THE ASSOCIATION OF MITOCHONDRIAL DNA MUTATION G3316A AND T3394C WITH DIABETES MELLITUS

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ABSTRACT

Mutation in the mitochondrial DNA (mtDNA) is known as a monogenic causative factor for A3243G mtDNA mutation in the pathomechanism of type 2 diabetes mellitus (T2DM). A point mutation at nucleotide position G3316A and T3394C in the mtDNA NADH dehygrogenase 1 (ND1) gene has been reported in T2DM, categorized as Single Nucleotide Polymorphism (SNP. However, those two SNPs have been also found in normal population. The role of mtDNA mutation is still not yet studied widely among Indonesian population. We therefore investigated the contribution of mtDNA mutation and diabetes mellitus (DM). Blood DNA was screened from 451 of T2DM cases collected from DM patients at Dr. Soetomo Hospital during 2001-2003. The G3316A and T3394C were detected using PCR and digested with HaeIII restriction enzyme. Fortunately, we found two pedigrees harboring G3316A and two pedigrees with T3394C point mutation. Family studies of those pedigrees showed that DM with those G3316A and T3394C had a significant odds ratio 5.2 (95% CI: 1.222-2.,134) and 3.185 (95% CI: 1.025-9.893), respectively, compared to the sample taken from the same social and environmental background. We suggest that there are two types of mtDNA mutation associated with DM. The first is pathogenic mutations that lead to severe defect in oxidative phosphorylation (OXPHOS), represented by A3243G, and thus causal for DM. The second is SNPs that presumably alter tissue capacity for OXPHOS in such a way contributing to the polygenic T2DM as a presponding factor. A conclusion can be made that is G3316A and T3394C are SNPs that have a role as polygenic components to the pathomechanism of DM.

Keywords: *mithocondrial DNA mutation, monogenic, polygenic, diabetes mellitus, maternally inherited, single nucleotide polymorphism, A3243G, G3316A, T3394C.*

INTRODUCTION

Diabetes mellitus (DM) is a complex polygenic disorder, characterized by a disturbance in insulin production by the pancreatic β cell or in the ability of target tissues to respond to insulin. The importance of the mitochondrial genetic factor(s) in its pathogenesis has long been suggested, and several mutations in the mitochondrial DNA (mtDNA) are indeed expressed as DM. Mitochondrial Diabetes Mellitus (MDM) represents a significant subset of adult onset DM. MtDNA is extrachromosomal and inherited along the maternal line. It codes for 13 polypeptides of the oxidative phosphorylation (OXPHOS) complexes, essential for the function of the OXPHOS machinery. Together with nuclearly coded protein subunits imported from the cytosol, the mitochondrial translation products are assembled to form a functional mitochondrial respiratory chain and the ATP synthase.

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MtDNA also codes for 22 tRNAs plus two rRNAs essential for the expression of the protein-coding genes. Mutations in the mtDNA, therefore, primarily affect the enzyme complexes of OXPHOS.

Many mtDNA mutations that have been suggested to be associated with DM (C1310T, A1382C, G1438A, A1202G, A3252G, A3256T, A3264C, A3271C, T3290C, C3303T, G3316A, T3394C, A8296G, A8344G, G11778A, A12026G, C12258A, T14577C, T14709C, T16189C, etc (Zeviani et al., 1991; Silvestri et al., 1992; Morten et al., 1993; Hirai 1996, et al., ; Suzuki et al., 1997a; Kameoka et al., 1998; Lynn et al., 1998; Poulton et al., 1998a; Bruno 1999 et al.,; Ohkubo et al.,, 2001; Tawata et al., 1998; Tawata et al.,, 2000), only one, a A3243G substitution in the $tRNA^{leu}$ gene, is in fact firmly established to be causal for DM (Campos et al., 1995, Kadowaki et al., 1995, van den Ouweland et al., 1994, Alcolado et al., 1994; van den Ouweland et al.,, 1992). Others are only provisionally associated with DM because they have been found in single cases only, albeit syndromic with other neurological signs common to mitochondrial disorders, or subject to controversy because they are found also in the normal population. Two of the latter are a G3316A (found in 3.4% of patients with DM type 2 [DMT2] in Japan [Odawara *et al*, 1996]) and a T3394C (reported in 4.9%

of Japanese DMT2 patients [Hirai *et al*, 1996]), resulting in tyrosine to histidine and alanine for threonine amino acids changes in the ND1 subunit of the respiratory complex I respectively. Subsequent publications, mainly from China, however, disputed the association of these mutations with DM as both are found also, with the same frequency, in the normal control population (Ji et al., 2001). In the present study, we seek for more definitive evidence for the association of the G3316A and T3394C mtDNA mutations with DM.

PATIENTS AND METHODS

Sample collection and family study

A number of 451 patients aged 30 to 82 years with DM type 2 (DMT2) were randomly selected from Diabetes Outpatient Clinic of Dr. Soetomo Hospital, Surabaya, Indonesia. Presence of DMT2 was based on clinical criteria: onset of diabetes after the age of 30 years without treatment with insulin in the first year after diagnosis. Control (104 individuals) included at non-maternally related members of the pedigrees as well as unrelated individuals (83) at random from the same population sampling frames. Blood were taken with informed consent as part of a diagnostic work up of the patients and the study was cleared by the Ethical Committee of Airlangga University School of Medicine, Dr. Soetomo Teaching Hospital on June 7 2001. All samples were then subjected to DNA analysis using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method to detect the presence of G3116A and T3394C mutation. Two families harboring the mtDNA G3316A and another two families with T3394C Single nucleotide polymorphism (SNPs) along the maternal lines were included in the present study.

DNA extraction and detection of the mtDNA mutation

Total DNA was isolated from blood samples using standard procedure as described previously (Pramoonjago et al., 2000). The mtDNA T3394C and G3316A were detected by PCR-RFLP. A 902 bp fragment of the mtDNA between nt 2826 – nt 3728 was PCR amplified using the primer pair of L2826 (5'GAGCAGAACCCAACCTCCGAGCAG3') and H3728 (5'GATTGTTTGGGCTACTGCTCGC3') in a programmable thermal cycler (Perkin Elmer 9700 GeneAmp System). The PCR was carried out for 30 cycles of denaturation at 95^{o}C for 1 min (first cycle at 95^{o}C for 5 min), annealing at 56^{o}C for 90 sec and elongation at 72^{o}C for 2 min 30 sec. The amplification product was then digested with HaeIII restriction enzyme (New England Biolabs, Beverly, Mass, USA) and the DNA fragments were separated by electrophoresis on a 2% agarose gel. The G to A substitution at nt 3316 was detected by the loss of an HaeIII restriction site. The T to C substitution at nt 3394 was detected by the presence of additional restriction site at nt 3392.

Confirmation by DNA sequence analysis

The presence of the two base substitutions were confirmed by DNA sequence analysis. MtDNA fragment was PCR amplified using the primer pair as described above. PCR products were purified using the QIAquick purification kit (QIAGEN GmBh, Hilden, Germany), and were sequenced with H3502 and L2826 as the sequencing primers; cycle sequencing reactions were performed as indicated by the manufacturer with BigDye Ready termination Cycle Sequencing Ready Reaction Kits and analyzed using a model 377 automatic DNA sequencer (Applied Biosystems, Perkin Elmer, Norwalk, Conn., USA).

Statistical analysis

Association analysis using ODDS Ratio analysis of total number of DM in the pedigrees with G3316A and T3394C, compared to the sample taken from the same social and environmental background was done to reveal whether those mutation as a predisposing factor of DM.

RESULTS

Our study identified two patients with G3316A mutation and also two patients with T3394C mutation from totally 451 who entered this study. Thus, the frequency of both mutations is 0.44% (2/451). PCR-RFLP of G3316A mutation is shown in figure 1, T3394C mutation in Figure 2, and confirmation by DNA sequencing was done in both cases (Figure not shown). Clinical manifestation of both mutation were all type 2 diabetes.

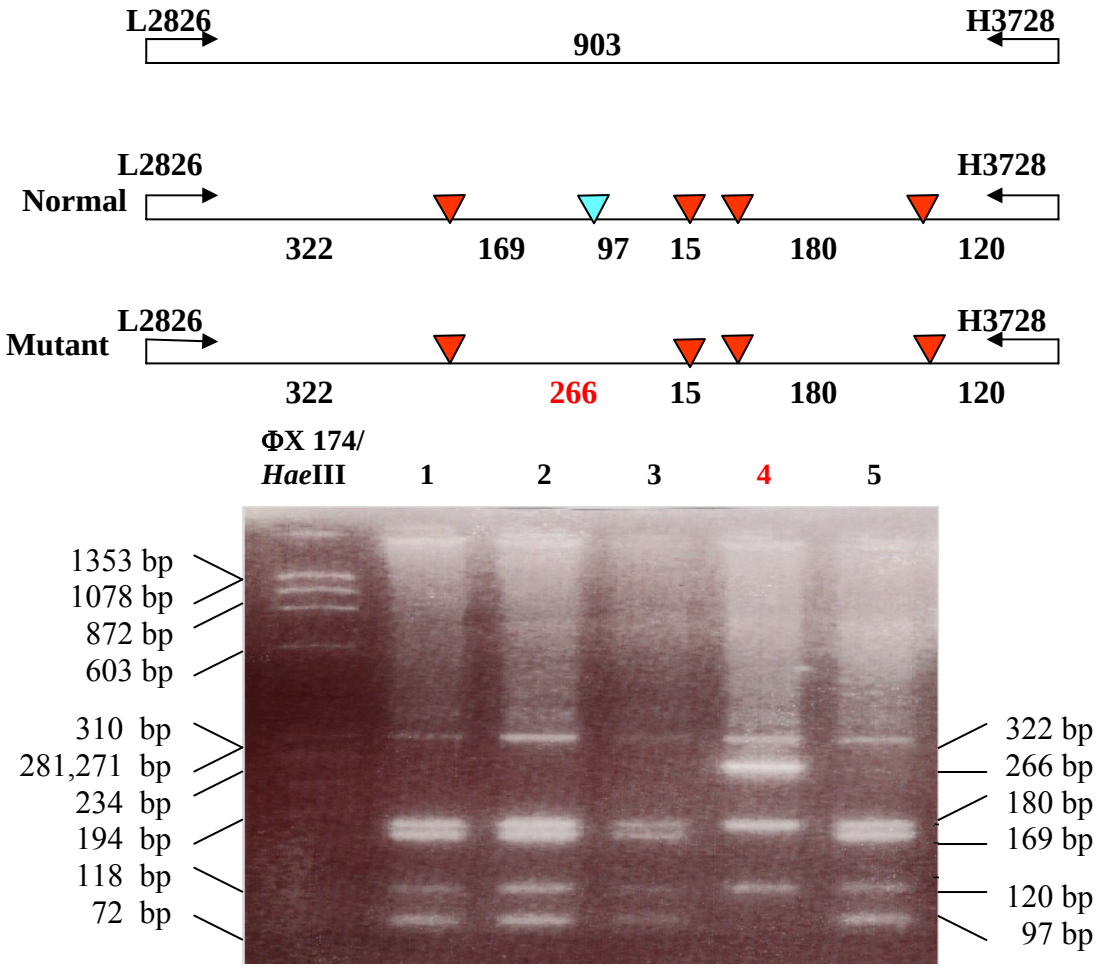


Figure 1. Detection of mtDNA mutation G3316A by PCR-RFLP using *Hae*III restriction enzym. A 902 bp fragment of the mtDNA between nt 2826 – nt 3728 was PCR amplified using the primer pair of L2826 (5'GAGCAGAACCCAACCTCCGAGCAG3') and H3728 (5'GATTGTTTGGGCTACTGCTCGC3'). *Wild type* has six restriction fragment. Gel electrophorosis column 4 is a profile of 3316G, patient with G3316A mtDNA mutation. Control marker is DNA ϕX 174/HaeIII.

Note: bp = base pair

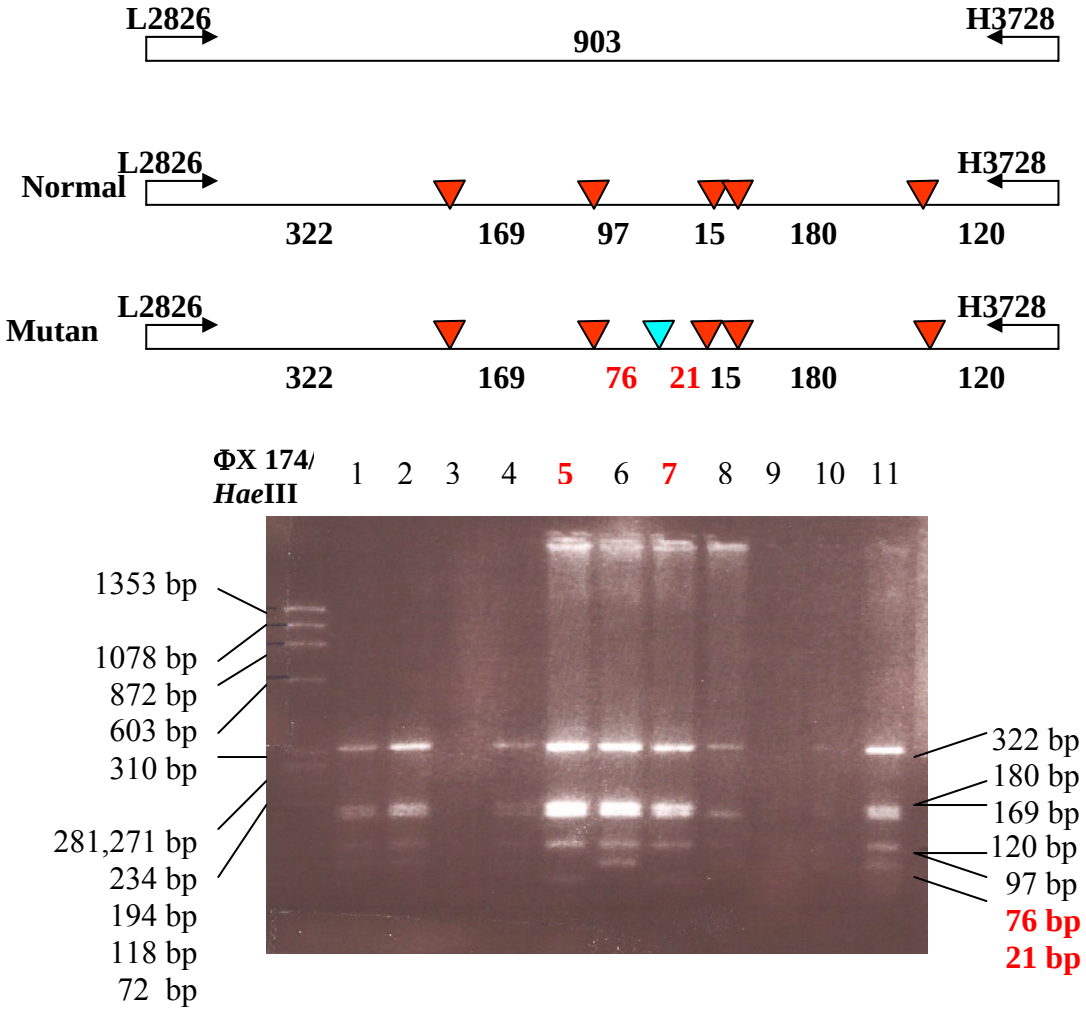


Figure 2. Detection of mtDNA mutation T3394C by PCR-RFLP using *Hae*III restriction enzym. A 902 bp fragment of the mtDNA between nt 2826 – nt 3728 was PCR amplified using the primer pair of L2826 (5'GAGCAGAACCCAACCTCCGAGCAG3') and H3728 (5'GATTGTTTGGGCTACTGCTCGC3'). *Wild type* has six restriction fragment. Gel electrophorosis column 5 and 7 is a profile of 3394 T, patient with T3394C mtDNA mutation.. Control marker is DNA ϕX 174/HaeIII.

Association analysis of those pedigrees showed that DM with those G3316A and T3394C had a significant odds ratio 5.2 (95% CI: 1.222-22.134) and 3.185 (95% CI: 1.025-9.893), respectively, compared to the sample taken from the same social and environmental background.

DISCUSSION

The evidence for the association of these mutations with DM is strong in some cases such as for mutations in $tRNA^{leu}$ in the neighborhood of the A3243G, but weak for many others, such as mutation in G3316A and T394C. Two of these SNPs are found in the normal population but are significantly associated with DM and suggested as a predisposing factor.

Both G3316A and T3394C mutations were in the coding region of ND-1 gene that encodes NADH dehydrogenase subunit 1, as a component of mitochondrial OXPHOS complex 1. G3316A mutation cause an amino acid replacement from tyrosine (TAT) to histidine (CAT) and T3394C mutation substitutes alanine (GCC) for threonine (ACC). Because both G3316A and T3394C mutations resulted in an amino acid change and might have affected enzyme activity of NADH dehydrogenase, it

could be a defect for OXPHOS process inside β cell and cause a DM. Unlike the European or Japanese populations, which are relatively homogeneous, the populations of Indonesian archipelago are very diverse in their social and economic structures. Combined with the fact that there is a progressive transition from a traditional to an industrial life-style in many urban centers and also a relatively large population of migrants from this region in the industrialized western countries, this region provides an ideal setting for a study on the role of mtDNAs SNPs such as G3316A and T3394C as a predisposing factors for DM and on the nutritional aspects of the SNPs expression.

Our study has different approach of study design to prove whether SNP in G3316A and T3394C as a causative factor of DM. Given the fact from previous studies that the prevalence is low, it is almost impossible to draw a strong causative conclusion based on prevalence study. Family study of pedigrees with mutation from serial cases could give an estimation of association from OR analysis, and the conclusion of association could be drawn which is never been reported by previous reports. Our strategy to add a control group from the neighbor of mutation carrier is aimed to get the result of OR in a significant 95% confidence interval. The result of the present study come out that both G3316A and T3394C mutation have a prevalence of 0.44% and it has an association with DM with significant OR. Previous studies showed conflicting results with only one prevalence study has a significant higher prevalence of G3316A mutation of DM population compared to control group (3.4% versus 0.98%) reported by Nakagawa et al. (1995), and a similar result of T3394C mutation (4.9% versus 1.3%) reported by Hirai et al., (1996). The other studies of G3316A mutation revealed no significant differences between mutation group and control as reported by McCarthy (1996) which was shown by 7/142 versus 1/85, p = 0.51, Ji (2001) reported 16/716 versus 5/181, and also reported by Wan Lam et al., (2001). A no significant difference of T3394C mutation between diabetes group and control also had been reported by Matsuura et al. (1999) and Ohkubo et al., (2001). Thus, the positive association of this present study gives a new evidence as a strong predisposing of the pathogenesis theory of β-cell defect in DM patients with G3316A and T3394C mutation that is really based from OXPHOS dysfunction, such conclusion is still not well established before.

In conclusion, we suggest that they are two types of mtDNA mutation associated with DM. The first type are pathogenic mutations that lead to severe defect in OXPHOS, represented by A3243G, and thus causal for MDM. The second are SNPs that presumably alter tissue capacity for OXPHOS in such a way contributing to the polygenic DMT2 as a predisposing factor. We provided evidence that G3316A and T3394C are such SNPs.

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