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Research Article
Universal Primers for Amplification of TNF-α -308 Promoter Region

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Abstract

Background and Objective: Genetic variation in the form of a single nucleotide polymorphisms (SNPs) in the tumor necrosis factor alpha (TNF-α) promoter region is known to influence the regulation of TNF-α production, transcription and translation and has been linked to several diseases. Primer sequences that amplify DNA flanking the -308 sequence are not universal, therefore, research on SNP conducted in this area still uses different primer pairs. The purpose of this research was to design and optimize universal primers to amplify DNA sequences covering the TNF-α -308 promoter area for other researchers to study the presence of SNPs in the -308 nucleotide and beyond. **Materials and Methods:** The peripheral blood samples for DNA preparation were obtained from 3 participants. The DNAs were extracted using available commercial kit. The candidate of universal primers were designed using BLAST and Primer3 softwares. Amplification of DNA region flanked by the designed primer pairs was performed using PCR method using available commercial kit. **Results:** The study showed that there were significant differences between the 5 primary pairs studied. From the 5 pairs of primers, the TNF-α 1 primer pair (TNF-α 1F: AACCAGCATTATGAGTCTC and TNF-α 1R: AACAACTGCCTTTATATGTC) and the TNF-α 2 primer pair (TNF-α 2F: TGAAACCAGCATTATGAGT and TNF-α 2R: AACAACTGCCTTTATATGTC) produced single, distinct, sharp and thick bands. **Conclusion:** From this study it can be concluded that TNF-α 1 and TNF-α 2 primer pairs have the potential to be used as universal primers to study the SNPs in the TNF-α -308 promoter region.

Key words: TNF-α, tumor necrosis factor alpha, -308, promoter, single nucleotide polymorphism, SNP

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tumor necrosis factor alpha (TNF-α) was a cytokine protein produced in the body by many different types of cells[1]. This protein plays a role in the body's immune functions such as anti-tumor, anti-microbial activity and mediates inflammation[2]. Some researches have shown that there were genetic variations in the form of single nucleotide polymorphisms (SNPs) in the promoter region[3], which regulates TNF-α production, transcription, as well as affects its reaction related to disease[4]. The SNPs in the TNF-α promoter sequence were found at -308 (G/A), -238 (A/G), -857 (C/T), -1031 (T/C) and -1376 (T/C)[5].

Some studies related to the polymorphism of the TNF-α gene promoter -308 had been carried out to see its relation to various diseases[6,7]. Polymorphisms at this position greatly influenced the number of TNF-α expression. Some diseases associated with polymorphism -308 were stroke[5], liver cell carcinoma[8], kidney[9] and diabetes[10].

One of the method that facilitate the analysis of the SNPs is polymerase chain reaction (PCR)[11]. This method requires short DNA sequences called the primers, which function to start the polymerization stage[12]. In order for amplification to work properly and produce good results, the primer sequences were amongst the important factors needed to be considered[13]. In analyzing polymorphism in -308, the primers used varied greatly[8,10]. The primer sequences were generally made through softwares and tested directly in the amplification process[13]. However, if the designed primers were inaccurate and not optimized properly, then DNA sequences generated by PCR cannot be compared accurately (less efficiently). It may also lead to mispriming and produce several unwanted PCR products. There has been lack of information regarding universal primers to amplify DNA sequences in the -308 region. Therefor, the aim of this research was to design and optimize universal primers for TNF-α promoter region which can be used as universal primers for the detection of SNP in -308.s

MATERIALS AND METHODS

Primer design: The design of universal primers in TNF-α -308 promoter region was carried out *in silico*. This stage began with downloading TNF-α gene DNA sequences from GenBank. Then, the universal primer candidates were designed using BLAST and Primer3 softwares. The primers design was carried out following the procedure provided by the softwares. General characteristics such as melting temperature, percentage of guanine and cytosine (GC) and nucleotide length of candidates for universal primer were adjusted. Melting temperature was set at a range of 52-58°C, the percentage of guanine and cytosine in the range of 40-60% and the length of nucleotides in the range of 15-30 nucleotides[13]. Five primer pairs were generated for the purpose of this study. Primer sequences were synthesized by a provider of synthetic primers manufacturing services.

DNA sample preparation: The peripheral blood from three humans were used as samples. About 1 mL of the blood were put into anticoagulant tubes containing ethylenediaminetetraacetic acid (EDTA). The DNAs from the blood samples were extracted using Genomic Mini Kit (Geneaid). The extraction procedure was carried out in accordance with the instructions. The isolated DNA was stored in the refrigerator until used.

DNA amplification: For each primer, 3 DNA samples from three different persons were used, resulting in 15 reactions. MyTaq™ HS Red Mix (Bioline) polymerase chain reaction (PCR) kit was used to amplify the target regions. The following protocol was for a standard 50 µL reaction: 200 ng of DNA, 1 µL of primer (20 µM each), 25 µL 2x MyTaq HS Red Mix and the volume was adjusted to 50 µl by adding dH_20. All of the PCR reaction samples were amplified with a thermo-cycler machine with different temperature settings at each stage. The initial denaturation stage was carried out at 95°C for 2 min, followed by 34 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at temperature set by primer design and elongation for 1 min at 72°C, followed by final elongation for 1 min at 72°C. The amplification results were elecrophorized on 1% agarose gel and photographed.

RESULTS AND DISCUSSION

Primer design: The primer pairs were design using BLAST and Primer3 softwares based on TNF-α gene promoter sequences retrieved from GenBank. Five candidates universal primer pairs generated using these softwares are presented in Table 1. These primer pairs were optimized to evaluate their efficiency and accuracy using PCR method to amplify the TNF-α promoter region.

Optimization of designed primers: The results of DNA optimization were visualized by electrophoresis using 1% agarose gel. In the visualization of the amplification results using 5 primers, there are 2 primer pairs (TNF-α 1 and TNF-α 2) that showed single, clear and sharp

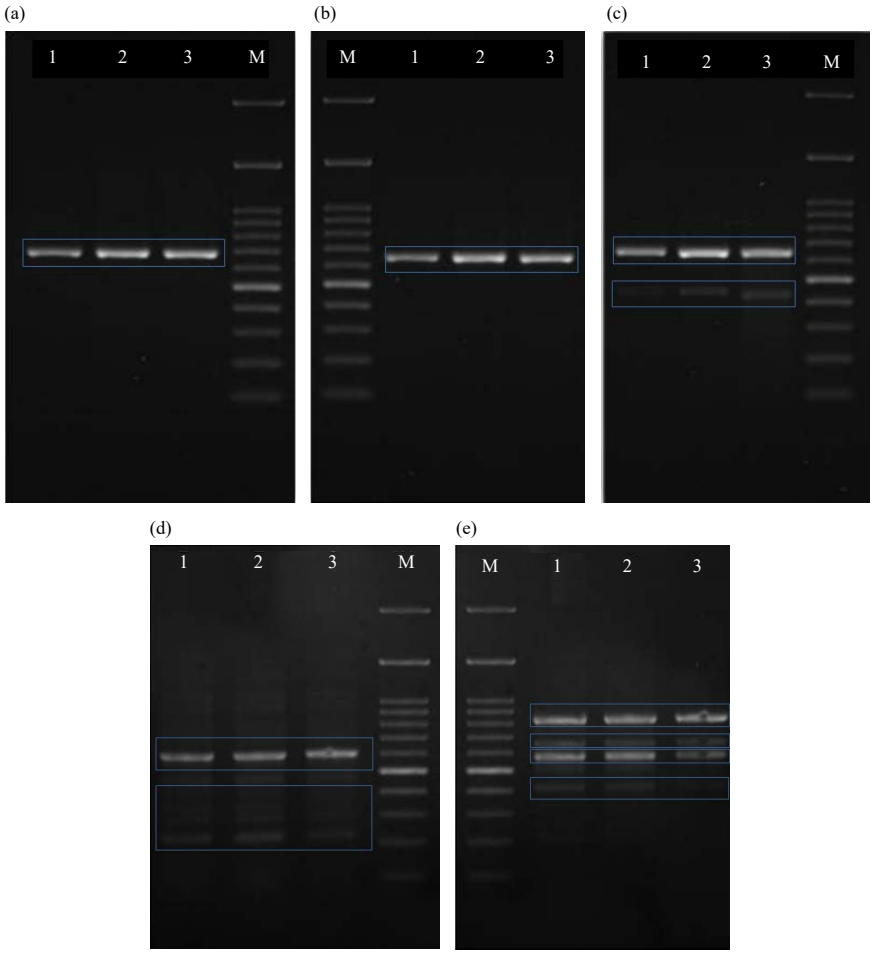

Fig. 1(a-e): Agarose gel visualization of PCR amplification resulted from optimization of the designed primers, (a) Primer TNF-α 1, (b) Primer TNF-α 2, (c) TNF-α 3, (d) TNF-α 4 and (e) TNF-α 5

bands that showed single, clear and sharp bands and did not form multiple bands (Fig. 1). The length of the visualized band corresponds to the length of the band produced by the primer based on the primers designed *in silico*. The amplification results using primer pairs TNF-α 3, TNF-α 4 and TNF-α 5 did not generate single bands, but spread and multiple bands.

Primer design: Primers are short oligonucleotide molecules with define sequences complementary to the target DNA which function to limit the area to be amplified and serve as an extension point for the DNA polymerase[14]. The designed primers determine the success of amplification, therefore, the design of primer is an important aspect to obtain specific, effective and efficient PCR products. Primer annealing temperature must be specific for successful amplification. Five candidate universal primer pairs has been generated based on criteria suggested by Lorenz[13] using BLAST and Primer3 softwares (Table 1).

The forward primers are aimed to amplify in the forward direction and the reverse primer are aimed to amplify in the reverse direction[15]. These primers hybridize to specific locations on the target DNA sequence between the primer binding sites to be amplified[16]. The primer design reduce the complexity and time-consuming effort, particularly if generates a large number of hits[17].

Optimization of designed primers: The DNA samples used in the amplification process were derived from human peripheral blood. Blood samples still contain blood components that can interfere with the amplification process using PCR. The DNA extraction process was carried out to obtain pure DNA samples. The success of the primer design could be seen in the success of the initiation by the primer in the amplification process. If DNA is specifically amplified in the region flanked by the primers, it can be said that primer design and optimization are successful.

Table 1: Five candidate universal primer pairs of designed using BLAST and Primer3

Primer pairs	Sequences	Softwares	Annealing temperature (°C)
TNF-α 1			
Forward	AACCAGCATTATGAGTCTC	Primer3	51.12-51.97
Reverse	AACAACTGCCTTTATATGTC		
TNF-α 2			
Forward	TGAAACCAGCATTATGAGT	Primer3	51.35-51.72
Reverse	GGGAAAGAATCATTCAACC		
TNF-α 3			
Forward	CTGAAACCAGCATTATGAGT	BLAST	53.28-53.43
Reverse	GGGAAAGAATCATTCAACCA		
TNF-α 4			
Forward	ATGAAAGAAGAAGGCCTG	Primer3	51.25-51.70
Reverse	AAAGAATCATTCAACCAGC		
TNF-α 5			
Forward	ATTATGAGTCTCCGGGTCAG	Primer3	55.96-56.13
Reverse	GGGTCAGTATGTGAGAGGAA		

The result (Fig. 1) showed that primer pairs TNF-α 1 and TNF-α 2 were able to amplify the flanked DNA -308 TNF-α promoter region accurately, however primer pairs TNF-α 3,TNF-α 4 and TNF-α 5 showed multiple bands. This can be caused by several factors such as temperature, chemical reaction, nucleotide composition and position and type of nucleotide mismatch[18]. The temperature factor is considered not very influential on the results of amplification, because the primer annealing temperature has been adjusted to a good temperature range according to the theory. Another factor that can cause primer attachment errors (primer mispriming) is the repetition of dinucleotides which can increase primer entropy so that it causes non-specific primer annealing[19].

In addition to this, it can also be indicated that the 3 'end of the primer can make an error attaching to the wrong location and then polymerized by DNA polymerase to produce undesired PCR products[20]. Well-complemented primers at end position 3' can initiate the amplification process well[21,22]. Amplification using TNF-α 3, TNF-α 4 and TNF-α 5 primer pairs still needs other optimization steps such as hot start and touch down. This process can improve the accuracy of the primer attachment to the right area to produce the desired PCR products. However, this process requires additional time and cannot be ensured to produce the right PCR product. Amplification using TNF-α 1 and TNF-α 2 primers does not need further optimization because the original visualization has shown that the primer pairs were able to amplify the TNF-α promoter region well and produce the desired products in accordance with the design results. This finding will provide convenience for researchers conducting SNPs research in TNF-α promoter region, especially SNP in -308.

CONCLUSION

Based on the results of the design and optimization of the primers carried out on 5 pairs of primers, 2 pairs of primers are recommended to be used as universal primers to amplify the TNF-α -308 promoter region: TNF-α 1 (TNF-α 1F: AACCAGCATT ATGAGTCTC dan TNF-α 1R: AACAACTGCCTTTATATGTC) and primer TNF-α 2 (TNF-α 2F: TGAAACCAGCATTATGAGT and TNF-α 2R: GGGAAAGAATCATTCAACC).

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