toward environmental factors such as diet, exercise, smoking and presence of comorbid diseases such as diabetes and hypertension [2]. It is well known that the Renin Angiotensin –Aldosterone system (RAAS) as a circulating or hormonal system that regulates blood pressure, electrolyte and fluid homeostasis [3]. The development of hypertension in coronary disease is also due to the effect of RAAS activation. Several studies analyzed the association of angiotensinogen (AGT) gene variants in relation to hypertension and coronary disease [3]. Among the variant of AGT, adenine - to – cytosine transition at nucleotide -20 of the 5’ upstream core promoter region affects the transcription activity of AGT mRNA and possibly involved in the development of essential hypertension (EHT). Another variant A for G substitution at position -6 in the core promoter of the gene has been significantly associated with elevated plasma AGT levels, AGT gene transcription and EHT [3]. Another variant of AGT is also the M235T polymorphism which is associated with increased plasma AGT level. The polymorphism is localized in exon 2 and methionine at position 235 is replaced by threonine. The AGT T235 allele was reported to be significantly associated with EHT in Caucasians [4, 5], in whites with a positive family history of hypertension and in Japanese population [6].

Polymerase chain reaction (PCR) has revolutionized the detection of nucleic acids. This method is the most common technique for DNA amplification and is able to detect as little as single copy of DNA or RNA. Fluorimetric detection of PCR products has simplified readout and made possible “Real Time” technique that allow amplifications to be monitored employed ethidium bromure, which in turn was replaced by more sensitive dyes like SYBR Green [7]. However, conventional PCR-based testing formats involve multiple complex steps and therefore require considerable expertise to perform [8]. Thus, Real Time PCR, used for many different purposes, especially for quantifying nucleic acids and for genotyping [9]. Due to the property of Real Time PCR, the process of amplification is monitored in Real Time by using fluorescence techniques. Real Time PCR offers quick and reliable detecting of any target sequence during the detection of genetic polymorphisms [10]. Real Time PCR instruments measure fluorescence and can detect the SYBR Green dye when it is intercalated with double-stranded PCR product formation [11].

This article describes a method for SNP detection and determines an alternative polymorphism strategy. Real Time PCR is a much more rapid and reliable method than the classical technique for SNP analysis and especially AGT polymorphisms. The aim of this study is to compare the MS-PCR and Real Time PCR methods in order to determine the M235T, the A(-6)G and the A(20)C polymorphisms.

Material and methods

Study population

The study population consisted on two groups. The control subjects made of 120 healthy volunteers (80 men and 40 women) with no history of CVD, diabetes or cerebrovascular diseases. Their mean age is 40±7 years. 150 consecutive patients (115 men and 35 women) with angiographically documented CVD were enrolled from Cardiovascular Department of University Hospital Fat-touma Bourguiba of Monastir, Tunisia. The mean age of this group is 61.15±10.64 years.

Genotyping of M235T, A(-6)G and A(20)C by Mutagenically Separated PCR (MS-PCR)

Genomic DNA was amplified by MS-PCR techniques [12] in which both normal and mutant alleles are amplified in the same tube, using length allele-specific primers (table 1) [13]. These primers were designed with one forward primer and two reverse primers. PCR reactions were conducted in a 25 μL reaction volume containing 2.5 μL of 10x PCR buffer, 1.5 mM MgCl2, 10 μM of dNTPs, 10 μM each of three primers and 0.5 units Taq polymerase. The first denaturation step (94°C for 5 min) was followed by 36 cycles, each of 94°C for 30 s, 61°C (M235T) or 59°C (A(-6)G) or 58°C (A(20)C) for 30 s and by a final extension.

Determination of M235T, A(-6)G and A(20)C by Real Time PCR

Detection of M235T, A(-6)G, A(20)C polymorphisms were carried out by Real Time PCR (MyiQ™, BIO RAD). PCR and melting curve analysis were performed in a final volume of 14 μL containing 300 ng of DNA, 1 μl of PCR mastermix (iQ™ SYBR® Green Supermix), 4 μL PCR-grade water and 10 μl of different allele specific primer used in the MS-PCR technique (table 1).

The cycling program involves a first denaturation step (3 min at 95°C) was followed by 2 × 40 cycles (each of 20 s at 65°C and 40 s at 60°C), 3 × 1 min at 95°C and by 4 × 1 min at 55°C. Melting curve analysis shows the temperature at which a double- stranded amplicon dissociates into single-stranded DNA, thus releasing the SYBR Green I resulting in decrease inflorescences.
RT-PCR and detection of angiotensinogen polymorphisms

Table 1. Polymorphism in the AGT gene.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primer sequences (Forward/ reverse, F/R)</th>
<th>Fragment size (pb) (MS-PCR)</th>
<th>Melting temperature (°C) (Real Time PCR)</th>
</tr>
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<tbody>
<tr>
<td>M235T</td>
<td>F: 5’AGC AGA GAG GTT TGC CTT ACC T 3’</td>
<td>MM: 118</td>
<td>M: 87</td>
</tr>
<tr>
<td></td>
<td>R1: 5’GTT CAT GCA GGC TGT GAC GAG TGG C 3’</td>
<td>TT: 98</td>
<td>T: 86</td>
</tr>
<tr>
<td></td>
<td>R2: 5’GAT GGA AGA CTG GCT GCT CCC AGA C 3’</td>
<td>MT: 118 and 98</td>
<td></td>
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<tr>
<td>A(-6)G</td>
<td>F: 5’ GTG TCG CTT CTT GCA TCT GTC CTT G 3’</td>
<td>AA: 187</td>
<td>A: 83</td>
</tr>
<tr>
<td></td>
<td>R1: 5’TAC CCA GAA CAA CGG CAG CTT CTT CCA CT 3’</td>
<td>GG: 212</td>
<td>G: 89</td>
</tr>
<tr>
<td></td>
<td>R2: 5’CCG GTT ACC TTC TGC TGC TGT AGA GCC CAG AAC AAC GGC AGC TTC TTC CAT C 3’</td>
<td>AG: 187 and 212</td>
<td></td>
</tr>
<tr>
<td>A(-20)C</td>
<td>F: 5’ACC TTCTGCTGTAGTAACCCAG 3’</td>
<td>AA: 82</td>
<td>A: 87</td>
</tr>
<tr>
<td></td>
<td>R1: 5’CAC CCC TCA GCT ATA AAT AGG CCC 3’</td>
<td>CC: 102</td>
<td>C: 85</td>
</tr>
<tr>
<td></td>
<td>R2: 5’CCT CCG AGG AGC TCC ATC CCG TCC CCT CAG CTA TAA ATA GCG CA 3’</td>
<td>AC: 82 and 102</td>
<td></td>
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</table>

Results

Genomic DNA was isolated from peripheral blood samples and the genotyping of AGT polymorphisms (M235T, A(-6)G and A(-20)C) were performed by MS-PCR and by specific primers. In this study, 97 MM genotypes and 53 TT genotypes were performed with the MS-PCR technique (figure 1A). Then, 78 (-20)AA and 72 (-20)CC were detected by the MS-PCR (figure 1B). So, 42 (-6)AA, 99 (-6)GG and 9 (-6)AG are displayed in figure 1C. The same DNA samples were exposed to Real Time PCR technique and each sample was analyzed with regard to melting temperature. MM and TT genotypes are indicated in figure 2A. (-20)AA and (-20)CC genotypes are displayed in figure 2B and figure 2C demonstrates the (-6)AA, (-6)GG and (-6)AG genotypes. In M235T genotyping, the melting temperature of M allele was at 87°C, while the melting of T allele was at 86°C. Thus, in AGT A(-20)C polymorphism, the melting temperature of A allele was at 87°C, while the melting of C allele was at 85°C. Finally, in A(-6)G genotyping, the melting temperature of A allele was at 83°C, while the melting of G allele was at 89°C.

No difference was found between the Real Time PCR and MS-PCR genotypes results. In our patients and similar to the MS-PCR, the MT genotypes and the (-20) AG subjects were also not observed by the Real Time PCR. All MS-PCR and Real Time PCR results were shown in table 2.

Figure 1. Agarose gel photographs of AGT M235T, A(-6)G and A(-20)C polymorphisms. A: determination of M235T polymorphism MS-PCR method (separated on a 5% ethidium bromide – stained agarose under UV light) M-100bp DNA molecular weight marker; lane- 1,2- MM genotype, lane - 3,4- TT genotype. B: determination of A(-6)G polymorphism MS-PCR method (separated on a 5% ethidium bromide – stained agarose under UV light) M-100bp DNA molecular weight marker; lane- 1,2- (-6)AA genotype; lane - 3,4- (-6)GG genotype; lane - 5,6- (-6)AG genotype. C: determination of A(-6)G polymorphism MS-PCR method (Separated on a 5% ethidium bromide – stained agarose under UV light) M-100bp DNA molecular weight marker; lane- 1,2,3- (-20)AA genotype; lane - 4,5- (-20)CC genotype.
Figure 2. Real Time PCR graphics of AGT M235T, A(-6)G and A(-20)C polymorphisms. A: melting curve analysis of AGT M235T polymorphism with SYBR Green dye I. Arrows demonstrates MM and TT genotypes at different melting curve. Water was used as a negative control. B: melting curve analysis of AGT A(-6)G polymorphism with SYBR Green dye I. Arrows demonstrates (-6)AA, (-6)GG and (-6)AG genotypes at different melting curve. Water was used as a negative control. C: melting curve analysis of AGT of A(-6)G polymorphism with SYBR Green dye I. Arrows demonstrates (-20)AA and (-20)CC genotypes at different melting curve. Water was used as a negative control.

Table 2. Comparison of MS - PCR and Real Time PCR methods for determining AGT gene polymorphisms.

<table>
<thead>
<tr>
<th></th>
<th>MS-PCR</th>
<th>Real Time PCR</th>
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<tbody>
<tr>
<td>M235T</td>
<td>97</td>
<td>97</td>
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<tr>
<td></td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>A(-6)G</td>
<td>42</td>
<td>42</td>
</tr>
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<td></td>
<td>99</td>
<td>99</td>
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<td>9</td>
<td>9</td>
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<tr>
<td>A(-20)C</td>
<td>78</td>
<td>78</td>
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<td>72</td>
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</table>

Discussion

The AGT gene has been associated with heart disease progression. So, several AGT variants were discovered and some of them were found to be associated with an increase in plasma AGT and hypertension [14]. Recent advances in genotyping technology and insights into disease mechanisms have increased interest in the genetic of cardiovascular disease [15]. AGT polymorphisms are a genetic risk factor for CVD. Rapid and reliable genotyping is an important in the determination of the predisposition of the individual to CVD [16]. Lately, denaturing gel electrophoresis, sequencing, and restriction endonuclease digestion are used for determination of the AGT polymorphisms.

According to the various studies, the PCR-RFLP and the MS-PCR are the usual methods for detecting AGT M235T, A(-6)G and A(-20)C polymorphisms [17]. However, PCR-RFLP process requires at least 4-6 h. In addition, PCR-RFLP and MS-PCR techniques include various post amplification handling steps. The same steps are carried out approximately in 20 to 30 min by Real Time PCR [17]. On the other hand, post amplification handling step may associate with end product contamination. Besides, this process might result in an incomplete enzyme digestion in PCR-RFLP technique. In Real Time PCR with no post-amplification processing, these potential problems are eliminated [17].

In our study, AGT region was amplified with site specific primers and PCR products were analyzed using MS-PCR method. In AGT polymorphism genotyping, Real Time PCR takes advantages of the fluorescent peculiarity of SYBR Green I dye and the melting curve analysis that allows the detection and distinction of different length of PCR product [18]. Thus, determination of AGT polymorphisms can be carried out in a rapid and reliable way.
Apart from SYBR Green I dye study, hybridization probe technique was carried out for the first time by Somogyvari et al. [19] for genotyping of the angiotensin-converting enzyme gene insertion/deletion polymorphism. They used marked fluorescing and LCR-640 probe at this technique. However, using fluorescing or other specific probes makes the technique very expensive. Consequently, in our study we prefer using SYBR Green I dye, AGT variants and two different allele – specific upstream primers and a common downstream primer used for MS-PCR [9]. The DNA samples were performed by Real Time PCR technique and each sample was analyzed with regard to melting curve. We found accurate genotype results for M235T, A(-6)G and A(-20)C polymorphisms at a single reaction.

Thus, because of fluorescent property of SYBR Green I dye, sensitivity of Real Time PCR and appropriate primers and PCR conditions, AGT polymorphisms (M235T, A(-6)G and A(-20)C) genotyping were performed seamlessly. In summary, the Real Time PCR based method provides a rapid and sensitive way for detection of AGT M235T, A(-6)G and A(-20)C polymorphisms in clinical samples. We genotyped all samples and found very good concordance MS-PCR results. In conclusion, no differences were observed between the Real Time PCR and MS-PCR results. Accordingly, advances in molecular genetics are rapidly adding genetic tests to the armamentarium of diagnostic and predictive tools available for the management of cardiovascular disease. Genetic tests offer many advantages over traditional tests. They do not require invasive sampling, have high accuracy, and can be done at any time in life, whether or not symptoms of disease are present.

**Conclusion**

In Real Time PCR, we genotyped the AGT M235T, A(-6)G and A(-20)C polymorphisms using length allele specific primers designed with one forward primer and two reverse primers. This method offers an appropriate option for reliability and labor intensiveness. The Real Time PCR is useful, rapid and not expensive genetic test for especially AGT polymorphisms associated with blood pressure regulation, heart failure, heart attack and hypertension.

**Conflicts of interest:** none.

**References**


