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Variants Analysis of Human Mitochondrial Genome Mutation: Study on Indonesian Human Tissues

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Abstract: Comparison of variants of mutations that occur in the Indonesia human mitochondrial genome between tissues which origins are from layers of endoderm, mesoderm, and ectoderm have been investigated. In this study, analyzed the diversity of the mtG in various tissues of different origins, namely layer of entoderm, mesoderm, and ectoderm are compared with sequence of *revised Cambridge Reference Sequence* (rCRS). Determination of mtDNA mutations carried by the isolation procedure followed the protocol of DNA purification from tissues, amplification by using the technique repli-G, determining the nucleotide sequence of the mtG Sanger dideoxy method, and mtDNA *in silico* sequence analysis. Here we showed that mutation variants between tissues in a normal human individual (not associated with diseases caused by mtDNA mutations) are monomorphic. The results of mutation analysis of mtG variants to rCRS showed that nucleotide mutations in several regions, and found eight variants of the novel mutations that have not been reported in MITOMAP.
Keywords: Variants, mtG, mtDNA mutation, repli-G.

Introduction

The research results of mitochondrial DNA (mtDNA) of human with unique properties have been widely used in various disciplines, including studies of the evolution, population genetics, bioinformatics, genetic diseases, and forensic science.[1-2] Significant information in the related research is the nucleotide sequence of mtDNA of mtG D-loop is a hypervariable region because mtDNA mutation rate is relatively higher than other regions. Hypervariable properties the mtDNA has been associated with the comparison of nucleotide sequences and mtDNA mutations between individuals, ethnic/tribes, and age of human being.[3-5] However, how the pattern of mutations occuring variants of the mtG in various tissues of different origin such as a layer of endoderm, mesoderm, and ectoderm in certain individual has not been reported.

The changes of structure and function of mitochondria may cause mitochondrial disease. Decline in mitochondrial function could be due to a decrease in respiratory function of the enzyme complex that could inhibited respiration reactions (EXPHOS) in the formation of ATP[6-7] or caused by free radical attack of ROS (*reactive oxygen species*) in various tissues.[8-9] Mitochondrial diseases are caused by mutations in genes in the mitochondria and mtDNA genetic changes affect the and function of mitochondria which then affects the reaction of oxidative phosphorylation, mitochondrial biogenesis and other metabolic pathways.[10-12]

In this study, the differences in mtG nucleotide mutation compared to rCRS and analyzed mtDNA homology to a particular individual. Human tissue samples were analyzed through the isolation of mtDNA template, PCR process using the repli-G technique, and DNA sequencing by the Sanger dideoxy method. The results of mtDNA analysis further carried out to determine novel mutation or which have not been reported with mutations in these variants compared to the MITOMAP database.

Materials and Method

To obtain primary data, samples of some of Indonesian human tissues derived from several different layer are analyzed. Research step conducted through the efficient design of primers for amplification of mtG, purification step of DNA isolation from tissues, amplification by using repli-G technique, nucleotide sequence determination by Sanger dideoxy method, and the final step is to perform mutation *in silico* analysis of variants

Preparation of template mtDNA

Samples used in this study is a sample of forensic autopsy tissues of human origin derived from layer of endoderm, mesoderm, and ectoderm. Samples were stored in eppendorf tubes 1.5 mL in frozen state (-20 ^{o}C), to prevent damage of mtDNA. Tissue samples were taken from the Department of Forensic Medicine and Medicolegal, Hasan Sadikin Hospital/Faculty of Medicine, University of Padjadjaran.

Isolation of human mtDNA

Mitochondrial DNA isolation procedures from tissues following the protocol of DNA purification from tissues (*QIAamp DNA Mini Kit*). Tissue samples were weighed using analytical balance to be isolated samples 25 mg (1 mg of tissue equivalent to 0,2-1,2 µg DNA). For spleen tissue samples are used only 10 mg because of the abundance of mtDNA in this tissue.[13-14]

***In vitro* amplification of repli-G**

For amplification of the mtG, pipette 5 µL DNA template from isolated product using the QIAamp DNA Mini Kit, and entered into tube 200 µL. Added to 20 µL sample volume using RNAase. Then added 29 µL of reaction buffer repli-G, then mixed with vortex. Incubating the samples for 5 min at 75 ^{o}C, then cooled the sample to temperature 25 ^{o}C. Add repli-G midi DNA polymerase to the DNA, mixed and vortex. Incubation of samples at temperature 33 ^{o}C for 8 hour, and then repli-G midi inactivation of DNA polymerase by heating the samples for 3 min at 65 ^{o}C. Save the mtDNA that has been amplified in the freezer at temperature -20 ^{o}C. The amplified mtDNA was analyzed by using PCR, the samples need to be delusional by comparison 1:1000 in TE buffer. Used 2-3 µL of mtDNA that have been deluded for each time PCR.[15]

Design of mtG primers

Design of primers for amplification of mitochondrial genome fragments and internal primers for direct sequencing is done using Perlprimer simulation software to simplify the determination annealing temperature. Mitochondrial genome template was prepared by using the repli-G technique. Template genome is divided into ten fragments of mtDNA (A, B, C, D, E, F, G, H, I, and M) with genomic template amplification performed using the primers pairs shown in Table 1.

PCR reaction and processes

Ten fragments amplification reaction catalyzed by DreamTaq PCR Mastermix containing DNA DNA Polymerase DreamTaq enzyme (Fermentas). PCR process is done by Automatic engine thermal (Perkin Elmer) by 30 cycles. The early steps of the PCR process is the initial step denaturation at 95 °C for 3 min, then go to program PCR cycles with each cycle comprising three steps, namely denaturation step performed at temperature 95 °C for 30 second, annealing step is conducted at temperature 50 °C for 30 second and extention or polymerization step conducted at temperature 72 °C for 60 second. End of all cycles carried out additional polymerization process at the temperature 72 °C for 10 min.[16] The results of the PCR amplification is then analyzed by agarose gel electrophoresis using *sub^{TM} DNA electrophoresis cell* instrument. Electrophoresis process was performed in TAE buffer 1x as medium voltage conductor currents at 75 volts for 45 min. Control marker used was 1 kb ladder marker with size of 250 bp, 500 bp, 750 bp, 1000 bp, 1500 bp, 2000 bp, and 4000 bp. The results of electrophoresis visualized with UV lamp series 9814-312 nm (Cole Parmer).

Table 1. Data pairs of primers for amplification of mtG fragments.

Fragment	Forward primers		*Reverse* primer		Fragment size (kb)
	Name	Position (5'→3')	Name	Position (5'→3')	
A	Afor	458-479	Arev	2491-2473	2.1
B	Bfor	2324-2341	Brev	4252-4234	2
C	Cfor	4189-4215	Crev	6225-6208	2.1
D	Dfor	6046-6055	Drev	8095-8076	2.1
E	Efor	7925-7944	Erev	9916-9899	2
F	Ffor	9752-9770	Frev	11774-11757	2.1
G	Gfor	11624-11644	Grev	13639-13616	2.1
H	Hfor	13551-13568	Hrev	15434-15417	1.9
I	Ifor	15311-15328	Irev	824-807	2.1
M	Mfor	15978-15997	Mrev	429-409	1

Direct sequencing and analysis of mtG sequences

For the determination of the nucleotide sequence of mtG used PCR primers and several internal primers, as shown in Table 2. Map of the primers for fragment amplification, amplification and sequencing primers, as wells as fragments and the sequencing could be seen in Figure 1. mtG analysis performed using the application EditSeq, Seqman, and MegAlign in the DNASTAR program. Determination of variations in nucleotide mutation is accomplished using the Human mtDNA Analyzer version 1.2 and compared with MITOMAP. While the verification of the substitution mutation using spreadsheet program v.3 and v.4.

Table 2. Forward, reverse, dan internal primers data for sequencing.

Primers	Position (5'→3')	Oligonucleotide sequences
Afor	458-479	CCTCCCACTCCCATACTAACTAA
Bfor	2324-2341	TTCTCCTCCGCATAAGCC
Cfor	4189-4215	CCACTCACCCTAGCATT
Dfor	6046-6055	GGCAACCTTCTAGGTAACGA
Efor	7925-7944	GGCGGACTAATCTTCAACTC
Ffor	9752-9770	CGAGCTTCCCTTCACCATT
Gfor	11624-11644	TCTTCAATCAGCCACATAGCC
Hfor	13551-13568	CGCCTGAGCCCTATCTAT
Ifor	15311-15328	ATTGCAGCCCTAGCAACA
Arev	2491-2473	GGGGTAAGATTTGCCGAGT
Brev	4252-4234	GGGGAATGCTGGAGATTGT
Crev	6225-6208	AGGGAGGTAAGAGTCAGA
Drev	8095-8076	TAAGCCTAATGTGGGGACAG
Erev	9916-9899	GCTTCGAAGCCAAAGTGA
Frev	11774-11757	TGTGAGTGCGTTCGTAGT
Grev	13639-13616	GTTGACCTGTTAGGGTGAGAAG
Hrev	15434-15417	GGGCGTCTTTGATTGTGT
Irev	824-807	ATCACTGCTGTTTCCCGT
11Fg	6730-6749	GATCACAGGTCTATCACCCTA
2F	1138-1156	GAACACTACGAGCCACAGC
5F	2995-3013	GGATCAGGACATCCCGATG
8F	4832-4849	CACCCCTCTGACATCCGG
23F	14227-14246	CCCATAATCATACAAAGCCC
17F	10394-10414	CTGAACCGAATTGGTATATAG
Xin	5530-5550	CAGACCAAGAGCCTTCAAAGC
FBr	12048-12065	TCACACGAGAAAACACCC
Mfor	15978-15997	CACCATTAGCACCCAAAGCT
Dmt2L	8251-8270	GCCCGTATTTACCCTATAGC
Mrev	429-409	CTGTTAAAAGTGCATACCGCC

* Primer nucleotide sequence arranged in an efficient and evoid the overlapping mtDNA sequence [6, 14, 18, 20]

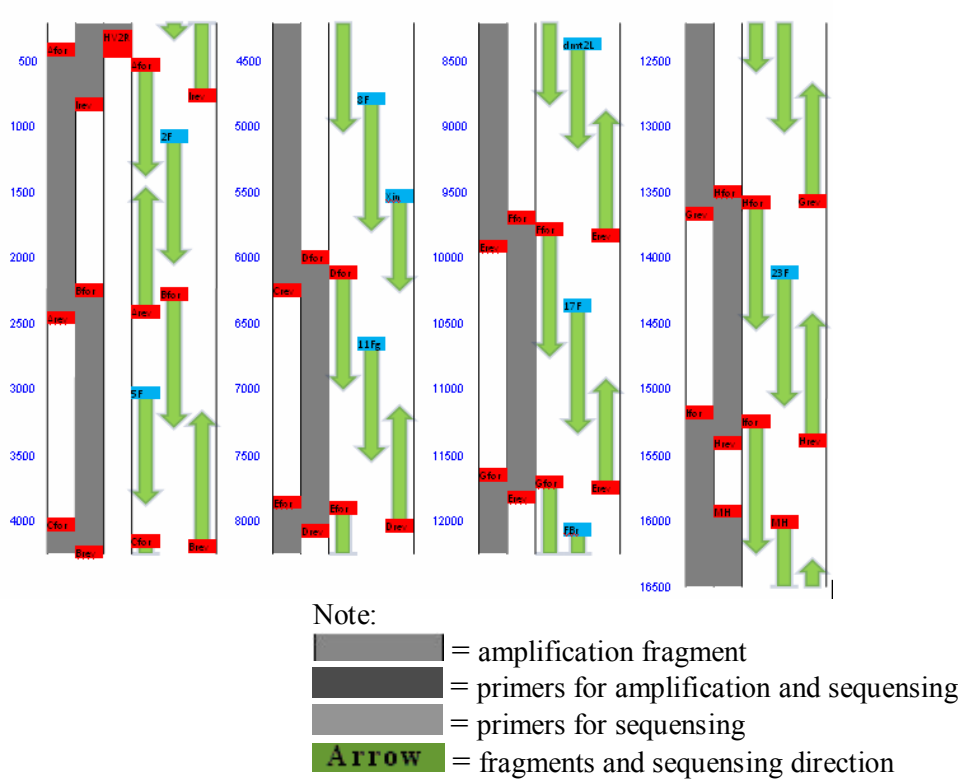

Figure 1. Map of human mtG primers

Results and Discussion

The results of ten fragments amplified

The process of DNA amplification performed using PCR method at annealing temperature 50 °C for amplification of the ten fragments (A, B, C, D, E, F, G, H, I, and M). Amplification results of ten fragments could be seen in Figure 2. The results of PCR amplification resulted in DNA bands of bright and consistent for each PCR fragment. PCR process by using repli-G could be minimize DNA contamination.[14] Six tissue from certain individual (individual A) was chosen to determine sequence of the Indonesian human mitochondrial genome from different layers of endoderm, mesoderm, and ectoderm. The results of electrophoresis of human DNA samples that have been successfull lysis of mtDNA through the process of isolation, PCR, and then electrophoresed consisting of tissues from liver, pancreas, spleen, kidney, brain, and skin. Electrophoresis results showed that the whole fragment is indicated by the bands light of the appropriate size. Electrophoresis results showed fragment A size of 2.1 kb, fragment B of 2 kb, fragment C of 2.1 kb, fragment D of 2.1 kb, fragment E of 2 kb, fragment F of 2.1 kb, fragment G of 2.1 kb, fragment H of 1.9 kb, fragment I of 2.1 kb, and fragment M of 1 kb (Figure 2).

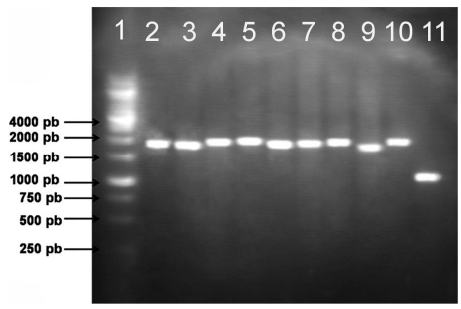

Figure 2. Characterization of ten fragments of the mitochondrial genome on 2 % agarose gel using 1 kb ladder marker.

Table 3. Variant nucleotide mutations that cause changes in the individual A.

Nucleotide position	mtG region	rCRS	Heart	Pancreas	Kidney	Spleen	Brain	Skin
650	12S	T	c	c	c	c	c	c
1438	12S	A	g	g	g	g	g	g
2706	16S	A	g	g	g	g	g	g
3873	ND1	A	g	g	g	g	g	g
4769	ND2	A	g	g	g	g	g	g
5213	ND2	C	g	g	g	g	g	g
5581	Int	A	g	g	g	g	g	g
6755	CO1	G	a	a	a	a	a	a
6863	CO1	A	g	g	g	g	g	g
7028	CO1	C	t	t	t	t	t	t
7894	CO2	A	g	g	g	g	g	g
8653	AP6	A	g	g	g	g	g	g
8853	AP6	A	g	g	g	g	g	g
8860	AP6	A	g	g	g	g	g	g
8886	AP6	G	a	a	a	a	a	a
9190	AP6	C	t	t	t	t	t	t
9531	CO3	A	g	g	g	g	g	g
10370	ND3	T	c	c	c	c	c	c
10373	ND3	G	a	a	a	a	a	a
11440	ND4	G	a	a	a	a	a	a
11719	ND4	G	a	a	a	a	a	a
12654	ND5	A	g	g	g	g	g	g
12705	ND5	C	t	t	t	t	t	t
13474	ND5	T	c	c	c	c	c	c
15211	CYB	C	t	t	t	t	t	t
15326	CYB	A	g	g	g	g	g	g
16111	D-loop	C	t	t	t	t	t	t
16168	D-loop	C	t	t	t	t	t	t
16172	D-loop	T	c	c	c	c	c	c
16183	D-loop	A	c	c	c	c	c	c
16189	D-loop	T	c	c	c	c	c	c
Total mutations			31 variants					

The results of direct sequencing of mtG

All sequences data analyzed with DNASTAR be in the form of .Seq and AB1 files, then do the analysis of nucleotide sequence homology with rCRS sequence using the DNASTAR program: Megalign and Seqman application, to determine the variant mutation in the tissue layers of the Indonesian human individual. Comparison of nucleotide mutations in tissue samples from the origin endoderm, mesoderm, and ectoderm with rCRS can be seen in Table 3 from individual A.

An example of mutation analysis of mtG variance and different with rCRS as shown in Figure 3, which is six tissue individual A, namely the liver and pancreas tissue (endoderm), spleen and kidney (mesoderm), brain and skin (ectoderm). These tissues were sequenced using internal primers A for with the amplification of the fragmen A. Analysis results after using the program Megalign and reinforced by observing the position of the mutation spectrum using the Seqman program contained mutation at position 650, where there is a difference of thymine to cytosine nucleotide (t650c). Mutations of this nucleotide substitution mutations, including the type of transition. The study results of mtG mutation, mutation that occur in human generally is mutation of nucleotide substitution transition.[17-18] Mutation substitution occurs if there is a substitution of nucleotide bases to other nucleotides without altering the length of mtDNA. These mutation occur only in the nucleotide position. Causes of the nucleotide transition mutations include base installation errors, and the mutagenic base analog.[4] Based on the analysis, substitution mutations can be restored to normal by doing a reverse process of transition or transversion, hence the substitution effect is not permanent.

The analysis further verified using the Seqman program compared with one normal variant, where no mutation at position 650 (same as rCRS). This is done to prove the identity of the mutations are valid, including the position and type of mutation that occured. mtDNA sequence data of mutations on the overall mtG regions on rCRS can be seen in Table 3 for individual A. Each tissue were sequenced using 27 internal primers so as to include the whole human mtG nucleotide base pairs. Both the amplification and sequencing primers used for the redesigned so that more efficient. The analysis of the individual A there are 31 variant nucleotide mutation.

Variant of individual A mutations compared with rCRS there are 31 variants of the mutation and the mutation comparison between each tissue in particuler individual is monomorphic. This conclusion is very important in the field of forensic medicine, especially in analyzing crime victims and criminal offenders. mtG regions that are potentially different to rCRS other than D-loop region is rRNA, ND2, CO1, ATP6, ND3, ND4, ND5, CYB region. Observation and tracking due to mutations in mtDNA protein-coding regions in the mtG is of particular important to the relationship between mtG mutation on expression of biochemical and clinical manifestations.
Whereas mtDNA mutation observed among individuals who are not descendants of the mother lineage showed that the mtDNA mutation rate was different. This is because over the life cycle of human mtDNA mutations accumulate in somatic cells, and increased number of mtDNA mutations in tissues that carry an important factor in the aging process. [1,8] The high mtDNA mutations is caused by high levels of reactive oxygen species as by products of oxidative metabolism of mitochondria and the absence of an effective DNA repair systems in cell organelles. Accumulation of mtDNA carrying the mutation would affect the capacity of tissues of oxidative energy metabolism, so that capacity will decrease with increase of age. The effects of this reduction proposed would manifest especially in tissue require a lot of ATP for its function, and seen as a decrease ability of the heart muscle and brain function. Further mutation analysis results and mtG regions mutated as shown in Figure 4. The results of mutation analysis of the overall mtG regions contained the substitution of transition and transversion mutations, which are scattered in several coding regions genes and non-coding region or D-loop, but by the number of mutations that are very different proportion of each region.

Figure 3. The analysis of nucleotide mutations in the A fragment by using primers Afor. The analysis showed mutation change at position 650, where in the substitution mutation: Thymine to Cytosine (T650c).

Table 4. Novel mutations that have not been reported in the MITOMAP database.

Mutation position	rCRS	Individual Δ	Type of mutation	Gen position in mtG
650	T	c	Ti	12S
3873	A	g	Ti	ND1
5213	C	g	Tv	ND2
6863	A	g	Ti	CO1
7894	A	g	Ti	CO2
8653	A	g	Ti	ATP6
10373	G	a	Ti	ND3
15211	C	t	Ti	Cytb

Ti : transition mutation
Tv: transversion mutation

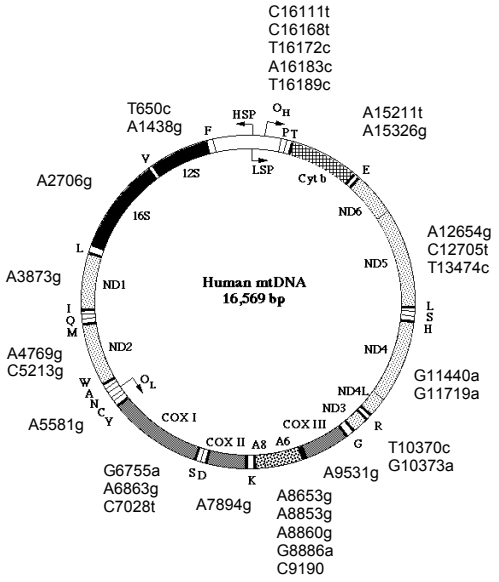

Figure 4. Map of nucleotide mutations in the mitochondrial genome of the human individual A.

Novel mutations in MITOMAP

Analysis of variants of new mutations that have not been reported, carried out by inserting the mutated nucleotide mutations positions of individual mutations in the nucleotide bases variant MITOMAP database. MITOMAP database provides various data that is informative, such mutations have been reported, the disease associated with mutations in mtDNA, researchers are reporting, data related to the sample origin (ethnicity), and other information. Information about mtDNA mutations associated with disease is very important and useful for the medical world as facilitate tracking of medical information on mutations associated with mitochondial diseases. This is of course very important for the future of the revolution in medicine as it relates to *beautique medicine* (www.mitomap.org).[17,19,21]

Comparative results of nucleotide mutations in the MITOMAP database (www.mitomap.org) resulted eight novel mutations that have not been reported. From the eight variants of the mutations that occur along the mtG region, no new mutations in the D-loop, eight new mutation in the gene-coding regions. This is interesting because mutations that occur in coding regions of genes that could alter the amino acid generally could be lead to certain mitochondrial disease.

Nucleotide sequence accession numbers.
Nucleotide sequences analysis found in this study have been deposited to GenBank (*National Center for Biotechnology Information*/NCBI) under *accession numbers*: FN357127-FN357136.

Collection of unpublished mtDNA variants in MITOMAP
From this research, novel mtDNA mutation from Indonesian human have been registered in MITOMAP database, with the access code number: 20120119001, 20120124001, 20120124003, 20120124004, 20120124005, 20120124007, 20120124008, dan 20120124009.

Conclusions

The analysis shows there is mutation of nucleotide mutations in several mtG fragments. Nucleotide mutation between tissues in one individual showed similiar pattern in both the D-loop (control region) and coding regions of genes in certain individual. This suggests that mutations in one individual of the three layer such as endoderm, mesoderm, and ectoderm are monomorphic. In addition, eight novel mutations found have never been reported in MITOMAP. mtG mutations are T650c, A3873g, C5213g, A6863g, A7894g, A8653g, G10373c, and C15211t. Most type of mutations that occur in the mtG is transition substitution mutations. Mutation variants that showed difference of rCRS are in region such as D-loop, rRNA, ND2, CO1, ATP6, ND3, ND4, ND5, CYB. These regions can be selected for studies in forensic medicine, population genetics, and bioethno - anthropology.

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