Maternally inherited diabetes patients from Manado and Jakarta possess A3243G mutation by PCR-Allele’s specific amplification

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Abstract

Mutations in mitochondrial DNA (mtDNA) associate with diabetes that the most common mutation is the A3243G mutation in \textit{tRNA}^{Leu(UUR)} gene. Previous studies have suggested that this mutation is associated with maternally inherited diabetes, but only when additional features of mitochondrial disease are present. The frequently accompanying feature of maternally inherited diabetes is hearing impairment or deafness (MIDD). This mutation generally occur in heteroplasmic forms, with low levels in leukocytes and high levels in postmitotic tissues (e.g., skeletal muscle and \(\beta\)-cells). However, muscle biopsy cannot be used as a routine examination, especially in diabetic patients. Current methods have limited sensitivity and may lead to potential misclassification of patients with low levels of heteroplasm. Therefore, the aim of the research was to investigate the role of mtDNA defects in maternally inherited diabetes by first screening for the A3243G mutation by restriction assay, followed by PCR-Allele’s Specific Amplification. mtDNA was obtained from leukocytes of 103 diabetes patients which attained from Manado and Jakarta. A total of 47 patients had maternally inherited diabetes either alone (group 1, \(n=14\)) or with one or more additional features of mitochondrial disease, including deafness and neuromuscular disease (group 2, \(n=33\)). The negative control subjects are 56 patients diabetes with hadn’t maternally inherited diabetes and neuromuscular disease. Fourteen patients (eight from group 1) carried the A3243G mutation. Here, we identified that maternally inherited diabetes possess A3243G mutation, this is contrary to previous studies. We conclude, therefore, that screening for mtDNA mutations should be considered in patients with maternally inherited diabetes alone and/or additional features of mitochondrial disease, such as deafness.

Keywords: Maternally inherited diabetes, A3243G mutation, PCR-Allele’s specific amplification

Introduction

Mitochondrial gene mutation play a role in the development of diabetes mellitus. The defects in mitochondrial DNA (mtDNA) as pathogenic factor for diabetes, the most common mutation is A-to-G mutation at np 3243 (A3243G) in the \textit{tRNA}^{Leu(UUR)} gene (Narbone, et al., 2001; Lynn, et al., 1998). The mitochondrial A3243G mutation is associated with multiple clinical presentations including maternally inherited diabetes and deafness (MIDD) (Maassen, et al., 2004) and mitochondrial encephalomyopathy, lactic acidosis with stroke-like episodes (MELAS) (Chinnery, et al., 1999). Alan et al. (2002) have suggested that mtDNA defect is associated with maternally inherited diabetes, but only when additional neuromuscular features are present, such as deafness, myopathy, cardiomyopathy, cataract, ataxia, etc.

The molecular mechanism of the A3243G mutation affects insulin secretion may involve an attenuation of cytosolic ADP/ATP levels leading to a resetting of glucose sensor in the pancreatic \(\beta\)-cell, such as in maturity-onset diabetes of the young (MODY)-2 patients with mutations in glucokinase (Maassen et al., 2004). Furthermore, one would expect that all mtDNA mutations that affect ATP synthesis lead to diabetes.

Pathogenic mutations generally occur in heteroplasmic forms and large variations between various tissues are seen within patients. The ratio of mutant to wild-type mtDNA (\% heteroplasm) varies across tissues (Shanske et al., 2004), with low levels in blood cells (leukocytes) and high levels in postmitotic tissues (Alan et al, 2002). Because of random segregation of mutant and normal mtDNA into daughter cells, the high rate of replication in rapidly dividing tissues such as blood cells tends to reduce the proportion of mutated mtDNA in comparison with wild
type. Slower dividing (postmitotic) tissue such as muscle, neurons, and endocrine organs, usually show higher levels of mutated mtDNA and are often clinically affected. Unfortunately, tissues (e.g., pancreas) containing the highest levels of heteroplasmy are not easily accessible for diagnostic testing. However, muscle biopsy is an invasive method that cannot be used as a routine examination, especially in diabetic patients.

Direct sequencing is the gold-standard method used to detect mutations, but can only reliably detect % heteroplasmy >25%, which is rare in leukocytes. Approximately 1.5% of diabetic patients is found A3243G when mtDNA is extracted from leucocytes (Narbone, et al., 2001). Current methods to detect the presence of the mutation have limited sensitivity and may lead to potential misclassification of patients with low levels of heteroplasmy. Therefore, the aims of this research is to search the role of mtDNA defects in Indonesian maternally inherited diabetes by using PASA (PCR-Allele’s Specific Amplification) method. We have investigated the role of mtDNA defects in maternally inherited diabetes alone and/or additional features of mitochondrial disease, such as deafness, this is contrary to previous studies. We have identified maternal inheritance of A3243G heteroplasmy mutation in one of maternally inherited diabetes and deafness patient families.

Materials and Method

Subject

The subjects were 103 diabetes patients which attained from Manado and Jakarta. A total of 47 patients had maternally inherited diabetes either alone (group 1, n = 14) or with one or more additional features of mitochondrial disease, such as deafness, is deafness, this is contrary to previous studies. We have identified maternal inheritance of A3243G heteroplasmy mutation in one of maternally inherited diabetes and deafness patient families.

Isolation DNA

Total DNA was extracted from peripheral blood leukocytes using TE buffer, lysis buffer; and Proteinase K.

Cell lysis

Five hundred microliter of whole blood were depigmented using 500 μl TE buffer (Tris-Cl 10 mM; EDTA 0.1 mM; pH 8.0) several times until the solution were clear. Subsequent to preparation step, each sample was lysed using 300 μl 1X lysis buffer (50 mM Tris-Cl, pH 8.3, 1 mM EDTA, 0.5% Tween-20, 200 μg/ml proteinase K) (Noer, et al., 1994). Solution was incubated at 55°C for 2 hours, followed by heat inactivation of the proteinase K at 95°C for 10 minutes. All lysates were stored at -20°C.

Amplification of DNA

The fragment of mitochondrial DNA encompassing np 3243 were amplified by PCR with Taq DNA polymerase. PCR was carried out in a total volume of 25 μL containing 50 ng of extracted DNA, 200 μM each dNTP, 2.0 mM Tris-HCl (pH 9.0), 50 mM NaCl, 0.1% TritonX-100 MgCl₂, and 1.0 U of Taq polymerase. The forward primer was 5’-AGG ACA AGA GAA ATA AGG CC-3’ (D2), and the reverse primers were 5’-AAC GTT GGG GCC TTT GCG TA-3’ (D1) and 5’-TGG CCA TGG GTA TGT TGT TA-3’ (DR). The DNA was initially denatured at 94°C for 5 min and subjected to 30 PCR cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 50 s, and ended with 72°C for 10 min (Zhang et al., 2002). The PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide.

RFLP analysis

294 bp fragment amplified by the primers D1 and D2 was digested by Apal to identify A-to-G mutation at np 3243 and electrophoresed on 1.5% agarose gel.

PASA

In PASA, the reverse primer DR was at nucleotide positions 3319–3300 for the amplification of both wild-type mtDNA and the A3243G mutation. The forward primers for PASA analysis of the A3243G mutation are listed in Table 1. 190 bp fragment amplified by the primers D2 and DR was used as internal control. The amplification conditions were 30 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min and extension at 72°C for 1 min, with initial denaturation at 94°C for 5 min and final extension at 72°C for 4 min. The PCR products were electrophoresed on 1% agarose gel.

In addition, PASA used to genotype examination (Table 2) for deafness (A3243G, C12258A), cataract (A3243G, C12258A), myopathy (A3243G, A3302G), cardiomyopathy (A3243G, A3260G) and gastrointestinal dismotility (A3243G, G8313A) in diabetes patients.
Table 1 Forward primers (nucleotide positions 3223–3243) used in PASA-three bases mismatch analysis.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’→3’)</th>
<th>Size of fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type sequence</td>
<td>AGG GTT TGT TAA GAT GGC AGA</td>
<td>97</td>
</tr>
<tr>
<td>PASA primer for wild-type A3243G</td>
<td>AGG GTT TGT TAA GAT GGC tc^a</td>
<td>97</td>
</tr>
<tr>
<td>PASA primer for mutant A3243G</td>
<td>AGG GTT TGT TAA GAT GGC tgc^a</td>
<td>97</td>
</tr>
</tbody>
</table>

^a The underlined lowercase bases indicate the mismatches.

Table 2 Genotype examination for deafness, cataract, myopathy, cardiomyopathy, and gastrointestinal dismotility in diabetes patients.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’→3’)</th>
<th>Size of fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type C12258A forward</td>
<td>CCC CAT GTTTAA CAT GGC CCCTTTAA CAT ccctg^a</td>
<td>148</td>
</tr>
<tr>
<td>C12258A reverse</td>
<td>GAA TTA GGG AAG TCA GGG TTA GG</td>
<td>339</td>
</tr>
<tr>
<td>wild-type G8313A</td>
<td>GAG CCC ACT GAA CTA ACT TAG</td>
<td>189</td>
</tr>
<tr>
<td>mutant G8313A</td>
<td>GAG CCC ACT GAA CTA ACT atag^b</td>
<td></td>
</tr>
<tr>
<td>G8313A reverse</td>
<td>TTG GAG GTG GGG ACT AAT AGA</td>
<td></td>
</tr>
<tr>
<td>wild-type A3302G</td>
<td>GAG GGC CAA TTC CTC TTC TTA A</td>
<td></td>
</tr>
<tr>
<td>mutant A3302G</td>
<td>GAG GGC CAA TTC CTC TTC T atg^a</td>
<td></td>
</tr>
<tr>
<td>A3302G reverse</td>
<td>GGG GCT CTT TGGA AGA G</td>
<td></td>
</tr>
<tr>
<td>wild-type A3260G</td>
<td>AGC CCG GTA ATC GCA TAA</td>
<td>101</td>
</tr>
<tr>
<td>mutant A3260G</td>
<td>AGC CCG GTA ATC GCA atcg^e</td>
<td></td>
</tr>
<tr>
<td>A3260G reverse</td>
<td>TGG GTA CAA TGA GGA GTA GGA G</td>
<td></td>
</tr>
</tbody>
</table>

^a The underlined lowercase bases indicate the mismatches and ^b The red dashed indicates the deletion of two nucleotides.

Results and Discussion

Subject

Of the 103 diabetes patients, we clinically classified (based on medical records) 14 patients had maternally inherited diabetes alone (group 1), 33 patients had additional features of mitochondrial disease, including deafness, myopathy, cardiomyopathy, cataract, and gastrointestinal dismotility (group 2), and 56 patients as the negative control subjects hadn’t maternally inherited diabetes and neuromuscular disease.

RFLP

The PCR product amplified by the primers D1 and D2 was 294 bp in length. After digestion with ApaI, it was separated into 182 bp and 112 bp if it had a substitution of A to G at np 3243 to constitute the recognition site for ApaI. The RFLP analysis showed a heteroplasmic A3243G mutation (Figure 1). Among the 103 subjects, only two patients with maternally inherited diabetes and deafness (MIDD) had A3243G mutation in mtDNA. The positive results from RFLP could be the positive control for PASA analysis.

Figure 1 Mutation analysis of mtDNA at nucleotide position 3243 in the tRNA_Leu(UUR) gene by PCR and digested with ApaI. Among the 103 subjects, only two patients with maternally inherited diabetes and deafness (MIDD) had A3243G mutation in mtDNA (lane 5). Lane 1 indicates the DNA size marker (pUC19/Hinfl).
PASA

The PASA products amplified by the wild-type and mutant forward primers and the reverse primer DR were 97 bp in length. Control internal was 190 bp fragment amplified by the primers D2 and DR (Figure 2). In PASA result, fourteen out of 103 patients diabetes had A3243G mutation in mtDNA. They are eight with maternally inherited diabetes (MID) alone and six with maternally inherited diabetes and deafness (MIDD) included two the positive results from RFLP which could be the positive control for PASA analysis.

![Figure 2](image)

Mutation analysis of mtDNA at nucleotide position 3243 in the tRNA^{Leu(UUR)} gene by PASA. Among the 103 subjects, fourteen patients with maternally inherited diabetes alone and MIDD had A3243G mutation in mtDNA (lane 2). Lane 4 indicates wild type, lane 3 indicates internal control, and lane 1 indicates the DNA size marker (100 base pair ladder).

Genotype examination

Genotype examination was done by PASA in 103 diabetes patients (Figure 3), base on the point mutations which associated with deafness (A3243G, C12258A), cataract (A3243G, C12258A), myopathy (A3243G, A3302G), cardiomyopathy (A3243G, A3260G), and gastrointestinal dismotility in diabetes patients by PASA. Only A3243G mutation was investigated in fourteen diabetes patients, they are eight with maternally inherited diabetes alone and six with MIDD. Lanes 2, 3, 4, 5, and 6 indicate wild-types for A3243G, A3260G, A3302G, G8313A, and C12258A, respectively. Lane 1 indicates the DNA size marker (100 base pair ladder).

![Figure 3](image)

Genotype examination for deafness, cataract, myopathy, cardiomyopathy, and gastrointestinal dismotility in diabetes patients by PASA. Only A3243G mutation was investigated in fourteen diabetes patients, they are eight with maternally inherited diabetes alone and six with MIDD. Lanes 2, 3, 4, 5, and 6 indicate wild-types for A3243G, A3260G, A3302G, G8313A, and C12258A, respectively. Lane 1 indicates the DNA size marker (100 base pair ladder).

Maternal heredity pattern of A3243G mutation

In Figure 4, A3243G heteroplasmy mutation in one of maternally inherited diabetes and deafness patient families was inherited from mother to two children. Those were contrary to the negative control using normal human which it was no product PASA by mutant primer and conversely, 97 bp fragment amplified by wild-type primer.

![Figure 4](image)

Study of maternal heredity of A3243G mutation in one of MIDD patients and two her sons by PASA. Lanes 2, 4, and 6 indicate mutant primers for mother and two her children. Lanes 3, 5, 7 indicate wild-type primers for mother and two her children. Lanes 8 and 9 indicate mutant and wild-type primers for negative control (normal). Lane 1 indicates the DNA size marker (100 base pair ladder).

Three mismatches at the two nucleotides immediately 5' to the mutation site were then introduced (Table 1 and Table 2), which greatly improved the specificity. Thus, modified forward primers were used for this study. At the mutation site, the wild-type primer containing an “A” would perfectly
match the wild-type target sequence but would be a weak "AC" mismatch with the mutant target sequence. Similarly, the mutant primer containing a "G" would be a perfect match with the mutant target sequence but would be a weak "GT" mismatch with the wild-type target sequence. Therefore, introduction of a strong CC mismatch at the penultimate nucleotide is expected to increase the primer specificity.

A total of 47 patients had maternally inherited diabetes (MID) either alone (group 1, n = 14) or with one or more additional features of mitochondrial disease, including deafness and neuromuscular disease (group 2, n = 33). The negative control subjects are 56 patients diabetes with hadn’t MID and neuromuscular disease. Fourteen diabetes patients carried the A3243G mutation, they are eight with MID alone (group 1) and six with MIDD (group 2). Here, we identified that MID alone posses A3243G mutation, this is contrary to previous studies. Previous studies have suggested that this mutation is associated with maternally inherited diabetes, but only when additional features of mitochondrial disease are present. Because, based on one of mtDNA characteristic that is maternally inherited both are the normal and mutant mtDNA. In addition, the result of previous research used direct sequencing to detect mutations, but can only reliably detect % heteroplasmy >25%, which is rare in leukocytes. Therefore, the samples in previous research using skeletal muscle derived mtDNA (% heteroplasmy ~ 90%) (Alan et al., 2002).

Heteroplasmy A3243G mutation which found in diabetic patients was approximatively 1.5% when mtDNA is extracted from leucocytes (Narbone, et al., 2001). Some methods to detect the presence of the mutation have limited sensitivity and may lead to potential miscategorization of patients with low levels of heteroplasmy. Therefore, PASA-three bases mismatch method was used to detection of heteroplasmy mutations in blood cells. Also, we have identified maternal inheritance of A3243G heteroplasmy mutation in one of maternally inherited diabetes and deafness patient families. These result showed that PASA method has more sensitivity than PCR-RFLP method.

Conclusions

In conclusion, we identified that maternally inherited diabetes posses A3243G mutation, this is contrary to previous studies which have suggested that this mutation is associated with maternally inherited diabetes, but only when additional features of mitochondrial disease are present. Conversely, based on our own observations and characteristic of mtDNA, we considered that patients with maternally inherited diabetes alone had mtDNA defect.

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References


