Restriction Enzymes ApaI Analysis to Find A3243G Mutation in Indonesia Diabetes Mellitus Type II Patients

Richie Agusta Iwan Chandra[1], Sriwidodo[1], Ajeng Diantini[1], and Iman P. Maksum[2]
[1]Pharmacy Faculty Universitas Padjadjaran, Sumedang, Indonesia
[2]Chemistry Departement Mathematics and Natural Science Faculty Universitas Padjadjaran, Sumedang, Indonesia
Email: richie.chandra@unpad.ac.id; sirwied2004@yahoo.com

***Abstract*—The Use of PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) to find out the potential of mitochondrial DNA mutation at A3243G in type II Diabetic Patient has been done. Peripheral blood from 100 Indonesian type 2 diabetic subjects was selected randomly for this experiment. Peripheral blood lymphocyte was isolated, lysed, and it was in vitro amplified by PCR using a pair D1/D2 primers. PCR products were 294 base pair (bp) fragments which were then purified by ethanol precipitation method and characterized by restriction enzyme ApaI. Heteroplasmic A3243G mutation which was identified in 2 Subject (0,02%) was shown by 3 electrophoretic bands, 2 restriction products of APAI, i.e a 182 bp fragment and a 112 bp fragment; also a full fragment 294 bp, this means show that PCR-RFLP technique was approved for identifying heteroplasmic A3243g mutation in a $tRNA^{leu}$ gene mtDNA type 2 DM subject.**

***Index Terms*—DNA mutation, mtDNA A3243G, MIDD, restriction enzyme ApaI, PCR-RFLP**

I. Introduction

Diabetes Mellitus (DM) is a metabolic disease caused by insufficient insulin synthesis, increased insulin breakdown or impaired insulin actions [1]. If left unattended, will cause various fatal effects, including disease associated with the heart and kidney. Severe damage on certain body parts may require surgical removal (amputation) [2].

DM is classified into two main groups, type I and type II. Approximately 85% of diabetes in the world is Type II diabetes [3]. Number of patients with type II diabetes is expected increase rapidly in the future [4]. According to the recent research conducted by International Diabetes Federation (IDF) in 2000, prevalence rate of DM patients in Indonesia of 4.6% (5.6 million patient) occupy the sixth place globally and it is estimated an increase to 8.2 million in 2020 [5].

DM type II diabetes is a disease that does not rely on insulin / Non-Insulin Dependent Diabetes Mellitus (NIDDM) and clinical appearance is influenced by both genetic factors and environmental factors. Genetic factors play an important role in the onset of type II Diabetes Mellitus disease. Mutations in mitochondrial DNA (mtDNA) of human became one of the causes of type II diabetes, because oxidative phosphorylation in mitochondria plays an important role in insulin secretion by β-cells of the pancreas as a response to glucose and other nutrients in the body [6]. MtDNA mutations in this gene cause a form of diabetes, known as Maternal Inherited Diabetes and Deafness (MIDD). This form of diabetes can be diagnosed above 25 years in the form of impaired insulin secretion and is often followed by a weakening of the sense of sight and or hearing [4]. MIDD has a very specific pattern of inheritance, through the maternal lineage without the presence of recombination of paternal line. This is because only eggs carry mtDNA when fused with sperm cells [4].

Clinical studies of mtDNA point mutation A3243G in DM patients have been conducted in various countries. Mutations are found with different presentations, including in Taiwan it is found in 0.15% of the entire population of patients with Diabetes Mellitus [7], whereas in Poland, number of patients with A3243G mutations are not known for sure [4]. Point mutations in the mtDNA A3243G is also found in Japan 2.9% [8], in England 0.75% [9] and in Croatia 10% [10]. While in Korea 22.3% patient with mitochondrial disease had point mutation on the A3243G mtDNA [11]. In Spain it has been found in 18% of children patients had three heteroplasmy mutations including A3243G [12]. This mutation not only affects the synthesis of tRNAleu but also interfere with the binding mechanism of transcription termination factor that may lead to disruption of the synthesis of mitochondrial proteins [6]. A3243G mutation in tRNAleu in DM patients in different populations indicate the importance of studying these mutations and find which methods are most effective, efficient, economical as well in identifying these heteroplasmy mutations.

Maksum *et al*., (2005) [13] identified two A3243G heteroplasmy mutations from 100 samples of patients with Diabetes Mellitus Type II of Bandung population by Restriction Fragments Length Polymorphysm (RFLP) method using the restriction enzyme ApaI cutting The sticky end, GGGCCC. Accordingly, further researchs are needed in order to determine the extent of the role of

Manuscript received October 23, 2014; revised December 10, 2014.

doi: 10.12720/jomb.4.6.492-496

mutations in the mtDNA A3243G point in the pathogenesis of DM disease. In this research, search of potential mitochondrial DNA point mutations A3243 in patients with Diabetes Mellitus using PCR-RFLP (Polymerase Chain Reaction-Restriction Length Fragmnets Polymorphysm). In this research, a sample with different population, Jakarta patients with type II Diabetes Mellitus as an area with a multiethnic and Manado as the region with the highest prevalence rates of diabetes.

II. Method

A. Preparation of Template DNA

mtDNA template are prepared by lymphocyte cell lysis method with lysis buffer consisting of 50 mM Tris-HCl pH 8.5; 1 mM EDTA pH 8.0; proteinase K 0.04 mg/mL and 0.5% Tween-20. Lymphocytes obtained by washing the 200 mL of blood samples with 1000 mL TE buffer, then centrifuged for 1 minute at speed of 12.000 g, washing was repeated until a white pellet is obtain. White pellets were added with ddH2O, lysis buffer (50 mM Tris-HCl pH 8.5; 1 mM EDTA pH 8.0; and 0.5% Tween-20), and 0.04 mg/mL proteinase K, the reaction mixture was incubated at 60 ℃ for one hour, followed by protein inactivation process in an incubator at a temperature of 95 ℃ for 10 minutes.After inactivation of the protein, the mixture was centrifuged for five minutes at 12,000 g, and the supernatant was taken.

B. MtDNA in Vitro Amplification (PCR)

Amplification of 294 bp fragment of mtDNA gene $tRNA^{leu}$ performed by PCR using a primer pair (primary D1 and D2). The reaction mixture containing 1 unit of the enzyme Taq DNA Polymerase, mtDNA lysis template results, D1 and D2 primers respectively 1 µM, PCR buffer 10x (10 mM Tris-HCl, pH 9.0; 50 mM NaCl; Triton X-1000 0.1 %), 200 µL dNTPs, 2µM $MgCl_2$, and sterile ddH2O. The process will be carried out in a PCR machine Automatic Thermal Cycler as many as 30 cycles.

C. Purification of DNA Result from PCR

The PCR result was performed by ethanol precipitation method.

D. Characterization of PCR Product with the Enzyme ApaI (RFLP)

Purified mtDNA template are further reacted with ddH2O, L buffer 10x, and the enzyme ApaI (15 units) in eppendorf tubes and incubated in a water bath at a temperature of 37 ℃ over night.

E. Analysis of the Results of PCR-RFLP

Next, it is analyzed by agarose gel electrophoresis 2% (w/v) using a Mini subTM DNA electrophoresis cell. In each of the wells formed, a 10 mL sample of the PCR product was inserted and mixed with 2 mL of loading buffer (50% sucrose, 0.1 M EDTA pH 8.0, 0.1% blue bromfenol pH 8.0). The process of electrophoresis is performed in TAE buffer 1x as a current medium conductor with voltage of 75 volts for 20 minutes. Marker used was that with a band each measuring 100 bp to 4000 bp. The results of electrophoresis were visualized with a UV lamp series 9814-312 nm.

III. Results and Discussion

A. Isolation of mtDNA Templates

Blood cells are targeted as sample due to the sufficient number of mitochondria organelles in blood cells compared to many other cells, such as muscle cells, sperm tail cells, and hair root cells (Thorpe, 1984). Moreover, it is relatively easy for blood sampling and has been used as a sample of previous research that has been done by Ohkubo *et al*. (2001), Lee *et al*. (1977), and Malecki *et al*. (2001) to analyze the mtDNA A3243G mutation associated with diabetes mellitus in Japan, Korea, and Poland. Samples were taken randomly from patients who tested positive for type II diabetes mellitus, aged above 25 years.

The mtDNA template was isolated by lysis of blood cells that have been collected. Blood cells were washed repeatedly using TE buffer in order to obtain the white pellets of lymphocytes cells, while red blood cells are dissolved in TE buffer discarded. Red blood cells contain iron, and will form a complex in the presence of EDTA originated from TE buffer (Tris HCl-EDTA), Tris-HCl gives an alkaline pH 8.0 and EDTA itself acts as chelating agents. Red blood cells containing iron were discarded because it can interfere with the action of the enzyme DNA polymerase during PCR process. Lymphocytes are then lysis with Maniatis method adapted from Sambrook *et al*. by using lysis buffer (Tris HCl 2M pH 8.0; 0.5M EDTA pH 8.0; Tween-20), and proteinase K. Tween-20 in the lysis buffer acts like detergents that have hydrophobic tails and hydrophilic heads. Hydrophobic tail of Tween binds with the phospholipid, a component of the cell membrane which is hydrophobic, causing the damaged of cell membrane integrity, while Proteinase K can destroy peptide bonds of the cell membrane and mitochondrial membrane, hence, both of the constituents work simultaneously in the breaks down of both the cell membrane and the mitochondrial membrane. This disruption was done, so that the DNA present in the mitochondrial matrix can be released, while the enzyme nuclease activity that can destroy the DNA when it is out of the cell system is inhibited by the presence of EDTA in the lysis buffer. Lysis was carried out at 56 ℃ for an hour so that all the cells lysis, while the temperature of 60 ℃ is the optimum working temperature of proteinase K (Sambrook *et al*., 1989). After one hour, the samples were incubated at 95 ℃, 10 minutes for inactivation of proteinase K, to prevent fragmentation of enzyme DNA polymerase in the PCR process. Samples were then cooled and centrifuged at 12.000 g for 5 min, then, the supernatant was taken. Mitochondrial DNA consisting of 16.565 bp will be in the supernatant, because of the relatively low molecular weight compared to the nuclear DNA consisting of 3.5

billion base pairs. Hence, after centrifugation nuclear DNA tends to exist in the sediment and mtDNA in the supernatant.

B. Template Amplification of mtDNA in Vitro by PCR

The results of template lysis, was further amplified to obtain tRNAleu gene in vitro by PCR using the primer pair D1 and D2 with the nucleotide sequence based on the previous study (Zhang *et al* 2002). Primary D2 consists of 20 primers acting as a forward primer that will stick to the 3130-3149 positions in the light strand of the DNA template and primer D1 which also consists of 20 primers that acts as a reverse primer attached to the heavy strand of mtDNA template at position 3423-3404. As a result of the amplication, a 294 bp was found which is the difference of the primary extension. Besides the primer, several other components are necessary to conduct the PCR process, such as PCR buffer to resists in pH changes, dNTP which acts as a source of bases during elongation while enzyme Taq DNA polymerase works to extend the primer. Before the sample is added, all of the other components of the PCR was made mastermix for a number of desirable reactions. It aims to prevent the loss of significant components due to pipetting errors, especially the enzyme Taq DNA polymerase.

The PCR process is carried out at pH 8.00 which resembles the pH in the process of in vivo DNA replication by adding PCR buffer containing Tris HCl 100mm, KCl 500mm, and 15 mM $MgCl_2$. The PCR process is carried out in three phases: denaturation, annealing, and extension or elongation of primer. First and foremost, the mtDNA template was denatured to single strands at 94℃ for 30 seconds per cycle, followed by annealing stage or stages of primer attachment to a template performed at a temperature of 57℃ for 30 seconds per cycle. Annealing temperature is selected based on Tm of the primer D1 and D2 at 62℃ and 58℃ respectively. Most laboratories consider annealing stage around 3-5ºC below the primer's Tm to optimize the PCR process (Newton and Graham, 1997). In The third stage, where the elongation of primer was carried out at 72℃ for 30 seconds per cycle which is the optimize working temperature of the enzyme Taq DNA polymerase, a DNA polymerase enzyme isolated from the bacterium in the hot water of *Thermus aquaticus*.

The PCR process was carried out in 30 cycles because the limitation of enzyme Taq DNA polymerase after 25-30 PCR cycles according to Newton and Graham (1997). After 30 cycles of PCR, it is supposed to obtain approximately 2n of tRNAleu gene fragment as an amplification product. This concludes about 1,073,741,824 copies subtracted with the mtDNA fragments that were not fragments of 294 base pairs absent from the first, and the third second cycle. After 30 cycles, an additional extension cycle at 72℃ for 10 minutes was performed. This was done to enhance the extension phase (elongation) Results from PCR were then analyzed by agarose gel electrophoresis 1.5% (w/v) using EtBr staining similar to the analysis by Zhang *et al.* (2002) and Sambrook *et al*. (1989). Migration speed of DNA fragments by agarose gel depends on several factors. According to Helling *et al.* (2002) double-stranded DNA molecules migrated in the agarose gel with a speed proportional to log10 the total number of base pairs.

Ready made agarose gel was then soaked with TAE buffer in a horizontal minigel electrophoresis apparatus, and filled with 10 mL sample resulted from the PCR, a negative control and a positive control into the wells.Previously a 2 mL of loading buffer consisting of blue and sucrose bromfenol were added. Loading buffer serves to increase the density of the sample, while the sucrose contained in the loading buffer serves as a weight and blue bromfenol will provide color (stain) on the sample to facilitate entry into the gel wells. In addition, the loading buffer dye which, when placed in an electric field will move towards the anode at a certain speed. Blue Bromfenol migrates at a velocity of ~ 2.2-fold in the agarose gel compare to xylene cyanol FF (Sambrook *et al*.). 100bp each measuring 100 bp to 4000 bp Control marker was used as a standard in the analysis of the results from the PCR. Loading buffer is added also into the 100 bp Marker and electrophoresed along with the samples to determine whether or not the results of PCR are being amplified. This is determined by comparing the location of the samples bands with the location marker band on the gel after electrophoresis. Electrophoresis was performed at a voltage of 80 volts for 45 minutes, with this voltage and time the fragment had been carried away from the well. Once the running gel process is completed, the gel was visualized under a UV light.

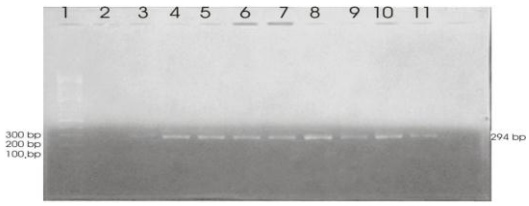

Figure 1. Results of PCR fragments using the primer pair, D1 and D2

All samples gave results to DNA fragments amplification sized 294 base pairs of $tRNA^{leu}$ mtDNA genes. It can be seen from the appearance of the band in the samples that lies parallel between the 200 bp and 300 bp marker.Positive control produces a band that is located at similar position with the sample, while the negative control did not generate bands on gel electrophoresis indicating the absence of contaminants in the PCR process has been done. The band on gel electrophoresis results turned out to have different thicknesses depending on the concentration. Thick band indicates the high concentration of DNA fragments produced by PCR with an upper limit of 200ng/µL, while a faint band have a concentration of about 60 ng/µL. The low concentration of mtDNA fragments is affected by several factors, including the imprecise lysis process, the unconscientious process of making mastermix or perhaps because of less optimal of annealing temperature. The figure of mtDNA in vitro by PCR can be seen in Fig. 1

C. Purification of MtDANA Template

PCR results were then purified in advance by using ethanol precipitation method before characterized by

Apa1 restriction enzymes. Purification aims to eliminate the remnants of PCR components and other impurities that can interfere with subsequent analysis.

D. Characterization of mtDNA PCR Template with Apa1

A total of 10 µL DNA purification results were further characterized to determine the mtDNA A3243G mutation by Apa1 restriction enzyme from the bacterium *Acetobacter pasteurianus sub* that has the nucleotide recognition GGGCCC. In the Eppendorf tube, pure DNA was added with buffer L consisting of 100mm Tris-HCl pH7,5; $MgCL_2$; and 10mM dithiothreitol and 15 units of restriction enzyme. One unit of Apa1 enzyme is the total amount of enzyme needed for a perfect cut of 1 µg DNA in a total volume of 50 mL using buffer L at a temperature of 37℃ for 1 hour. The buffer serves to maintain the pH required in order for the enzyme to work optimally and Mg 2+ is used as a cofactor for the enzyme Apa1. Mixed buffer, enzyme, DNA template, and ddH2O were then incubated in the waterbath at a temperature of 37℃ for 16-18 hours.

The results of incubation were then analyzed again by agarose gel electrophoresis 2% (w / v). Greater agarose concentration were used to analyze DNA fragments that have a fairly short chains, ie fragments of 182 bp and 112 bp, necessitating an agarose gel that has a better degree of separation. Electrophoresis was performed under the same conditions used in electrophoresis using TAE buffer as in the analysis of PCR results. Pure PCR result that was not reacted with the enzyme was electrophoresed as a control and also Apa1 100bp marker as a standard marker. The cutting results of the electrophoresis results of Apa1 restriction enzyme on 100 samples showed that the sample had A3243G mutation. This is indicated by the presence of three bands on the agarose gel electrophoresis characterization results, 2 bands resulting from Apa1 restriction enzymes, and 1 intact band of 294 bp.

Fragmentation of mtDNA 294 bp of tRNAleu gene by Apa1 restriction enzyme into two fragments 182 bp, and 112 bp indicates the presence of mutations A3243G in 2 samples. This happens because the A3243G mutation causes the formation of 6 nucleotides recognition site recognized by Apa1 restriction enzyme at 3242-3247 base pairs sequence, namely GGGCCC. While for normal subjects, GAGCCC sequence is present in that position, where concludes that there are no cut by the Apa1 restriction enzymes, because it is not the specific recognition Apa1.

The Apa1 cuts, results in blunt ends to form, explains the cut right in the middle at the recognization site on the double-stranded 6 nucleotide sequence. The present of 294 bp whole band in PCR-RFLP sample results by Apa1 restriction enzyme indicates that these mutations are heteroplasmy(a mixture of mutated mtDNA and normal mtDNA in the cell).

Furthermore, for the positive samples containing heteroplasmy A3243G mutations were then further explored on patient medical history data and genealogy. This was done to observe the clinical picture owned by patients with type 2 diabetes mellitus in Jakarta and Manado that have these mutations, as well as to prove that the heteroplasmy mutation is inherited maternally. This is to assist future research on the relationships between the effects of maternal and phenotype MIDD mutation. MIDD is characterized by non-obese diabetic patient with no experience of ketoacidosis, and a disturbance in hearing (deafness). The figure Characterization of mtDNA PCR Template with Apa1 can be seen in Fig. 2

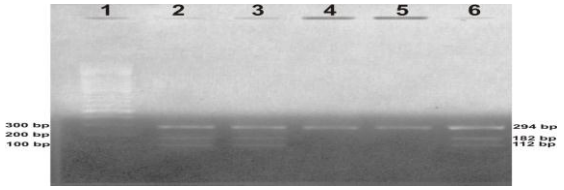

Figure 2. The results of PCR-RFLP fragmentation with Apa1 restriction enzyme

IV. Conclusion

The conclusion of this research 2 patients that have these mutation from 100 diabetes mellitus patients in Indonesia. This mutation is not significance in diabetes mellitus pathogenesis.

References

[1] T. McKee and J. R. McKee, *Biochemistry: An Introduction*, 2nd Ed., WCB/McGraw-Hil, New York, 1999.

[2] S. Suyono, *Buku Ajar Ilmu Penyakit Dalam. Jilid I. Edisi ke-3*. Balai Penerbit FKUI, Jakarta, 1996.

[3] G. C. Weir and J. L. Leahy, *Diabetes Mellitus Theory and Pragtice: Pathogenesis of Non-Insulin-Dependent (Type II) Diabetes Mellitus*, 4th Ed. Ellenberg and Rifkin's, Elsevier, 1990.

[4] M. Malecki, T. Klupa, K. Wanic, J. Frey, K. Cyganek, and J. Sieradzki, "Search for mitochondrial A3243G tRNA leu mutation in Polish patients with type 2 diabetes mellitus," *Med Sci Monit.* vol. 7, no. 2, pp. 246-250, 2001.

[5] Anonim, *Konsensus Pengelolaan Diabetes Melitus Tipe 2 Di Indonesia*, PERKENI, Bandung, 2002.

[6] T. Kadowaki, H. Kadowaki, Y. Mori, *et al*. "A subtype of diabetes mellitus a mutation of mitochondrial DNA," *NEJM*., vol. 330, pp. 962-968, 1994.

[7] C. W. Liou, C. Huang, and Y. H. Wei, "Molecular analysis of Diabetes mellitus associated A3243G mitochondrial DNA mutation in Taiwan cases," *Diabetes Res. and Clinical Practice*, vol. 54, no. 2, pp. 39-43, 2001.

[8] K. Ohkubo, A. Yamano, M. Nagashima, *et al*. "Mitochondrial gene mutations in the tRNA $^{leu(UUR)}$ region and diabetes: Prevalence and clinical phenotypes in Japan," *Clinical Chemistry*, vol. 47, pp. 1641-1648, 2001.

[9] K. Owen, A. Sride, S. Ellard, and A. T. Hattersley, "Etiological investigation of diabetes in young adults presenting with apparent type 2 diabetes," *Diabetes Care*, vol. 26, no. 7, pp. 2088-2093, 2003.

[10] I. M. Kleiner, E. P. Medvidoic, I. P. Renar, *et al*., "A pilot study of mitochondrial DNA point mutation A3243G in a sample of croatian patient having type-2 diabetes mellitus associated with maternal heritance," *Acta Diabetol*. vol. 41, pp. 179-184, 2004.

[11] J. Chae, H. Hwang, C. Lim, *et al*. "Cllinical features of A3243G mitochondrial tRNA mutation," *Brain & Dev*. vol. 26, pp. 459-462, 2004.

[12] J. Uusimaa, S. Finnila, A. M. Remes, *et al*., "Molecular epidemiology of childhood mitochondrial encephalomyophathies in finnish population; sequence analysis of entire mtDNA of 17 children reveals heteroplasmic mutation in tRNA Arg. tRNA Glu, and tRNA Leu (UUR) genes," *Pediatrics*, vol. 112, no. 2, pp. 443-450, 2004.

[13] I. P. Maksum, A. Subarnas, T. Subroto, and B. Prihartanto, "Penggunaan PCR-RFLP dan teknik DNA rekombinan untuk analisis diabetes melitus tipe II: Laporan penelitian hibah bersaing XII," 2004.

Richie A. I. Chandra was born in Indoesia 15th August 1984. Graduated from Pharmacy Bachelor program Universitas Padjadjaran, Bandung, Indonesia, 2007 and Pharmacy Master program from Universitas Gadjah Mada, Yogyakarta, Indonesia, 2009.
His current job is as a LECTURE in Departement of Pharmaceutical Faculty Pharmacy Universitas Padjadjaran. Research interests in biotechnology and Nanotechnology.
Chandra is a member of Cosmetic Societies Scientist Indonesia.

Sriwidodo was born in Indoesia 30th March 1974. Graduated from Pharmacy Bachelor program Universitas Padjadjaran, Bandung, Indonesia, 1997 and Pharmacy Master program from Universitas Padjajdaran, Bandung, Indonesia 2007.
His current job is as a LECTURE in Departement of of Pharmaceutical Faculty Pharmacy Universitas Padjadjaran. Research interests in Pharmaceutical, and Biotechnology

Ajeng Diantini was born in Indoesia 12th March 1964. Graduated from Pharmacy Bachelor program Universitas Padjadjaran, Bandung, Indonesia, 1987 and Pharmacy Master program from Institut Teknologi Bandung, Bandung, Indonesia, 1993 dan Doctoral program from Universitas Padjajdaran, Bandung, Indonesia 2009.
Her current job is as a LECTURE in Departement of Pharmacology Faculty Pharmacy Universitas Padjadjaran. Research interests in Clinical Pharmacy and Pharmacology.