



Detection of ATPase 6 Gene Mutations in Mitochondrial DNA of Type 2 Diabetes Mellitus Patients: A PCR–Sequencing Study

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Abstract

Mitochondrial diabetes is a form of diabetes caused by mutations in mitochondrial DNA (mtDNA). These mutations impair oxidative phosphorylation (OXPHOS) and ATP synthesis, which reduces glucose-stimulated insulin secretion. Because its clinical features overlap with those of type 2 diabetes mellitus (T2DM), mitochondrial diabetes is often diagnosed as T2DM. The G9053A mutation in the ATPase 6 gene is a nonsynonymous mtDNA variant that has been predicted to affect ATP synthase function. In this study, a fragment of the ATPase 6 gene was amplified by the Polymerase Chain Reaction (PCR) and analyzed by Sanger sequencing. In four blood samples from T2DM patients, the G9053A point mutation (guanine to adenine) was detected in samples B21 and B22. Sample B36 carried a cytosine to thymine substitution at position 8970 (C8970T), a synonymous change at codon 148 (serine). No mutation was detected in sample B43. G9053A was the predominant variant in this small group, and both carriers had a maternal history of T2DM. A larger study with non-diabetic controls is needed to determine whether G9053A is associated with mitochondrial diabetes.

1. Introduction

Mitochondrial diabetes is a form of diabetes caused by mutations in mitochondrial DNA (mtDNA). It is often misdiagnosed as type 2 diabetes mellitus (T2DM) because the clinical features overlap. These mutations may disrupt the oxidative phosphorylation (OXPHOS) that pancreatic β -cells need for insulin secretion in response to glucose [1, 2]. T2DM is characterized by chronic hyperglycemia resulting from insulin resistance and insufficient insulin secretion [2]. Impaired mitochondrial OXPHOS has also been linked to this condition [3].

Mitochondria are responsible for most cellular ATP production through oxidative phosphorylation. mtDNA is prone to mutation, and mtDNA mutations can impair this process [4]. The m.9053G>A variant in the MT-ATP6 gene results in a serine-to-asparagine substitution at position 176 (p.Ser176Asn). Previous bioinformatics analysis has suggested that this substitution may affect

ATP synthase function; however, its clinical significance remains uncertain and requires further experimental validation [3].

Mutations in mtDNA can be identified by amplifying the target region with the Polymerase Chain Reaction (PCR) and sequencing the product. PCR works by repeatedly duplicating specific DNA fragments with the help of polymerase enzymes at high temperatures [5]. In this study, a fragment of the mitochondrial ATPase 6 gene was screened for mutations in blood samples from T2DM patients. The PCR products were examined by agarose gel electrophoresis and then sequenced to obtain the nucleotide sequence of the ATPase 6 gene.

This study aims to confirm the presence of the G9053A point mutation in blood samples from T2DM patients using PCR and Sanger sequencing. The results provide preliminary data for evaluating G9053A as a

candidate marker of mitochondrial diabetes in T2DM patients.

2. Experimental

The research was conducted at the Central Laboratory, Biomolecular Section, Padjadjaran University, using the Polymerase Chain Reaction (PCR) method, an in vitro technique for DNA amplification [5]. The PCR products were visualized by agarose gel electrophoresis and sequenced to identify variants against the reference sequence [6].

This study involving human blood samples was approved by the Research Ethics Committee of Universitas Padjadjaran under Ethical Approval Number 533/UN6.KEP/EC/2022 (Registration Number: 2201050098). Written informed consent was obtained from all patients prior to sample collection.

2.1. Materials and Equipment

The materials used in this research included blood samples from T2DM patients obtained from Dustira Cimahi Hospital; Nuclease-Free Water (NFW); ATPase 6 gene forward primer (5'-AGGCACACCTACACCCCTTA-3') at 20 pMol/μL; ATPase 6 gene reverse primer (5'-AGGCGACAGCGATTCTTAGG-3') at 20 pMol/μL; Tris-HCl pH 8.5 and pH 9; 5% PEG solution (Sigma Aldrich, analytical grade); glacial acetic acid; disodium EDTA (analytical grade); agarose; GoTaq® Green Master Mix (Promega); SYBR Safe DNA Gel Stain; and a 100 bp GeneRuler DNA ladder. The equipment used included a Polymerase Chain Reaction (PCR) system, electrophoresis apparatus (gel chamber, gel matrix, and power supply; Bio-Rad Mini-Sub Cell Horizontal Electrophoresis and PowerPac Basic Supply), and a Sanger dideoxy sequencing service.

2.2. Sample Selection

Four blood samples from T2DM patients were selected from a group previously screened by qPCR for the G9053A mutation in the ATPase 6 gene. The quantification cycle (Cq) in the FAM channel was used as an indicator of the mutant allele. Two samples with low FAM Cq values and two samples with high FAM Cq values were selected for sequencing.

2.3. Primer Design

Primer design was performed by downloading the ATPase 6 gene sequence (base pairs 8527-9207) from the NCBI database. Forward and reverse primers were selected based on standard primer design criteria, and the resulting sequences were validated using the Primer-BLAST tool. Primers that met all requirements were synthesized by Macrogen.

2.4. Optimization of PCR Conditions

The annealing temperature for the ATPase 6 primers was optimized by gradient PCR over a range of 55°C to 60°C. The PCR conditions consisted of an initial denaturation at 95°C for 5 minutes, followed by 38 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C

for 30 seconds, and extension at 72°C for 1 minute. A final extension was carried out at 72°C for 10 minutes.

2.5. Amplification of the ATPase 6 Gene Using the PCR Method

The amplification was carried out in 0.2 mL Eppendorf tubes containing 0.6 μL forward primer, 0.6 μL reverse primer, 7.5 μL 2× GoTaq® Green Master Mix, 1 μL template DNA, and 5.3 μL NFW. Thermal cycling was performed under the conditions described in Section 2.4.

2.6. Analysis of PCR Results Using Agarose Gel Electrophoresis

PCR products were analyzed on a 2% agarose gel, made by dissolving 0.8 g agarose in 40 mL of 1× TAE buffer in a 100 mL Erlenmeyer flask and heating until fully melted. The solution was then cooled to approximately 60°C before adding 3 μL of SYBR Safe DNA gel stain. After homogenization, the agarose solution was poured into a gel tray with a comb and allowed to solidify.

A 100 bp GeneRuler marker (3 μL) was loaded into the first well, followed by 5 μL of PCR product into the subsequent wells. Electrophoresis was performed at 100 V for 30 minutes using 1× TAE buffer as the running buffer. DNA bands were visualized under UV illumination and documented using a digital camera. The PCR bands were compared with the DNA ladder to estimate the size of the amplified fragments.

2.7. Nucleotide Sequence Determination Using the Sanger Dideoxy Method

For sequencing, PCR products were prepared in a final volume of 20 μL in microtubes and sealed with parafilm. The samples were then submitted for sequencing, which was performed by Genetics using the Sanger dideoxy method, following the BigDye™ Terminator protocol.

2.8. Analysis of Results

Data analysis was conducted by comparing the sequencing results of the samples with the rCRS (Revised Cambridge Reference Sequence; GenBank accession NC_012920.1) using the SeqMan™ software version 4.0.0 from DNASTAR. The rCRS reference sequence and the sample sequences were imported into SeqMan™, and the software automatically identified and marked nucleotide positions that differed from the rCRS.

3. Results and Discussion

3.1. Samples

Peripheral blood was selected as the sample source because it is readily accessible, and leukocytes provide a convenient source of genomic and mitochondrial DNA for molecular analysis. Blood samples are also relatively easy to obtain [7]. Four blood samples from T2DM patients were used in this study, identified as B21, B22, B36, and B43. All selected patients were individuals undergoing routine treatment at Dustira Cimahi Hospital. Notably, samples B21 and B22 were obtained from patients with a maternal history of T2DM.

Table 1. Forward and reverse primer properties

Primer	Sequence (5'-3')	Length (nt)	GC (%)	T _m (°C)
9053_Foward	AGGCACACCTACACCCCTTA	20	55	60.18
9053_Reverse	AGGCGACAGCGATTTCTAGG	20	55	59.90

3.2. Primer Design

Primer design was conducted using the NCBI database and the nucleotide sequence of the mitochondrial ATPase 6 gene in *Homo sapiens*, located within the base pair range 8527–9207. The sequence was entered into the Primer-BLAST tool to generate primer pairs that met standard primer design criteria. The selected primer pair is shown in Table 1.

3.3. Optimization of the Annealing Temperature

The annealing temperature was optimized by testing several temperatures in a gradient PCR. The annealing temperature (T_a) was determined based on the melting temperature (T_m) of the primers, which generally falls within the 50–60°C range [8]. The ATPase 6 forward and reverse primers used in this study had melting temperatures of 60.18°C and 59.90°C, respectively.

The gradient PCR process consisted of three main stages: (1) pre-denaturation, where double-stranded DNA was denatured at 95°C for 5 minutes, followed by 38 cycles of denaturation at 95°C for 30 seconds; (2) annealing to the template DNA at 55°C for 30 seconds; (3) primer extension, where Taq polymerase extended the primers at 72°C for 60 seconds, followed by a final extension step at 72°C for 10 minutes. The polymerase extends DNA at approximately 35–100 nucleotides per second [9].

In the optimization, a gradient annealing test was conducted at six temperatures (55°C, 56°C, 57°C, 58°C, 59°C, and 60°C), each differing by 1°C. Electrophoresis using a 100 bp marker showed clear DNA bands under UV illumination, with the amplified fragment appearing at approximately 230 bp. The optimal annealing temperature was identified as 55°C, producing the strongest and most distinct band. Greater band intensity was interpreted as indicating a higher relative amount of amplified DNA.

3.4. Amplification of the ATP6 mtDNA Fragment by PCR

Amplification of the ATP6 mtDNA fragment was conducted using the PCR method, in which DNA is duplicated through repeated cycles of denaturation, annealing, and extension [10]. The reaction mixture consisted of 7.5 µL GoTaq® Green Master Mix, 0.6 µL forward primer, 0.6 µL reverse primer, 5.3 µL NFW, and 1 µL of lysed DNA template. The GoTaq® Green Master Mix is a premixed reagent containing Taq DNA polymerase, dNTPs, MgCl₂, and buffer to support the synthesis of new DNA strands. PCR was performed for 38 cycles, beginning with an initial denaturation at 95°C for 5 minutes. Each cycle consisted of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds, followed by a final extension at 72°C for

10 minutes. The extension step proceeded at an estimated polymerase rate of 35–100 nucleotides per second.

3.5. Analysis of PCR Results by Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to determine the size of PCR fragments based on the migration of DNA molecules through the gel matrix, where smaller fragments travel faster due to lower resistance. Agarose serves as a molecular sieving matrix for DNA separation [11]. A 2% gel was prepared to produce pore sizes suitable for resolving the expected DNA fragments, as the agarose concentration influences migration rates [12].

To prepare the gel, 0.8 g of agarose was dissolved in 40 mL of 1× TAE buffer and heated in a microwave until fully melted and clear. The solution was then cooled to approximately 60°C before adding 3 µL of SYBR Safe DNA Gel Stain, which enables visualization of DNA bands under UV light. The stained solution was mixed thoroughly, poured into a mold fitted with a comb, and allowed to solidify. Once the gel hardened, the comb was removed.

The solidified gel was placed in the electrophoresis tank and submerged in 1× TAE running buffer until the gel surface was covered by about 1 mm. Each well was loaded with 5 µL of PCR product, except for the first well, which was loaded with 3 µL of a 100 bp GeneRuler DNA ladder used as a molecular size reference. Electrophoresis was performed at 100 V for 30 minutes. Excessive voltage or heat can cause DNA denaturation and band shifting, so stable conditions were maintained [11]. After electrophoresis, the DNA bands were visualized under UV light to assess fragment size based on comparison with the DNA ladder, as shown in Figure 1.

The electrophoresis results shown in Figure 1 indicate that all samples produced a DNA band of approximately 230 bp, based on comparison with the DNA marker. Each sample lane showed a single main band, with no distinct non-specific products. The presence of a single band at approximately 230 bp was consistent with the expected amplicon size and indicated specific amplification under the PCR conditions used.

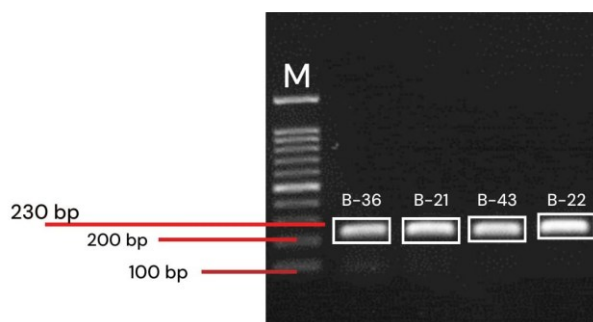


Figure 1. Agarose gel electrophoresis results

3.6. Nucleotide Sequence Determination by Dideoxy Sanger Sequencing

DNA sequencing determines the order of nucleotides in a DNA fragment [6]. PCR-amplified template DNA is sequenced to identify the encoded nucleotide bases, allowing further genetic identification. Once the nucleotide sequence of a gene is known, the amino acid sequence of the resulting protein can also be determined [9]. This study used the dideoxy Sanger method, a sequencing technique based on chain termination by dye-labeled ddNTPs during *in vitro* DNA synthesis by DNA polymerase [3].

The sequencing reaction requires template DNA and a sequencing primer. Data were processed using DNASTar software, including the EditSeq and SeqMan applications. EditSeq is used to import and prepare sequence files, including data from NCBI, while SeqMan is used to align the sample sequences with the reference sequence (rCRS) [3].

The electropherogram produced from sequencing shows colored peaks representing each nucleotide: black for guanine (G), green for adenine (A), red for thymine (T), and blue for cytosine (C). Alignment of the reference and sample nucleotide sequences using the SeqMan™ program version 4.00 revealed changes in several nucleotide positions across the four samples from patients with T2DM. These changes are indicated by

peaks that differ from the reference base at the same position [13].

Based on the sequencing results from the four samples, different outcomes were observed. In samples B21 and B22, a G9053A mutation was detected, indicated by a nucleotide change from guanine (G) to adenine (A), as shown in Figures 2 and 3. This mutation results in an amino acid change from serine to asparagine at position 176 (S176N) of ATPase 6 [3]. In sample B36, a cytosine (C) to thymine (T) substitution was found at position 8970 (Figure 4). This substitution is located at the third position of codon 148 and does not change the encoded serine, so it is a synonymous (silent) variant. The C8970T variant is a previously reported synonymous MT-ATP6 variant, not a novel sequence change. In contrast, sample B43 showed no detectable mutation in the sequenced region, as shown in Figure 5. The identified variants are presented in Table 2.

Table 2. Sequence variants identified in the MT-ATP6 gene

Sample	Nucleotide change	Amino acid change
B21	G9053A	S176N
B22	G9053A	S176N
B36	C8970T	None (synonymous, Ser148)
B43	No mutation detected	Not applicable

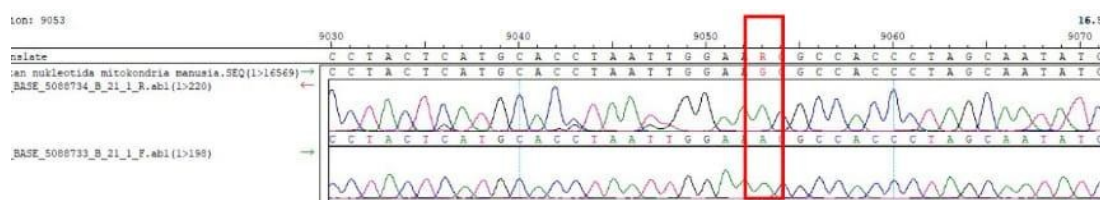


Figure 2. Results of sequencing analysis on blood sample B21

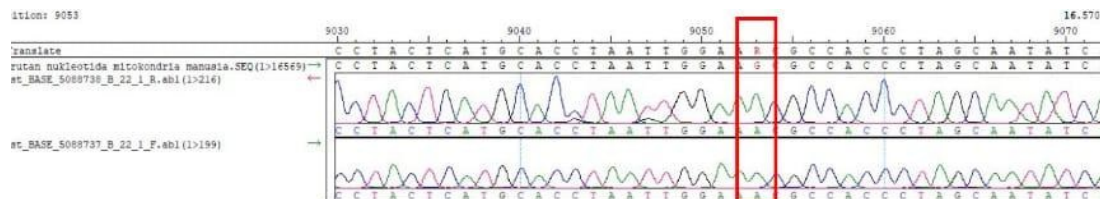


Figure 3. Results of sequencing analysis on blood sample B22

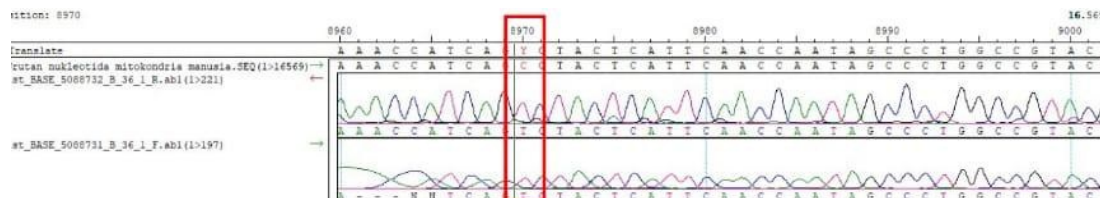


Figure 4. Results of sequencing analysis on blood sample B36

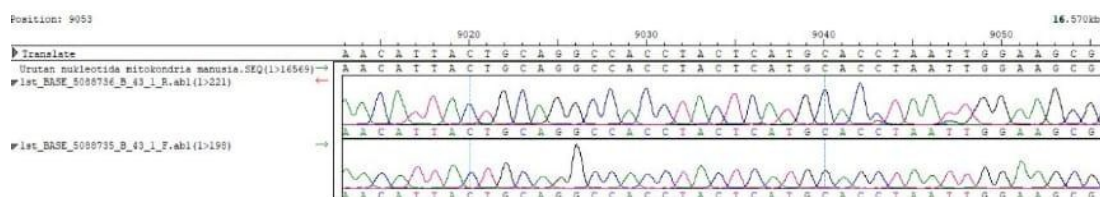


Figure 5. Results of sequencing analysis on blood sample B43

The identified G9053A and C8970T variants are located in the mitochondrial ATPase 6 gene (MT-ATP6), which encodes subunit a of Complex V (ATP synthase). This complex produces most of the ATP in the cell. ATPase 6 contributes to proton transport across the inner mitochondrial membrane, and amino acid-changing mutations in this gene can disrupt proton flow, reduce ATP synthase efficiency, and decrease cellular ATP production, potentially leading to energy deficiency and cellular damage [14]. This mechanism is relevant to G9053A, which replaces serine 176 with asparagine. C8970T does not change the amino acid sequence, so it is unlikely to affect ATP synthase function.

Previous studies report that mitochondrial mutations are more commonly found in individuals with a family history of mitochondrial disease [7]. In this study, two samples (B21 and B22) carried the G9053A mutation, and both came from patients with maternal histories of T2DM. G9053A is less common than A3243G, the mtDNA mutation most often reported in mitochondrial diabetes [15]. A bioinformatics study predicted that G9053A alters ATP synthase function and may be related to T2DM [3].

This study has several limitations. Only four patients were analyzed, and they were selected on the basis of their qPCR results rather than at random. No non-diabetic controls were included, so the frequency of G9053A in patients could not be compared with that in the general population. The heteroplasmy level of the mutation was also not measured. The results, therefore, show that G9053A is present in these patients, but they do not establish an association between G9053A and mitochondrial diabetes. A larger case-control study is needed to test this association.

4. Conclusion

Conventional PCR amplified the target fragment of the ATPase 6 gene in all four samples, and Sanger sequencing identified two variants. G9053A (S176N) was found in samples B21 and B22, both from patients with a maternal history of T2DM. The synonymous variant C8970T was found in sample B36, and no variant was detected in sample B43. These results confirm the presence of G9053A in this small group of T2DM patients. Whether G9053A is associated with mitochondrial diabetes should be tested in a larger study that includes non-diabetic controls.

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