AT1 Receptor A1166C and AT2 Receptor –1332A/G Gene Polymorphisms: Efficient Genotyping by Single-Tube PCR

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Angiotensin II type 1 receptor (AT1) and angiotensin II type 2 receptor (AT2) genes have been investigated in recent years as potential etiologic candidates for cardiovascular and renal diseases. The pathogenic implications of AT1 A1166C and AT2 A–1332G gene polymorphisms have been shown. Here we describe a rapid and reliable method for detecting both AT1 and AT2 gene polymorphisms by a single-tube PCR, to reduce analysis time and simplify the genotyping procedure. In contrast to previously described methods, our method does not require hybridization, primer extension, or nested PCR for geno-

typing. In most previous studies concerning gene polymorphisms of RAS, both AT1 and AT2 receptor gene polymorphisms were investigated. The advantage of our method is that it makes it possible to detect both of these polymorphisms in a duplex PCR. The procedure described is convenient for routine laboratory use with manual sample processing, and offers the potential for further automation as well. Its simplicity makes it practical for large-scale screening of individuals and families at risk for cardiovascular or renal diseases. *J. Clin. Lab. Anal.* 19:84–86, 2005. © 2005 Wiley-Liss, Inc.

Key words: angiotensin II receptors; polymorphisms; duplex PCR

INTRODUCTION

Renin-angiotensin system (RAS) genes have been investigated in recent years as potential etiologic candidates for cardiovascular and renal diseases. Various pathologic conditions, such as kidney damage and heart failure, are characterized by persistent activation of RAS, and therefore the study of these genes may be of particular relevance. Most of the known effects of angiotensin II (Ang II), the powerful effector peptide of RAS, are mediated by at least two different receptor types that are involved in the regulation of cell growth, fibrosis, and inflammatory response: Ang II type 1 receptor (AT1) and Ang II type 2 receptor (AT2) (1).

The substitution of a cytosine for an adenosine at position 1166 in the 3' untranslated region of the human AT1 receptor gene has been identified (2). In intron 1 of the AT2 receptor gene, the A-G transition at position –1332 (3) (also assigned as +1675G/A (4)) has been determined.

The pathogenic implications of these polymorphisms have been investigated and associated with hypertension (2,5,6), left ventricular hypertrophy (7–9), coronary heart disease risk (10), and congenital urinary anomalies (3,11,12).

To further investigate the possible roles of these polymorphisms in clinical studies, we developed a rapid and reliable method for genotyping both AT1 A1166C and AT2 A–1332G gene polymorphisms by a single-tube PCR, which reduces analysis time and simplifies the genotyping procedure.

MATERIALS AND METHODS

Genomic DNA was isolated from whole-blood samples collected with EDTA and purified by the proteinase K/phenol (Sigma, Taufkirchen, Germany) extraction method (13). For amplification of the region containing AT1 A1166C gene polymorphism, two primers were used: forward AT1F 5'-GCAGCACTT-CACTACCAAATGGGC-3', and reverse AT1R

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5'-CAGGACAAAAGCAGGCTAGGGAGA-3' (14). The AT2 gene fragment was amplified with sense primer AT2S 5'-GGA AGG TAG AAC ATA CAT TAA ATG-3' and antisense primer AT2AS 5'-AGA GAA ACA GCA GCT AAA GAA TT-3', as described by Nishimura et al. (3) but modified by us. The antisense primer was designed to introduce an A-C substitution at position -1327 in the amplicon, which in combination with the -1332G allele creates an *EcoRI* restriction site (3). To determine the optimum annealing temperature, we performed PCR at three different annealing temperatures: 56°C, 58°C, and 60°C. We found that 58°C gave the best amplification products for both AT1 and AT2 DNA fragments. PCR was also performed with and without hot-start and with different *Taq* polymerases (MBI Fermentas, Vilnius, Lithuania; Amersham Biosciences, Uppsala, Sweden, Ampli Taq Gold Polymerase, PE, Applied Biosystems, Warrin, UK). All of the combinations used resulted in a sufficient yield of the expected PCR products. The PCR was performed in incubation buffer (50 mM KCl, 10 mM Tris-HCl, pH 8) with 3.33 mM MgCl₂, 125 μM of each dNTP (MBI Fermentas), 200 ng of template DNA, using 11 pmol of both AT1F and AT1R primers and 20 pmol of both AT2S and AT2AS primers, and 0.5 U of *Taq* polymerase (MBI Fermentas). The final reaction volume was 30 μl. Reactions were run in Touch Down™ (Hybaid, Teddington, UK) under the following conditions: initial denaturation at 93°C for 4 min, 30 cycles at 94 °C for 45 sec, 58 °C for 45 sec, 72 °C for 45 sec, and final extension at 72 °C for 5 min. Ten microliters of the PCR product were first tested on a 1.8% agarose (Sigma) gel to confirm the presence of 256-bp DNA fragment containing AT1 A1166C base substitution and 120-bp fragment containing -1332 A/G AT2 gene polymorphism. The samples were then double digested with both *BsuRI* (which cuts the AT1 gene polymorphic site) and *EcoRI* (which cuts the AT2 gene polymorphic site) restriction endonucleases in the 2 × Y⁺/Tango™ buffer (MBI Fermentas). After digestion with 5 U of both restriction enzymes for 4 hr at 37 °C, 6 μl of each sample were loaded per well on an 8% polyacrylamide gel (19:1) (Sigma) for genotyping. The genotypes were confirmed by two independent researchers who were blinded to subject outcome, and by repeated genotyping. Additionally, genotypes for both gene polymorphisms were confirmed in separate reactions and by a previously published method (15). The electrophoretic conditions for the polyacrylamide gel were 2 hr at 12 V/cm in 1 × Tris-borate-EDTA (Sigma). The gels were stained with silver nitrate (Sigma) and visualized by GDS8000 gel documentation system (Ultra Violet Products Inc., Upland, CA).

Fig. 1. Genotyping of AT1 receptor A1166C and AT2 receptor A/G-1332 gene polymorphisms in a single-tube PCR, on 8% polyacrylamide gel. M-ΦX174 DNA /*HinfI* ladder (MBI Fermentas). AT2 receptor A/G-1332 genotypes are indicated below lanes (GG, G/–, AG, AA). Sizes of bands after double digestion with *BsuRI* (MBI Fermentas) and *EcoRI* (MBI Fermentas) are shown on the left, and genotype assignments for AT1 receptor A1166C are indicated above each lane (AA, AC, and CC).

RESULTS AND DISCUSSION

The digestion of PCR products containing the AT1 1166C allele results in the presence of a 233-bp band, while undigested PCR products with allele A are 256 bp long. The digestion of AT2 amplified DNA fragments with allele G at position -1332 yields a 91-bp band, while undigested amplification products with allele A are 120 bp long.

Since the PCR was carried out in a single tube, all primers were designed to avoid complementarities between each other and the presence of nonspecific restriction sites for *BsuRI* and *EcoRI* in the PCR products. Figure 1 shows the typical band pattern for genotyping of AT1 and AT2 gene polymorphisms by the current method.

The method we developed is less time-consuming than other techniques, and is cost-effective and convenient for routine clinical laboratory use involving manual sample processing. In contrast to previously described methods, our method does not require hybridization (8), primer extension (6), or nested PCR (11) for genotyping of the AT2 gene polymorphism. We concluded that there is no need to perform hot-start PCR or to use any specific *Taq* polymerase or TaqStart antibodies. Although different allele-specific methods that exclude the use of restriction enzymes have been developed for genotyping of the AT1 A1166C polymorphism (15,16), we chose simultaneous double digestion of AT1 and AT2 DNA fragments as the most suitable technique for our single-tube genotyping method.

In most previous studies concerning gene polymorphisms of RAS, both AT1 and AT2 receptor gene polymorphisms were investigated. The advantage of

our method is that it makes it possible to detect both of these polymorphisms in a duplex PCR. Its simplicity makes it practical for large-scale screening of individuals and families at risk for cardiovascular or renal diseases.

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