Advances in Health Sciences Research, volume 40
2nd International Conference on Contemporary Science and Clinical Pharmacy 2021 (ICCSCP 2021)

Primer Design of SNP rs4506565 Transcription Factor 7 like 2 (*TCF7L2*) Gene to Detect Type-2 Diabetes Mellitus

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ABSTRACT
Diabetes Mellitus is a chronic disease caused by a deficiency or insufficient insulin that increases the levels of blood glucose. Diabetes Mellitus causes several complications that can increase the numbers of mortality. SNP rs4506565 Transcription Factor 7 Like 2 (*TCF7L2*) gene reported to be strongly correlated with Type-2 Diabetes Mellitus (T2DM) in ethnic Caucasian, East Asian, and South Asian. SNP rs4506565 is indicated by polymorphism at point 112,996,282 from nucleotide Adenine (A) to Thymine (T). Amplification-Refractory Mutation System Polymerase Chain Reaction (ARMS PCR) was used to detect the existence of polymorphism by its specific primer. The objective of this study was to construct a particular primer to identify the SNP rs4506565 *TCF7L2* gene using ARMS PCR. This research was a descriptive study to figure the specific primer of the PCR reaction. The research process begins with isolating the DNA sample, followed by primer construction using Geneious software, doing PCR to amplify the specific area, and DNA sequencing. Based on the research, four primers have been designed to detect the SNP rs4506565 *TCF7L2* gene. The primers were rs4506565-F, rs4506565-R, rs4506565-F(A) as a specific primer for the allele A, and rs4506565- R(T) to detect the allele T. The reaction produced three fragments of 650bp, 271bp, and 417bp. It can be concluded that the primer can be used in a small laboratory to detect the presence of rs4506565 *TCF7L2* gene and identify the association between variant *TCF7L2* gene SNP rs4506565 with T2DM as a preventive tool of T2DM in other ethnic.

Keywords: *Primer, TCF7L2, SNP rs4506565, Type 2 Diabetes Mellitus*

1. INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a metabolic disease characterized by high glucose levels (hyperglycemia) caused by a deficiency of insulin secretion and/or insufficiency. Poorly managed diabetes leads to severe complications and early death. T2DM is associated with long-term damage, dysfunction, and failure of various organs like eyes, nerves, kidneys, heart, and blood vessels [1]. The prevalence of DM in the world in 2019 was around 9.3%, while Indonesia is ranked seventh with an estimated number of DM patients around 10.7 million in 2019 and predicted to increase to 13.7 million in 2030 [2]. T2DM has certain factors that can increased the risk, include: age, weight, inactivity, gave birth to a baby weighing more than 4000 g, race and ethnic, family history, BMI more than 23 kg/m2, high blood pressure, and dyslipidemia [3]

A marker can use to prevent and reduce the incidence of T2DM. One of the most studied markers is Single Nucleotide Polymorphism (SNP) in a gene; SNP is expected to associate with the risk of T2DM in patients. Several potential genes involved in T2DM susceptibility have been studied previously [4]. The gene with the strongest association with T2DM is Transcription Factor 7 Like 2 (*TCF7L2*) [1], [5], [6], [7]. The *TCF7L2* gene encodes the High Mobility Group (HMG) box-containing transcription factor that plays an important role in the Wnt signaling pathway. The Wnt signaling pathway produces incretin hormones that control the expression and function of several hormones in glucose homeostasis, such as GLP-1, GIP, and insulin [1], [8], [9]. Variations in the *TCF7L2* gene such as rs4506565 are estimated to affect the conversion of proinsulin to insulin [9], decrease incretin hormone production, and have a relationship with reduced insulin sensitivity throughout the body and hepatic insulin [1].

Variant *TCF7L2* gene rs4506565 was reported to be associated with T2DM in ethnic Caucasian, East Asian, and South Asian [10], [11], [12]. Polymorphism was

characterized by a point mutation from Adenine (A) to Thymine (T) (A>T) at position 112,996,282. Slight variation in genes contributes to an alteration in the function of their protein product. These influences on protein function, in turn, may affect some parameters including insulin secretion and increase an individual's overall risk of developing T2DM. SNP rs4506565 is located in the intron region between exons 5 and 6, which does not undergo translation, but the polymorphism is suspected to affect the transcription and splicing processes of the gene [8].

The study aimed to determine a specific primer to identify SNP rs4506565 so that it can be used as an accurate method to detect SNP. The technique used was ARMS PCR with two pairs of primers. The PCR results will then be sequenced to confirm the validity of the designed primer. The discovery of the primer of SNP rs4506565 *TCF7L2* gene can be used small laboratory to identify the association between the presence of SNP and T2DM in other ethnic. Ethnic Minangkabau, Indonesia is the target group that wants to be studied because of the high incidence of T2DM and the people's diet that can increase the risk of T2DM.

2. METHODS

This research is a descriptive study that describes the primer design results to detect SNPs' presence and the primers' ability to amplify the expected area. Identification of the existence of SNPs and sample genotypes was carried out using the ARMS PCR method. Primers were designed based on the *TCF7L2* gene sequence using the Geneious program. In this study, two pairs of primers were used to detect the A allele and the T allele. The size of the ARMS PCR product was analyzed by agarose electrophoresis.

2.1. Materials

The samples were obtained from veins and stored in a tube containing EDTA (0.1 mmol/L). The study received ethical clearance from Faculty of Medicine Andalas University with reference number 176/KEP/FK/2015. The DNA isolation was done using Purelink Genomic DNA kits (Invitrogen). The component that used in the ARMS PCR reaction were DNA template, primers for internal control SNP rs4506565-F, SNP rs4506565-R, allele A specific primers rs4506565-F(A), allele T specific primers SNP rs4506565-R(T), GoTaq Green master mix 2X (Promega), MgCl2 0.5mM, nuclease-free water (Promega), and PCR tube. The materials for electrophoresis are agarose gel (Vivantis), Red safe 20,000X (Intron Biotechnology), VC 100bp plus DNA Ladder (Vivantis), DNA loading dye (Vivantis), Tris-Borate-EDTA (TBE) buffer 0.5X which is every 1 L contains 108 g of tris and 55 g of boric acid in 800 ml of distilled water and 40 ml of 0.5 M Na2EDTA.

2.2. DNA isolation

DNA isolation was carried out using the Purelink Genomic DNA kits protocol consisting of proteinase K, RNase A, pure link genomic lysis/binding buffer, pure link genomic wash buffers I and II, pure link genomic elution buffer, 96-100% ethanol. The results of DNA isolation were detected using 1.5% agarose electrophoresis with a voltage of 100 V for 30 minutes.

2.3. Primer Design

The primers were designed using the Geneious program based on the Transcription Factor 7 like 2 (*TCF7L2*) gene sequence (Acc. nr. NG012631) from the http://ncbi.nlm.nih.gov site. The primer designed must cover a specific condition, for example, having a melting temperature (Tm) in the range of 50-60ºC and not having a tendency to form hairpin structures and/or stable self-dimers. The selected primers were checked on the website http://ncbi.nlm.nih.gov/tools/primer-blast to ensure primer specificity. The examination was carried out to check the possibility of primer miss-priming with other regions on the *TCF7L2* gene. If the primer has been checked, then the primer design results are ready to be synthesized.

2.4. Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS PCR)

Determination of the presence of alleles T and A in the sample was carried out using ARMS PCR. The formula that used to determine the existence of allele A and T SNP rs4506565 were GoTaq green master mix 1X (Promega, USA), primer rs4506565-F 0.5µM, primer rs4506565-R 0.5µM, primer rs4506565-F (A) 0.5µM, primer rs4506565-R(T), MgCl2 0.5 mM, water-free nuclease, and 0.5µL DNA molds. The PCR conditions in initial denaturation are at 95°C for 3 min and denaturation of each cycle at 95°C for 30 s. The annealing temperature was 63°C for 45 seconds, and the product elongation was 72°C for 45 seconds for 5 minutes for a total of 35 cycles, and the final elongation at 72° C for 5 minutes.

2.5. Electrophoresis

Agarose gel is made by weighing the amount of agarose mixed with 0.5X TBE, then heated until a homogeneous solution is obtained. The agarose gel concentration used to detect DNA isolates, and ARMS PCR product was 1.5% (1.5 g agarose in 100 mL TBE). In the agarose solution, 8 µL of red-safe was added to visualize the DNA when exposed to UV light. The solution is poured into a mold that has been fitted with a column comb. The agarose gel is placed in an electrophoresis container containing a 0.5X TBE solution.

In detecting DNA isolates, a mixture of 3 µL of DNA isolate and 2 µL of DNA loading dye was inserted into the gel well. The containers' electrodes are connected to a power supply with a voltage of 100 V for 30 minutes. In detecting ARMS-PCR products, the amounts of samples and marker were inserted into gel wells were 7 µL and 4 µL, respectively, with a voltage of 120 V for 65 minutes. DNA from agarose electrophoresis was visualized using the Gel doc.

2.6. Sequencing

The results of ARMS PCR samples were then sequenced to prove whether the amplified gene was correct and the primer was successful in amplifying the targeted area

2.7. Data Analysis

The data were analyzed qualitatively to know the primer construction results and the primer's ability to amplify the desired area.

3. RESULTS AND DISCUSSION

SNPs rs4506565 *TCF7L2* gene have a significant association with T2DM risk in some ethnic, included Caucasian, East Asia, and West Asia [10], [11], [11]. A designed primer to detect SNP rs4506565 can be used to detect the presence of rs4506565 *TCF7L2* gene and determine the association between the SNP and T2DM in ethnic Minangkabau. Therefore, SNP might be used as genetic marker for T2DM and as a preventive tool for T2DM. The sample in this study was obtained from a vein. DNA sample was isolated by the Purelink Genomic DNA kits protocol and the DNA was analyzed using 1.5% agarose gel electrophoresis to determine the effectiveness of DNA isolation (Figure 1). The basic principle of DNA isolation is to break down and extract the tissue to obtain the required DNA [13].

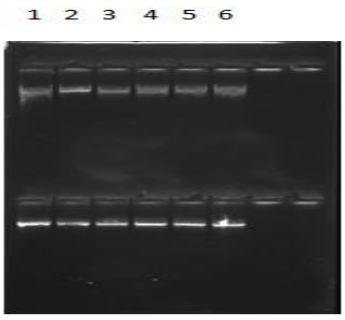


Figure 1 The DNA band from the first elution (top) and the DNA band from the second elution (bottom) in sample T2DM patient

An appropriate primer was needed to determine the presence of SNPs using a PCR reaction. A good primer has a size of 18-22 bp, Tm in the range of 52-58ºC, tolerance for differences in Tm between primers is 3-5ºC, percentage of GC in the field of 30-60%, and does not form self-dimers and hairpins. Hairpins and self-dimers are secondary structures resulting from the interaction of intermolecular and intramolecular primers that can interfere with PCR results [14], [15].

Primers used in the research were rs4506565-F and rs4506565-R as internal control reactions, rs4506565-F(A) as allele A specific primers, and allele T specific primer rs4506565-R(T). The SNP should be 300-500 bp from the rs4506565-F and rs4506565-R. The 3' end of the primer will specifically recognize the template DNA, so the allele A and the allele T should be at the 3' end of the rs4506565-F(A) and rs4506565-R(T) primers. The designed primers were rs4506565-F (5'-CAG CAT GGA CTA AGG ACT TTA C-3'), rs4506565-R (5'-TTC ACC AGC ACT GAT CAC T-3'), allele A specific primer rs4506565-F(A) (5'-ATA TGG CGA CCG AAG TGA TA-3'), and allele T specific primer rs4506565-R(T) (5'-CTT GAC AAG GGC CCC AA-3').

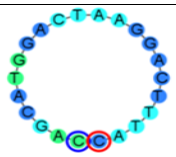
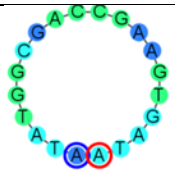
The designed primer has a size of 18-22 bp, Tm is in the range of 56-60ºC, the difference in Tm between primers is 4^0C, %GC is in the range of 45-55%, does not form a hairpin structure and/or self-dimer. Then the primer used in the study followed the good primary criteria (Table 1).

The specificity of the designed primers was confirmed using the software http://ncbi.nlm.nih.gov/tools/primer-blast to avoid possible miss-priming of the primers with other regions in the *TCF7L2* gene [16]. Based on the study, the primer can attach the desired region, and the allele A and T specific primers can identify the SNPs in the gene.

Based on the design results, the internal control primer will produce a fragment with size of 650 bp. In determining A allele, the primer pair rs4506565-F(A) and the primer rs4506565-R binds to the template DNA resulting a fragment with a theoretical size of 471 bp. In the determination of T allele, the primer pair rs4506565-F and the primer rs4506565-R(T) binds to the template DNA resulting in a fragment with a theoretical size of 271 bp.

The ability of the designed primer to amplify the desired area can be tested by a PCR reaction. The principle of the PCR reaction is to multiply the DNA sequence exponentially using a specific primer, then electrophoresed to identify the DNA band [17]. ARMS PCR is a PCR method that can be used to detect mutations or polymorphisms in DNA. In the ARMS PCR method, three primers are used; one of the primers will specifically identify the presence of an allele. The 3' end of the specific primer attaches to the mutated template DNA. Identification of the presence of alleles was determined based on the resulting PCR product [18].

Table 1 Primer Characteristics for Determining SNP rs4506565 of the *TCF7L2* gene

Primer	DNA Fold	Primer Characteristic
rs4506565-F	 5' CAGCATGGACTAAGGACTTTAC 3'	Length: 20 bp %GC: 50 Tm: 57,4 Tm hairpin and self-dimer: none
rs4506565-R	 5' TTCACCAGCACTGATCACT 3'	Length: 18 bp %GC: 50 Tm: 57,1 Tm hairpin and self-dimer: none
rs4506565-F(A)	 5'ATATGGCGACCGAAGTGATA 3'	Length: 22 bp %GC: 40,9 Tm: 55,2 Tm hairpin and self-dimer: none
rs4506565-R(T)	 5' CTTGACAAGGGCCCCAA 3'	Length: 18 pb %GC: 50 Tm: 55,1 Tm hairpin and self-dimer: none

At the time of PCR, it is necessary to determine the optimal conditions of examination, including the amount/ concentration of the mixture/ mix used and the optimal time and temperature when the reaction occurs. Each component of the PCR has an essential function in the PCR reaction.

The DNA obtained from the PCR reaction was electrophoresis on agarose gel to prove the DNA fragments size. Electrophoresis is an effective way to separate DNA fragments with sizes ranging from 100 base pairs to 25 kb. DNA molecules will be separated based on size, where the distance traveled will be inversely proportional to the weight of the DNA [19]. In this study, 1.5% agarose was used. To determine the location of the DNA on the agarose gel, red-safe was added to visualize the DNA when it was placed on the Doc gel.

The mutated sample will produce two fragments with different sizes, while the normal sample will only produce one fragment beside the control fragment. The ARMS PCR method can be used to diagnose a disease caused by mutations in a gene [17]. Visualization of the electrophoresis results of PCR products using primer pairs can be seen in Figure 2, where the determination of the A allele presence will obtain fragments with sizes of 650 bp and 417 bp, while the presence of the T allele is characterized by a 650 bp and 271 bp fragment. The size of the fragments obtained in this study is close to the theoretical size of the ARMS PCR product.

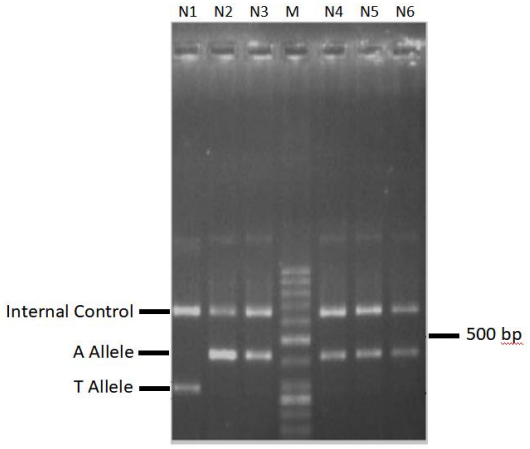


Figure 2 Visualization of ARMS-PCR assay for A and T allele rs4506565 identification. PCR product were visualized using agarose gel electrophoresis 1.5%; N1, N2, N3, N4, N5, N6 = sample code for normal controls; M= DNA Marker. N1 has TT Genotype, N2-N6 have AA genotype.

The data above shows that the optimized ARMS PCR reaction can be used to identify the presence of the SNP rs4506565 in the *TCF7L2* gene. In addition, several samples were sequenced to ensure the accuracy of the ARMS PCR method (Figure 3). The sequencing results showed that the correct amplified gene *TCF7L2* and primer were attached to the desired region.

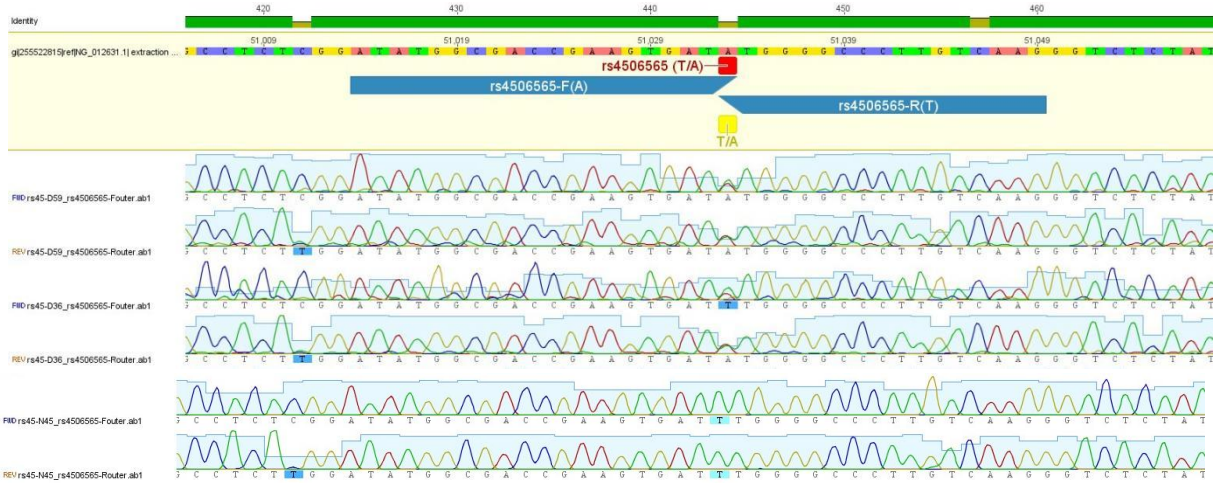


Figure 3 Sequencing results of samples with genotype AA, AT, and TT

Based on this study, the designed primer can be used to determine the presence of SNP rs4506565 in the sample. The association between the presence of SNPs and the risk of T2DM can also be determined. The association between SNPs and T2DM was determined by comparing the genotype frequencies of SNPs in normal and T2DM subjects. So, if there is an association, the SNP can be used as a marker for an early detection of T2DM. An early T2DM detection is expected to reduce complications that occur in patients.

SNP rs4506565 were located in intron region between exon 4 and 5. In eukaryotes, intron-non coding portions of the sequence- must be removed and exon -protein coding part- should join to produce a mature RNA that can be translated into protein. The exact mechanisms by which a genetic variation within the intron of *TCF7L2* gene confers susceptibility of T2DM remain to be elucidated. Genetic variations in intron region would affect the action of *TCF7L2* -alter gene expression- through the regulation of alternative splicing. Alternative splicing markedly affects human development, and its miss-regulation underlies many human diseases. In this process, particular exons of *TCF7L2* gene may be included within or excluded from the final, that give rise to different protein isoforms in different tissues or disease condition [8], [20], [21].

4. CONCLUSION

Based on the study results, it can be concluded that four primers have been successfully constructed, namely rs4506565-F, rs4506565-R, rs4506565-F(A) as a specific primer for the A allele, and rs4506565- R(T) to detect the T allele. The primer was able to identify the presence of the SNP rs4506565 of the *TCF7L2* gene using the ARMS PCR method and identify the association between variant *TCF7L2* gene SNP rs4506565 with T2DM as a preventive tool of T2DM in other ethnic.

ACKNOWLEDGMENT

The authors would like to thank all who were involved and contributed to this research.

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