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Studies on Specific Nucleotide Mutations in the Coding Region of the ATP6 Gene of Human Mitochondrial Genome in Populations of Papuan Province-Indonesia

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Abstract: Studies of mitochondrial DNA (mtDNA) variants of normal human and related to the disease has been conducted. Several variants of human mtDNA nucleotides in Papuan-Indonesian has been identified that include genes that encode proteins: ND2, ND4, ND5, CO1, and tRNAs and the region that do not encode protein (D-loop). The aims of this study is to create a database of normal human variants of Papuan and its translation products, while the aim in particular is the amplification of fragments of 4.1 kb of human mtDNA and determine the nucleotide sequence. Here we showed that the number of nucleotide sequences of the three Papuan samples of human muscle cells that successfully determined the sequence is 3195 bp, which includes genes of ND2, COII, tRNA-Lys and ATP8 to 100% homology to sequences rCRS, and ATP6 gene there are two mutations are found in two samples G9053A and G9192A on one sample. G9053A mutation causes amino acid change serine to asparagine, whereas G9192A did not cause amino acid changes. Based on comparison of the MITOMAP data, all samples showed no presence of nucleotide variants associated with diseases.

Key words: mtDNA mutation, ATP6 gene, direct sequencing, Papuan population.

INTRODUCTION

Mitochondria are organelles in eukaryotic cells that have a very important role in maintaining cell viability, this is related to its function as an energy in form of ATP compounds. Most of the energy needs of cell generated through a series of oxidation-reduction reactions that occur in multisubunit enzyme complexes I, II, III, IV, V which is integrated in membrane of the mitochondria, this process is aerobic because it involves oxygen (O_2) and ultimately produce compound high energy ATP (Mathews, C.K., and Van Holde, K.E., 1999; Wallace, D.C., 1997).

Complete nucleotide sequence of mitochondrial DNA of human as much as 16,569 base pairs (bp) are arranged in a circle (circular) was first published by Anderson in 1981 (Anderson *et al*., 1981), later called consensus Anderson or Cambridge and became a reference standard in interpreting nornal nucleotide variant and variants associated with diseases (Marzuki *et al*., 1991). The structure consists of human mtDNA genes that encode proteins that two genes ribosomal RNA namely 12S and 16S rRNA, 22 tRNA genes, and 13 genes that encode 13 protein subunit of 70 polypeptides of complex respiratory enzymes and proteins are not coding regions, D-loop and some region between genes.

High mutation rates in mtDNA because mitochondria do not have a repair system for the replication process. mtDNA has no protective proteins (histons) and presence of oxygen free radicals as by products of oxidative phosphorylation reaction (Watson *et al*., 1987; Lewin, 1997; Wallace, D.C., 1999; Albert *et al*., 1994). High mtDNA mutation rate can lead to differences in nucleotide sequence (polymorphisms) of mtDNA between individuals is also high (Wallace, 1989). MtDNA mutations can occur in normal variants and variants associated with disease. Mutations in the normal variant usually occurs in the mtDNA that do not encode proteins or in genes that encode proteins but amino acid changes do not affect the function of an enzyme that translates (Marzuki *et al*, 1991; Lertrit *et al*., 1994; Barnes, 1994). Mutations in the variance associated with the disease usually affects the translation process (Noer *et al*., 1991; Moraes *et al*., 1999) resulting in a different translation products as well as changing the amino acids that have an important function of the enzyme.

The study of human mitochondrial DNA variant associated with both normal and disease continue to be made. Several variants of human mtDNA nucleotides in Indonesia has been identified that include gene coding

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for proteins (ND2, ND4, ND5, CO1, and rRNAs) and regions that do not encode protein (D-loop). Study of D-loop region of homology in individuals from three to seven generations of descendants of the mother line and approximately 98 % homology to the D-loop on individuals from various ethnic groups in Indonesia has been demonstrated (Ngili *et al*., 2012). Similarly, the determination of the nucleotide sequence of 0.9 kb, genes ND2 and CO1 (5274-6203) and the 1.6 kb fragment of the ND4 and ND5 genes have been successfully determined (Cann *et al*., 1987; Lertrit *et al*., 1992).

Determination of nucleotide sequence performed by direct sequencing is the method of determining the nucleotide sequence of the PCR fragments directly without going through the process of cloning. Direct sequencing performed using four primer namely C_L, C_2R_2, C_2R_1, dan C_H (Marzuki *et al.,* 1991; Noer, A.S., 1991). The results of this study are expected to complete the database normal variant of Indonesian human mtDNA.

MATERIALS AND METHODS

Template Preparation of mtDNA:

Samples used in this study were human forensic samples of Papuan ethnic of Indonesia. Dilute the sample preparation is done by freezing the muscle cells at room temperature, then weighed 100 mg of muscle cells and enter into a sterile 1.5 mL eppendorf tubes. Muscle cells cut into small pieces using a sterile surgical scissors, then washed with TE buffer pH 8 (10mM Tris-HCl pH 8 (Pharmacia Biotech) and 1mM EDTA pH 8 (J.T. Baker)) several times until the resulting while pellets (Sambrook *et al*, 1989). After incubation the reaction mixture was centrifuged using a microcentrifuge (Eppendorf Centrifuge 5417C) at 20,000 g for 3 min and then taken the supernatant used as template for PCR reaction (Noer *et al*., 1994).

In vitro Amplification of mtDNA with the PCR Method:

In this research the amplification of mtDNA fragments of 4 kb; which includes the genes ND2, CO1, CO2, ATP8, ATP6, and CO3 by PCR using primers C_L dan C_H. Nucleotide sequence of each primer is C_L 5'-GGAGGCCTGCCCCCGCTAACCGGC-3', primer C_H 5'-CTGGGTTTTCTATATGATAGGCATGT-3' (27 nt), (Noer A.S., 1991; Marzuki *et al*., 1991). PCR reaction was performed in 0.2 mL tubes (eppendorf), with a reagent consisting of 10 μL mtDNA template lysis, 20 pmol of each primer, 5 μL 10x PCR buffer (Amersham Pharmacia Biotech: 500 mM KCl, 15 mM $MgCl_2$, and 100 mM Tris-HCl pH 9.0 at room temperature,), 1.25 units of *Taq* DNA polymerase enzyme (Amersham Pharmacia Biotech), 200 μM dNTP mix (Amersham Pharmacia Biotech) and added sterile ddH_2O so that the volume is reached 50 50 μL (Noer, 1991; Marzuki *et al*., 1991; Qiagen, 1995). The process of PCR using the *GeneAmp PCR System 2400* (Perkin Elmer) machine by 30 cycles with the stages of the process as follows: initial denaturation at 94 °C (2 min), then go to program PCR cycles with each cycle consisting of three stages, namely stages of denaturation at a temperature 94 °C (1 min), *annealing* 55 °C (1 min) and *extension* or polymerization at temperature 72 °C(3 min) (Noer, A.S., 1991; Marzuki *et al*., 1991, Newton, C.R., and Graham,A., 1997; Innis, A.M., and Gelfand, H.D., 1990; Ngili *et al*., 2012). At the end of all cycles carried out additional polymerization process at temperature 72 °C for 10 min, which aims to improve the polymerization reaction that not perfect. DNA PCR results are stored at –20 °C prior to further processing.

Agarose Gel Electrophoresis:

PCR amplification of DNA results are then analyzed by agarose gel electrophoresis 0.8 % (b/v) (Boehringer Mannheim) (Noer, A.S., 1991) using a *mini sub* ™ *DNA electrophoresis cell* (Biorad). DNA size marker used is λ/*Hin*dIII (Amersham Pharmacia Biotech), which has 8 bands (each measuring 23,130 bp, 9,416 bp, 6,557 bp, 4,364 bp, 2,322 bp, 2,027 bp, 564 bp, and 125 bp). The results of electrophoresis visualized with UV lamp series 9814-312 nm (Cole Parmer). Prediction of the size and concentration of DNA PCR products can be done by comparing the position and thickness of the bands which was analyzed by PCR results of the marker bands whose concentration had been determined previously (Sambrook *et al*, 1989).

The Results with GFX PCR Purification Kit:

Purification of the PCR products is done by using GFX kit (*GFX* ™ *PCR DNA and gel band purification kit, Amersham Pharmacia Biotech*). The initial step in this purification process is the concentration of fragment 4 kb mtDNA PCR products with a vacuum concentrator (Eppendorf Concentrator 5301) until the volume of 15 μL. The 15 μL concentration results electrophoresed with 0,8 % agarose gel in TBE buffer 0.5x (Merck: tris-borat 0,09 mM and EDTA 0,002 mM). Electrophoresis is performed at a voltage of 80 volts for 60 min in 0.5x TBE buffer solution and used as DNA marker λ/*Hind*III. Determination of the concentration of DNA purification performed with 0.8 % agarose electrophoresis and standard DNA marker λ/*Hind*III (Amersham Pharmacia Biotech, 1999).

Direct Sequencing Through the Sanger Dideoxy Method:

Sequencing method used is the method of dideoxy Sanger using *Automatic DNA Sequencer* instrument with *Dye Terminator Labeling* method. Reagents used derived from *ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit* (Perkin Elmer). Stages of mtDNA direct sequencing performed on the study include: (1) direct sequencing reactions of the purification of PCR products using primers C_L, C_H, C_2R_1 and C_2R_2, (2) purification of sequencing results with sephadex G-50 column, (3) electrophoresis polyacrylamide gel, and (4) reading electrophoregram and analysis of sequencing results.

Sequencing Reaction:

A total of 3 μL GFX purification of template DNA (300 ng) added each of the 1,2 μL primer (2.4 pmol), which used the sequencing primers C_L 5'-GGAGGCCTGCCCCCGCTAACCGGC-3' (24 nt), C_H 5'-CTGGGTTTTACTATA TGATAGGCATGT-3' (27 nt), C_2R_1 5'-AGATTTTCGTTCATTTTGGT-3' (20 nt), and C_2R_2 5'-CCCCCGGTCGTGTAGCGGTGAAAG-3' (24 nt) (Noer, A.S., 1991; Marzuki *et al*., 1991), then add to the volume of ddH_2O sterile until 9 μL. After the reaction mixture was homogenized inserted into the PCR machine (*Thermal cycler 480 Perkin Elmer*) for denatured at 96 °C for 30 seconds and then stopped a moment to do the added of 6 μL reaction mixture containing A-*dye terminator*, G-*dye terminator*, C-*dye terminator*, and T-*dye terminator*, dNTP, Tris-Cl (pH 9,0), $MgCl_2$, pyrophosphate thermostable and *Amplitaq DNA Polymerase*. Sequencing reaction was homogenized using a pipette tip, and then resumed with the process as much as 25 cycles of PCR. PCR conditions for each cycle of denaturation conducted at temperature of 96 °C for 30 sec, annealing at a temperature 48 °C for 15 sec, and extension at 60 °C for 4 min (Perkin Elmer, 1995).

Purification of Sequencing Results with Sephadex G-50 Column:

DNA purification is done by using the amplification kit Sephadex G-50 column (Sephadex G-50 DNA Grade F-Micro Spin™ G-50 columns). Column containing Sephadex G-50 was vortex until homogeneous and then followed by centrifugation at 3,000 g for 1 min in microcentrifuge series 5417C, forming a solid gel (column). Concentrating the DNA pellet was added 4.5 μL *Loading buffer* (Merck: 5 parts formamide and 1 part 25 mM EDTA pH 8.0 containing 50 ng/mL blue dextran) and then denatured at 92 °C for 2 min and cooled in ice, so be prepared for the DNA samples electrophoresed in polyacrylamide gel (Perkin Elmer, 1995).

Polyacrylamide Gel Electrophoresis:

Electrophoresis performed on polyacrylamide gel (Merck: 40 mL polyacrylamide 6%, 200 μL ammonium sulfate, and 23 μL TEMED). Before use polyacrylamide gel wells were washed with 1xTBE buffer (Merck: tris-borate 0.09 mM and EDTA 0.002 mM). After that, 4 μL sample of DNA inserted into each well. Electrophoresis performed at 32 Ampere and voltage current from 1300 to 1400 Volt for 14 h (Perkin Elmer, 1995).

Analysis of Sequencing Results:

Analysis of the results is done by comparing the nucleotide sequence data with electrophoregram data using DNASTAR program, and then determine the size and nucleotide sequence is read as well as the nucleotide sequence by using the program editseq. The nucleotide sequence of all samples is further processed by DNASTAR Megalign program to determine the homology between the sample and CRS sequence. Determination of translation products made using the program editseq based on human mitochondrial genetic codons and the properties of the translation products were analyzed using DNASTAR protean program (DNASTAR, 1997).

RESULTS AND DISCUSSION

Penyiapan Templat mtDNA:

In this research used three forensic samples of muscle cells forensic autopsy samples of normal variants men aged between 40-50 years. Three human samples obtained from individuals in the tribes of Jayapura, Papuan Province, Indonesia. Muscle cells cut into small pieces, then washed with TE buffer pH 8 several times until the resulting white pellets. Washing with TE buffer aims to eliminate red blood cells and other impurities that attach to the muscle cells by binding to metal ions (eg. iron ion in hemoglobin) using EDTA as anticoagulant agent (Sambrook *et al*, 1989).

Lysis of the white pellets are chemically performed using lysis buffer and Tween-20 (Merck). Tween-20 is a non-ionic detergent to form micelles in solution and its molecular structure has a hydrophilic part is composed of ester or alcohol and a hydrophobic part which is a hydrocarbon compound. Interaction between the hydrophobic micelles Tween-20 with phospholipids compound makes the cell membrane of the membrane phospholipid compounds soluble from mixed micelles with tween-20, and the heating at temperature of 55 °C

led to the structure of cell membranes become damaged. The addition of the enzyme proteinase K 10 mg/mL aim to degrade the DNase enzyme and other proteins. The enzyme is then deactivated proteinase K at temperature of 95 °C for 5 minutes. DNA extracts used as templates for PCR (Noer *et al*., 1994; Crimi *et al*., 2002; Reider *et al*., 1998).

Fragments of 4024 bp of mtDNA Amplification by PCR:

PCR is a technique for *in vitro* amplification of spesific regions bounded by a pair of DNA primer. Primers used in this study is the C_L and C_H (Noer, A.S., 1991; Marzuki *et al*., 1991), the sequence and nucleotide position are listed in Table 1. MtDNA region were amplified between nucleotide positions 5223 to 9247 which includes the size of 4024 bp genes *ND2, COI, COII, ATP8, ATP6* and *COIII*.

Table 1: Nucleotide sequence and position of primers C_L dan C_H. Both primers used in PCR reactions for amplification of 4024 bp fragment of human mitochondrial DNA (Marzuki *et al.,* 1991; Noer, 1991).

Primer Name	5'→3' sequences	Position	Nucleotide size
C_L	GGAGGCCTGCCCCCGCTAACCGGC	5223	24
C_H	CTGGGTTTTACTATATGATAGGCATGT	9247	27

PCR reaction using Taq DNA polymerase enzyme with standard buffer from Amersham Biotech and the process lasts for 30 cycles with denaturation phase of 94 °C (1 min), annealing 55 °C (1 min), and extension 72 °C (3 min) (Noer, A.S., 1991; Marzuki *et al*., 1991). The results of agarose gel electrophoresis 0,8 % (b/v) in 1xTAE solution at a voltage of 80 volts for 45 min showed the existence of a 4 kb band, and used as a standard DNA λ/*Hin*dIII.

4 kb Fragment Purification Using GFX PCR Kit:

4 kb fragment of PCR results from three Indonesia human samples was further purified using the GFX kit. Function of GFX kit is to remove salts, enzymes, residual nucleotides and PCR primers, while the GFX kit is the working principle of the spesific DNA binding with a glass fiber matrix and using chaotropic reagent which can denature proteins and dissolve the agarose the DNA was eluted with sterile ddH_2O (Amersham Pharmacia Biotech, 1999), and prepared as DNA template for direct sequencing process.

Direct Sequencing and Analysis of Sequencing Results:

In this study the determination of the nucleotide sequence is done by direct sequencing is the method of determining the nucleotide sequence of the PCR fragments directly without going through the process of cloning. The reason for the direct sequencing method is faster process and the PCR result is a dominant nucleotide sequences of the DNA population. This method can not determine the mutation in the DNA that is not dominant, for example, mutations in DNA heteroplasmy. Sequencing was performed using four primers are C_L, C_2R_2, C_2R_1, and C_H, which is the design of previous researchers (Marzuki *et al.,* 1991; Noer, 1991) as shown in Table 2.

Table 2: Sequence data, position, size and place of attachment of DNA strands (hybridization) primer. These four primers used in sequencing reactions to determine these sequence of human mitochondrial DNA Papuan, Indonesia (Marzuki *et al.,* 1991; Noer, 1991).

Primer Name	5'→3' sequences	Position	Size	Hybrid strands
C_L	GGAGGCCTGCCCCCGCTAACCGGC	5223	24 nt	H
C_2R_2	CCCCCGGTCGTGTAGCGGTGAAAG	8156	24 nt	L
C_2R_1	AGATTTTCGTTCATTTTGGT	8540	20 nt	L
C_H	CTGGGTTTTACTATATGATAGGCATGT	9247	27 nt	L

Based on data analysis of electrophoregram sequencing results of the three samples of Papuan human mitochondrial DNA is known the total number of nucleotide successfully determined the sequence is 3195 bp, which includes the nucleotide sequence of sequencing results of the primers C_L = 204 pb, C_2R_2 = 294 pb, C_2R_1 = 248 pb, and C_H = 319 pb. The results of comparison with the nucleotide sequence of CRS (Anderson *et al.,* 1991) contained two mutations in the results of these sequencing using primers C_H, base C to T mutation at nucleotide position 9053 and 9192, while in other regions showed homology 100%. The results include partial sequencing of the gene as much as 204 bp ND2, COII as much as 294 bp, tRNA-Lys and ATP8 as much as 248 bp and 319 bp ATP6, respectively.

C9053T or G9053A mutation occured in two samples of P1 (Papuan 1) and P2, while the C9192T or G9192A mutation occurs only in one sample that is only sample P3. C9192T or G9192A mutation occurs because of changes in base cytosine (C) to base thymine (T) at position 9192 of the P3 sample. While the C9053T or G9053A mutation occurs because of change the cytosine to thymine base at position 9053 of the sample P1 and P2.

Nucleotide Sequence Analysis of 8902-9220 Genes ATP6 and Translation Products:

The results of sequencing with the primer C_H in the direction of H strand of mtDNA (Fig. 1A), because the fragment C_H (8902-9220) is part of the ATP6 gene, whereas the ATP6 gene transcription in the direction of L strand of mtDNA, then the determination of the translation product (Fig. 1C) carried out on the reverse complement (Fig. 1b) of the nucleotide sequence of the fragment C_H. Determination of translation products using DNASTAR editseq program based on human mitochondrial DNA genetic codon and the translation of human ATP6 gene from the NCBI database.

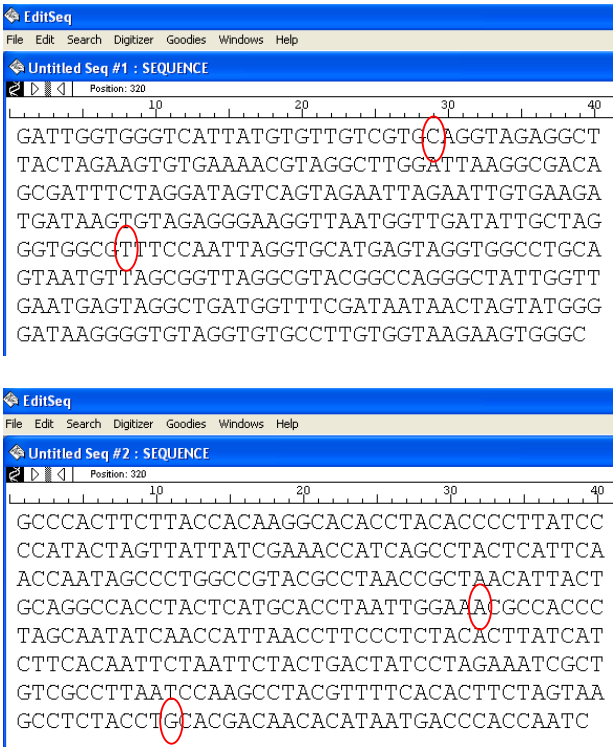

Fig. 1: Determination of translational products of the nucleotide sequence of the fragment C_H (8902-9220). [A] P1 nucleotide sequence of the same sample by sample P2 contained a C9053T mutation, the same position with CRS, while no mutation C9053A P3 samples. [B] The results of reverse complement of samples P1 and P2 be the mutation C9053T to C9053A and sample P3 G9192A mutation into G9192A. [C] The product translational produce 105 amino acid, where 101 amino acid is the translation product ATP6 remaining COIII gene, mutation of serine (S) to asparagines (N) occurs in the amino acid 51.

Comparison of nucleotide variants-amino acid between the three samples the results of the experiments with the normal variants of the consensus (Marzuki *et al.,* 1991; Strachan and Read, 1999) and *database* MITOMAP (www.mitomap.org) showed that the mutation G9192A has never been found, while other researcher have found G9053A mutation of previous investigators from other ethnic groups in normal human variant in the population of Papua, Indonesia.

Based on the results of the analysis showed that the 101 amino acid translation product C_H fragment is part of the enzyme ATP synthase 6. Total number of amino acids in the enzyme ATP synthase 6 is 226 amino acids, amino acids 101 to occupy the center of the C terminal, as shown in Fig. 2

1 MNENLFASFI APTILGLPAA VLIILFPPLL IPTSKYLINN RLITTQQWLI KLTSKQMMTM
61 HNTKGRTWSL MLVSLIIFIA TTNLLGLLPH SFTPTTQLSM NLAMAIPLWA GTVIMGFRSK
121 IKNALAHFLP QGTPTPLIPM LVIIETISLL IQPMALAVRL TANITAGHLL MHLIGSATLA
181 MSTINLPSTL IIFTILILLT ILEIAVALIQ AYVFTLLVSL YLHDNT

Fig. 2: Amino acid sequence of the enzyme ATP synthase 6. Amino acid sequence of the translation products occupies the middle to end of the C terminal (blue) ATP synthase enzyme, mutation of serine (S) to asparagines (N) occurs at position 176 (red).

G9053A mutation in samples P1 and P2 causes the second base of codon mutation AGC to AAC and based on mitochondrial genetic code led to changes in amino acid serine (S) to asparagine (N) occurs at position 51 or position 176 translational product on the enzyme ATP synthase 6. While the G9192A mutation in sample P3 mutations cause the third base of codon CTG (CUG) to CTA (CUA) based on mitochondrial genetic code does not cause amino acid changes, both CUG and CUA is a codon from leucine (L). Change in the third base of a codon normally do not cause amino acid changes, this is due to the wobbling (non-standard base pairs) on the third base codons that are recognized by the base of the anticodon of tRNA is flexible so that causes one kind of tRNA molecule can recognize more than one codon (Lewin, B., 1997; Thyagarajan *et al*., 1995; Wallace *et al*., 1988).

The enzyme ATP synthase subunit 6 is part of the F_O ATPase. This enzyme to be one component of the proton channel that plays a role in proton transfer from the outer side of the membrane into the mitochondrial matrix. Mechanism of proton transfer and ATP synthesis occurs in the enzyme ATP synthase subunit complex F_OF_1 or complex.

Analysis of Serine Into Asparagine Mutation at Position 176 of the Enzyme ATP Synthase 6:

Data showed the results of several amino acid mutations occurred in the enzyme ATP synthase 6 in both normal and variant associated with diseases such as LHON and MERRF. G9053A mutation causes amino acid change serine to asparagines at position 176 instead of the enzyme ATP synthase 6 as a new mutation was found in normal human variant. Comparison with literature data of mutations G9053A, G9053A mutation was flanked by two other mutation are A9053G and G9055A. G9055A mutation (Marzuki *et al.*, 1991; Cheng *et al*., 1994; Holt *et al*., 1990) which causes amino acid change alanine (GCC) to threonine (ACC) at position 177. These mutations occur in a normal variant, the mutation of serine to asparagines at position 176 of the samples P1 and P2 can also occur in normal variant. Based on this analysis it can be estimated that approximately 176-177 positions are not important for the function of the position of the enzyme ATP synthase 6. While the A9052G mutation that changes serine (AGC) to glycine (GGC) is still a new discovery that is associated with disease (Crimi *et al.*, 2002; Solano *et al*., 2001; Redd *et al*., 1995).

Effect of variant amino acid serine to asparagines at the translational product is not yet determined, but it appears that the function of the translation products did not change because all the samples are normal variants. Serine amino acid change to asparagines does not change the hydrophilic properties, thus changing this amino acid is not expected to alter the conformation of the enzyme ATP synthase 6 significanly (Thorpe, 1984; Cheng and Kolmodin, 1997; de Vries *et al*., 1993; Lamminen *et al*., 1995).

Conclusion:

In this study identified the existence of two transitions mutations, G9053A and G9192A in mitochondrial DNA ATP6 gene of three human in the population of Papua, Indonesia that has no family relationship. G9192A mutation is known that a new mutation that has not been repoted. All samples showed no nucleotide variants associated with disease.

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