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Original Article

DEVELOPMENT OF NEW AS-PCR BASED ANALYTICAL APPROACH FOR DETECTING THE SINGLE NUCLEOTIDE POLYMORPHISM OF *AGTR.1* GENE

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ABSTRACT

Objective: Angiotensin II is a potent vasoactive peptide that causes blood vessels to constrict, resulting in increased blood pressure. It acts through at least two types of receptors. The angiotensin II receptor type 1 gene (*AGTR1*) encodes the type 1 receptor (AT1). The AT1 receptor mediates the major cardiovascular effects of angiotensin II. A single nucleotide polymorphism (SNP) in the 3'-untranslated region of the *AGTR1* gene (A1166C) has been linked in several studies with essential hypertension. A new analytical approach based on allele-specific polymerase chain reaction (AS-PCR) was developed in this study to detect this SNP.

Methods: Allele-specific primers were designed by using appropriate software to allow the PCR amplification only if the nucleotide at the 3'-end of the primer complements the base of the wild-type or variant-type DNA sample. The primers were then tested for uniqueness using the Basic Local Alignment Search Tool search engine. The developed method was tested on 21 samples.

Results: The developed method accurately detected the genotype of the SNP. Our results were validated using the reference method for detecting A1166C SNP, PCR-restriction fragment length polymorphism (PCR-RFLP). The use of AS-PCR technique reduced both time and cost of the A1166C *AGTR1* genotyping. Moreover, the AS-PCR test is more suitable as it reduces the false results due to incompletely digested PCR products, which can be a problem with PCR-RFLP technique.

Conclusion: The use of this method will enable researchers to carry out genetic polymorphism studies for the association of A1166C SNP in *AGTR1* gene with essential hypertension and other heart diseases without the use of expensive instrumentation and reagents.

Keywords: Essential hypertension, SNP, Angiotensin II type 1 receptor gene (*AGTR1*), Renin-angiotensin system, AS-PCR

INTRODUCTION

The renin–angiotensin system (RAS) or the renin–angiotensin–aldosterone system (RAAS) is a hormone system that regulates blood pressure and water (fluid) balance. When renal blood flow is reduced, juxtaglomerular cells in the kidneys convert the prorenin already present in the blood into renin and secrete it directly into circulation. Plasma renin then carries out the conversion of angiotensinogen released by the liver to angiotensin I [1]. Angiotensin I is subsequently converted to angiotensin II by the enzyme angiotensin-converting enzyme. Angiotensin II is a potent vasoactive peptide that causes blood vessels to constrict, resulting in increased blood pressure. Angiotensin II also stimulates the secretion of the hormone aldosterone from the adrenal cortex [2]. Aldosterone causes the tubules of the kidneys to increase the reabsorption of sodium and water into the blood. This increases the volume of fluid in the body, which leads to elevated blood pressure [3]. Fig. 1 below illustrates the pathways of the RAS system.

Angiotensin II acts through at least two types of receptors. The gene *AGTR1* encodes the type 1 receptor AT1 which is a class of G protein-coupled receptors [4]. AT1 is found in the heart, blood vessels, kidney, adrenal cortex, lung and brain [5]. The AT1 receptor mediates the major cardiovascular effects of angiotensin II. Effects include vasoconstriction, aldosterone synthesis, and secretion, increased vasopressin secretion, cardiac hypertrophy, augmentation of peripheral noradrenergic activity, vascular smooth muscle cells proliferation, and decreased renal blood flow, renal renin inhibition, and renal tubular sodium reuptake, modulation of central sympathetic nervous system activity, cardiac contractility, central osmoregulation and extracellular matrix formation [6].

A polymorphism in the 3'-untranslated region of the *AGTR1* gene (A1166C) has been linked in several studies with essential hypertension. In wild-type A1166, a translational repression occurs, and the AT1 receptor is translated in normal levels. On the other side, with the variant-type C1166 translational repression does not happen and as a result, the AT1 receptor is translated with elevated levels which lead to the increase of the receptor function [7].

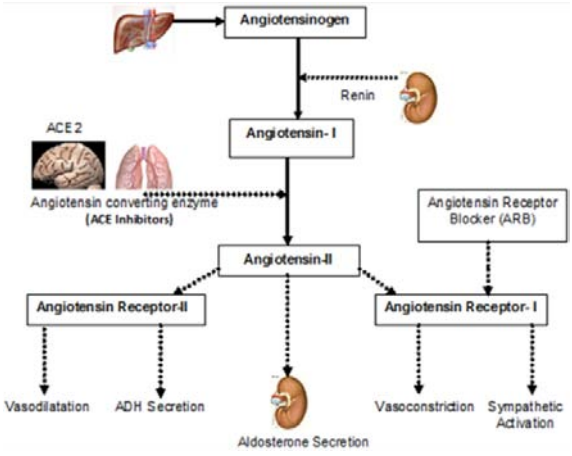

Fig. 1: Schematic illustration of the renin-angiotensin system (RAS)

Allele-specific polymerase chain reaction (AS-PCR), also known as the amplification refractory mutation system (ARMS) or PCR amplification of specific alleles (PASA) is a PCR-based method which can be employed to detect a known SNP [8]. The concept of AS-PCR was initiated by Newton *et al.* [9], approximately six years after PCR was invented. In this approach, the specific primers are designed to permit amplification by DNA polymerase only if the nucleotide at the 3'-end of the primer perfectly complements the base of the variant or wild-type sequences.

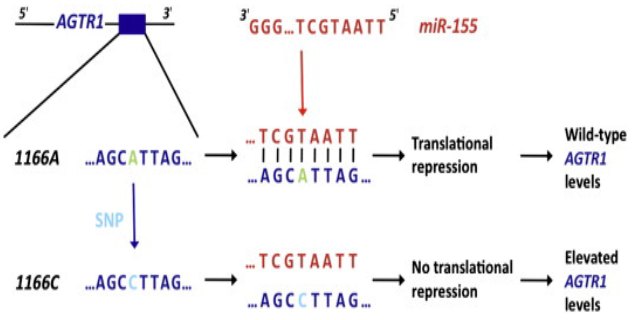

Fig. 2: Schematic illustration of the A1166C SNP in the *AGTR1* gene

After the PCR and electrophoresis, the patterns of specific PCR products allow the differentiation of the SNPs. Several approaches have been used to detect the presence of specific PCR product. Some are based on probe hybridization which requires specific labeled probes [10] and melting curve analysis [11] which requires nucleic acid stains. AS-PCR has been utilized widely in many areas of study such as pharmacogenetics [12], genetic disorders [13, 14], microbiology [15] and others.

The AS-PCR approach in determining SNP is relatively cheaper than other methods. The crucial aspects in creating a working AS-PCR-based genotyping system are primer design and optimizing PCR. Once the optimized protocol has been achieved, the execution of AS-PCR is relatively simple, similar to the conventional PCR.

The aim of this study was to develop, validate and utilize an AS-PCR method to determine the *AGTR1* (A1166C) SNP genotype.

MATERIALS AND METHODS

DNA samples

Subjects were recruited from the Cancer Research Center of Aleppo University, Aleppo, Syria. 5 ml of blood were collected from each subject. Genomic DNA was extracted from whole blood using the GF-1 Blood DNA Extraction Kit (Vivantis, Malaysia) according to the manufacturer's instructions. DNA samples were stored at-20 °C until they were used. Samples were inspected for their SNP genotypes using both the reference PCR-RFLP method [16] and the developed AS-PCR. Results were compared and discussed in this paper.

Sequence data

Data for the *AGTR1* gene sequence and the SNP region were retrieved from the Genebank of the NCBI website (National Center for Biotechnology Information).

AS-PCR

The genotyping method used to detect the selected SNP was developed using AS-PCR. Primers were designed with the aid of Primer designer software, version 2.0 and searched for uniqueness using the NCBI BLAST search engine [17]. The primers were designed to amplify a 448 bp DNA fragment of the *AGTR1* gene (Genbank reference number NC_000003.11). The sequences of the primers are shown in table 1.

Table 1: Sequence of primers for genotyping of the polymorphism of *AGTR.1* gene

Primer name	Sequence (5'-3')
AGTR-W	TTCACTACCAAATGAGCA
AGTR-V	TTCACTACCAAATGAGCC
AGTR-rev	CGACTACTGCTTAGCATA

The AS-PCR consists of two complementary reactions (two tubes) and utilizes 3 primers. The AGTR-rev primer is constant and complementary to the template in both reactions, while the forward primers differ at their 3' terminal residues and are specific to either the wild type (AGTR-W) or the variant type (AGTR-V). Only one of these forward primers is used per tube.

PCR was carried out using a gradient thermocycler (TaKaRa, Japan).

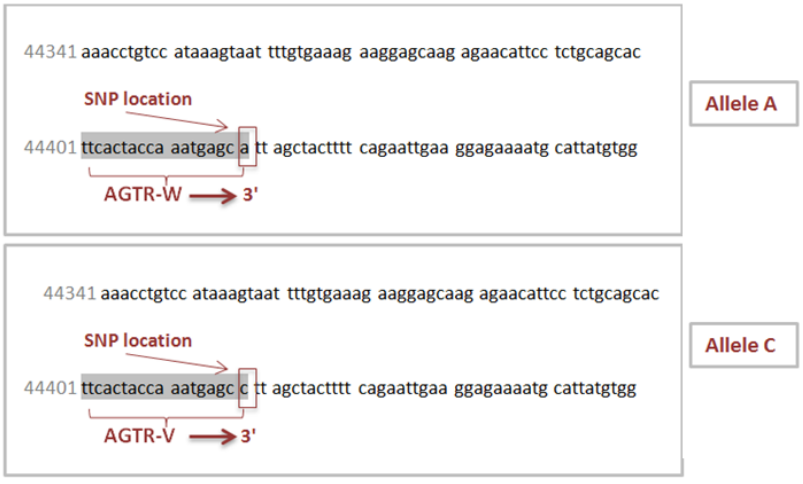

Fig. 3: A part of the *AGTR1* gene sequence around the SNP site. The highlighted sequence shows the designed primers which are specific at their 3' site to the genotype of the SNP

Each PCR reaction was carried out in two tubes, one containing the wild type forward primer and the other contained the variant type. The final volume of all PCR protocols was 25 µl contains: 1X ViBuffer A (500 mM KCl, 100 mM Tris-HCl "pH9.1 at 20 °C" and 0.1% TritonTM X-100), 2 U of Taq DNA polymerase (Vivantis, Malaysia), 0.4 µM of each primer, 6 mM of $MgCl_2$, 0.2 mM of dNTPs, and 25 ng of DNA. The PCR cycling was performed with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of amplification; 94 °C for 30 sec, 53.2 °C for 30 sec and 72 °C for 60 sec. The final extension was performed at 72 °C for 10 min.

Agarose gel electrophoresis

Genomic DNA and PCR-Products were separated on 0.8% and 2% agarose gel respectively. Using 1X TEA buffer and applying field strength of 5 v. cm^{-1}. The DNA was stained with ethidium bromide and visualized using a video documentation system (Nise UV; Daihan Scientific Co., Korea).

Validation and accuracy of method

21 DNA samples were genotyped for *AGTR1* A1166C SNP using the AS-PCR protocol developed in our research, and the method was validated by Restriction Fragment Length Polymorphism (PCR-RFLP) strategy which is the reference method for detecting the A1166C SNP in *AGTR1* gene [16].

RESULTS

To optimize a successful PCR protocol, we started from the reference PCR-RFLP protocol and developed it to be optimal for our designed primers. The most challenging problem we faced was to increase the PCR product to have a strong band on the agarose gel without the presence of unspecific PCR products. We used the PCR gradient technique to determine the optimal conditions for annealing temperature and Mg^{2+}concentration. Amplification and analysis of the SNP were performed successfully as showed in fig. 3. The presence of PCR bands with a size of 448 bp, which corresponds with the theoretical size (Genbank reference number NC_000003.11), indicated the genotype of the samples. For each DNA sample two PCR reactions were performed; the first with the AGTR-W primer and the second with the AGTR-V primer. The SNP genotype can be determined according to the number of the resulted amplicons as explained in table 2. The genotypes for the samples were 14 for AA and 7 for AC. The results that were obtained from the developed AS-PCR method were all consistent with genotype data obtained using the reference PCR-RFLP technique.

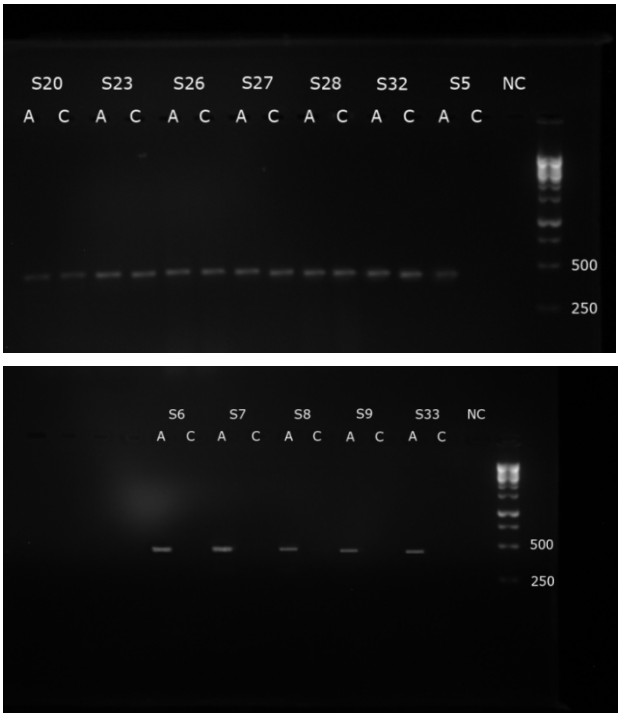

Fig. 4: The electrophoresis profiles for some of the successful amplifications. Samples 5, 6, 7, 8, 9 and 33 have the homozygous genotype AA, while samples 20, 23, 26, 27, 28 and 32 has the heterozygous genotype AC. The ladder we used is GeneRuler 1 kb DNA Ladder, Fermentas

Table 2: The size of amplicons and the genotypes for A1166C SNP in the *AGTR1* gene

Wild type tube	Variant type tube	Genotype
448 bp amplicon	No band	AA
448 bp amplicon	448 bp amplicon	AC
No band	448 bp amplicon	CC

DISCUSSION

As mentioned, AS-PCR consists of two complementary reactions (two tubes) and utilizes 3 primers. Therefore, three primers were designed (AGTR-W, AGTR-V and AGTR-rev). The forward primers, AGTR-W and AGTR-V, are allele-specific primers as the nucleotide at the 3'-end of each allele-specific primer perfectly matches the SNP site (AGTR-W primer is complementary to the wild-type allele, while AGTR-V primer is complementary to variant type allele); only one of these primers is used per tube. The reverse primer AGTR-rev is constant and complementary to the template in both reactions. If the sample is a homozygous variant or homozygous wild type, amplification will only take place in one of the two tubes. If the sample is heterozygous, amplification will be seen in both tubes.

The PCR was optimized by adjusting the concentration of the primers, $MgCl_2$ and annealing temperatures. The optimum annealing temperature and $MgCl_2$ concentration were determined using a gradient PCR. Under optimized PCR components and conditions, the patterns of PCR bands that form after agarose gel electrophoresis enable an effective characterization of the SNP genotype. The inclusion of a negative external control, a reaction containing all PCR components except the DNA sample, was to confirm the absence of contamination and false positive results.

There are several methods that can be used to detect SNPs, such as PCR-restriction fragment length polymorphism (PCR-RFLP), high resolution melting (HRM), pyrosequencing and probe hybridization-based techniques. PCR-RFLP has some disadvantages such as the necessity of an incubation period for enzymatic digestion by restriction endonuclease to separate the restriction fragments, false results due to incompletely digested PCR products and the cost of the restriction enzymes [18]. The other methods mentioned above are faster and easier to determine SNPs [19, 20] but these methods are expensive because they require the use of high technology instrumentations and costly reagents.

The AS-PCR method developed in this study only requires basic equipment such as a conventional thermal cycler and a gel documentation system which are available in most genetic laboratories. It is cost-effective as it does not use fluorescent nucleic acid stains or hybridization probes. This makes it suitable to be used in studies where lack of funding, equipment or expertise may be a factor.

CONCLUSION

The use of this method will, therefore, enable researchers to carry out genetic polymorphism studies for the association of A1166C SNP in *AGTR1* gene with essential hypertension and other heart diseases without the use of expensive instrumentation and reagents.

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CONFLICT OF INTERESTS

Declared none

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