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Mutation of mtDNA ND1 Gene in 20 Type 2 Diabetes Mellitus Patients of Gorontalonese and Javanese Ethnicity

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Mitochondrial gene mutation plays a role in the development of type two diabetes mellitus (T2DM). A point mutation in the mitochondrial gene Nicotinamide adenine dinucleotide dehydrogenase 1 (mtDNA ND1) gene mainly reported as the most common mutation related to T2DM. However, several studies have identified another SNP (single-nucleotide polymorphisms) in the RNA region of mtDNA from patients from specific ethnic populations in Indonesia. Building on those findings, this study aimed to use PCR and DNA sequencing technology to identify nucleotides in RNA and ND1 fragment from 20 Gorontalonese and 20 Javanese T2DM patients, that may trigger T2DM expression. The results showed successful amplification of RNA along 294 bp for all samples. From these samples, we found two types of point mutation in Javanese patients in the G3316A and T3200C points of the rRNA and ND1 gene. In samples taken from Gorontalonese patients, no mutation were found in the RNA or ND1 region. We conclude that T2DM was triggered differently in our two populations. While genetic mutation is implicated for the 20 Javanese patients, T2DM pathogenesis in the Gorontalonese patients must be traced to other genetic, environmental, or behavioral factors.

Key words: mtDNA ND1 fragment gene, type 2 diabetes mellitus, Javanese, Gorontalonese

INTRODUCTION

Type 2 Diabetes Mellitus (T2DM) is a chronic disease of metabolic disorder caused by insulin resistance in target membranes (Lim *et al*. 2011). In Asia, Indonesia reports the highest number of T2DM patients (Ibrahim *et al*. 2010). An increase in the incidence of T2DM is predicted throughout the world, from 285 million cases in 2010 to 439 million in 2030 (Shaw *et al*. 2009).

The pathogenesis of T2DM is influenced by several factors including environment, food and genetics factors. Genetic factors are explored in much of the current research, which conduct molecular studies of the DNA of T2DM patients. T2DM has been traced in some patients to mutations in mitochondrial DNA (mtDNA). Puspitaningrum *et al.* (2014a) tried to identify nucleotide sequence variation in the hypervariable region of 1D-Loop mtDNA, in T2DM patients and their offspring, theorizing that mutations in this region may be precipitating cause of diabetes.

Mitochondrial DNA is circular double chain DNA 16.569 base pairs long, that encompasses 2 rRNA genes, 22 tRNA genes and 13 subunit protein genes for a complex respiration chain (Alexeyev *et al*. 2004). The mtDNA mutation most commonly linked by research to T2DM is found in tRNA-leu, at site 3243 (Zhong *et al.* 2000; Poulton *et al*. 2002). However, Puspitaningrum *et al.* (2014) did not find this mutation or any others in the tRNA of 20 Javanese T2DM patients studied.

The risk of T2DM is depends greatly on the population under consideration; in general diabetes risk is 1 to 5%; however, it is 6 to 7% in the US with even higher risk if the patient has one or more affected siblings (Nussbaum *et al.* 2007). Therefore, it is important to study the prevalence of T2DM in specific population, and in particular to determine whether there are genetic factors that contribute to pathogenesis of T2DM. Genetic factors have been

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traced along some ethnics lineages in Indonesia, including the Gorontalonese ethnic.

The Gorontalonese population lives in the northern part of Sulawesi Island, generally enjoying an intact natural environment and traditional diet. But, city hospital have reported a marked increase in the prevalence of T2DM since 2006, in pace with the development of Gorontalo City. This study using 20 Gorontalonese patients as one of two subjects group of interest, whose DNA is examined for evidence of a potential genetic basis for acquiring T2DM.

In Indonesia, the Javanese ethnic also shows relatively higher rates of T2DM. There are 57.7% more Javanese T2DM patients than there are Minangnese, Manadonese, and Torajanese patients. Moreover, the incidence of T2DM among the Javanese is rising, from 0.83% in 2006 to 0.96% in 2007 and 1.25% in 2008 (Central Java Province Health Services 2008). In 2007, Wates Hospital in Yogyakarta Province treated 124 T2DM patients per month; and in 2012 the hospital saw an average of 160 patients of T2DM per month. Despite the fact that Javanese are disproportionately afflicted with T2DM, molecular genetic research on Javanese patients has not yet been undertaken. We therefore selected Javanese patients as the second ethnic group of enquiry for our investigation of genetic mutations in RNA and in ND1 region.

Analysis of RNA and ND1 genes was conducted by a technique of sequencing PCR products. Genetic sequences obtained from this process can serve as a reference for further research. The framework of our research rests on prior studies that found variation—due to mutation—in the RNA and ND1 sequences of T2DM patients. We sought evidence of the same or similar variation in the DNA of our study subjects. Our subjects were 20 Javanese T2DM patients at Wates Hospital, Yogyakarta; and 20 Gorontalonese patients from Aloei Saboe Gorontalo City Hospital. The sequences that we identify can also be used as a references for genetic research regarding T2DM patients of other ethnic background in Indonesia.

MATERIALS AND METHODS

The protocol for this research has been approved by the Ethics Committee University of Indonesia document number 532/PT02.FK/ETIK/2012. Our research subjects included one group of 20 T2DM patients from Aloei Saboe Gorontalo City Hospital, and another group of 20 T2DM patients from Wates Hospital, Yogyakarta. Subjects ranged in age from 35 to 60 years old. DNA samples were taken from each subjects, and secondary data was collected about diet, family clinical background, and daily activities.

DNA extraction and PCR replication took place in the Biochemistry and Molecular Biology Laboratory, Biology Department, Faculty of Mathematics and Natural Science, Universitas Negeri Jakarta, Indonesia. DNA sequencing analysis was carried out in the Laboratory of Clinical Technology, Faculty of Health Science, Yamaguchi University School of Medicine, Japan.

Blood Sampling and DNA Extraction. The blood taken from the subjects in each hospital were saved in refrigerator until the DNA extraction process. Mitochondrial DNA was extracted from 3 mL of whole blood using a PROMEGA Extraction Kit® for whole blood with several modifications. Modifications included the use of isopropyl alcohol to take the DNA from the blood cells. The extracted DNA samples were stored at -20 ^{o}C before analysis.

Polymerase Chain Reaction (PCR). PCR was used for amplification of the RNA and ND1 region located on mtDNA. PROMEGA Master Mix® for PCR was used with a primer of Mt3243 forward 5'-AGG ACA AGA GAA ATA AGG CCT-3' and Mt3243 reverse 5'-AAC GTT GGG GCC TTT GCG T-3'. PCR products of RNA and ND1 region with 294 bp size only, were carried out in a total volume of 50 µL. The DNA was first denaturized at 94 ^{o}C for 3 min and then subjected to 30 PCR cycles at: 94 ^{o}C for 30 sec, 60 ^{o}C for 30 sec, and 72 ^{o}C for 30 sec. Amplified products were confirmed *via* 2% agarose gel electrophoresis.

Sequencing. Approximately, 2 µL of purified PCR product--purified using the protocol by Qiagen®--was amplified in a total reaction volume of 20 µL containing the Big Dye terminator reaction buffer, each of the primers, and molecular grade water. The amplified gene was precipitated after several alcohol washes at 95 and 70%, dried in a vacuum centrifuge, resuspended in Hi-Di formamide and loaded into ABI 3130xl Sequence Analyzer (Applied Biosystems) for sequencing. Sequence editing and analysis was conducted using Sequencher® 5.0.1. software for Macintosh®.

RESULTS

Fragments of RNA Genes as Amplified with PCR Technique. The amplified area of reverse and forward primer mt3243 is shown in Figure 1. The blue nucleotide sequence shows the coding area of 16S rRNA, while the red one shows the coding area of tRNA Leucine. The black sequence is the gene