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Detection of the NFKB1 Gene in Patients with Type 2 Diabetes Mellitus and Non-Diabetic Individuals Using the Polymerase Chain Reaction Method

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Abstract

Type 2 diabetes mellitus is a metabolic condition characterized by elevated blood sugar levels resulting from reduced insulin release by β pancreatic cells. The primary form of NF-kB is Nuclear Factor kappa Beta subunit-1 (NFKB1), a gene that encodes the DNA-binding protein (p50). The NFKB1 gene contributes to oxidative stress and mild inflammatory processes, which may exacerbate diabetes. The Polymerase Chain Reaction (PCR), an enzymatic procedure, amplifies a nucleotide sequence to identify whether patients with type 2 diabetes mellitus or non-diabetic individuals possess the NFKB1 gene. This study aimed to detect the presence of the NFKB1 gene in patients with type 2 diabetes mellitus and non-diabetic individuals. The study employed a descriptive research method with a purposive sampling technique conducted at the molecular biology laboratory of Sekolah Tinggi Ilmu Kesehatan Nasional. The respondents included 9 patients with type 2 diabetes mellitus who were participants of the *Prolanis* (Chronic Disease Management Program) at Puskesmas Wonosari I Klaten and 9 non-diabetic individuals from the Family Welfare Empowerment (*PKK*) of Kadilangu Village, Baki District, Sukoharjo Regency, Central Java Province. The results revealed that the NFKB1 gene was detected after electrophoresis and visualized at 176 bp (base pairs). While the qualitative presence of the NFKB1 gene in DNA was confirmed, the level of gene expression in terms of transcription and translation remained unknown.

Keywords: Type 2 Diabetes Mellitus, NFKB1 Gene, PCR

Introduction

Diabetes mellitus is a group of metabolic disorders characterized by increased levels of glucose in the blood (hyperglycemia) and an insufficiency in the production or action of insulin produced by the pancreas in the body (Asmat, Abad, & Ismail, 2016). Diabetes is classified into four types (Care & Suppl, 2019), with three main types: Type 1 diabetes mellitus, Type 2 diabetes mellitus, and gestational diabetes. Type 2 diabetes mellitus accounts for approximately 90% of cases (Cole & Florez, 2022).

Multifactorial polygenic diabetes mellitus (Type 2 diabetes) is believed to arise from the interplay of multiple genes and environmental factors (Rheinheimer et al., 2017). Insulin resistance and beta cell dysfunction are linked to inflammation in Type 2 diabetes mellitus, expanding the scope of immune metabolism (Ali et al., 2022). In Type 2 diabetes, high blood sugar initially results from body cells being unable to respond optimally to insulin, a condition known as insulin resistance. When it develops, the hormone becomes less effective, leading to increased insulin production. Over time, the production may decrease as pancreatic beta cells fail to meet the body's demands (Webber, 2021).

The regulation of several genes encoding mediators of inflammation, the cell cycle, apoptosis, viral replication, and various autoimmune illnesses depends on the Nuclear Factor Kappa Beta (NF-kB) (Behera et al.,

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2020). Five members of the NF-kB family have been identified: NFKB1 (p105/p50), NFKB2 (p100/p52), RelA (NFKB3/p65), RelB, and c-Rel (Sun & Zhang, 2007). The NFKB1 gene, mapped at 4q23-q24, consists of 24 exons. Nuclear Factor Kappa Beta subunit-1 (NFKB1) is a protein-coding gene and is the primary form of NF-kB. This gene encodes a 105 kD protein, which undergoes cotranslational processing by the 26S proteasome to produce a 50 kD protein. The 105 kD protein functions as a specific transcription inhibitor of the Rel protein, while the 50 kD protein serves as the DNA-binding subunit of the NF-kB protein complex (Gene, Factor, & Subunit, 2023). According to Raza et al. (2022), the NFKB1 gene is implicated in mild inflammation and oxidative stress processes, which may contribute to complications in diabetic conditions, such as diabetic nephropathy in Type 2 diabetes mellitus (Oguntibeju, 2019). Polymorphisms in NFKB1 are associated with long-standing inflammatory diseases resulting from diabetes complications. Studies conducted by Gautam et al. (2017) indicate that NFKB1 polymorphism (rs28362491) serves as a genetic marker for developing diabetic nephropathy in patients with Type 2 diabetes mellitus. As a risk factor for diabetic nephropathy in Type 2 diabetes mellitus, the NFKB1 gene is activated in hyperglycemic conditions, where excess glucose in the blood damages the small blood capillaries in the kidneys (Yuniarti et al., 2021). Long-term hyperglycemia activates the NFKB1 gene, causing oxidative stress and inflammatory reactions that trigger the release of proinflammatory cytokines, such as Tumor Necrosis Factor-alpha (TNF-α). Inflammatory reactions and excessive cytokine release can affect glomerular capillaries and kidney function (Jin et al., 2023).

One method for replicating an organism's DNA is the Polymerase Chain Reaction (PCR). This method has been widely used, particularly in diagnostics requiring high sensitivity and specificity. It has diverse applications, such as gene cloning, DNA computation, genetic fingerprinting, diagnosing infectious diseases, and detecting genetically determined conditions (Eling et al., 2014).

The present research participants included patients with Type 2 diabetes mellitus and non-diabetic individuals. Type 2 diabetes patients were members of the *Prolanis* (Chronic Disease Management Program) diabetes program at Puskesmas Wonosari I, aged 30–60 years. Puskesmas Wonosari I is a health center located at Jl. Pakis - Daleman, Dusun I, Bentangan Village, Wonosari Disctrict, Klaten Regency, Central Java Province. Non-diabetic participants were selected from members of the Family Welfare Empowerment (*PKK*) of Kadilangu Village, RT 2/RW 1, Baki District, Sukoharjo Regency, who had no history of diabetes.

Based on the above background, this study aimed to investigate the presence of the NFKB1 gene in patients with Type 2 diabetes mellitus and non-diabetic individuals.

Research Methods

Ethical clearance

This study passed the ethical review of the Research Ethics Committee of Universitas Muhammadiyah Purwokerto (*KEPK-UMP*) under the registration number KEPK/UMP/57/VIII/2023.

Tools and materials

a. Tools

The tools utilized in this research were tourniquet, holder, micropipette, centrifuge tube, microcentrifuge, spin-down, vortex, microwave, Erlenmeyer flask, tube racks, scales, spatula, ultra-low temperature freezer, dry bath, screw-cap tube, centrifuge tube rack, casting tray, cryogenic box, PCR tube, refrigerated centrifuge, PCR thermal cycler, UV-Vis spectrophotometer, bowl, monitor, CPU, electrophoresis chamber, and gel documentation system.

b. Materials

The materials used in this research were 70% alcohol, cotton, EDTA vacuum tube, EDTA blood sample, yellow tip, blue

tip, collection tube, GS column, aluminum foil, plastic clip, filter paper, distilled water (*aquabidest*), PBS (Phosphate-Buffered Saline), wash buffer, elution buffer, Proteinase K, GSB buffer, absolute ethanol (96-100%), buffer W1, TE buffer, 1× TBE (Tris-Borate-EDTA), DNA ladder, GelRed, loading dye, agarose powder, master mix, nuclease-free water (NFW), forward primer 5'-GCTGCTGTCATCTGTTGGAA-3', and reverse primer 5'-CAATGCTTCAGGGATTTGGT-3' (NCBI, June 24, 2023).

Procedure

a. Sample Preparation

A blood sample of 200 μl was inserted into a 1.5 ml microcentrifuge tube, followed by the addition of 200 μl of Phosphate Buffered Saline (PBS). The mixture was homogenized. Next, 20 μl of proteinase K was added, homogenized using a vortex, and incubated at 60°C for 5 minutes (Geneaid Kit).

b. Isolation of DNA

200 μl of GSB buffer was added to the sample, homogenized by vortexing, and incubated at 60°C for 5 minutes, with the tube inverted every 2 minutes. Subsequently, the sample was promptly vortexed for 10 seconds and spun down. In the DNA binding stage, 200 μl of absolute ethanol (96–100%) was added to the sample, and the mixture was transferred to a GS column fitted into a collection tube using a pipette. The sample was centrifuged at 14,000 × g for 1 minute. The GS column was then placed in a new collection tube after the flow-through from the previous tube was discarded. 400 μl of buffer W1 was added to the GS column, and the sample was centrifuged at 14,000 × g for 30 seconds. The liquid was discarded, and the GS column was reattached to the collection tube. In the next step, 600 μl of wash buffer (with absolute ethanol added) was introduced into the GS column and centrifuged at 14,000 × g for 30 seconds. The liquid was discarded, and the GS column was reattached to the collection tube. The column matrix was dried by centrifugation for 3 minutes at 14,000 × g. Afterward, the dried GS column was transferred to a clean 1.5 ml microcentrifuge tube. 100 μl of pre-heated elution buffer was added to the center of the GS column matrix, and the mixture was allowed to sit at room temperature (15°–25°C) for 3 minutes to ensure complete absorption. Finally, the mixture was centrifuged for 30 seconds at 14,000 × g to extract pure DNA. The resulting DNA isolate was saved for further analysis (Geneaid Kit).

c. DNA Qualitative Test

Agarose powder was placed into an Erlenmeyer flask, and 50 ml of 10× TBE buffer was added. The mixture was heated in a microwave at medium-high power for 2 minutes, with homogenization every minute, until fully dissolved. The clear agarose solution was poured into an agarose casting tray and cooled to room temperature. Once solidified, the comb was carefully removed, and the gel was set up in an electrophoresis device (Maftuchah, Winaya, & Zainudin, 2014). For DNA electrophoresis, 10 μl of sample (composed of 5 μl DNA isolate, 3 μl loading dye, and 2 μl gel red) was loaded into the wells. The cables were connected to a power source, ensuring the magnetic field was properly aligned. The voltage and running time were set to 90 volts for 30 minutes (Maftuchah, Winaya, & Zainudin, 2014).

d. DNA Quantitative Test

20 μl of DNA isolate was pipetted and diluted with 3,980 μl of distilled water, then homogenized by vortexing for 15 seconds. The absorbance was measured using a UV-Vis spectrophotometer at 260 nm and 280 nm wavelengths. 4 ml of

distilled water was used as a blank (cuvette I), and 4 ml of the diluted DNA sample was placed in cuvette II. The results of the DNA quantitative test were calculated using the following formulas:

- Concentration of DNA:
  λ260 x 50 ng/μl x dilution factor
- Purity of DNA:
  $\frac{\lambda 260}{\lambda 280}$

e. Amplification of the NFKB1 Gene

For PCR, 12 μl of master mix, 2 μl of forward primer, 2 μl of reverse primer, 5 μl of DNA template, and 4 μl of nuclease-free water were pipetted into a sample tube. The PCR program was set with the following parameters: pre-denaturation at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing at 61.1°C for 30 seconds, extension at 72°C for 1 minute, final extension at 72°C for 5 minutes, and a final hold at 4°C. The primers used for this PCR examination were Forward primer: 5'-GCTGCTGTCATCTGTTGGAA-3' and Reverse primer: 5'-CAATGCTTCAGGGATTTGGT-3' (NCBI, June 24, 2023).

Research Results and Discussion

A. Results

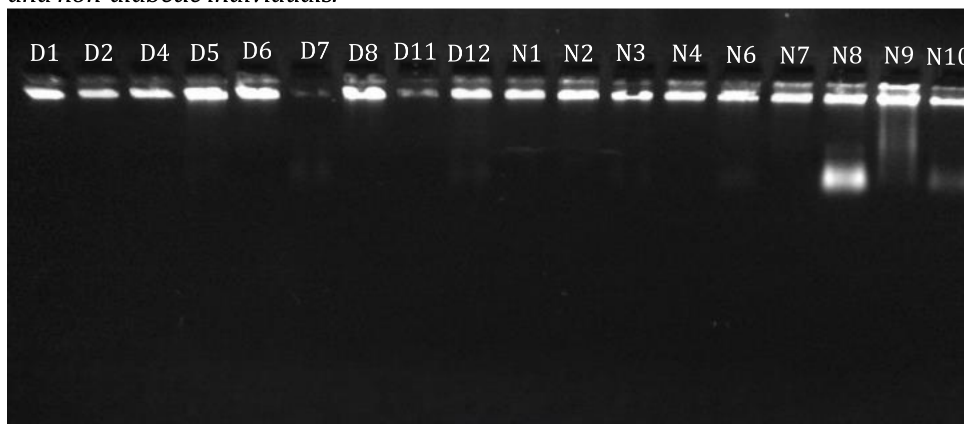
The detection results of the NFKB1 gene included 9 samples from patients with Type 2 diabetes mellitus at the *Prolanis* (Chronic Disease Management Program) at Puskesmas Wonosari I Klaten, as well as 9 samples from non-diabetic individuals from the Family Welfare Empowerment (*PKK*) of Kadilangu Village, Baki District, Sukoharjo Regency. This analysis was conducted at the Molecular Biology Laboratory of Sekolah Tinggi Ilmu Kesehatan Nasional. Following the isolation phase, qualitative and quantitative tests were performed, yielding the following results:

1. DNA Qualitative Test

The results of DNA isolation, visualized using gel electrophoresis and a gel documentation system, are shown in Figure 1. All samples were successfully extracted.

Figure 1

Qualitative test results of DNA isolates from blood samples of patients with Type 2 diabetes mellitus and non-diabetic individuals.



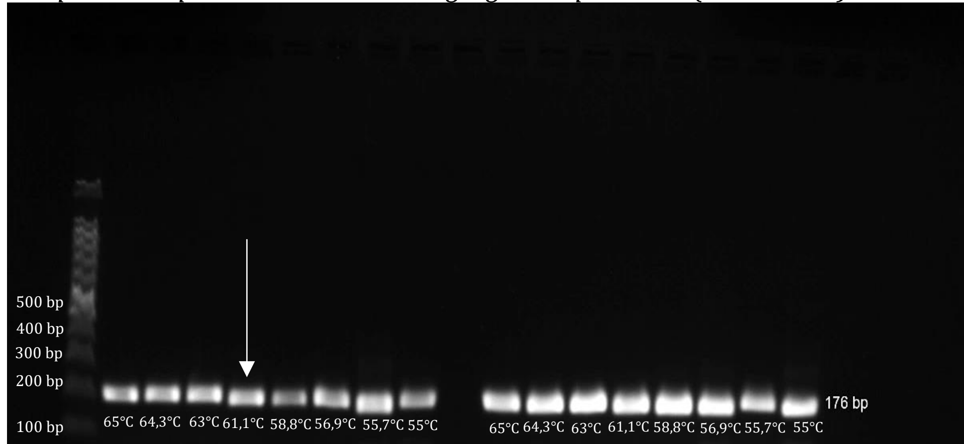
Description:
D1 – D12: DM Samples
N1 – N10: Non-DM Samples

2. Temperature Optimization

The temperature optimization results for NFKB1 gene amplification indicated that the optimal annealing temperature was 61.1°C, with successful amplification at 176 bp. Temperature selection was based on the sharpness and clarity of the bands, as well as the absence of double bands.

Figure 2

Temperature optimization results using eight temperatures (55°C – 65°C).

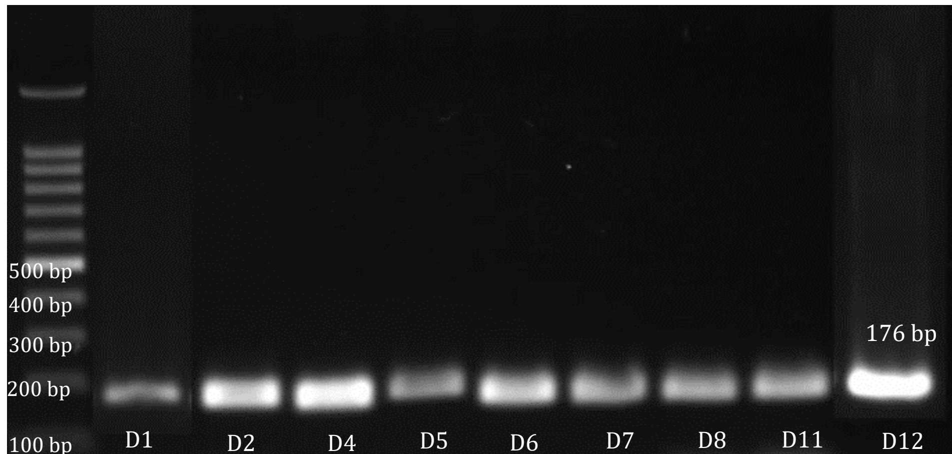


3. PCR visualization of the NFKB1 gene in patients with Type 2 diabetes mellitus

Figure 3 shows the PCR visualization results of the NFKB1 gene in Type 2 diabetes mellitus patients at Puskesmas Wonosari I Klaten. The gene was successfully amplified and visualized at the target size of 176 bp.

Figure 3

PCR visualization of the NFKB1 gene in Type 2 diabetes mellitus patients at Puskesmas Wonosari I Klaten.

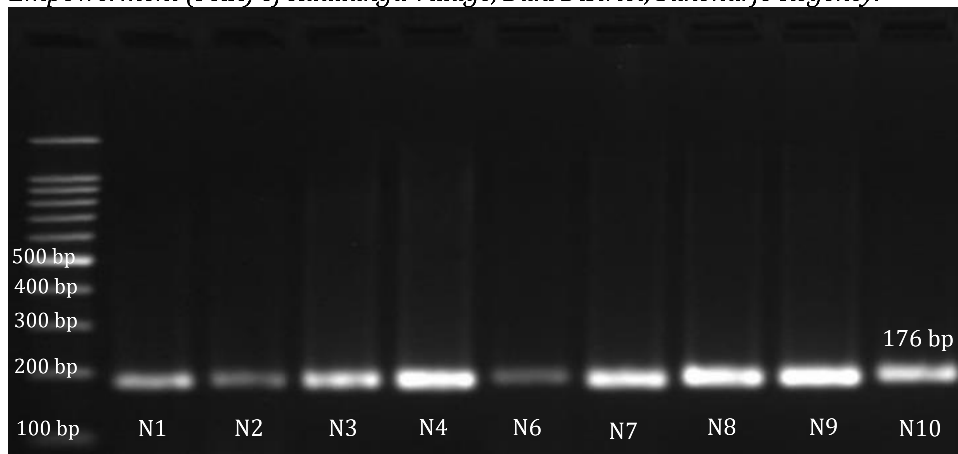


4. **PCR visualization of the NFKB1 gene in non-diabetic individuals**

Figure 4 presents the PCR visualization results of the NFKB1 gene in non-diabetic individuals from the Family Welfare Empowerment (PKK) of Kadilangu Village, Baki District, Sukoharjo Regency. The gene was successfully amplified and visualized at the target size of 176 bp.

Figure 4

PCR visualization results of the NFKB1 gene in non-diabetic individuals from the Family Welfare Empowerment (PKK) of Kadilangu Village, Baki District, Sukoharjo Regency.



B. Discussion

The present study aimed to ascertain whether the NFKB1 gene was present in nine samples of Type 2 diabetes mellitus patients from the *Prolanis* (Chronic Disease Management Program) at Puskesmas Wonosari I Klaten and nine non-diabetic individuals from the members of the Family Welfare Empowerment (*PKK*) of Kadilangu Village, Baki District, Sukoharjo Regency, visualized at 176 bp.

In Figure 1, which presents the DNA qualitative test from the isolation results, the visualization of bands with varying thicknesses could be observed. According to Wijaya, Darmawati, and Kartika (2018), the quantity of DNA successfully isolated impacts the thickness of the DNA bands. The more DNA that is isolated, the thicker the DNA band appears; conversely, the less DNA isolated, the thinner the band visualized on the agarose gel. In Figure 1, samples with the codes N8, N9, and N10 displayed thick DNA bands accompanied by excess bands and smears (impurities below the DNA band). These impurities might result from residual contaminants, such as proteins or other compounds, remaining from the isolation process (Iqbal, Buwono, & Kurniawati, 2016). A smear refers to a DNA band appearing as a stain or a faded, longitudinal pattern on an electrophoretic gel (Rahayu, Saryono, & Nugroho, 2015). Excess bands, which are additional bands visible on electrophoresis, are usually caused by contamination during isolation or by the degradation of DNA or RNA samples, which can produce fragments or pieces visible as extra bands (Iqbal, Buwono, & Kurniawati, 2016).

The annealing temperature, which affects the stability of DNA hydrogen bonds and sequence specificity, is a

critical parameter for the success of PCR amplification (Fraige, Travensolo, & Carrilho, 2013). The annealing temperature for each primer pair was determined using the melting temperature (Tm) provided by the supplier as part of the primer synthesis process (Aulia et al., 2023). For this study, the Tm of the forward primer was 55.5°C, and the reverse primer was 53.3°C (as indicated in the primer sheet). Following the Tm calculation, PCR temperature optimization was performed using eight temperature settings within the range of 55°C to 65°C. The electrophoretic visualization results (see Figure 2) showed that the primer amplified the DNA effectively, with DNA bands visualized at the expected 176 bp target. A temperature of 61.1°C was selected based on the clarity and firmness of the bands, as well as the absence of double bands. After determining the optimal temperature, PCR amplification was conducted with the following parameters: pre-denaturation at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, annealing at 61.1°C for 30 seconds, and extension at 72°C for 5 minutes. This process successfully amplified the NFKB1 gene in both Type 2 diabetes mellitus samples (see Figure 3) and non-diabetic samples (see Figure 4), with DNA bands visualized at the expected 176 bp target.

In Type 2 diabetes mellitus, increased blood sugar levels (hyperglycemia) result from the body's inability to respond properly to insulin, a condition known as insulin resistance. In this state, the hormone becomes ineffective, prompting increased insulin production. Over time, insulin production may become insufficient as pancreatic beta cells fail to meet the demand. A lack of insulin or an inability of cells to respond to it leads to hyperglycemia, a clinical indicator of diabetes (Tiurma & Syahrizal, 2021). Uncontrolled hyperglycemia in Type 2 diabetes mellitus can trigger chronic complications, such as microvascular and macrovascular diseases. Microvascular complications, such as diabetic nephropathy, are closely associated with uncontrolled inflammation and hyperglycemia, potentially leading to kidney failure (Satria, Decroli, & Afriwardi, 2018).

The Nuclear Factor kappa Beta subunit-1 (NFKB1) gene is a protein-coding gene that serves as the main form of NF-kB. Mapped at 4q24, the NFKB1 gene encodes the DNA-binding protein (p50) with a molecular weight of 50 kDa, which plays a key role as a regulator of inflammation (Yang et al., 2014). The NFKB1 gene is associated with oxidative stress and mild inflammatory processes, which may exacerbate diabetes. According to Raza et al. (2022), in Type 2 diabetes mellitus, the NFKB1 gene is downregulated or expressed at low levels. In contrast, in non-diabetic individuals, it is upregulated or expressed at high levels.

In the present study context, low expression of the NFKB1 gene in patients with Type 2 diabetes mellitus might result from elevated blood glucose levels, contributing to oxidative stress and inflammation. These factors could disrupt gene transcription, leading to reduced gene expression. In contrast, the NFKB1 gene was highly expressed in non-diabetic individuals, where it played a role in activating mild inflammatory signaling pathways as part of the immune response. However, the specific mechanisms causing decreased or increased NFKB1 gene expression in Type 2 diabetes mellitus and non-diabetic controls were still under investigation. Gene expression, a process involving gene transcription and mRNA production, ultimately affects protein synthesis (Mitsis et al., 2020). In this study, gene expression was quantitatively analyzed using Real-Time PCR.

Conclusion

Based on the research results, it can be concluded that the detection of the NFKB1 gene in nine blood samples from patients with Type 2 diabetes mellitus and nine non-diabetic individuals successfully identified the presence of the NFKB1 gene, visualized at 176 bp.

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