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DEVELOPMENT OF AMPLIFICATION REFRACTORY MUTATION SYSTEM PCR TO DETECT ANDROGEN RECEPTOR GENE G1773A (RS6152) POLYMORPHISM

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Androgen receptor (AR) is a ubiquitous receptor responsible for responses by androgen stimulus. Androgen, a hormone which will bind to the AR, is essentials for normal male sexual development. Nevertheless, one of the polymorphisms in the AR gene, G1733A (rs6152) have been associated with numerous clinical risks such as cardiovascular disease (CD), androgenetic alopecia, high prostate-specific antigen (PSA) levels, male infertility, recurrent spontaneous abortions and prostate cancer. This study aims to develop an alternative and cost-effective method to detect G1773A (rs6152) polymorphism. In this study, amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) using two pair of primers was used to detect the G1733A (rs6152) polymorphism. Primer design was done using http://primer1.soton.ac.uk/primer1.html online tools and then manually adjusted to increase the specificity. A total of 54 samples were screened using ARMS PCR and 2 representative samples of each allele from previous screening were used to validate the results using Sanger DNA sequencing. Among 54 subjects screened, we found 52 (96.3%) subjects carry G allele and 2 (3.7%) subjects carry A allele. No heterozygote was found in this study. The frequency of G allele was 96.97% and the frequency of A allele was 3.03%. Result validation using DNA sequencing was in agreement with ARMS-PCR method results. ARMS-PCR can be used as efficient alternative for genotyping of G1733A (rs6152) AR gene polymorphism.

Keywords: Androgen receptor, rs6152, ARMS-PCR, polymorphism

1. Introduction

Androgen, such as testosterone and dihydrotestosterone (DHT), were steroid hormones involved in the development of male reproductive organs, such as: embryogenesis; puberty and sexual maturation.(1) In addition, androgen also have diverse effect in bone and skeletal muscle, either in male or female.(2) The mechanisms of androgen action involves the enzymatic process of reduction of testosterone by 5α-reductase to DHT and followed by activation of Androgen Response Elements (ARE), a specific motif presents in promoter or enhancer which activation rely on androgen receptor (AR).(3) Nevertheless, the binding of testosterone, DHT and activation of ARE were mediated by AR.

The AR is a receptor belong to superfamily of DNA binding transcription factors which share both DNA sequence and functional homology such as steroid, thyroid, vitamin D, retinoic acid hormones and many others. AR gene is located on the X chromosome on Xq11-12. Significant effect due to the mutations in the AR may result in androgen insensitivity syndrome (AIS) which characterized by evidence of feminization of the male genitals at birth, abnormal secondary sexual development during puberty and infertility in individuals with

normal 46XY chromosomes. The clinical presentation of AIS is vary with different phenotype due to large amount of mutation and heterogeneity (missense mutations, nonsense mutations, splicing variant, deletions and insertions) in the AR gene.(4) One of the mutations in AR, G1733A (rs6152) have been associated with increased risks of many clinical outcomes such as androgenetic alopecia (AGA),(5) higher prostate specific antigen (PSA),(5) recurrent spontaneous abortion,(6) polycystic ovary syndrome,(7) cardiovascular disease(8) and prostate cancer aggressiveness.(9)

Currently, the most frequent method to detect G1733A (rs6152) polymorphism is by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) with the help of *StuI* restriction enzyme(5,8). This method involves two different process in which amplification of target DNA sequence and followed by cutting of the amplified band using specific enzyme. Here we introduce Amplification Refractory Mutation System (ARMS) which is a reliable, simple and fast method to detect the SNPs by using four primers in one PCR reaction.

2. Research Method

Samples and DNA extraction

A total of 54 subjects were used in this study. Informed consent was given to each subjects and ethical clearance were given by Universitas Tarumanagara Human Research Ethics Committee with ethical clearance number PPZ20222090. Blood DNA extraction were done using Genomic DNA Midi Kit (Geneaid, Taiwan). After extraction, DNA quantification were done by spectrophotometer (Thermo Fischer Scientific), each sample then diluted to approximately 50 ng/mL DNA concentration and kept at -20°C until further use.

Tetra arms PCR for rs6152

Four primers were designed by PRIMER1 online software (http://primer1.soton.ac.uk/primer1.html). Further customization was done manually using Bioedit software ver 7.2.5 (https://bioedit.software.informer.com/7.2/) to increase the specificity of the primers (Table 1). ARMS-PCR were done in total volume of 25 µL with 12.5 µL 2x myTaq TM mastermix (Meridian Bioscience, United Kingdom), 0.4 µM of FO primer, 0.4 µM of RO primer, 0.4 µM of FI primer, 0.4 µM of RI primer and topped with dH2O. The PCR conditions are initial denaturation at 95°C for 2 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 63°C for 30 seconds, extension at 72°C for 10 seconds and followed by final extention at 72°C for 5 minutes at the end of cycle. The PCR products were visualized by 1.5% agarose gel electrophoresis at 100 V for 45 minutes.

Table 1. Primers designed for rs6152 SNP detection

Primer	Sequence (5'- 3')	Product size
Forward inner (FI)	CAGCAGCGGGAGAGCGAGGTAG	G allele 245 bp
Reverse inner (RI)	AAGTGGGAGCCCCCGAGTCT	A allele 367 bp
Forward outer (FO)	CAAGCCCATCGTAGAGGCCC	Outer band 572 bp
Reverse outer (RO)	GCCAATGGGGCACAAGGAGT	

DNA Sequencing validation

DNA Sequencing method was employed to validate the result of ARMS-PCR. Four samples were chosen randomly from the G allele positive ARMS-PCR and all samples (n =2) from A allele positive ARMS-PCR, which is the minor allele, were amplified using only the outer primer (FO & RO) and then outsourced for the sequencing process (Apical Scientific Laboratory, Malaysia).

3. Result And Discussion

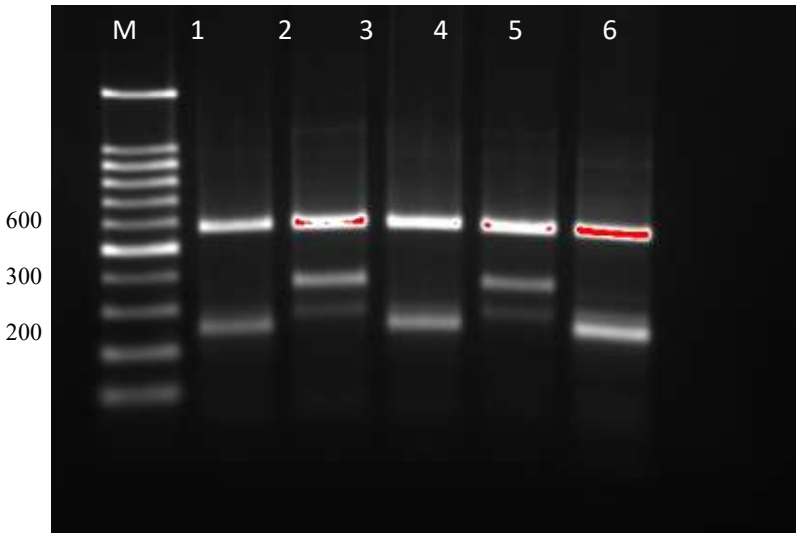

Figure 1. Electropherogram of rs6152 ARMS-PCR. lane M is for DNA ladder, lane 1, 3 and 5 are products of the G allele positive with two bands at 572 and 245 bp, lane 2 and 4 were products of the A allele positive with three bands at 572 bp, 375 bp and one unspecific band, lane 6 was negative controls

A representative for the rs6152 ARMS-PCR were shown in figure 1. The G allele shows two bands which are 572 and 245 bp, while A allele shows two band with different pattern which are 572 and 367 bp. Among 54 samples enrolled in this study, 52 (96.3%) were G allele positive and 2 (3.7%) were A allele positive. We cannot categorize the allele assignment by genotype (GG/GA/AA) due to rs6152 was sex linked SNPs. No heterozygotes in the female subjects were found in this study (table 1). The allele frequencies were 96.97% for the G allele and 3.03% for the A allele. The distribution of the genotypes in this study was not in Hardy-Weinberg equilibrium ($\chi^2 = 7.68287$; $P = 0.021$)

Table 2. Allele and genotype distribution in this study

Genotype	Distribution
Female (n=45)	
GG	44 (97.78%)
AA	1 (2.22%)
Male (n=9)	
G	8 (88.89%)
A	1 (11.11%)

Allele Frequency	
G	96 (96.97%)
A	3 (3.03%)

The representative results of DNA sequencing are shown in Figure 2. Two different samples with different result, the G allele positive (572 and 245 bp) were randomly chosen and both samples with A allele positive (572 and 375 bp), were used for validation by sequencing. The results of ARMS PCR agreed with the DNA sequencing results.

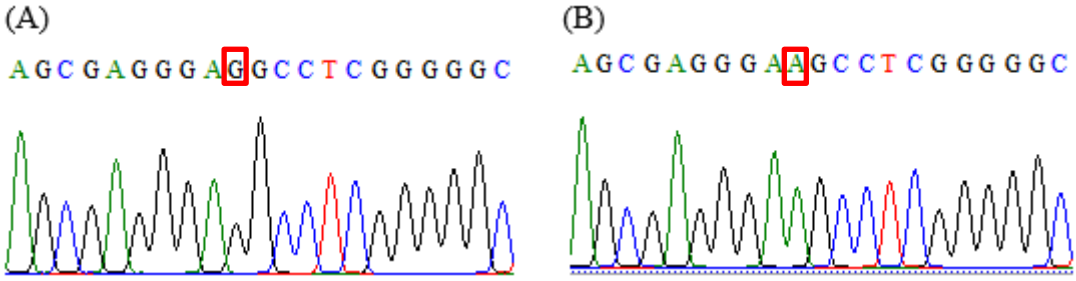

Figure 2. DNA sequencing result of rs6152. A, G allele positive; B, A allele positive

Discussion

Androgen receptor, which gene is located on X chromosome Xq11-12, is a receptor that stimulated by testosterone and 5α-DHT. Both hormone function as a ligand dependent nuclear transcription factor which affect the activation for the AR. AR is expressed widely in many cells and tissues. it modulates diverse range of biological mechanisms, including the development and maintenance of reproductive, cardiovascular, neural, musculoskeletal, immune and hemopoetic system.(10) Genome-wide association studies (GWAS) have identified a number of SNPs in this gene and its possible associations with higher risks of clinical outcomes such as prostate cancer,(11) AIS,(4) autism,(12) polycystic ovary syndrome (7) and many more. One of the SNP located in the AR gene, rs6152 is one of the mutation that have been associated with various clinical outcomes.(5–8)

ARMS PCR is already reported as a simple, fast and low-cost method in SNP genotype detection and this method already been compared to PCR-RFLP, which need restriction enzyme and a two process method. The application of ARMS PCR is already widely used in detecting various SNPs.(13–18) This method uses four primer in single PCR reaction in which a pair of the primers recognize one specific SNP.(19) The challenge in this method was the primer design and the optimization process. The primers used in this study were designed by PRIMER1 software. After the *insilico* primer design was recommended by the software, we use BLAST to see the specificity of the primers. The inner primer which was supposed to detect the G-allele shown the specificity for the *homo sapiens* chromosome 17. To overcome this issue, we altered the inner G-allele primer manually by adding 2 nucleotides to increase the specificity for the AR gene. The problem following the issues is the high GC content of each primers (ranged from 60-70%) due to the high GC of rs6152 site, however the Tm result from the high GC content didn't exceed 67. Nevertheless, to ensure a working PCR reaction, we choose a pre-mixed PCR mastermix which can handle high GC content reaction and known for its capability in multiplex PCR. ARMS PCR were not recommended for detection

high GC percentage region by Medrano and Oliveira, (19) but recent study shown the use of dimethyl sulfoxide as PCR enhancer or thermostable strand displacement polymerase can overcome the problem.(14) As shown in figure 1, The G allele (245 bp) and A allele (367 bp) shown the same intensity of band luminance which shown the PCR reaction is working. Subjects number 2 and 4 also shown an unspecific band with lower luminance and higher placement than the G allele band which can be ignored. Unspecific bands were common in ARMS PCR and various method may be employed to eliminate the unspecific band.(19) Although no heterozygote alleles were found in this study, this method also capable to detect heterozygote allele (GA) which was obtained by mixing 2 different samples with different alleles (data not shown). Lastly, to ensure the validity of the ARMS PCR for rs6152, we sequenced 4 random samples from samples shown ARMS PCR G allele positive and 2 samples of the ARMS PCR A allele positive in which the sequencing results were in concordant with the ARMS PCR results.

According to our knowledge, this is the second time the detection of rs6152 were done in Indonesia and the first study of rs6152 SNP prevalence for healthy people. The only known study for rs6152 were done by Sribudiani et al. (2018) which detect the prevalence of rs6152 SNP in prostate cancer patients. Our study shows lower minor allele frequency (MAF) of rs6152 (0.03) than the study reported by Sribudiani et al. (2018) in prostate cancer patients (0.0789).(20)

4. Conclusion

ARMS-PCR, is an effective, simple, reliable and low cost SNP detection method for rs6152 despite its high GC content site, which can be used as effective screening method as an alternative to PCR-RFLP. This study also shown low MAF (A allele) of rs6152 in Indonesian population.

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