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Research Article

Detection of mtDNA Mutations Using the Amplification Refractory Mutation System-Polymerase Chain Reaction Method in Type II Diabetes Mellitus Patients in Publich Health Center Poasia

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ABSTRACT

Background: *Diabetes Mellitus* (DM) is a heterogeneous disease caused by hereditary and ecological factors. One form of diabetes mellitus that is related to genetic factors is type 2 DM. type 2 diabetec melitus caused by dysfunction of insulin secretion, due to the presence of inhibition in the production of Adenosine Triphosphate (ATP) necessary in the process of its secretion by cells β glands of the pancreas. The dysfunction is related to the mutation of A to G at the 3243rd nucleotide position of the mitochondrial DNA tRNA gene. The purpose of the study was to find out whether there was a heteroplasmic mtDNA mutation in the genes of respondents to type 2 diabetes mellitus using the ARMS-PCR method.
Methods: The type of research used is descriptive research, the sample used in this study is a blood sample in respondents of type 2 diabetes mellitus. The method chosen in this study is the *Amplification Refractory Mutation System* (ARMS)-PCR Method which is a PCR application that uses a specific primer.
Results: According to this study, 20 of the 20 samples evaluated using the ARMS-PCR method were positive for the heteroplasmic A3243G mutation, which is defined by the presence of DNA bands measuring 200 bp on both tubes. These pathogenic mutations are inherited maternally and can cause a variety of disorders.
Conclusion: After analyzing all samples, this study has concluded that they all contain hetero plasma mutations. The suggestions provided in this study are expected to be useful for future researchers who employ samples from other public health centers and hospitals.

Keywords : *Diabetes Mellitus Type 2, A3243G mutation, Detection*

INTRODUCTION

Diabetes Mellitus (DM) is a heterogeneous disease caused by hereditary and ecological factors. One form of diabetes mellitus that is related to genetic factors is type 2 DM. Type 2 DM is caused by insulin secretion dysfunction, due to an inhibition in the production of ATP which is needed in the secretion process by the β cells of the pancreatic gland. This dysfunction is related to the mutation A to G at the 3243rd nucleotide position of the mitochondrial DNA (mtDNA) tRNA gene (1).

Type 2 diabetes mellitus is the most common type of diabetes and accounts for almost 90% of all diabetes in the world (2). Type 2 DM has symptoms that are almost similar to type 1 DM. Type 1 DM occurs due to autoimmune damage to pancreatic β cells which results in an absolute deficiency of insulin resulting in metabolic disorders. Autoimmune damage and destruction of pancreatic β cells in type 1 DM causes the need for a sensitive examination to see the function of pancreatic β cells (3). Type 2 DM is related to risk factors such as obesity, unhealthy diet, environmental factors, and genetic predisposition. Therefore, early detection and prevention are very important to reduce mortality and morbidity associated with type 2 DM (4).

According to the World Health Organization (5) diabetes is found in every population in the world, including low and middle income countries. The number of diabetes sufferers continues to increase, WHO estimates that there were 422 million adults with diabetes worldwide in 2014. The prevalence increased in adults from around 4.7% in 1980 to 8.5% in 2014, with this increase in countries middle income compared to high income countries. In addition, the International Diabetes Federation (IDF) estimates that 1.1 million children and adolescents aged 14-19 years suffer from diabetes mellitus without prevention and treatment. There will be at least 629 million people living with diabetes by 2045(2).

According to in Indonesia the prevalence of DM in women versus men is 1.7% versus 1.4%. Judging from the last 5 years, there has been an increase in the number of diabetes cases in women compared to men. The number of DM cases in Indonesia based on doctor's diagnosis at age > 15 years is 2%, this figure has increased compared to the prevalence at age > 20. Growth in Southeast Sulawesi with the prevalence of DM cases reaching 22,982 cases. There was an increase, especially in Kendari City, there were 3,297 cases. According to Riskesdas 2018, this percentage is higher than in 2013, where diabetes cases with an increase of 1.1% experienced a significant increase in 2018 (2).

Based on data obtained in the last three months in 2023 from the Poasia Health Center, in June there were 58 people, in July there was an increase of 60 people and in June there were 37 people. The services provided by the Poasia Health Center for diabetes mellitus sufferers are in accordance with standards, namely around 1,282 cases. Poasia is the public health center with the highest incidence of diabetes mellitus, compared to 14 public health center in Kendari City (6).

Mitochondria consist of a matrix containing mostly water, DNA, RNA, protein synthesis machinery, and enzymes that play a role in the oxidation of fatty acids, pyruvate, and the citric acid cycle (7). Mitochondrial DNA (mtDNA) encodes important components for cellular energy

production. Mutations in mtDNA can cause degenerative diseases in humans. The tRNA (Leu) A3243G mutation is one of the most common mtDNA mutations and is associated with various clinical phenotypes, including diabetes mellitus, hearing loss, cardiomyopathy, and mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)(8–10).

The A3243G tRNALeu (UUR) gene mutation is a common diabetogenic (7,11,12) point mutation in mtDNA. This mutation can cause loss of mitochondrial transcription from translation which ultimately disrupts the function of the respiratory chain. This mutation is detected in 0.5-1.5% of diabetics with type 2 DM. Characteristics of the A3243G mtDNA mutation is a heteroplasmy mutation with a relatively low amount of mutant DNA . Apart from that, this mutation has a universal genetic code with heteroplasmy and heteroplasmic properties. One of the most common point mutations found is the A to G transition at position 3243 in the tRNALeu(UUR) gene. This A3243G mutation causes maternal inherited diabetes with deafness or MIDD (Maternally Inherited Diabetes and Deafness) and MELAS (Mitochondrial Encephalomyopathy With Lactic Acidosis And Stroke-Like Episodes) which is a subtype of type 2 diabetes mellitus (13,14). Patients with the A3243G mutation in mtDNA generally attack in adulthood, are non-obese, develop deafness, or have a history of diabetes in the maternal lineage accompanied by (15,16) .

Research on the A3243G mtDNA mutation has been carried out in several countries. In England it was found that diseases caused by the A3243G mutation were responsible for other diseases, reported besides MELAS (mitochondria, lactic acidosis, and stroke-like episodes) including deafness, accounting for 80% of cases due to mtDNA gene mutations (17). in Japan the A3243G mutation was found in 2.9% of DM patients (18). in Hungary, mutations in mtDNA A3234G were found in the DM patient population, reaching 2.22% (19). In France, the A3243G mutation was found with the existence of a mutation hotspot (20). In Pakistan, mtDNA mutations were found in patients who had certain criteria, namely out of 39, 34 were detected as having mtDNA (21).

The aim of this study was to find out whether there was an A3243G mtDNA mutation in type 2 DM sufferers using the ARMS-PCR technique. The scientific benefit of this research is that it is useful as reference material and reference for further research related to mtDNA mutations in type 2 DM sufferers, especially in the medical laboratory technology department.

METHODS

Research Design

The type of research used in this research is descriptive.

Population and Sample

The population in this study was people with type 2 diabetes mellitus in the at the Poasia Health Center, Kendari City. The sample for this study was part of the population, namely 20 respondents suffering from type 2 diabetes mellitus.

Sources and Methods of Data Collection

Research tools

The tools used in this research are as follows:

Table 1. Research Equipment

No	Tool's name	Function
1.	Thermal cycler	For DNA amplification
2.	PCR tube	As a solution
3.	Centrifuge	To separate DNA particles from the supernatant
4.	Micropipette	To move samples
5.	Agarose mold	To make agarose gel
6.	Microfugation tube	To store DNA samples
7.	Analytical scales	To weigh the materials used
8.	Vortex	To homogenize the solution
9.	Micropipette tip	To accommodate the fluid to be transferred

Research materials

The materials used in this research are as follows:

Table 2. Research Materials

No	Material	Function
1	Whoole Blood	As a sample
2	Buffer TAE 1X Ph 8,0	For solution
3	DNA	For standard DNA
4	Primer D1,-AAC GTT GGG GCC TTT GCG-3,DN,-GGG TTT GTT AAG ATG GCA GA-3,DMt,-GGG TTT GTT AAG ATG GCA TG-3	For DNA replication
5	Master PCr mix (DNA polymerase, dNTPs, MgCl2, loading dye	For PCR solution mixtures
6	Proteinase K	To lyse the membranes on blood cells
7	My taq red mix	For DNA extraction
8	Aquabides	As a solvent
9	Kit geneaid	As DNA purification

Research Procedures

Respondent Criteria

Individuals with type 2 diabetes were eligible for this study provided they met the following criteria: 1) Type 2 diabetes patients who have been diagnosed, treated, or examined at the Puskesama Poasia Kendari and are willing to participate; 2) fasting blood sugar levels >= 126 mg%; and 3) 2-hour blood sugar levels PP >=200 mg%. Respondents who refuse to participate in this survey are not eligible.Blood sample collection. Whole Blood DNA isolation is carried out based on the Geneaid SYNCTM 2020 protocol.

Blood sample collection (Phlebotomy)

Blood sample collection was carried out by research members who are ATLM (Medical Laboratory Technology Experts). The Blood then isolated by geneaid DNA extraction kit. The process involved transferring up to 200 µl of whole blood into a 1.5 ml microcentrifuge tube and adjusting the volume to 200 µl with PBS. Then, 20µl Proteinase-K was added and mixed by

pipetting, and the mixture was incubated at 60°C for 5 minutes. Next, 200 µl of GSB Buffer was added, and the tube was vortexed and incubated again at 60°C for 5 minutes with inversion every 2 minutes. After that, 200 µl of absolute ethanol was added and mixed with the lysate sample immediately by homogenizing for 10 seconds. If any precipitate appeared, break it up as much as possible with a pipette. Finally, the GS Column was placed in a 2 ml Collection tube.DNA amplification. Transfer the mixture to the GS Column and centrifuge at 12.000 rpm for 1 minute. Discard the 2 ml Collection Tube containing the flow-through and transfer the GS Column to a new one. Add 400 µl of W1 Buffer, centrifuge, discard the flow-through, and repeat with adding 600 µl of Wash Buffer. Centrifuge for 3 minutes to dry. Elute purified DNA by adding 100 µl pre-heated Elution Buffer to the center of the column matrix. Let stand for 3 minutes to be completely absorbed and then centrifuge at 12.000 rpm x g for 30 seconds (22).

DNA Amplification

ARMS PCR method uses three primers: universal primer (D1), normal primer (DN), and mutant primer (DM). Two tubes are used: the first with D1 and DN, and the second with D1 and DMt. Prepare PCR tubes and pipette 25 µL of mastermix, DNA 5 µL, add 1µL for each primer and 11 µL of the ddH20. The PCR process, initial denaturation, is carried out at 94°C for 5 minutes, followed by three stages in each cycle: denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute. An additional extension process is run at 72°C for 10 minutes at the end of cycles. Run 30 cycles in the Major Cyclers PCR machine. PCR products were verified using a 2% agarose gel electrophoresis at 100 volts for 30 minutes and visualized with UV Transilluminator (23).

RESULTS

1. Respondent characteristics

a. Distribution by age

The data is presented in the form of a Table 1 displaying the different age groups of the respondents and the number of people in each group.

Table 3. Distribution of respondents based on age

No	Age Range	Total	Percentage (%)
1	46-56	9	45
2	57-67	8	40
3	68-78	3	15
Total		20	100

The age range of the participants in this study was calculated from their birth until the time of conducting this research in years. Upon analyzing Table 3, it was discovered that the group of respondents between the ages of 46-56 years had the highest prevalence of DM type

2, with 9 individuals accounting for 45% of the total number of participants in this category.

b. Distribution respondents by gender
Based on observation of research data, the number of respondents based on gender is shown in Table 4.

Table 4. Distribution of respondents by gender.

No	Gender	Total	Percentage (%)
1	Female	9	45
2	Male	11	55
	Total	20	100

According to Table 4 male respondents had the highest prevalence of type 2 DM, with 11 individuals accounting for 55% of the total in this category.

2. Analysis of Amplification Refractory Mutation System (ARMS)

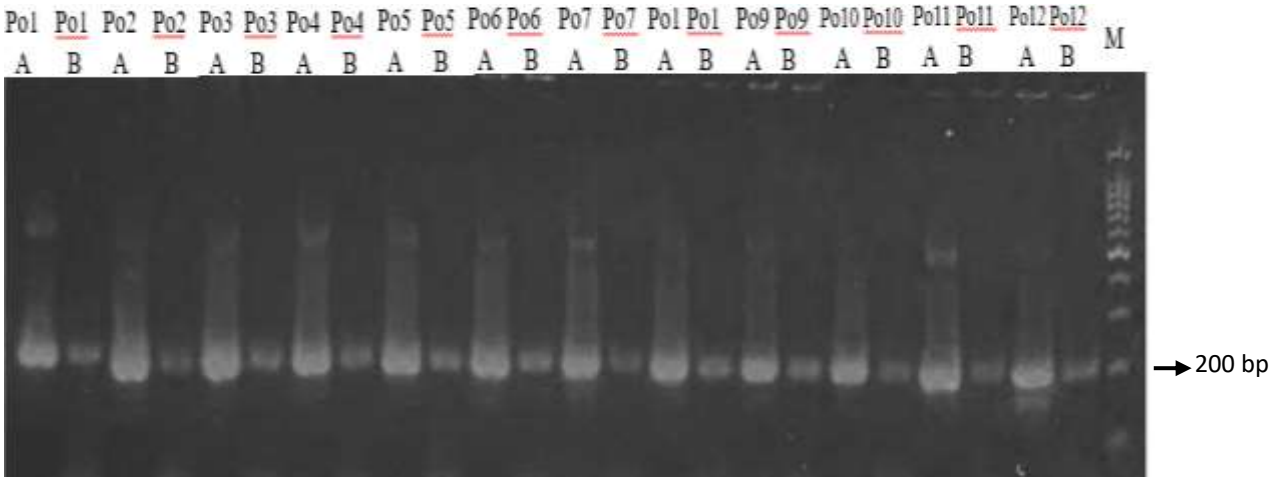

Figure 1. Electrophoresis results show heteroplasmy mutation in all samples. Po1-Po12 samples, code A tubes with D1 and DN primers, and the code B with D1 and DMt primer. M = Marker 100 bp DNA ladder Tiangen.

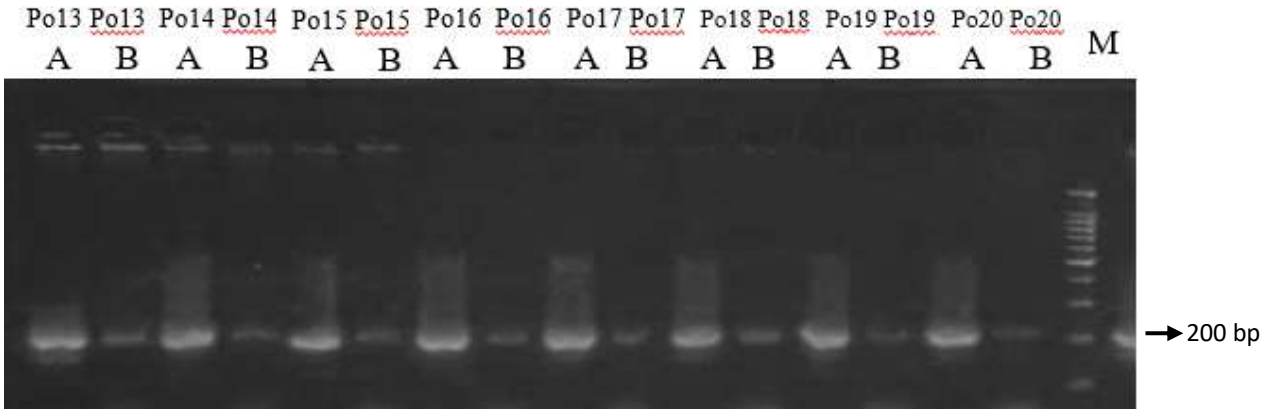

Figure 2. Electrophoresis results show heteroplasmy mutation in all samples. Po13-Po20 samples, code A tubes with D1 and DN primers, and the code B with D1 and DMt primers. M = Marker 100 bp DNA ladder Tiangen.

The results depicted in Figure 1 and Figure 2 indicate the presence of heteroplasmy in all samples, as evidenced by the appearance of a 200 bp DNA band in both tube A and tube B.

DISCUSSION

Type 2 DM is one of the fastest growing public health problems throughout the world. Although environmental factors clearly contribute to the recent increase in disease prevalence, there is growing evidence that genetic factors also have an important impact (1). Diabetes is caused by various mutations in mitochondrial DNA, and these mtDNA mutations are associated with defective insulin secretion. Mutation of nucleotide 3243 in the tRNALeu(UUR) genome sequence causes β-cell instability and impaired insulin secretio (24) . The A3243G mutation in the tRNA gene Leu(UUR) is the most frequently identified mtDNA mutation that correlates with different modes of diabetes (15,24).

The samples taken as mtDNA templates were venous blood cells from 20 subjects suffering from type 2 diabetes mellitus. Blood cells were chosen as samples because these cells have quite a large number of mitochondrial organelles. Another reason is that blood samples are relatively easy to take and have been used as samples in studies that have been carried out to analyze mtDNA mutations associated with diabetes mellitus (18). After a blood draw, the collected samples were isoalated to obtain the DNA components. These samples were then processed using amplification techniques to increase their amount and make them suitable for further analysis. Finally, the amplified samples underwent thorough electrophoresis.

The result of this study shown all of sample are hetero plasma (Figure 1 and Figure 2). This is show high incident of hetero plasma since similar study was conducted in Indonesia to investigate

This finding suggests that the samples are likely to exhibit patogenenic mtDNA A3243G mutation.

mitochondrial mutations in diabetic individuals that included 1,500 diabetic patients from Jakarta, Jogjakarta, and Surabaya revealed, has not found any A3243G mutation (7).

All respondents with heteroplasmy mutations were women and man (Table 2). The mtDNA A3243G mutation is often inherited maternally (8–10). According to a study by Finsterer (2007)(8), a mother with a high mutation burden has an increased likelihood of having a child with a genetic disorder. Clinical studies in Japan conducted by Morovvati (2002) (25), have shown that the A3243G mutation is also associated with chronic progressive external ophthalmoplegia, diabetes mellitus (DM), hypertrophic cardiomyo, neuropathy, mental retardation, sensory nerve hearing loss, and other types of Mitochondrial encephalomyopathy. In family 1, the mother aged >45 years who had the A3243G mutation was diagnosed with DM and treated with insulin at the age of 41 years. Generation 1 of the mother, a 16 year old female, was diagnosed with the A3243G mutation, and she was diagnosed with MELAS based on the typical clinical symptoms and mtDNA mutations of the mother and child. The mother had been diagnosed with heart disease beriberi at the age of 20 years and mtDNA analysis showed the A3243G mutation in Patients 1 and 2 and their mother.

Nonetheless, there have been a few exceptional cases where children inherited paternal mtDNA. This suggests that the mtDNA in sperm can occasionally persist after fertilization and remain present in the developing embryo a phenomenon referred to as "paternal leakage" (12). Therefore, both parents have the potential to pass on this mutation to their offspring, but paternal leakage is exceedingly rare.

CONCLUSIONS

Based on this study, it can be concluded that all samples carry out heteroplasmy mtDNA A3243G mutation

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