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A G-to-A Substitution at Nucleotide Position 3316 in Mitochondrial DNA Is Associated with Japanese Non-Insulin-Dependent Diabetes Mellitus

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By directly sequencing amplified DNA from 30 patients with non-insulin-dependent diabetes mellitus (NIDDM) who had diabetic family members, we identified a G-to-A mutation at np 3316 in the ND-1 gene of the mitochondrial DNA (mtDNA). DNA from 254 patients with NIDDM or impaired glucose tolerance (IGT), from 154 patients with insulin dependent diabetes mellitus (IDDM) and from 207 non-diabetic control subjects was screened for this substitution. The mutation existed in 5 of 254 Japanese NIDDM or IGT patients (2.0%), but not in other subjects. Its prevalence was significantly higher in NIDDM than in other patients or non-diabetic control subjects. The association of the G-A mutation at np 3316 with glucose intolerance suggests the importance of this area for the development of diabetes.

Diabetes mellitus is a heterogeneous disorder which afflicts about 5% of the world population. Up until now, abnormalities in the insulin gene[1], insulin receptor gene[2],[3] glucokinase gene[4], adenosine deaminase gene[5] and glucose transporter gene[6] have been shown to be associated with diabetes mellitus. Mitochondrial gene is also one of the candidates for diabetes susceptibility genes, because ATP production in the beta cell of the pancreas is thought to be important for the insulin secretion[7]. Mitochondrion supplies most of the energy necessary for normal cell functions through oxidative phosphorylation, and its malfunction may lead to mitochondrial myopathies[8] or cardiomyopathy[9]. Recently, mitochondrial DNA deletion of 10.4kb[10] and an A-to-G mutation at nucleotide position np 3243[11] have been reported to cosegregate with diabetes mellitus. This 3243 mutation exists in about 1% of randomly selected non-insulin dependent diabetes mellitus (NIDDM)[12], but exists rarely in autoimmune IDDM or autoimmune thyroid diseases which are often associated with IDDM[13]. The patients with this mutation may have insulin resistance[14],[15], as well as insulin secretory defects, causing elevated plasma glucose[16]. The mutation is situated in $tRNA^{Leu(UUR)}$ gene, which is thought to be a genetic hot spot for pathological mutations[17]. We selected 30 diabetic patients who have at least three family members with glucose intolerance, from randomly selected NIDDM patients and searched for new mutations around the $tRNA^{Leu(UUR)}$ gene. We identified a G-to-A substitution at np 3316 of the mitochondrial ND-1 gene in one patient with NIDDM. We investigated if this polymorphism is associated with glucose intolerance.

SUBJECTS AND METHODS

Subjects. We randomly selected patients with glucose intolerance from among the individuals who visited the outpatient clinic at the University of Tsukuba or its affiliate hospitals. The subjects consisted of 254 patients with

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TABLE 1
The Profiles of the Patients with Non-insulin-dependent Diabetes Mellitus, Impaired Glucose Tolerance, or with Normal Glucose Tolerance

	Number	Age	Sex (M/F)	3316 bp polymorphism	% of polymorphism
NIDDM	230	55.0 ± 13.9	121/109	3	2.0
IGT	24	50.3 ± 13.6	12/12	2	
IDDM (1)	38	37.8 ± 21.2	16/22	0	0
IDDM (2)	116	8.51 ± 4.90[a]	49/67	0	0
Nondiabetic control	207	47.7 ± 16.5	77/130	0	0

[a] Age at diagnosis.

glucose intolerance: 230 NIDDM and 24 IGT, 154 IDDM, and 207 non-diabetic control subjects (Table 1). Informed consent was obtained from all of the subjects, who were unrelated Japanese. Diagnosis of NIDDM, IGT or normal glucose tolerance was based on the criteria of the World Health Organization[18]. We regarded patients as having IDDM if their diabetes mellitus was diagnosed under 30 years-old and they were receiving more than 25 U of insulin or their daily excretion of urinary C-peptide reactivity was below 10 μ g or 331 μ mol (IDDM 2). Those who exhibited typical episodes of IDDM associated with positive islet cell antibodies were included. We also recruited 38 patients who developed typical episodes of juvenile-onset (mean±SD: 8.51±4.90) IDDM with ketoacidosis, from outpatient clinics of pediatrics department (IDDM 1).

Methods. DNA was extracted from peripheral leukocytes[19]. A part of the mitochondrial gene between 3130 and 3423 was amplified by polymerase chain reaction (PCR)[12]. Primers for PCR were: (H strand) AGGACAAGAGAA-ATAAGGCC (3130-3149) and (L strand) CACGTTGGGGCCTTTGCGTA (3423-3404). We also used an extra set of primers[20] for confirmation. The PCR conditions were: 30 cycles of denaturation at 94° C for 1 min, annealing at 65° C for 1 min, and extension at 72° C for 2 min, with an initial extra 4 min denaturation at 94° C. A G-to-A mutation at np 3316 alters GGCC to GACC (3315-3318), disrupting the HaeIII cleavage site. The amplified fragments (294bp) were digested by HaeIII into three smaller fragments (18 bp, 266 bp and 10 bp) instead of four (18 bp, 169 bp, 97 bp and 10 bp) if the G-to-A substitution exists. Digested fragments were separated on an 8 % polyacrylamide gel. For those fragments which failed to be cleaved, the nucleotide sequences were confirmed by directly sequencing[12] (Figure 1). Three NIDDM patients of the five subjects carrying this mutation were checked for hearing disorders by an audiometry performed by an otolaryngologist.

RESULTS

By direct sequencing analysis, we identified a G-to-A mutation at np 3316 of the ND-1 gene of the mtDNA, encoding one of the seven mitochondrially encoded subunits of respiratory-chain complex I (Figure 1). This substitution did not change codon (Trp^{TGG} to Trp^{TGA}) and was homoplasmic unlike many pathogenic mutations. We investigated if this mutation is associated with glucose intolerance. The mutation existed in 5 of 254 NIDDM+IGT patients (2.0%) and did not exist in 154 IDDM patients nor in 207 non-diabetic control subjects. Its prevalence in NIDDM patients was significantly higher than other subjects ($\chi^2=7.165$, $P=0.0074$) or non-diabetic control subjects ($\chi^2=4.119$, $P=0.0424$). Interestingly, three of the five patients carrying the mutation (60%) had sensorineural or mixed type hearing loss. One patient had hearing loss over 200kHz, and two in all frequencies observed (125Hz-8000Hz).

Clinical Features of the Three Patients with the 3316 Mutation

Patient 1. The 44-year-old female was confirmed as having NIDDM at 36 years old but its onset may be earlier, as she gave birth to a baby of 5000 grams in weight at 28 years-old. Her maternal aunt and grand-mother had NIDDM, and the proband's only brother, aged 38, also had NIDDM. His children did not have NIDDM, but the proband's eldest daughter had an episode of polyuria and polydipsia at 18 years-old, and diagnosed as diabetes mellitus and currently treated with 10-20 units of insulin. Her family history suggests that their diabetes

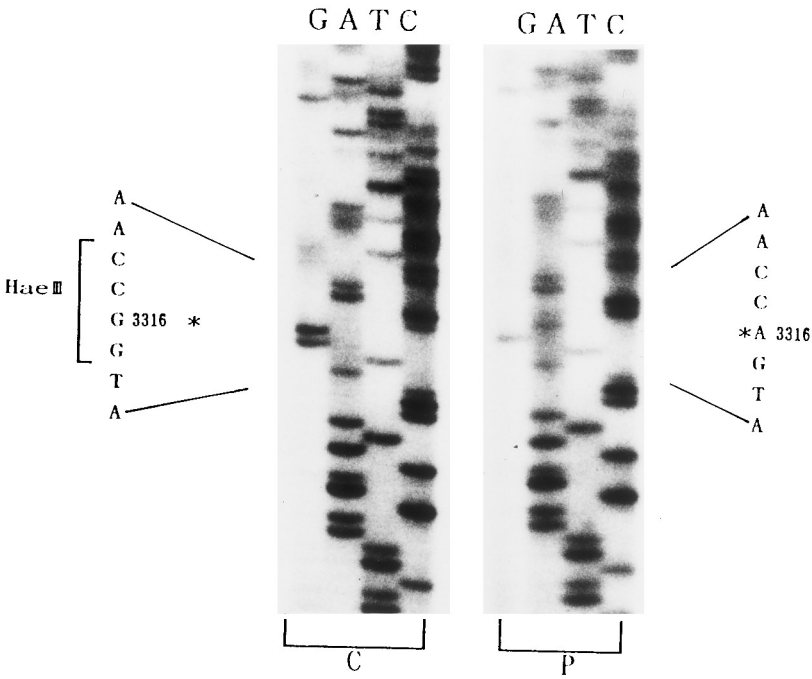

FIG. 1. A G-to-A transition at np 3316 of the mitochondrial DNA was identified by directly sequencing amplified products from a patient with glucose intolerance. Though the mutation was homoplasmic and did not change the codon, it was associated with glucose intolerance. The recognition site of Hae III is also indicated. (P, patient with a G-to-A substitution; C, control subject)

was maternally inherited at least for 4 generations. The proband has been treated with diet alone, but her daughter required insulin treatment. The proband had unilateral sensorineural hearing impairment. She was free from diabetic neuropathy assessed by physical examinations and nerve-conduction studies, suggesting that her hearing loss is not caused by diabetic neuropathy. Her hemoglobin A_{1C} reduced from 11.9 % to 7.1 % with an oral hypoglycemic agent, without any improvement of hearing ability even with prednisone.

Patient 2. NIDDM of the 59-year-old male was diagnosed at the age of 57. He has had bilateral hearing disorders since he was 20 years old. He is now treated with as much as 50 units of mixed type insulin because of the associated liver cirrhosis. He had two sisters, one of whom had NIDDM, but it was not clear whether her deceased mother had NIDDM, but she had a cerebral apoplexy. His hemoglobin A_{1c} was around 9.0-9.5 % (normal range: 4.9-6.5).

Patient 3. This 61-year-old female had bilateral sensorineural hearing loss since she was 55. Her NIDDM was diagnosed at 59, and her glycemic control has been fair with an oral hypoglycemic agent. Her family history was unclear. The family histories of hearing impairment were unclear in these patients with this mutation.

DISCUSSION

We identified a G-to-A substitution at np 3316 in the ND-1 gene of the mtDNA. Although this did not change codon and was homoplamic unlike many pathological gene mutations, it existed with significantly high prevalence in patients with NIDDM or IGT compared with in other subjects or non-diabetic subjects. Many pathological mutations are heteroplasmic: coexistence of mutant and wild type DNA. But homoplasmic mutation at np 11778 of the

mitochondrial genome has been shown to be causatively associated with about 50-70% of the patients with Leber's hereditary optic neuropathy (LHON)[8]. This mutation has been confirmed to be pathogenic by analyzing 1600 maternally related family members through generations[21], which indicates that homoplasmic mutations may also be pathogenic. Of the 5 patients carrying 3316 mutation, three had adult onset NIDDM and two had IGT. At least three of them had hearing impairment. Previous report indicates that about 40 % of diabetic patients with 3243 bp mutation had sensorineural hearing loss[22], which proved to be important for their correct diagnosis. We cannot exclude the possibility that their hearing disorders are coincidental. However, the fact that the 3316 mutation exists adjacent to position 3243, suggests a possible important role of 3316 mutation in the etiology of their hearing loss like 3243 mutation. Many polymorphisms may exist in the mitochondrial gene, because of its high mutation rate caused by the increased susceptibility to the superoxide, as the gene is not protected by nuclear proteins like histone. Most of the polymorphisms existing in the mitochondrial gene are non-pathological, and it is not clear if this polymorphism is causatively associated with diabetes and hearing impairment. The area around $tRNA^{Leu(UUR)}$ is thought to be a hot spot for pathological mutations[8],[17] for mitochondrial myopathies. The 3243 mutation has been shown to be associated with MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes)[8] or NIDDM[11]. The 3243 mutation is situated within the binding site of the termination factor (TERF)[23], which terminates transcription and produces the shorter of the two transcripts. The 3243 bp mutation leads to the production of abnormally processed protein, accumulation of which results in the decrease in the oxygen consumption[24]. The tridecamer sequence (3237-3249) and its flanking sequences (3165-3316) have been reported to be important for in vitro termination of transcription at the 3′ end of the 16S rRNA gene[23],[25]. The 3316 mutation exists at its end. This substitution may induce comformational changes of this area, resulting in the impairment of the binding capacity of the termination factor. It is also possible that this substitution links with some other really pathological gene abnormalities, which cosegregate with glucose intolerance. To search for another possible mutation coexisting with the 3316 substitution, we directly sequenced the amplified fragments encompassing 200 bp upstream and downstream of the 3316 mutation of the five patients carrying the mutation. We could not find any mutations that cosegregated with the 3316 mutation. As heteroplasmic mutations may not be detected by direct sequencing method, we applied a very sensitive PCR-restriction fragment-length polymorphism method[20] with condensation of PCR products, which detects less than 1% of heteroplasmic mutation, and screened these 5 patients for 3243 bp mutation[12] and 8834 bp mutation[26], which have been confirmed to cosegregate with NIDDM by familial analyses. However, we could not find any of these mutations in the patients. In conclusion, the prevalence of the G-to-A substitution at np 3316 of the mitochondrial genome was significantly higher in NIDDM including IGT compared with in other subjects or non-diabetic subjects. Further studies are needed to know whether it is involved in the mitochondrial dysfunction or not. But our observations suggest that the G-to-A mutation at np 3316 is associated with glucose intolerance and the region around the $tRNA^{Leu(UUR)}$ gene seems to be a pathological hot spot for diabetes mellitus as well as for mitochondrial myopathies.

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