A Novel Unstable Mutation in Mitochondrial DNA Responsible for Maternally Inherited Diabetes and Deafness

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OBJECTIVE—The m.3243A>G mutation in mitochondrial DNA (mtDNA) is responsible for maternally inherited diabetes and deafness (MIDD). Other mtDNA mutations are extremely rare.

RESEARCH DESIGN AND METHODS—We studied a patient presenting with diabetes and deafness who does not carry the m.3243A>G mutation.

RESULTS—We identified a deficiency of respiratory chain complex I in the patient's fibroblasts. mtDNA sequencing revealed a novel mutation that corresponds to an insertion of one or two cytosine residues in the coding region of the *MT-ND6* gene (m.14535_14536insC or CC), leading to premature stop codons. This heteroplasmic mutation is unstable in the patient's somatic tissues.

CONCLUSIONS—We describe for the first time an unstable mutation in a mitochondrial gene coding for a complex I subunit, which is responsible for the MIDD phenotype. This mutation is likely favored by the m.14530T>C polymorphism, which is homoplasmic and leads to the formation of an 8-bp polyC tract responsible for genetic instability.

Diabetes Care 34:2591–2593, 2011

The most common form of maternally inherited diabetes and deafness (MIDD) is associated with the m.3243A>G mutation in mitochondrial DNA (mtDNA), which is located in the *tRNA^Leu* gene (1). The mutation that affects up to 1% of diabetic patients leads to both impaired glucose-induced insulin secretion (2) and progressive β-cell loss (3). However, in some rare cases characterized by a highly suggestive phenotype but without m.3243A>G mutation, geneticists should look for other diabetes-prone variants (4). Here, we describe a patient presenting an MIDD phenotype who carries a novel unstable mutation in the mitochondrial *MT-ND6* gene responsible for a deficiency in the respiratory chain complex I.

RESEARCH DESIGN AND METHODS

Case report
The patient is a normal-weight (BMI, 22.5 kg/m^2), 77-year-old male suffering from diabetes since the age of 45. Insulin had to be introduced at the age of 69 because of poor metabolic control under maximal oral treatment. At admission, his HbA$_{1c}$ was 9% despite two injections of NPH insulin. He was switched to an intensified insulin treatment by injections of premixed insulin analogs three times a day (0.87 units/kg/day, Lispro/NPH, 50/50 and 25/75; Lilly, Indianapolis, IN). Thereafter, HbA$_{1c}$ ranged between 6.8 and 7.6%. He reported a history of myocardial infarction 20 years ago and an acute congestive heart failure episode 12 months ago. Since this episode, he was treated with an ACE inhibitor, anticalcic drug, and furosemide. At admission, the heart echography and pro-brain natriuretic peptide value (39 pg/mL) were in the normal range. Plasma creatinine (86 μmol/L) and microalbuminuria (<2 mg/L) were normal. Fundus examination detected only signs of mild background diabetic retinopathy. Macular pattern dystrophy was absent. The patient suffered from chronic bilateral hearing impairment and required permanent use of a conventional hearing aid. Audiogram showed that the defect preferentially affected high frequency perception (Supplementary Fig. 1).

Oxidative phosphorylation spectrophotometric measurements
Respiratory chain complexes I–V and citrate synthase activities were measured in fibroblasts obtained from the patient's forearm skin explant. Fibroblasts were spun down (2,500g × 5 min) through a digitonin (0.01%) and Percoll (5%) solution. Rotenone-sensitive NADH quinone reductase (complex I), malonate-sensitive succinate quinone 2,6-dichloroindophenol sodium salt hydrate (DCPIP) reductase (complex II), antimycin-sensitive quinol cytochrome *c* reductase (complex III), cyanide-sensitive cytochrome *c* oxidase (complex IV), oligomycin-sensitive ATP hydrolase (complex V), and citrate synthase were measured by spectrophotometry using a dual-beam spectrophotometer (Cary 50 UV-visible spectrophotometer; Agilent Technologies, Massy, France) with standard procedures (5). Decylubiquinol quinone

derivative was used to measure complex III (5). All measurements were performed at 37°C. Protein levels were determined by the Bradford method using BSA as a standard.

mtDNA analysis
Blood and tissue samples were obtained after the patient had given informed consent. PCR restriction fragment–length polymorphism analysis was used to look for the m.3243A>G mutation (6). Surveyor analysis was performed as previously described (7). For quantitation of heteroplasmy, the forward unlabeled primer spanned mtDNA nucleotides 14443–14462 and the reverse labeled primer spanned nucleotides 14578–14559. Total DNA was

amplified by PCR for 20 cycles (95°C/30 s, 60°C/30 s, and 72°C/30 s) (8). Fluorescent PCR products were separated by electrophoresis using an ABI 3130 genetic analyzer (Applied Biosystems, Foster City, CA) and were studied using the GeneMapper v4.0 software.

Western blotting
Total protein extracts (50 μg) were separated on an acrylamide gel by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore, Saint-Quentin, France). A cocktail of anti-human total oxidative phosphorylation complex antibodies (Mitosciences, Eugene, OR) was used at 1/1,000, and detection was performed as previously described (9).

RESULTS—Our patient was suspected to suffer of mitochondrial diabetes based on the combination of a high-frequency sensorineural hearing impairment and other cases of diabetes and/or deafness in the family (Fig. 1A). We did not find the m.3243A>G mutation in leukocytes, fibroblasts, hair follicles, buccal mucosa, or urinary cells. Enzymatic spectrophotometric measurements of the individual respiratory chain complexes revealed a decrease in complex I activity in fibroblasts (Fig. 1B). Western blot analysis did not reveal any significant reduction in the amount of respiratory chain proteins as compared with the control (Supplementary Fig. 2). By sequencing the mitochondrial genome in fibroblasts, we
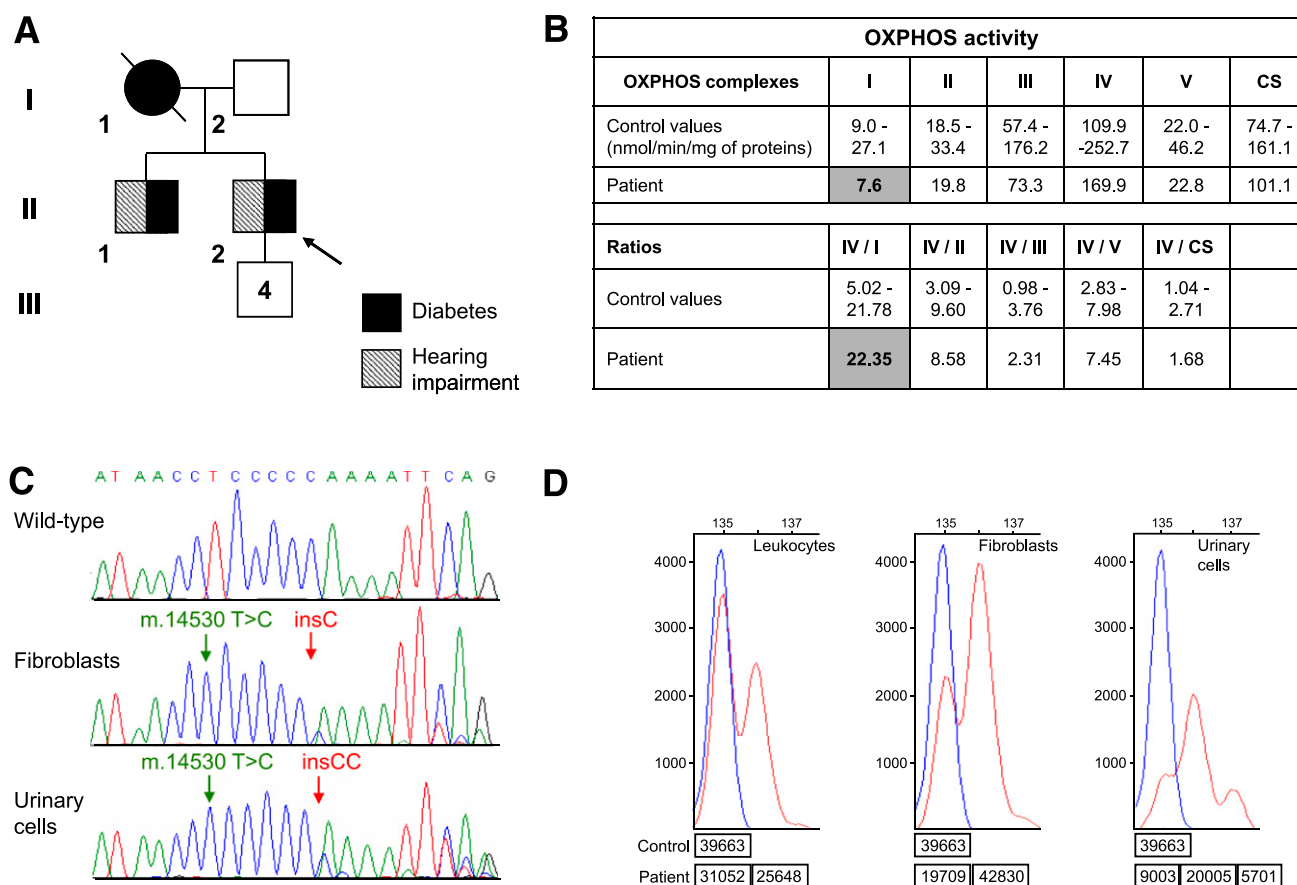

B

OXPHOS activity						
OXPHOS complexes	**I**	**II**	**III**	**IV**	**V**	**CS**
Control values (nmol/min/mg of proteins)	9.0 - 27.1	18.5 - 33.4	57.4 - 176.2	109.9 -252.7	22.0 - 46.2	74.7 - 161.1
Patient	**7.6**	19.8	73.3	169.9	22.8	101.1

Ratios	**IV / I**	**IV / II**	**IV / III**	**IV / V**	**IV / CS**	
Control values	5.02 - 21.78	3.09 - 9.60	0.98 - 3.76	2.83 - 7.98	1.04 - 2.71	
Patient	**22.35**	8.58	2.31	7.45	1.68	

Figure 1—*Identification of a novel unstable mutation in mtDNA leading to diabetes and deafness. A: Pedigree of the family. B: Respiratory chain enzyme activities in cultured skin fibroblasts. Results are expressed 1) as absolute values for controls or patient (in nanomols of substrate per minute per milligram of proteins) and 2) as activity ratios compared with complex IV, which best reflects the balanced respiratory enzyme activity. The abnormal values are in bold. OXPHOS, oxidative phosphorylation. CS, citrate synthase. C: Sequencing electrophoregrams showing the m.14535_14536insC mutation in fibroblasts and the m.14535_14536insCC mutation in urinary cells. The homoplasmic m.14530T>C polymorphism is indicated. Sequencing electrophoregram from a control individual is also presented. D: Quantitation of heteroplasmy by semiquantitative PCR test (8). The size of the expected wild-type product is 135 bp. Blue peaks (control individual) and red peaks (patient) are superimposed. A supplementary fragment of 136 bp, corresponding to a C insertion, was found in all tissues tested in the patient. Another fragment of 137 bp, corresponding to a CC insertion, was also found in urinary cells and hair follicles. Peak areas are indicated in rectangles. Mutant load was calculated by dividing the 136-bp peak area by the sum of the 136-bp and 135-bp peak areas (m.14535_14536insC) and by dividing the sum of the 136-bp and 137-bp peak areas by the sum of the 135-bp, 136-bp, and 137-bp peak areas (m.14535_14536insCC). The level of mutation was 45% in leukocytes, 68% in fibroblasts, and 74% in urinary cells. Data corresponding to buccal mucosa (59%) and hair follicles (51.5%) are not shown.*

found a heteroplasmic insertion of a cytosine residue (m.14535_14536insC) in the *MT-ND6* coding region (Fig. 1*C*). The presence of this mutation, which was also detected in leukocytes and buccal mucosa, was confirmed by digestion with the Surveyor nuclease (7) (Supplementary Fig. 3). In hair follicles and urinary cells, we found a supplementary insertion of two cytosines (m.14535_14536insCC) (Fig. 1*C*). In all cases, insertions cause a frameshift at the 46th amino acid, leading to a premature stop codon in *MT-ND6* (F46fsX61 and F46fsX53, respectively). This results in truncated polypeptides that lack the three last transmembrane domains of ND6. These mutations likely lead to unstable truncated products and abolish assembly of complex I (10). In fibroblasts, complex I deficiency was secondary to a high level of mutations (68%) (Fig. 1*D*). We also found the homoplasmic polymorphism (m.14530T>C) in all tissues.

CONCLUSIONS—Determination of the mitochondrial origin of diabetes is important for genetic counseling and clinical care (6). Nevertheless, it is frequently impossible to confirm the diagnosis when the m.3243A>G mutation is not found (4). Muscle biopsy is an invasive method that cannot be used as a routine examination, especially in diabetic patients. Our observation reveals that analysis of fibroblasts can identify a respiratory chain deficiency and direct molecular screening. Identification of an isolated complex deficiency led us to sequence mtDNA genes coding for the complex I subunits. We found that complex I deficiency is associated with an insertion of cytosine residues in the coding region of the *MT-ND6* gene (m.14535_14536insC). This insertion is heteroplasmic and unstable because of the number of supplementary cytosine residues, which differs from one somatic tissue to another. This novel mutation is associated with a homoplasmic polymorphism at nucleotide 14530 that replaces a thymine by a cytosine residue, leading to the formation of an 8–base pair (bp) polyC tract. Several polyC repeats are found in the D-loop or in intergenic regions of mtDNA (11,12) and are unstable by a genetic standpoint. In the MIDD family, Janssen et al. (13) have shown that two repeats, a 6-bp polycytidine tract at nucleotide 568 in the D-loop and a 9-bp CCCCCTCTA sequence in the intergenic COII-tRNA^Lys region, are variable in length. Both repeats are stably inherited in a maternal way, but in proband's fibroblasts, the average length of the polycytidine tracts is increased at both locations, indicating a fibroblast-specific instability. Nevertheless, the contribution of mtDNA length variants to the phenotype was difficult to determine in this family because they were located in noncoding regions. In contrast, the insertion of cytosine residue(s) found in our patient is responsible for a frameshift in *MT-ND6*, leading to a deficiency in complex I. The patient also carries the m.14530T>C polymorphism corresponding to a neutral mutation (G48G) that is found in 1 of 2,704 individuals in the Human Mitochondrial Genome Database (14,15). It is likely that the formation of the polyC repeat secondary to this polymorphism in the *MT-ND6* coding region plays a role in the genetic instability leading to the observed complex I deficiency.

In conclusion, we describe for the first time an unstable mutation in mtDNA leading to the MIDD phenotype. This observation also illustrates the interest to study fibroblasts when the m.3243A>G mutation is not found.

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