Extra-Pancreatic Manifestations in Diabetes Secondary to Mitochondrial DNA Point Mutation Within the tRNA$^{Leu(UUR)}$ Gene

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OBJECTIVE — A point mutation in the mitochondrial genome has been identified as a cause of diabetes and deafness. We report two pedigrees with an A-to-G transition at nucleotide 3243 of mtDNA within the tRNA$^{Leu(UUR)}$ gene and focus our investigations on other localizations of the anomaly, particularly muscle and retina.

RESEARCH DESIGN AND METHODS — Muscular localization has been studied in probands by invasive and noninvasive methods, including muscle biopsy (evaluation of the proportion of mutated mtDNA in comparison to blood cells, measurement of respiratory chain complex activities and histological and histochemical aspects) and ^{31}P-nuclear magnetic resonance (NMR) spectroscopy. Ophthalmic and angiographic examination of retina, electroretinography, and visual evoked potentials were performed in five subjects.

RESULTS — This mutation, previously described in patients with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), was expressed more abundantly in muscle than in nucleated blood cells. This low expression in blood cells could hamper the diagnosis for some patients. In addition, despite poor clinical expression, muscle was found to be highly affected. Ragged red fibers and dystrophic mitochondria were observed in muscle biopsy. Histochemical assays showed decreased activity of respiratory chain complexes, and ^{31}P-NMR in vivo data further confirmed the defect of muscle oxidative processes. Exercise-induced lactate production was increased. Finally, in both families, an atypical "salt and pepper" pigmentary retinopathy was observed without consequences on visual acuity.

CONCLUSIONS — In diabetes secondary to 3243 mtDNA mutation, infraclinical muscular and ocular lesions are frequent. These two locations of the disease, which are easily investigated by simple methods, can help in the diagnosis of this new type of diabetes.

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MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes; NIDDM, non-insulin-dependent diabetes mellitus; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; RRF, ragged red fibers.

Numerous cases of maternally inherited diabetes and deafness secondary to a point mutation of mtDNA leading to an A-to-G transition at position 3243 in the tRNA leucine gene have been reported (reviewed in 1). This mutation has also been found in patients with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes) (2). In this report of two families with diabetes-deafness syndrome, we would like to insist on the presence of infraclinical expression of other localizations of the mutated mitochondria similar to that observed in other mitochondriopathies. Alteration of oxidative processes in muscle and atypical pigmentary retinopathy were also detected in our pedigrees. These localizations, which are easily accessible by relatively noninvasive methods, could be of interest in the diagnosis of similar cases, particularly if detection of the mutation in blood cells is failing.

RESEARCH DESIGNS AND METHODS

Family 1

The pedigree of the family is presented in Fig. 1. The proband (II2), a 49-year-old woman, was identified as having the combination of insulin-requiring diabetes, deafness, and retinal abnormalities. Non-insulin-dependent diabetes mellitus (NIDDM) was diagnosed at 25 years of age. Fifteen years later, oral hypoglycemic drug failure led to insulin therapy (1.5 U/kg). Bilateral neurosensorial deafness was diagnosed at 30 years of age and "salt and pepper" pigmentary retinopathy at 42 years. Her mother (I2) died at 75 years of age, was diabetic from age 40 and deaf from age 60. Retinal abnormalities were observed but not defined. The proband's sister (II1), 56 years of age, was diagnosed with NIDDM at 47 years of age and was deaf at age 49. Ophthalmic examination showed pigmental abnormalities in the retina. She also suffered from cardiomyopathy. One of her daughters (III1), who

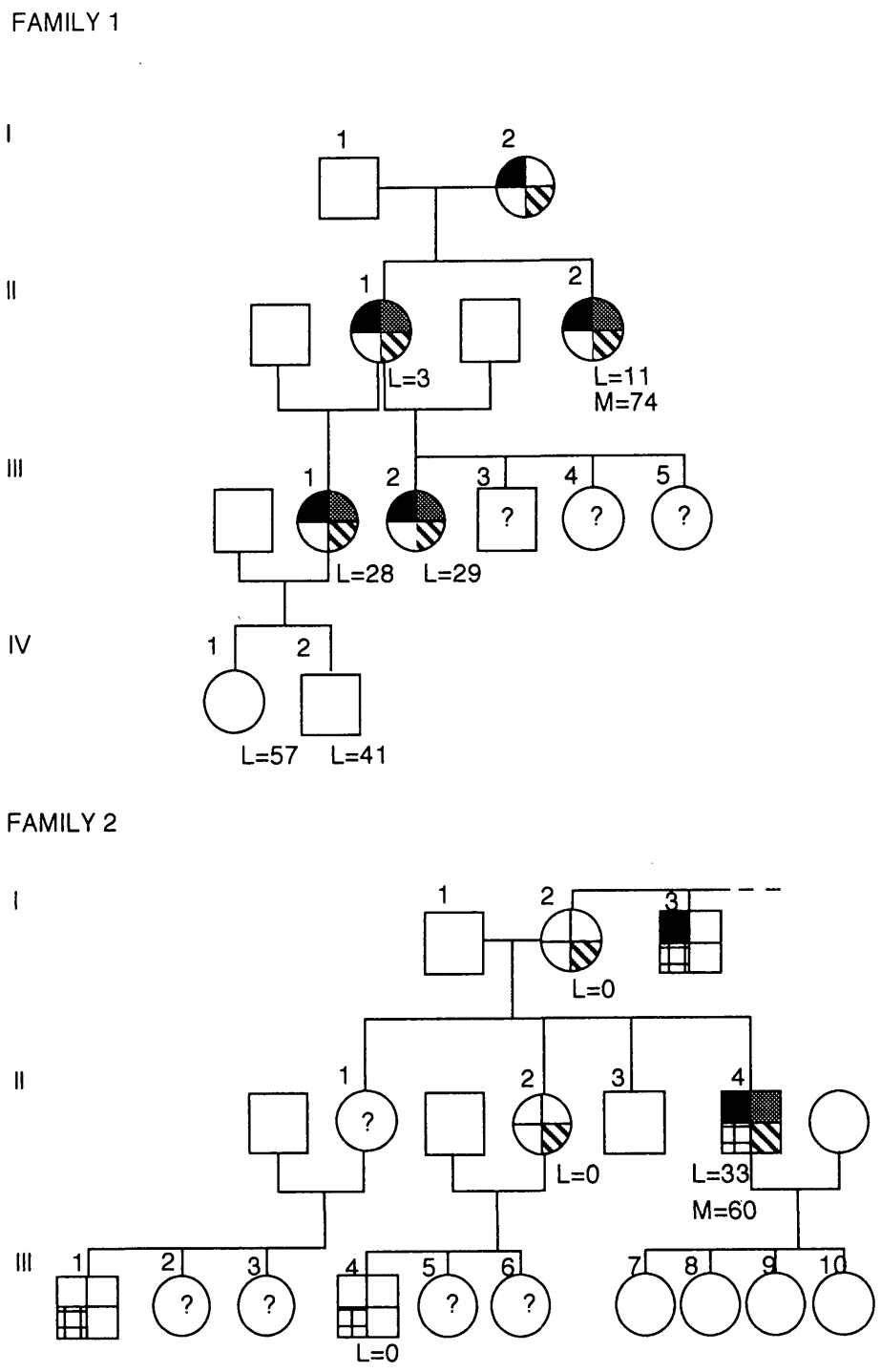

Figure 1—*Pedigrees of the two families. Open symbols represent clinically nonaffected patients.* ■, *Glucose intolerence or diabetes;* ▩, *pigmentary retinopathy;* ▧, *hearing loss;* ▦, *cerebral abnormalities; ?, not investigated. Numbers are percentages of mutated DNA in either lymphocytes (L) or muscle (M).*

was 36 years old, was not diabetic but was glucose intolerant and also suffered from neurosensorial deafness and retinal abnormalities. Another daughter (III2) (32 years of age) and the children of III1 (IV1 and IV2, 10 and 7 years old, respectively) were studied. III2 exhibited hearing loss, glucose intolerance, and retinal abnormalities. The two children were healthy.

Family 2

The pedigree of family 2 is also presented in Fig. 1. The proband (II4), a 42-year-old male, was identified by the combination of insulin-dependent diabetes, deafness, myocardiopathy, basal ganglia calcifications, and atypical pigmentary retinopathy. Diabetes and deafness were diagnosed at 23 years of age. Insulin therapy was administered immediately. He received 0.9 U/kg body wt of insulin, which was administered twice a day. Cardiomyopathy, intracranial calcifications, and retinopathy were diagnosed at age 40. Unfortunately, information concerning other members of the family was less precise than that of family 1 because some of them lived in another country and others refused the investigation. The mother (I2) was deaf. A maternal uncle suffered from NIDDM and died prematurely from an unexplained stroke. One sister (II2) of the proband was deaf and had a son with a cognitive decline (III4). Another sister had a child with a cognitive decline (III1).

mtDNA analysis

Total DNA was isolated from blood and frozen skeletal muscle by standard procedures. For Southern blot analysis, 5 μg of total DNA was digested with *Pvu* II (nt 2656), electrophoresed through a 0.8% agarose gel and transferred to nylon membrane. The filters were hybridized with the pGT 10/12.2 probe containing a 14032 bp *Eco* RI mtDNA insert cloned into pBB3 vector (3). The nylon filters were prehybridrized, hybridized, washed, and exposed according to the supplier (Amersham, U.K.). A 398 bp fragment of mtDNA was amplified using the polymerase chain reaction (PCR) technique over 35 cycles. Primers for the reaction were 5′-TCACAAAGCGCCTTCCCCCG-3′ (nt 3153—3172) and 5′-GCGATGGTGAGAGCTAAGGTC-3′ (nt 3551—3535).

The amplified fragment, encompassing the 3243 $tRNA^{Leu}$ mutation site, was labeled with (α^{32}P)dCTP added just before the last cycle to prevent underes-

timation of the proportion of mutant mtDNAs caused by heteroduplex formation. The 398-bp fragment was digested with *Apa* I, which cleaves the DNA at the additional restriction site created by mutation into 300- and 98-bp fragments. The digested fragments were subsequently electrophoresed on an 8% polyacrylamide gel, and then the gel was dried and exposed in a cassette. The relative proportions of mutant and wild-type mtDNA were determined by densitometric analysis, using a Studioscan (Agfa).

Pathological and functional study of muscle

In both probands, muscle mitochondrial functions were assessed by in vitro and in vivo methods as previously described (4). Muscle samples obtained from the deltoid were used for histochemistry and electron microscopy examination and measurement of respiratory chain complex activities. In addition, lactate production during an ergometric exercise test was measured. Finally, ^{31}P-nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker 47/30 Biospec according to previously published methodology (4,5).

RESULTS

mtDNA analysis

This analysis was performed on muscle of the proband of each family and in blood samples of five relatives of family 1 (II1, III1, III2, IV1, and IV2) and three relatives of family 2 (I2, II2, and III4). In everyone tested in family 1 and one person in family 2, the A-to-G mutation at position 3243 of mitochondrial leucine tRNA was identified. The proportion of mutated mtDNA in blood cells exhibited large interindividual variation without correlation with the phenotypic expression (Fig. 1). Some subjects exhibited a low percentage of mutated mtDNA despite a full syndrome. Others had a large proportion of mutated mtDNA without clinical expression. One patient in each family had a very low percentage of abnormal mtDNA detectable in blood cells, which might not have been recognized if the test had not been performed in a family study. Indeed, after cleavage with *Apa* I, PCR-amplified mtDNA fragments are visualized on ethidium bromide agarose gel. The labeling with (α^{32}P) CTP, which increases the sensitivity of the method, is usually performed only for the quantitative evaluation of mutated mtDNA. The bands corresponding to the 3% of mutated mtDNA did not appear on ethidium bromide gel. Finally, three relatives of family 2, despite neurological expression, were absolutely negative with blood cells for the mutation.

Histochemistry and electron microscopy of muscle biopsy. By light microscopy, histopathological changes were identical in the three muscle biopsies performed. There was an increased number of ragged red fibers (RRFs), 20% in case 1 and 35% in case 2. Mitochondrial aggregates in the type 1 fibers were revealed by succinyl dehydrogenase (SDH) and NADH tetrazolium reductase stainings. Some RRFs and nonRRFs were negative for cytochrome oxidase. Glycogen and lipid contents were increased in abnormal fibers. Electron microscopy study revealed numerous enlarged mitochondria with either abnormal cristae (paracrystalline inclusions, concentric cristae) or abnormal matrix (osmiophilic lipid inclusions, granular structure). Accumulation of lipid droplets in subsarcolemmal and intermyofibrillar areas was obvious. Arterial and capillary vessels were normal, as were the mitochondria of endothelial cells.

Assays of individual respiratory complexes. The activity of complexes I and IV were decreased in both cases, being 29.3% and 26.9%, respectively, in patient II2 (family 1) and not detectable and 5.9 % of the mean control values in patient II3 (family 2). Activity of complex III was also decreased in patient II2 (family 1) to 28.8% of the mean control value. Complex II and SDH activities were found to be within normal range ($< x + 2$ SD) in both cases.

Ergometer exercise test. Maximal load accepted during the exercise period of the ergometer exercise test was 80 W. The maximal oxygen uptake was reduced representing 85% of the normal value (23.2 $ml \cdot mn^{-1} \cdot kg^{-1}$). Resting and peak blood lactate values were elevated (2.94 and 6.39 nmol/l, respectively). The disappearance rate of blood lactate during recovery was low (0.093 nmol/mn, control subjects 0.23–0.31 nmol/nm). This excess of lactate production before and during exercise indicated a limitation in the rate of mitochondrial oxidative phosphorylation and an early participation of glycolytic metabolism to energy production.

^{31}P-NMR spectroscopy of muscle. Table 1 summarizes the metabolic values recorded during the standardized ^{31}P magnetic resonance spectroscopy (MRS) protocol. At rest, for both patients all the parameters except PCr:Pi ratio were within the normal range. The low PCr:Pi ratio indicated a decreased energy state of the muscle at rest. This abnormal value was correlated to an abnormal increase of Pi measured at rest but not to any abnormality in ADP. During exercise—considering that metabolic changes, i.e., PCr breakdown, were linked to pH values measured at the end of exercise—intracellular acidosis was not within normal range for both patients, and PCr breakdown was abnormally high for patient 2. In human mitochondrial diseases, inefficient pyruvate metabolism by abnormal mitochondria leads to lactic acidosis through an increase in the rate of glycolysis required to maintain the phosphorylation of increased ADP concentrations. Some studies have reported that intracellular acidosis may be prevented in such diseases by an adaptative mechanism that enables removal of intracellular lactate into the bloodstream (6,7). Inappropriate glycolysis-induced pH decrease could reflect this adaptive mechanism. During exercise, PCr breakdown encompasses the balance between energy demand and ATP

Table 1—*High-energy phosphate concentrations and intracellular pH measured during the standardized 31-P MRS protocol*

	Rest				Exercise				Recovery	
	Pcr (mmol/l)	PCr:Pi	pH	ADP (μmol/l)	Pcr (mmol/l)	PCr:Pi	pH	ADP (μmol/l)	d(PCr:Pi)/dt	Proton efflux (mmol · l^{-1} · min^{-1})
Control subjects	(34.1–39.7)	(7.94–10.88)	(6.97–7.02)	(4–10)	(9.05–26.13)	(1.21–1.66)	(6.60–6.76)	(18.97–47.16)	(2.38–3.53)	(1.54–3.14)
Patient 1	35.51	5.40*	7.02	35.68*	14.53	0.63*	6.65	40.01	1.29*	29.28*
Patient 2	37.16	5.51*	7.00	7.17	8.31*	0.17*	6.75	138.88*	3.47*	18.89*

Data for controls subjects (n = 20) are presented as confidence interval from mean −3 SE to mean +3 SE. Values outside the normal range were considered abnormal ($P < 0.01$). At the end of exercise and during recovery, values measured for patients were compared with values recorded for control subjects matching the same intracellular acidosis at the end of exercise. Details for calculation of absolute concentrations and proton efflux are given in ref. 4, 5, and 8. *Outside the normal range.

synthesis ensured by both oxidative and anaerobic processes. As limited pH changes were observed, the abnormally high PCr consumption observed for patient 2 could reflect impaired oxidative processes. Interestingly, ADP measured at the end of exercise was significantly higher, further suggesting that oxidative processes allowing ADP phosphorylation are impaired. During recovery from exercise, kinetics of PCr:Pi recovery were delayed for patient 1, and the rate of proton efflux was faster for both patients. Previous studies have demonstrated that ATP resynthesis during recovery is exclusively oxidative and dependent upon the extent of intracellular acidosis (8). For patient 1, the slow recovery clearly demonstrated impaired aerobic metabolism, and increase in proton efflux confirmed the adaptive mechanism leading to limited pH changes during exercise (9). For patient 2, however, the kinetics of PCr:Pi recovery was within the normal range, suggesting that mitochondrial metabolism was not impaired. Nevertheless, the ADP accumulation measured during exercise could reflect abnormal ADP utilization by mitochondria. Considering the hyperbolic relation between rates of oxidative ATP synthesis and ADP, one could hypothesize, as suggested previously (9), that an abnormally high ADP could represent another type of adaptive mechanism aimed at improving the rate of ADP rephosphorylation during recovery.

Ophthalmic examination

In five subjects (family 1 : II1, II2, III1, III2, and the proband of family 2), atypical pigmentary retinopathy was observed. There was a granular, "salt and pepper" appearance of retinal pigmentary epithelium that was most marked on the posterior, leaving the periphery unchanged. Few well demarcated zona of retinal pigment epithelium (RPE) atrophy were present in the macular area. In these subjects, visual acuity was preserved. Electroretinography and visual evoked potential were not altered. No diabetic retinopathy was present.

CONCLUSIONS — The clinical picture of diabetes-deafness syndrome in our two pedigrees is similar to that previously described in the literature (reviewed in 1). The maternal transmission and the presence of the 3243 mutation of the mtDNA confirm the mitochondrial origin of the symptomatology. But our observations show that in some cases, the detection of the mutation in blood cells can fail, with a substantial risk of underevaluation of the prevalence of this abnormality. Mitotic segregation and heteroplasmy implies that the distribution of mutated mtDNA is very variable from one tissue to another. The high rate of replication of blood cells can decrease the proportion of mutated mtDNA in comparison to wild type. In our families, the two probands exhibited a lower proportion of mutated mtDNA in nucleated blood cells than in muscle. It is also noteworthy that in some subjects, the band corresponding to mutated mtDNA is very faint despite phenotypic expression and/or transmission of the abnormality to the descendant. If this analysis had not been performed in a family study, there would be a risk of false-negative response. Furthermore, in three subjects of family 2, despite neurosensorial involvement, no abnormal mtDNA was found in blood cells. This limitation of screening of the mutation based on the analysis of blood cells could be alleviated by the use of another tissue, such as muscle. Indeed, our observations show that even in the absence of obvious functional symptomatology, muscular defect is frequently observed. This muscular localization was detectable in three subjects in our series by ergometric test, NMR spectroscopy, and/or muscle biopsy. As suggested by the same point mutation, diabetes-deafness and MELAS share oxidative defects in skeletal muscles (10). In the literature, cases of diabetes have been described in family with typical MELAS (13). The very poor clinical expression of this muscular impairment is probably due to an adap-

tive phenomenon, as suggested by NMR spectroscopy. The apparently frequent muscular localization of mutated mtDNA in the mitochondriopathy-induced diabetes points toward a diagnosis based on invasive or noninvasive investigation of muscle with a lower risk of false negative. This approach would be a substitute for the impossibility of looking for the mutation without risk in the pancreatic β-cells. The presence of the mutation in target organs of insulin, such as muscle or liver, could be responsible for some peripheral insulin resistance. For instance, the exacerbation of glycolytic pathways in muscle and exercise lactate production could stimulate hepatic neoglycogenesis by Cori cycle. This phenomenon could participate in the diabetogenesis and progressive exhaustion of β-cells. Katagiri et al. (12), however, had shown that in some subjects with the 3243 mutation, insulin secretion slowly moves from hyperinsulinism to hypoinsulinism. Suzuki et al. (13) have observed a marked reduction of insulin release after either oral glucose or glucagon in families with MELAS, which suggests that the main anomaly is localized in the β-cells.

Finally, atypical pigmentary retinopathy has been found in four patients with a combination of diabetes and deafness. These retinal abnormalities have been rarely noted in the diabetes-deafness pedigrees reported in the literature, particularly from Japanese authors (1,12, 13). However, in the cases reported by Reardon et al. (14), there was a "salt and pepper retinopathy" similar to that described in our families. This retinopathy is quite different from pigmentary retinopathy because there is a preservation of the periphery. Electroretinogram and visual-evoked potentials were also normal. The lack of functional consequences, particularly on visual acuity, could partly explain this apparent rarity. Such an atypical pigmentary retinopathy has been initially described in another mitochondriopathy, the Kearns-Sayre syndrome (15). Histological study of retinopathy associated to the 3243 point mutation of mtDNA has been performed by Chang et al. (16), who found dystrophic mitochondria in cells with a high metabolic activity, such as retinal pigment epithelium, corneal endothelium, and extra ocular muscle. Since diabetic patients require serial ophthalmic examinations, diabetologists should be aware of the description of atypical pigmentary retinopathy, which must lead to the search for a mitochondriopathy.

These case reports of diabetes by mitochondriopathy confirm that other localizations of abnormal mitochondria are relatively frequent despite a low clinical expression. These localizations are predominantly represented by retina and muscle. Fortunately, these organs are easily accessible to investigation by relatively simple methods. They offer the opportunity to identify this etiology in some diabetic subjects.

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