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Diabetes mellitus carrying a mutation in the mitochondrial tRNA$^{Leu(UUR)}$ gene

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Summary We screened 214 Japanese NIDDM (non-insulin-dependent) diabetic patients with a family history of diabetes for mutations in the mitochondrial tRNA$^{Leu(UUR)}$ gene using polymerase chain reaction-restriction fragment length polymorphism and direct sequencing. Six patients were identified as having an A to G transition at position 3243 (3243 mutation), but no patients were detected with a T to C transition at position 3271, in the mitochondrial tRNA$^{Leu(UUR)}$ gene. These two mutations were not present in 85 healthy control subjects. It was disclosed that the patients' mothers were also affected by diabetes mellitus in five of the six cases. In these six affected patients, the 3243 mutation shows variable phenotypes, such as the degree of multiple organ involvement, intrafamilial and interfamilial differences in disease characteristics, and the degree of the involvement of MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes) phenotype. Endocrinological examinations revealed that those diabetic patients with the 3243 mutation show not only beta-cell dysfunction, but also a defect in alpha-cell function, which is considered characteristic of diabetes with the 3243 mutation. When compared with 50 selected diabetic control subjects without the 3243 mutation, whose mothers, but not fathers, were found to have diabetes, it was established statistically that those with the 3243 mutation possess the following clinical characteristics; 1) the age of diabetes onset is lower, 2) they have lean body constitutions, and 3) they are more likely to be treated with insulin than control subjects. We suggest that diabetes with the 3243 mutation possesses phenotypes distinct from those in common forms of diabetes. [Diabetologia (1995) 38: 193–200]

Key words NIDDM, genetics, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS), mitochondrial tRNA$^{Leu(UUR)}$ gene, maternal inheritance, PCR-RFLP.

Diabetes mellitus is a genetically heterogeneous group of disorders which share the common feature of glucose intolerance. It is well-known that inherited factors contribute to the development of non-insulin-dependent diabetes (NIDDM), as reflected by the high degree of concordance in monozygotic twin pairs [1]. Several candidate genes, including genes for insulin, the insulin receptor, glucokinase, glycogen synthase, and the facilitative glucose transporters, have been examined for their involvement in the pathogenesis of NIDDM; however, recent data has not shown a significant contribution of these genes to the pathogenesis of NIDDM [2–6].

It has also been suggested that maternal influences play an important role in the inheritance of NIDDM [7, 8] and that the intrauterine environment may also predispose to its development [9]. In this respect, it is of interest to focus on the mitochondrial gene and

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Abbreviations: NIDDM, non-insulin-dependent diabetes mellitus; IDDM, insulin-dependent diabetes mellitus; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; BMI, body mass index; ICA, islet cell antibody; ICSA, islet cell surface antibody; GAD, glutamic acid decarboxylase.

to analyse whether it is involved in the pathogenesis of NIDDM because, unlike nuclear DNA, mitochondrial DNA is transmitted solely from mothers. Mitochondrial diseases are essentially multisystemic and the term "mitochondrial encephalopathy" encompasses three distinctive syndromes [10]: mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) [11, 12]; myoclonus epilepsy associated with ragged-red fibers (MERRF) [13, 14]; and chronic progressive external ophthalmoplegia (CPEO) [15], including Kearns-Sayre syndrome (KSS) [16, 17].

In 1992, Ballinger et al. [18] reported familial diabetes with deafness caused by a 10.4 kb deletion in the mitochondrial DNA, followed by a report by van den Ouweland et al. [19] in which they showed a cosegregation of a point mutation in the mitochondrial tRNA$^{Leu(UUR)}$ gene in a large pedigree with maternally transmitted NIDDM and deafness and suggested that an A to G transition at nucleotide 3243 in this gene (3243 mutation) is a pathogenic factor for NIDDM. Subsequently, some laboratories reported the association of the 3243 mutation with diabetes which showed variable clinical characteristics including insulin-deficient NIDDM and slowly-progressive insulin-dependent diabetes mellitus (IDDM) [20–25]. In this report, we screened for two mutations in the mitochondrial tRNA$^{Leu(UUR)}$ gene, the 3243 mutation which has been reported in approximately 80 % of MELAS patients [26] and a T to C transition at nucleotide 3271 (3271 mutation) which was known to be pathogenic in approximately 10 % of MELAS patients [27]. We also analysed clinical characteristics and the endocrinological background of diabetic patients with the 3243 mutation.

Subjects, materials and methods

Patients. We examined 214 Japanese NIDDM patients (101 male and 113 female). The onset of diabetes ranged from 12 to 69 years of age, with age between 15 to 83 years at the time of investigation. They all had a positive family history of diabetes in which the first-degree relatives were affected by diabetes. In the 214 patients analysed in this study, 95 patients (44.4 %) had a diabetic mother, 42 (19.6 %) had a diabetic father, 26 (12.1 %) had both a diabetic mother and a father, and 51 (23.8 %) who did not have a diabetic parent but had at least one diabetic sister or brother. Thus, in 95 out of 214 patients diabetes might have been transmitted maternally. The diagnosis of diabetes was made according to World Health Organization criteria. All the patients gave their informed consent to the study and the investigation was performed in accordance with the principles of the Declaration of Helsinki.

Mitochondrial DNA analysis

PCR-RFLP analysis of mitochondrial tRNA$^{Leu(UUR)}$ gene. Genomic DNA was extracted from the patients' leucocytes by the standard sodium dodecyl sulphate-proteinase K method [28]. We also obtained genomic DNA from various tissues where the biopsy specimens were available. Prior to polymerase chain reaction (PCR), genomic DNA was digested with a restriction endonuclease Hind III which does not cut mitochondrial DNA within the region to be amplified.

To detect the 3243 mutation, the oligonucleotides for PCR were designed to amplify the region encompassing the nucleotides 3029 to 3456. The sequences of the PCR primers were; 5′-AAGGTTCGTTTGTTCAACGA-3′ (nucleotide 3029 to 3048 of the mitochondrial tRNA$^{Leu(UUR)}$ gene) for the upstream primer and 3′-AGCGAAGGGTTGTAGTAGCC-5′ (nucleotide 3456 to 3437) for the downstream primer [19].

For the identification of the 3271 mutation, we used modified PCR primers; 5′-CCGTAAATGATATCATCTCA-3′ (nucleotide 3171 to 3190) for the upstream primer and 3′-AATtcCAGTCTCCAAGTTAAGGAGAAGAAT-5′ (nucleotide 3301 to 3272) for the downstream primer, with a G-to-T mismatch at 3275 and a T-to-C at 3276 [27]. Because of this mismatch reverse primer, the PCR products carrying a transition mutation at 3271 possess an endonuclease recognition site for Afl II (5′-CTTAAG-3′) at nucleotide 3271 to 3276 and will be cleaved into two fragments (101 bp and 30 bp) by a restriction with this enzyme.

PCR was carried out by the standard protocol in a programable heat block (ASTEC Program Temp Control System PC-700 or Perkin Elmer GeneAmp PCR System 9600) with 50 ng of genomic DNA as a template. A denaturation step at 94 °C was followed by 30 cycles of denaturation (94 °C, 30 s), annealing (55 °C for the 3243 mutation or 52 °C for the 3271 mutation, 30 s) and primer extension at 72 °C for 60 s. The PCR products were then extracted and digested overnight with either the endonuclease Apa I, which cleaves the mutant sequence (GGGCCC) at position 3243 but not the wild-type sequence (GAGCCC), or the endonuclease Afl II which cleaves the sequence (CTTAAG), created with the modified primer and the presence of the mutation, at position 3271 but not the sequence (TTTAAG), with the wild-type sequence. Then, the digested samples were analysed by 5 % (for the 3243 mutation) or 15 % (for the 3271 mutation) polyacrylamide gel electrophoresis with subsequent staining with ethidium bromide.

To quantitate the ratio of digested (mutated) fragments, we carried out PCR with the conditions as described above except using γ-[^{32}P]-dATP labelled primers as described previously [29] and the digested fragments were electrophoretically separated through a 5 % polyacrylamide gel. The dried gels were autoradiographed using Kodak AR X-ray film and intensifying screen at −70 °C. The radioactivity of digested and undigested bands in gels were counted with a radioanalytic imaging system and the proportions of the digested fragment were calculated as percentage of the radioactivity observed in the bands which were not subjected to the digestion [29]. In the case of patients where the mutation was identified, we subcloned PCR products into the phagemid pBluescript KS(−) (Strategene, La Jolla, CA, USA) and confirmed the mutation by dideoxy sequencing using a Sequenase kit (USB, Cleveland, Ohio, USA).

Profiles of the affected patients (Table 1)

Patient 1. A 33-year-old female with retinopathy and neuropathy. She was diagnosed with diabetes at the age of 25 years and insulin treatment started 2 years later. Her mother is also diabetic (Fig. 1 a).

Table 1. Comparison of the clinical profiles of the patients with or without the 3243 mutation

Patient	Sex	Age (years)	Age at onset (years)	Height (cm)	BMI (kg/m^2)	Current treatment	Complication
With the 3243 mutation							
1	Female	33	25	150	18.0	Insulin	neuropathy retinopathy
2	Female	34	13	146	18.8	Insulin	retinopathy nephropathy
3	Male	52	27	168	21.9	Insulin	neuropathy nephropathy
4	Male	59	49	156	23.0	Sulphonylurea	neuropathy, hearing loss, cardiomyopathy
5	Female	62	40	147	16.2	Insulin	triopathy, hearing loss, lactic acidosis
6	Male	43	17	152	18.9	Insulin	triopathy, hearing loss, mental retardation
Mean ± SD		47.2 ± 12.4	28.5 ± 13.7[a]	153.2 ± 8.1	19.7 ± 2.8[a]		
Without the 3243 mutation (n = 50)							
Mean ± SD		51.8 ± 14.1	40.4 ± 13.1[a]	158.7 ± 10.0	23.2 ± 3.7[a]	Insulin (n = 14) Sulphonylurea (n = 21) Diet (n = 15)	

[a] Statistically different ($p < 0.05$)

Patient 2. A 34-year-old female who is lean and of short stature. At the age of 13 years, she complained of general fatigue, and was first diagnosed with diabetes at the age of 15 years. Spotty haemorrhages in her ocular fundi were found at the age of 17 years when insulin treatment started. She now also has mild nephropathy, but without neuropathy. Her mother was diagnosed with diabetes at the age of 30 years and died from renal failure due to diabetic nephropathy at the age of 40 years. Her younger sister also has diabetes.

Patient 3. A 52-year-old male of average height. He was first found to be diabetic on an annual check-up at the age of 27 years and has been treated with both an oral hypoglycaemic agent and insulin. Mild nephropathy and sensory dominant neuropathy have been found. His mother died from diabetes at the age of 47 years and both his maternal uncle and aunt died from diabetes at the age of 55 years. The elder brother of the proband who is now 55 years old was found to be diabetic at the age of 53 years.

Patient 4. A 59-year-old male. Diabetes was first diagnosed at the age of 49 years and he had been treated with an oral hypoglycaemic agent. At the age of 56 years, he was found to have dilated cardiomyopathy. His mother died 30 years ago but no clinical information about her could be obtained at the time of the investigation.

Patient 5. A 62-year-old female who was diagnosed to be hyperglycaemic at the age of 40 years. She has been treated with insulin for 20 years. Now she is suffering from diabetic triopathy and underwent haemodialysis for chronic renal failure due to diabetic nephropathy. Her mother was also treated with haemodialysis for the same reason and the youngest brother of the proband died from diabetic coma (Fig. 1 b).

Patient 6. A 43-year-old male, of short stature. At the age of 17, he complained of thirst and polyuria which led to the diagnosis of diabetes and has been treated with insulin since then. Diabetic retinopathy, severe enough to require photocoagulation, was present at the age of 36 and at present he has diabetic triopathy, mild mental retardation, and hearing loss. His mother was also diabetic and died from renal failure due to diabetic nephropathy at the age of 56 years.

In patients 1 to 5, the laboratory findings showed normal values except in the following: patient 5 showed abnormal values for serum lactate of 2.98 mmol · l^{-1} (normal range, 0.36–1.66) and pyruvate of 147.7 μmol · l^{-1} (normal range, 34.1–106.8). Immunological analysis for islet cell antibodies (ICA), islet cell surface antibodies (ICSA) and antibodies against glutamic acid decarboxylase (GAD) were negative in all cases; however, anti-insulin antibody was detected in patients 1 (14 %, normal < 7 %) and 2 (45 %).

Several ancillary examinations were undertaken in patient 6. Magnetic resonance imaging scans of the brain showed cerebral and cerebellar atrophy. He showed mental retardation and hearing loss which are often seen in cases of MELAS; however, an aerobic exercise test using an ergometer at 15 W for 15 min showed no particular elevation of lactate and pyruvate, and no metabolic acidosis as judged by arterial blood gas analysis was seen during the exercise. Electromyography was normal, as were nerve conduction velocities. There were no histological abnormalities, such as ragged-red fibers that show abnormal accumulations of mitochondria as purplish blotches; nor were there cytochrome C oxidase-negative fibers in the muscle, which are considered specific for mitochondrial myopathy. Insulin sensitivity was measured by the euglycaemic glucose clamp test [30] in which the glucose consumption rate was 4.6 mg · kg^{-1} · min^{-1} (average for NIDDM subjects; 4.0–6.0, of normal; 8.0–10.0) indicating the presence of moderate insulin resistance. In spite of some observed abnormalities, a definite diagnosis of MELAS [29] could not be made in patient 6 mainly because of the absence of convulsion, lactic-acidosis and stroke-like episodes and also of negative evidence in histological determination in muscle specimens.

Endocrinological investigations

Glucagon infusion test and arginine infusion test. The patients were studied after an overnight fast. Venous blood samples were taken 0 min before and 3, 6, 10, and 20 min after an intravenous bolus injection of 1 mg porcine glucagon (Novo Nordisk, Bagsvaerd, Denmark) [31] or 0 min before and 15, 30, 60, 90, and 120 min after an i.v. infusion of 10% L-arginine (Morishita Roussel, Osaka, Japan) (30 g) over a 30-min period [32, 33]. Plasma C-peptide concentration was measured by solid-phase radio immunosorbant assay (RIA) using a C-peptide RIA kit developed by Shionogi pharmaceutical Co. (Osaka, Japan). Plasma glucagon concentration was measured by RIA using a double-antibody technique using a glucagon kit "Dai-ichi" (Dai-ichi RI Co., Ibaragi, Japan).

Immunological investigations

ICA and ICSA were detected by indirect immunofluorescent staining of pancreatic sections on serial dilution of serum samples. Antibodies against GAD were measured by RIA using an anti-GAD RIA kit developed by Hoechst Japan Limited (Tokyo, Japan) [34].

Statistical analysis

We compared several clinical parameters between the two groups, with or without the 3243 mutation. Statistical analyses were performed using either unpaired Student's *t*-test or chi-square test.

Results

Screening for the mutations in the mitochondrial tRNA$^{Leu(UUR)}$ gene in Japanese NIDDM patients

Using mitochondrial DNA from the patients' leucocytes, we amplified the region encoding tRNAs by PCR and screened for the presence of the mutation in the tRNA$^{Leu(UUR)}$ gene.

3243 mutation. In 214 Japanese NIDDM patients examined, six (2.8%) were detected to have a mutation at this position. The PCR products from positive patients were simultaneously subcloned into phagemid and the presence of this mutation was confirmed by subsequent dideoxy sequencing (data not shown). In 85 non-diabetic control subjects, we did not find this mutation.

3271 mutation. Genomic DNA extracted from the peripheral blood of the MELAS patients with the 3271 mutation was amplified by PCR and the resultant PCR product was subcloned into plasmid, pCR-II (Invitrogen, San Diego, CA, USA). We used this cloned DNA (a gift from Dr. M. Tanaka, Nagoya, Japan) as a positive control in screening for the 3271 mutation. We found no 3271 mutation in the 214 patients analysed or 85 healthy controls (data not shown).

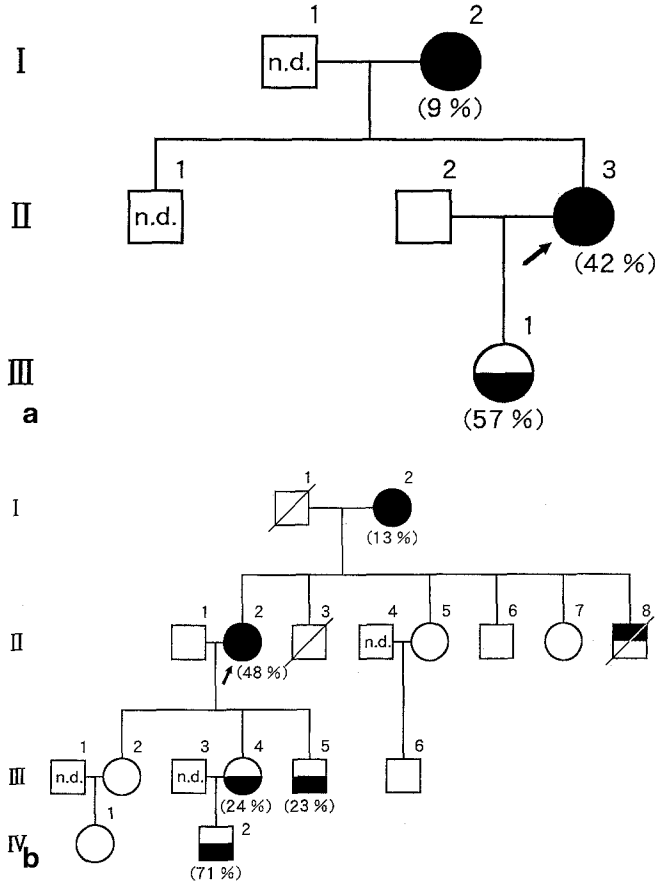

Fig. 1. Pedigree of patient 1 (**a**) and patient 5 (**b**). Roman numbers on the left of the figure indicate generation number and the numbers above the symbols indicate the identification number with that generation. The numbers below the symbols in parentheses represent percentage of mutant mitochondrial DNA in leucocytes. □ males; ○ females; ■ diabetes; ▬ mutation exists. n.d., not determined; slash, dead. Arrow indicates a proband

Statistical analysis of the characteristics of the patients with or without the 3243 mutation (Table 1)

As a control group we selected 50 patients without the 3243 mutation whose mothers, but not fathers, are affected by diabetes; out of 214 analysed NIDDM and we compared several parameters between two groups.

Among those parameters, including sex, height, body weight, body mass index (BMI), the age at onset of diabetes, current therapy, the frequency of the diabetic complications, and the capacity to secrete insulin, three factors were shown to be statistically different between the two groups. The average age at diabetes onset was 28.5 ± 13.7 years ($n = 6$, mean ± SD) in the mutation group and 40.4 ± 13.1 years ($n = 50$) in the control group, indicating

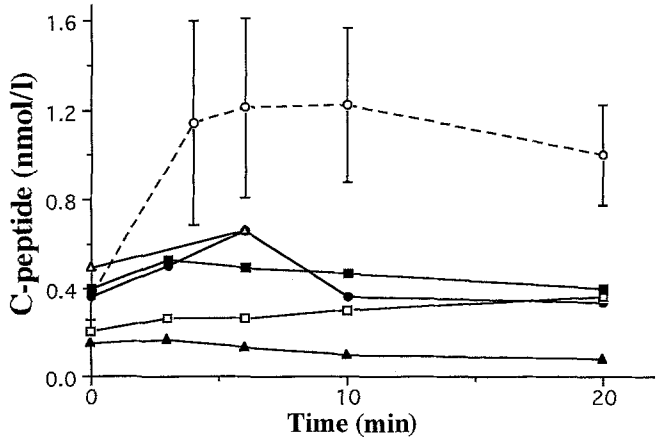

Fig. 2. Glucagon infusion test. Blood C-peptide concentrations after an intravenous bolus injection of 1 mg porcine glucagon in patient 1 (●), patient 2 (□), patient 3 (■), patient 4 (△), and patient 6 (▲). As a comparison, the mean ± 2SD in the normal control subjects (○) are quoted [31] and shown

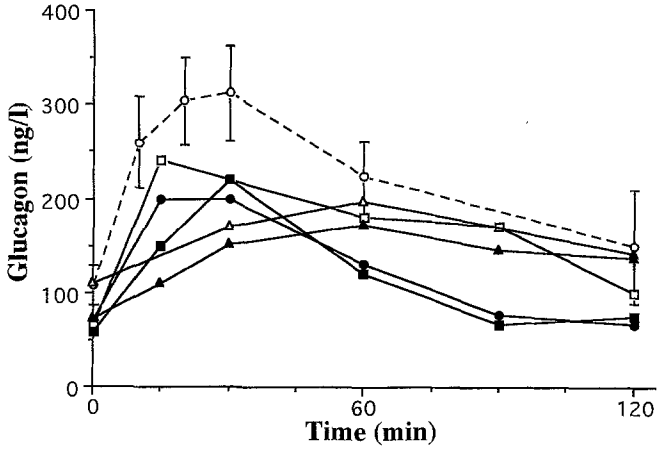

Fig. 3. Arginine infusion test. Blood glucagon concentrations after a drip infusion of 10% L-arginine (30 g) over a 30 min period in patient 1 (●), patient 2 (□), patient 3 (■), patient 4 (△), and patient 6 (▲). As a comparison, the mean ± 2SD in the control normal subjects (○) are quoted [32] and indicated

that patients with the 3243 mutation tend to become diabetic significantly earlier ($p < 0.05$). At the same time, five out of six patients with the 3243 mutation were treated currently with insulin while 36 out of 50 control subjects were not, suggesting that the subjects with the 3243 mutation are more likely to be given insulin treatment, compared with the subjects without the 3243 mutation ($p < 0.025$). The average BMI was 19.7 ± 2.8 kg/m^2 ($n = 6$, mean ± SD) in the group with the mutation and 23.2 ± 3.7 kg/m^2 ($n = 50$) in the normal group, suggesting that the group with the mutation showed relatively leaner body constructions than the group without the mutation ($p < 0.05$). However, other parameters failed to show statistical differences between the two groups. Four patients out of six affected subjects had sensory hearing loss; however, we could not estimate the frequency of hearing loss in the control group exactly because of the lack of detailed examination by the audiogram.

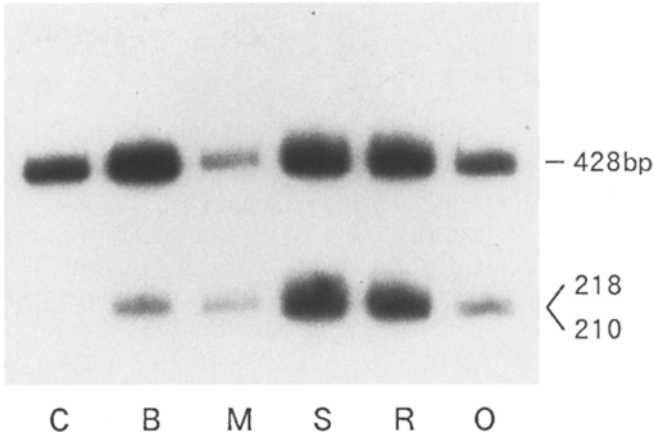

Fig. 4. Autoradiograph of restrictive analysis of mitochondrial DNA fragments with Apa I. A mitochondrial DNA fragment surrounding the tRNA$^{Leu(UUR)}$ mutation site was amplified by PCR using [γ-^{32}P]dATP-labelled primer. The radioactive fragments were digested with Apa I and electrophoresed. While normal mitochondrial DNA does not contain an Apa I recognition site at the position 3243 and does not yield digested fragments upon digestion with Apa I, the affected patient's sample yields two fragments. Positions of the undigested (428 bp) and digested PCR fragments (218 and 210 bp) are indicated. DNA was derived from blood (B), muscle (M), stomach (S), rectum (R) and oral mucosa (O) from patient, and from blood of a control normal subject (C)

Endocrinological examinations

Glucagon infusion test. A glucagon infusion test was carried out in the six affected subjects (Fig. 2). No remarkable rises in plasma C-peptide concentration were observed in the five subjects presented (patients 1–4, 6) throughout the examination in association with decreases of the plasma C-peptide levels at 6 min after glucagon infusion. Patient 5 was omitted from the examination because haemodialysis was being undertaken. These results suggested the presence of beta-cell dysfunction in the ability to secrete insulin against glucagon stimulation.

Arginine infusion test. An arginine infusion test was used to estimate alpha-cell function. All the patients analysed showed only small rises in plasma glucagon during the test (Fig. 3). At 30 min they showed more than 2 SD decreases in glucagon concentration below average values of normal control subjects, indicating the presence of alpha-cell dysfunction. Patient 5 was omitted because of undergoing haemodialysis.

Analysis of the cosegregation of the 3243 mutation in the affected pedigrees and the degree of heteroplasmy amongst different tissues

We carried out PCR using γ-[^{32}P]-dATP-labelled primers to estimate the proportions of the mutation by counting the radioactivity in each digested and undigested PCR fragment [29]. With this technique, we could also enhance the sensitivity of our PCR-RFLP system and succeeded in detecting a mutation in some cases of affected pedigrees where the mutation could not be detected by a simple staining of the gel with ethidium bromide (Fig. 4). Maximum heteroduplex formation between the wild type and the mutant DNA is assumed in the last step of PCR, interfering with the Apa I cleavage and thus with quantitation of the percentage of heteroplasmy [20, 29]. Therefore, we corrected the values obtained by counting according to the standard curves reported previously [35].

Analysis of pedigree 1 revealed that the mutations were detected in the proband's mother, the proband, and her daughter. The proportion of the mutated DNA from the peripheral leucocytes in the three individuals were 9.4 % ($n = 2$, 11.4, 17.3), 41.9 % ($n = 2$, 37.3, 46.4), and 57.0 % ($n = 2$, 53.2, 60.8), respectively, (Fig. 1 a). In spite of a high level of mutated DNA, the proband's daughter, who was 8 years old, showed no evidence of diabetes, judging from her fasting blood glucose level of 4.66 mmol/l, fasting insulin level of 33 pmol/l, and HbA_{1c} of 4.6 %.

In pedigree 5, the mutation was detected in leucocytes from the proband (II-2), her mother (I-2), her daughter (III-4), her son (III-5) and her grandson (IV-2) where the average proportion of mutated DNA were calculated as 48.0 ± 8.2 % (mean ± SD, $n = 3$), 13.2 ± 12.7 % ($n = 3$), 24.3 ± 2.5 % ($n = 3$), 23.4 ± 11.9 % ($n = 3$), and 70.8 ± 3.2 % ($n = 3$), respectively (Fig. 1 b). By contrast, even with this method, the mutation could not be detected in the probands' siblings (II-5, 6 and 7), her eldest daughter (III-2), and her granddaughter (IV-1). The proband's grandson (IV-2) who was 16 months old was also not diabetic although he showed the highest level of heteroplasmy in this pedigree. His 2-h postprandial blood glucose and insulin levels were 5.72 mmol/l and 32.4 pmol/l, respectively.

In patient 6, PCR-RFLP analysis was carried out with mitochondrial DNA from various tissues (Fig. 4). The proportions of the mutated DNA in tissues were 37.2 ± 9.2 % ($n = 5$) in leucocytes, 69.7 ± 8.1 % ($n = 5$) in skeletal muscle, 55.8 % ($n = 2$, 58.1, 53.5) in oral mucosa, 70.3 % ($n = 2$, 70.2, 70.4) in stomach, and 69.9 % ($n = 2$, 66.3, 69.9) in rectum. These results show that the degrees of heteroplasmy for this mutation were variable amongst different tissues.

Discussion

In this report, we screened 214 Japanese NIDDM patients for mutations in their mitochondrial DNA and identified an A to G transition at nucleotide 3243 in the mitochondrial tRNA$^{Leu(UUR)}$ gene in 6 patients (2.8 %). In the recent paper by Kadowaki et al. [22], it was reported that the frequency of the 3243 mutation in a Japanese NIDDM group with a family history of diabetes was 2 % (2 of 100 subjects), which was almost comparable with our result. Because the patient group analysed has a bias of having a positive family history, the exact frequency of this mutation among the overall Japanese diabetic population may be less than this percentage. However, the incidence of this 3243 mutation may be higher in the Japanese population than diabetes caused by other genetic mutations so far detected. For example, we could find only one mutation in the glucokinase locus in the patient group used in this report (M. Hashiramoto et al., unpublished data).

The disease phenotypes and laboratory investigations suggested the following characteristics in these six subjects: 1) the age of onset is relatively younger than that of common NIDDM. 2) They have generally lean body construction, judging from their body mass indices. 3) All but one of the patients required insulin to control their blood glucose levels and statistically tend to be given insulin treatment, compared with the control subjects. 4) Endocrinological examinations revealed a defect in insulin secretion from beta cells and an impairment in glucagon secretion from alpha cells of the pancreas. 5) Immunological examinations for islet-cell autoantibodies including islet cell antibodies (ICA), islet cell surface antibodies (ICSA), and anti-glutamic acid decarboxylase (GAD) antibody were all negative in all six patients.

Endocrinological examinations revealed the presence of the defects not only in beta cells but also in alpha cells of the affected patient's pancreas. All patients except patient 5, who was omitted because of undergoing haemodialysis, had a low response of plasma C-peptide concentrations against glucagon stimuli, indicating the presence of beta-cell defects in these patients. There are several possible explanations for the involvement of mitochondria in maintaining a proper glucose homeostasis. Oxidative phosphorylation in the mitochondria is thought to be particularly crucial in insulin secretion in pancreatic beta cells [36] since the basic energy state of the cells is maintained in the form of adenosine triphosphate (ATP) by oxidative phosphorylation in the mitochondria. Streptozotocin has been shown to inhibit mitochondrial respiratory chain function and lead to inhibition of insulin production by the pancreatic beta cells, analogous to the mechanism in which chronic oxidative phosphorylation deficiency inhibits insulin production, thereby producing diabetes in rats [37],

which suggests that pancreatic islets are also highly oxidative. These data together indicate that as inhibition of oxidative phosphorylation increases, mitochondrial ATP production declines until it falls below the minimum energy levels which are necessary for more oxidative tissues including beta cells to function. The fact that the biguanidine group of oral hypoglycaemic agents exerts its influence on glucose utilization by affecting the mitochondria [38, 39] is also considered supportive of such a mechanism.

Our five patients also showed more than 2 SD decreases below average values of normal control subjects in plasma glucagon concentration at 30 min against arginine infusion. However, it is well established that alpha-cell hyperfunction is usually observed in diabetic patients [33, 40] and that this hypersecretion of glucagon is improved by a normalized blood glucose level [40]. Therefore, it is possible that associated hypo-function of pancreatic alpha cells in secreting glucagon may be one of the characteristic features of diabetes with the 3243 mutation. The mitochondrial defects possibly impair not only the beta cells but also alpha cells. This contrasts with the pathophysiological aspects observed in IDDM where the beta cells alone are specific targets for the immunologically-regulated cytotoxicity [41]. In this respect, monitoring plasma glucagon after arginine infusion is useful to differentiate this disease entity from common IDDM or NIDDM.

We characterized the degree of heteroplasmy in various tissues from patient 6 and found that it was variable in the tissues examined. The percentage of the mutant DNA was higher in skeletal muscle than in the leucocytes, which is similar to the observation by van den Owelend et al. [19] who reported that NIDDM patients carrying the 3243 mutation without typical MELAS symptoms showed differences in the level of heteroplasmy between their blood and muscle. Patient 6 had a higher degree of heteroplasmy in his stomach and rectum, which was more comparable with that in his muscle, than in his leucocytes and oral mucosa. These findings are consistent with his complaints of gastro-intestinal symptoms, such as repeated diarrhoea and abdominal pain. Possibly the tissue specificity of symptoms results from the characteristics of mitochondrial genetics which play an important role in the pathogenesis of mitochondrial cytopathies as multiorgan disease. However, some investigators have reported a similar percentage of mutant genomes in both clinically affected and unaffected tissues [42] and that decreased respiratory chain complex activities measured in different tissues from MELAS patients do not correlate with the proportion of mutated mitochondrial genome [43]. Nevertheless, it should be considered that the different "thresholds" of vulnerability in the individual tissues and organs may possibly take part in the process of quantitatively similar impairments of oxidative phosphorylation. It is not known whether these parameters to estimate the clinical defects of individual tissues are sufficient to reflect the mitochondrial cytopathies. Altogether, it is strongly recommended that tissues other than blood be intensively examined for the presence of the mitochondrial mutations, especially in tissues where local symptoms are observed.

We analysed the relationship between the degree of heteroplasmy and the diabetes-related phenotypes in the subjects with the 3243 mutation. The degree of heteroplasmy in the patients' blood was not correlated with clinical features including the age of diabetes onset, current treatments, BMI, glucose tolerance, and ability to secrete insulin. It is noteworthy that affected children (III-1 of pedigree 1 and IV-2 of pedigree 6, Fig. 1 a and b) do not show overt diabetes at the point of study, suggesting that aging plays a pathogenetic role in diabetes caused by the 3243 mutation. It has been proposed that the severity of the oxidative phosphorylation defect and phenotype is determined by two parameters, ratio of mutant mitochondrial DNAs and age [36], and that deleterious mutations in the mitochondrial genome have also been proposed to be important in aging [44].

In the present study, we report that the 3243 mutation of the mitochondrial gene is of relatively high frequency (2.8 %) among Japanese NIDDM patients with a positive family history and that the 3243 mutation possesses distinct phenotypes from common forms of diabetes. Because those patients showing the distinct phenotypes are genotypically-related to the 3243 mutation, and the cosegregation of the mutation with diabetes onset is observed in most of those affected pedigrees, we suggest that the 3243 mutation indicates a specific diabetes subtype, which has some features in common with classic IDDM as well as with the classic NIDDM.

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