Identification of ATPase6 gene mutation from cimahi clinical isolates

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Abstract. Diabetes mellitus is a mitochondrial disease, caused by ATP deficiency. ATP was produced by the OXPHOS system in the mitochondria. Mitochondrial ATPase6 was one of important enzyme in oxidative phosphorylation process of ATP synthesis. The aim of this research was to provide information of ATP6 gene mutation that correlated to DMT2. A pair of primers was designed by in silico study. Blood samples were taken from DMT2 patients. Blood cells were lysed to obtain DNA template. Amplification of ATP6 gene was done by Polymerase Chain Reaction (PCR) technique. The amplicon was analyzed by 1% agarose gel electrophoresis. The gel showed 0,7 kb band of amplicon. The nucleotide sequencing showed that the amplicon was 681 base pairs. Analysis of phylogenetic showed that the sequence was 94,08% identical to homo sapiens ATP6 gene. Homology analysis between ATP6 gene from genbank and ATP6 gene fragments showed that there was a mutation 8860A>G. Amino acid analysis showed that 8860A>G change the amino acid T112A. Interestingly. It both happened in all samples, and it is a haplogroup. This is mean, it need further research to convince 8860A>G related to DMT2.

1. Introduction
Diabetes Mellitus type 2 (DMT2) is a metabolic disorder disease characterized by an increase in blood sugar due to altered insulin production by pancreatic beta cell and or impaired insulin function (insulin resistance)[1]. Diabetes mellitus is commonly called the silent killer because this disease can affect all organs of the body and cause various kinds of complaints. Diseases that will be caused include eye vision disorders, cataracts, heart disease, kidney disease, sexual impotence, lung infections, blood vessel disorders, strokes, etc.[2]. In Indonesia, the prevalence of people suffering from diabetes based on a doctor’s diagnosis is 1.5% [3]. DMT2 is associated with mutations in mitochondrial DNA (mtDNA). Because mitochondria affects insulin secretion from pancreatic cell β. The secretion of insulin by pancreatic cells is highly dependent on adenosine triphosphate (ATP) which is synthesized from oxidative phosphorylation in mitochondria. The essential proteins needed for oxidative phosphorylation are synthesized in mtDNA[4].

Mitochondria as energy producers in the form of adenosine triphosphate (ATP) are needed to maintain cell activity and integrity. In mitochondria, ATP synthesis can take place in the presence of ATP synthase. ATP synthase is an enzyme that synthesizes ATP through oxidative phosphorylation
reactions. In the ATP synthase structure there is a subunit area, the part is encoded by mitochondrial DNA (mtDNA), namely ATPase6. The presence mtDNA mutations especially in the ATP6 gene will affect ATP synthesis. Disruption of energy synthesis (ATP) is one of the causes of the development of certain diseases. ATP disruption could happen from the defect of the OXPHOS systems, it inhibited flow of electrons through the respiratory chain. It caused an imbalance of NAD+/NADH, oxidative stress and a potential reduction of the mitochondrial membrane [5]. In previous studies it has been reported that T2DM is associated with mutations in mitochondrial DNA (mtDNA), namely the new 9053G>A mutation in the ATP6 gene region in patients with type 2 DM and cataracts [6]. 8561C> G mutations have been reported in the ATP6 gene in two siblings with a phenotype consisting of cerebellar ataxia, peripheral neuropathy, diabetes mellitus, and hypergonadotropic hypogonadism [7]. Therefore, we need to find more information which provide the correlation between ATP6 gene mutation to T2DM or other mitochondrial disease.

2. Methods
The blood samples were collected from Dustira hospital, Cimahi-West Java. We had collected two blood samples from DM2T patients. The characteristic of the samples were female, having an age between 50-65 years, and had no complication yet. Samples were namely A and B. A pairs of primers ATPase6F 5' -ATGAACGAAAATCTGTTCGCTTCATTCATT-3' as forward primer and ATPase6R 5'- AATACACAACGCAGCTCCATCTCCGA-3' as reverse primer were designed based on ATP6 gene human sequences (GenBank NC_012920.1).

Blood cells were attempt by washing the red blood with TE buffer as much as 3 times. DNA template was obtained by cell lysis using buffer lysis. Amplification of DNA template was attempt by PCR technique. All primers were designed using DNASTAR version 4.0. PCR fragment were analysed by using 1% agarose gel electrophoresis. Nucleotide sequencing was based on dideoxy Sanger method by Macrogen Inc., Korea [8]. Analysis of ATP6 fragment sequences were done by using Blastn. Homological analysis and point mutation were done by Seqman software. Analysis of amino acid mutation were using ClustalX software.

3. Result and Discussion
ATP6 gene was amplified by PCR annealing gradient temperature. It was showed that the best of ATP6 gene fragment was at temperature 55°C. The fragment was 0,7 kb in size. (Figure 1). Database showed that ATP6 gene of human consist of 681 base pairs (bp). Therefore, the size of ATP6 fragment were approximately close to human ATP6 gene.

![Figure 1. Gradien annealing temperature PCR. M: 1kb DNA ladder. T1: 55°C, T2: 56,3°C, T3: 57,2°C, T4: 58,3°C, T5: 60,6°C, T7: negative control](image)

The result of identical analysis from samples were using blastn service. Sample A was 98,68% close to ATPase synthase 6 mRNA homo sapiens. While sample B was 98,84% close to homo sapiens
It was clearly showed that the primers is the right primers for amplifying ATP6 gene of human mitochondria.

Figure 2. Homological analysis samples. (a): Sample 1 and (b): sample 2. The similarity were showed in red box.

A point mutation could be identified by alignment analysis. In this report, we had aligned sample A, B, and ATP6 gene (GenBank NC_012920.1) to find DNA variation in ATP6 fragment. There was one DNA point mutation 8860A>G. The mutation was occur in both samples and same position (figure 3).

Analysis of amino acid alteration was using ClustalX. The nucleotide A at position 8860 was the first nucleotide for codon Threonine (T). Nucleotide substitution A to G had altered the codon from ACA into GCA, which lead to amino acid changes Threonine (T) which is polar amino acid to Alanine which is non polar amino acid (A) (figure 4).
Figure 3. Alignment of ATP6 fragment gene from both samples with ATP6 gene from GenBank. Point mutation were in black box. Both samples had 8860A>G.

Figure 4. Amino acid alignment of ATP6 gene (1) and ATP6 gene fragment. The amino alteration is A>G, marked in orange box.

Interestingly, the MITOMAP analysis of showed that 8860A>G in ATP6 gene was a haplogroup, which is mean all samples that we used is have the same ancestor [9]. Also, there are some articles mention 8860A>G mutation occur whether in people with DMT2, cancer, and hypertrophic cardiomyopathy (HCM) [10]. The rRCS nucleotide adenine (A) is predominant, but from the consensus it was G which were dominant. It was sometime led to misunderstanding the role of 8860 transition. It was analysed that 8860A>G were present higher in people with some diseasne, like abdominal aortic aneurysm, AD, PD, T2DM with or without angiophaty [11]. From this explanation, an alteration DNA at position 8860 in mtDNA is not clearly stated to be a genotype characteristic for DMT2. Hence, it is need further research to convince that 8860A>G mutation related to DMT2. We assume that based on our research, the point mutation connected to DMT2 were also probably located in other region of the mitoconhdrial genome.

4. Conclusion
ATP6 genetic analysis from DMT2 local isolate was conducted. Surprisingly, there is only one DNA point mutation 8860A>G found. The mutation were haplogroup, and is not clearly whether it is related to genotype characteristic of DMT2. Therefore, a further research is needed to convince that 8860A>G in ATP6 gene is related to alteration of ATP6 gene which cause DMT2.

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