

Mutation of mtDNA ND1 Gene in 20 Type 2 Diabetes Mellitus Patients of Gorontaloese 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 Gorontaloese 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 Gorontaloese 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 Gorontaloese patients must be traced to other genetic, environmental, or behavioral factors.

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

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 Gorontaloese ethnic.

The Gorontaloese 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 Gorontaloese 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 Gorontaloese 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 °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 °C for 3 min and then subjected to 30 PCR cycles at: 94 °C for 30 sec, 60 °C for 30 sec, and 72 °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

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ctgtacgaaa ggacaagaga aataaggcct acttcacaaa gcgccttccc ccgtaaatga
tatcatctca acttagtatt ataccacac ccaccaaga acagggtttg ttaagatggc
agagcccggg aatcgcataa aacttaaac ttacagtca gaggttcaat tcctcttctt
aacaacatac ccatggccaa cctcctactc ctcattgtac ccattetaat cgcaatggca
ttcctaatgc ttaccgaacg aaaaattcta ggctatatac aactacgcaa aggccccaac

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Figure 1. Nucleotide sequence of 16S rRNA, tRNA^{Leucine} and ND1 genes amplified by mt3243 reverse and forward primers. Blue: coding area of 16S rRNA gene, red: coding area of tRNA^{Leucine} gene, black: coding area of ND1 gene (Gene Bank NCBI, 2012).

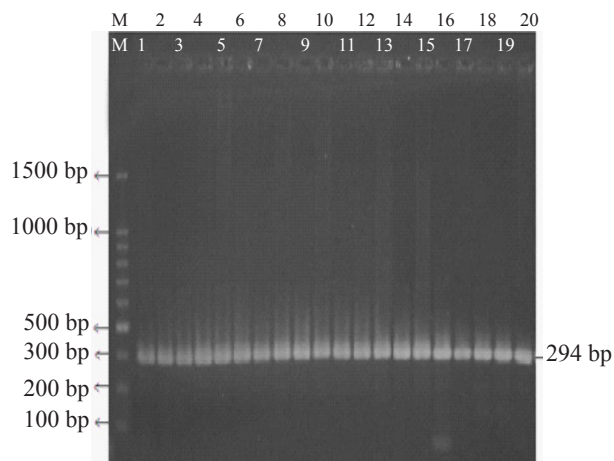


Figure 2. Visualization of PCR products of P1-P20 samples through electrophoresis of 2% agarose gel shows 294 bp bands. M: Marker of 100 bp DNA Ladder; 1-20: PCR products of tRNA gene fragments in T2DM patients samples of Javanese tribe.

ND1 area. Figure 2 shows a visualization of the RNA gene fragment produced during PCR. The resulting sequences were compared to the data base of human mitochondrial DNA using BLAST (Basic Local Alignment Search Tools) online on DDBJ (DNA Data Bank of Japan).

Mutation of T3200C and G3316A mtDNA in T2DM Javanese Patients. The sequencing data that we obtained revealed two mutations, in two Javanese T2DM patients. One mutation was to T3200C located in the 16S rRNA region. The arrow illustrates a base change from T to C (Figure 3). This mutation occurred in sample P16 from a T2DM patient. Figure 4 illustrates the second point mutation to G3316A on site 3316 in ND1 region. The arrow indicates a base change from G to A. There was no mutation or any base change detected in the tRNA of any of the subjects.

Type 2 Diabetes Mellitus in Gorontaloese Patients. The sequencing results of RNA and ND1 regions for all of the T2DM and control samples revealed no SNP. This result is in line with the clinical profiles of the subjects, none of whom had Maternally Inherited and Diabetes (MIDD) or exhibited symptoms known to be related to RNA

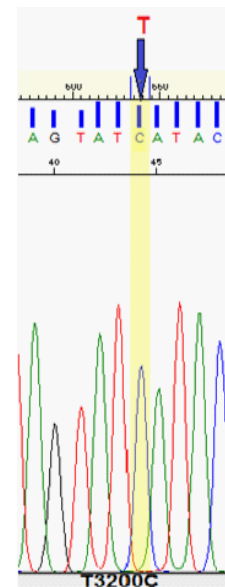


Figure 3. T3200C mutation in sample P16 occurs in site 3200 on gene 16 rRNA mtDNA. Mutation point shows a base change from T to C.

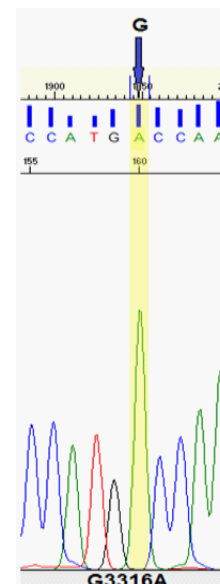


Figure 4. Mutation of G3316A from sample P10 in site 3316 on gene ND1 mtDNA. Mutation point shows a base change from G to A.

and ND1 mutation, i.e. Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis, and Stroke Like Episodes (MELAS).

DISCUSSION

T2DM Javanese Patients. Common symptoms of T2DM include fatigue, increased hunger and thirst, and frequent urination; 90% of the subjects in this study is also having obesity. Obesity may be caused by polyphagia and the type of metabolic syndromes experienced by T2DM patients (Sherwood 2010). Twenty percent of the research subjects had a family history of T2DM, which support evidence from prior studies of a genetic basis for diabetes (Codario 2005).

The use of oral medication by patients in this research showed 20% of Metformin users and 10% of Glimpiride users. While Glimepiride is an oral medication which can control blood sugar (Kanazawa *et al.* 2009; Ahad *et al.* 2011), as many as 75% of patients checked their blood sugar periodically. Periodical checks of blood sugar helps patients understand and be aware of their blood sugar level. This is necessary to keep their body in good shape.

A total of 65% patients said they kept a healthy diet, such as three tablespoons of rice (carbohydrate) in each meal. This can reduce the level of blood sugar. There were 15% of patients who regularly exercised for more than 30 min and the rest exercised only less than that. Thirty-minutes daily exercise is recommended by International Diabetes Federation (Haque *et al.* 2011). All subjects in this research, patients and non-patients, are of Javanese tribe. This was a necessary criterion as the research aimed to trace the genetic factors that caused T2DM in people of Javanese ancestry.

The Base Nucleotide Fragments of RNA and ND1 Gene Fragments in 20 T2DM Javanese Patients. All the samples taken from Javanese subjects—20 T2DM and five non-patients—were successfully matched using homolog analysis. Results showed that all samples analyzed were 100% identical in sequence to human mtDNA data in the Gene Bank (Figure 4). The two point mutations discovered during molecular analysis were located at gene site of 16S rRNA and in the ND1 fragment.

One mutation discovered was a T3200C mutation (Figure 4) in sample P16 from a T2DM patient. The T3200C mutation involved a base change from T to C on site 3200 gene 16S rRNA. A mutation in this gene affects ribosome generation and is implicated in the increased number of ribosomes found in T2DM patients. Nucleotide substitution from T to C results in abnormal change to the shape of ribosome, indicative of a disease (Tao *et al.* 2002). Previous researches on RNA mutation in T2DM patients also found mutations in gene 16S rRNA and ND1. The

mutations were found to be associated with the trigger of T2DM (Pranoto 2005).

Our research revealed a second mutation, of G3316A in sample P10 from a subject with T2DM. This was a point mutation that occurred in gene ND1, and was located on site 3316, involving a base change from G to A. This mutation affects the coded subunit 1 NADH dehydrogenase, a component of OXPHOS complex 1 in mitochondria. The mutation may affect enzyme activities of NADH dehydrogenase so it might cause T2DM (Pranoto 2005).

Absence of Mutation in RNA and ND1 in T2DM Gorontalones Patients. The A3243G mutation—the most common mutation in tRNA region related to T2DM (Porte & Sherwin 1997)—did not appear in 20 T2DM subjects and 20 normal controls. The A3243G mutation has been previously found in T2DM patients in populations from Japan (Katagiri *et al.* 1994; Ohkubo *et al.* 2001; Yorifuji *et al.* 2012); Egypt (Fawzi *et al.* 2006); Finland (Majamaa *et al.* 1998); among the Chinese Han population (Liao *et al.* 2008); and many more. However, other investigators have failed to find this A3243G mutation. Rigoli *et al.* (1997) did not detect any A3243G mutation among 231 T2DM patients from Southern Italy. Naveed *et al.* (2009) also had negative result when testing for the A3243G mutation among 39 T2DM Pakistani patients as well as 129 MIDD Polish patients (Ma *et al.* 2001), 10 Tamil Indian patients (Lepretre *et al.* 1998), and 112 T2DM Nigerian patients (Ameh *et al.* 2011).

Absence of mutation in our samples from 20 T2DM Gorontalones was probably due to several factors. Firstly, it may be that the sample size was too small. Previous investigations have discovered the A3243G mutation in only a very small proportion of the sample tested. Ohkubo *et al.* (2001) found that only 2.9% of the A3243G mutation from 240 T2DM patients had the A3243G mutation in a Japanese study. Kunsan *et al.* (1997) found that 0.5% of 207 T2DM Chinese patients had the mutation, and a study of 100,000 T2DM patients worldwide found only 5.71% with the A3243G mutation (Maasen & Kadokawi 1996). Meanwhile Gerbitz *et al.* (1995) estimated that the prevalence of this mutation is approximately 1.5% of the diabetic population worldwide.

Secondly, none of the T2DM patients in our study exhibit severe clinical characteristics, such as acute deafness, obesity, and stroke-like syndrome, that are often seen in MIDD and MELAS patient (Porte & Sherwin 1997; Zhong *et al.* 2000; Chen *et al.* 2004; Kanaumi *et al.* 2006). Previous studies have linked

the A3243G mutation to MIDD and MELAS (Onishi *et al.* 1993; Porte & Sherwin 1997; Solano *et al.* 2000; Shanske *et al.* 2004). Patients from our study were only exhibit more general symptoms of T2DM such as mild obesity, fatigue and increase appetite (Porte & Sherwin 1997).

Thirdly, previous investigations have reported that DNA taken from leucocytes had the lowest incidence of the tRNA A3243G mutation compared to DNA taken from skeletal muscle (Kelley *et al.* 2002), urinary sediment (Solano *et al.* 2000), skin fibroblast, hair roots, and cheek mucosa (Shanske *et al.* 2004). Other research suggest that A3243G mutation are less prevalent in blood DNA taken from the elderly because heteroplasmy levels of mutant mtDNA decrease in leucocytes upon aging (Hart *et al.* 1996; Sue *et al.* 1998). For the reasons above, we cannot state with certainty that the Gorontaloese T2DM patients in this study do not possess the tRNA A3243G mutation. Further investigation of DNA from other tissues is needed to get more conclusive data.

Alternative Causes of T2DM Prevalence in Gorontaloese. Prevalence of T2DM varies from population to population (Zimmet 1992). Many risk factors have been identified which influence the prevalence or incidence of T2DM, such as family history of T2DM, age, excess weight, increased abdominal fat, hypertension, lack of physical exercise, and ethnic background (van Tilburg *et al.* 2001). Many prior investigations have also demonstrated that lifestyle factors can function as an initiator of mutation in T2DM (Kahn *et al.* 1996; So *et al.* 2000; Watanabe *et al.* 2007).

Hospital record in Gorontalo shows that both the prevalence and incidence of T2DM in the city has increase about 15% per year since 2006. This might be linked to changes in socio-economic status, as Gorontalo City urbanized, and as rural citizens move to the city. As mentioned earlier, environment and lifestyle play an important role in the development of T2DM (Mohan *et al.* 2005). Changes in the socio-economic status of Gorontalo City residents (the capital city of Gorontalo Province) began to be felt in 2005. Gorontalo Province is recently formed jurisdictions that split off from the province of North Sulawesi in 2001. As capital of the new province, Gorontalo City experienced rapid development beginning in 2003. Growth resulted in an abundance of newly-build convenient stores, lifestyle center, modern market, malls, and fast food restaurants that changed the character of the city and the behavior of residents. Increased city development aligned increase in the prevalence of T2DM reported by

hospitals. In 2006 the average number of T2DM in the city was one thousand per year, up from 500 cases reported one year earlier in 2005. In 2012, the average number of T2DM reaching two thousand cases per year or four times higher than in 2006.

A similar correlation between T2DM incidence and rapid development has been described in India where prevalence of T2DM is only about 3% in rural areas (Ramachandran 2002) while in urbanized cities it approaches 16% (Raman *et al.* 1999). This large divergence in the prevalence of T2DM in rural vs. urban populations is probably a result of environmental and behavioral factors. The prevalence of T2DM also follows another demographic trend, increasing as ethnic groups migrate from the world's less developed regions to urban region or westernized regions (van Tilburg *et al.* 2001). As an illustration, the T2DM in ethnic Japanese residents in Hawaii was found to be higher than in Japan (Fujimoto *et al.* 1991) and the prevalence of T2DM in ethnic Chinese residents in Mauritius was 13% higher than in mainland China (Fujimoto 2000).

These previous findings are corroborated by the findings in our study. Almost all the T2DM subjects acknowledged heavy consumption of food and beverages that are high in trans-fats and/or have a high Glycemic Index (GI), without significant physical activity. Unsurprisingly, most of the diabetic subjects were obese, in contrast to the control subjects who maintained normal weight, and practiced healthier eating and fitness. Studies have identified several protective behaviors that can reduce the incidence of T2DM in women by as much as 90%. These include: maintaining a body-mass index of 25 or lower, eating a diet high in cereal fiber and poly-unsaturated fat and low in saturated and trans-fats and in glycemic load; regular exercise, abstaining from smoking, and moderate alcohol consumption (Hu *et al.* 2001). Such findings suggest that the majority of cases of T2DM could be prevented by weight loss, regular exercise, modification of diet, smoking cessation, and limited alcohol consumption. Weight control appears to offer the greatest benefit.

The result of our genetic research and analysis of samples from Javanese T2DM patients and control subjects, revealed a mutation in the mtDNA ND1 gene. No variation or mutation was found in the tRNA genes of this group. Two mutations were also found in T2DM patients, T3200C in gene 16S rRNA and G3316A in gene ND1 which were related to T2DM. T2DM patients samples did not have any mutation in tRNA gene, 16S rRNA, and ND1, which was probably cause by lack of exercise and unhealthy diet.

Meanwhile in Gorontalones subjects, we did not find any mutations. The high prevalence of T2DM among the Gorontalones ethnic could probably caused by another genetic, environmental, and/or lifestyle factors. From both ethnics, we conclude that there is different expression of T2DM among both ethnicity due to different genetic, environmental, and lifestyle trigger.

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