Screening for the Mitochondrial DNA A3243G Mutation in Children With Insulin-Dependent Diabetes Mellitus

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Since recent studies demonstrated the occurrence of the mitochondrial DNA (mtDNA) mutation A3243G in patients with adult-onset diabetes, an investigation was undertaken to determine the frequency of this mutation in a pediatric population with insulin-dependent diabetes mellitus (IDDM). DNA was extracted from peripheral blood of 270 pediatric patients with IDDM. The presence of the mtDNA A3243G mutation was screened for by minisequencing and mutation-specific *Apa*I endonuclease restriction after polymerase chain reaction (PCR) amplification of mtDNA. The A3243G mtDNA mutation was not found in any IDDM patients examined. This mutation is uncommon in children with IDDM from various ethnic and racial groups. Therefore, the contribution of the mutation to the pathogenesis of IDDM, if any, is minimal.

DIABETES MELLITUS is a spectrum of conditions with a wide variation in clinical presentation, but with the common feature of glucose intolerance. Conventionally, diabetes has been classified into two main types.[1] Type I, or insulin-dependent diabetes mellitus (IDDM), typically has an onset in childhood and is characterized by severely decreased insulin production. The current hypothesis is that type I diabetes is due to a chronic autoimmune disorder leading to destruction of pancreatic islet cells.[2] Type II, or non–insulin-dependent diabetes mellitus (NIDDM), usually occurs after the age of 40 and is characterized by hyperglycemia due to peripheral insulin resistance.[1]

The inheritance of diabetes has been the subject of intensive investigation. Early twin studies documented the genetic contribution to both type I and type II diabetes,[3,4] and family studies identified the autosomal dominant inherited maturity-onset diabetes of the young (MODY).[5] Recent efforts have attempted to define the molecular abnormalities and the mode of inheritance of the various forms of diabetes. HLA-DR haplotypes have been shown to be associated with IDDM, and have been used to predict predisposition in families with IDDM.[6] More recent molecular studies have identified mutations in the glucokinase gene as the underlying lesions in MODY type II.[7] In addition, linkage analysis has identified over 20 different genes that may be involved in predisposition to type I diabetes.[8]

In addition to investigation of nuclear genes in the causation of diabetes, researchers also have investigated the possible etiologic role of mitochondrial genes in diabetes mellitus. Previous epidemiologic studies have shown that NIDDM patients were more likely to have affected mothers than affected fathers.[9] Since mitochondrial DNA (mtDNA), unlike nuclear DNA, is inherited from the mother, the possibility exists that in some families diabetes is transmitted via mtDNA. If mtDNA abnormalities are involved in the etiology of type I or II diabetes, such findings would aid greatly in understanding its pathophysiology and in providing genetic counseling to families with mtDNA lesions.

In 1992, van den Ouweland et al[10] and Reardon et al[11] independently reported the occurrence of an A to G transition at nucleotide 3243 in the mitochondrial $tRNA^{Leu(UUR)}$ gene in patients from two unrelated families with maternally inherited diabetes mellitus and deafness. Since then, other investigators have reported families with maternally transmitted diabetes mellitus who had the A3243G point mutation.[12-19] Based on these reports, other investigators screened type II adult diabetic patients in Europe and Japan for the A3243G mtDNA mutation and found the lesion in 0.9% to 11% of the patients.[20-29] Due to heteroplasmy and replicative segregation of mtDNA, it is possible that mtDNA mutations such as A3243G contribute to the pathogenesis of IDDM. Thus, both a restriction digestion–based assay and a more sensitive solid-phase minisequencing method were used for screening the mtDNA A3243G mutation. In this communication, we describe the use of these assays for the molecular screening of 270 children with IDDM from different ethnic and racial groups for the A3243G mtDNA mutation. Notably, the mtDNA lesion was not detected in these IDDM patients, suggesting that the A3243G mutation did not underlie or contribute to the pathogenesis of IDDM in this population.

SUBJECTS AND METHODS

Patients

The study included 270 consecutive patients (265 unrelated families) with IDDM who presented for their regular outpatient check-up between May 1994 and March 1995 at Mount Sinai Medical Center's Program for Young People with Diabetes. The diagnosis of IDDM was based on the early age of onset of diabetes (4 months to 22 years), the presence of ketones in the urine, and the continuous requirement for exogenous insulin from the onset of diabetes.

There was a family history of IDDM in 26 families as defined by an affected first-degree relative of the proband. In five of these families, there were two affected siblings. Patient information including the age of onset of diabetes, family history, islet cell surface antibodies (ICSA), and insulin autoantibodies (IAA), as well as other symptoms suggestive of a mtDNA disease, was obtained from the Center's database and/or

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patient records. Excluded from the study were two liver- and two heart-transplant patients whose diabetes was induced by FK506 immunosuppression, one patient with systemic lupus erythematosus who developed IDDM while on high-dose steroids, and one patient with cystic fibrosis. Another child was excluded because she had a previously diagnosed mtDNA deletion associated with diabetes, hearing impairment, and renal Fanconi's syndrome.[30]

Molecular Screening for the A3243G mtDNA Mutation

mtDNA was extracted from the peripheral blood of each patient by standard techniques[31] and then analyzed for the A3243G mtDNA mutation by polymerase chain reaction (PCR) amplification and restriction analysis, essentially with the method used by Alcolado et al.[27] Briefly, each 50-μL PCR mixture contained 100 to 200 ng total patient DNA, 1 μmol/L each of the sense [5′ AGGACAAGAGAAATAAGGCC 3′ (nt 3130-3149)] and antisense [5′ TAGAAGAGCGATGGTGAGAG 3′ (nt 3558-3539)] oligonucleotide primers, 50 μmol/L of each dNTP, 50 mmol/L KCl, 10 mmol/L Trishydrochloride/pH 9.0, 0.1% Triton X-100, 1.5 mmol/L $MgCl_2$, and 1 U *Taq* polymerase (Perkin Elmer, Foster City, CA). The reaction mixture was incubated at 94°C for 4 minutes, and then 30 cycles of amplification were performed in a PTC-100 Programmable Thermal Cycler (MJ Research, Watertown, MA) with denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. PCR products were restricted with 10 U *Apa*I (New England Biolabs, Beverly, MA) and electrophoresed in 1.5% agarose (Ultra Pure grade; GIBCO BRL, Gaithersburg, MD) gels containing 0.1 μg/mL ethidium bromide, and then the restriction fragments were visualized under UV light. A positive control was analyzed with each group of assays.

Minisequencing for Detection of the A3243G Mutation

Quantitative solid-phase minisequencing for detection of the mtDNA A3243G mutation was performed according to the method used by Syvänen,[32] with the following modifications. The initial PCR amplification was performed using an antisense oligonucleotide that was synthesized with a 5′ Biotin-ON phosphoramidite (Clontech, Palo Alto, CA). Biotinylated PCR products were captured using streptavidin-coated microtiter plates (Boehringer Mannheim, Indianapolis, IN). The minisequencing oligonucleotide adjacent to the A3243G mutation was 5′ GGGTTTGTTAAGATGGCAG 3′ (nt 3224-3242). ^{3}H-dATP (DuPont NEN, Boston, MA; wild-type) or ^{3}H-dGTP (mutant) were added to a final concentration of 0.2 μmol/L (activity, 0.18 and 0.09 μCi/50 μL reaction volume, respectively). Pipetting of the initial PCR and minisequencing reactions was performed on a Biomek 2000 laboratory automation workstation (Beckman Instruments, Fullerton, CA). DNA samples with known heteroplasmy were supplied by Drs Alcolado (University Hospital, Cardiff, Wales) and DiMauro (Columbia University, New York, NY). Positive controls, with 88% and 5% heteroplasmy (as determined by the method of Moraes et al[33]), respectively, and a negative control (normal DNA) were included in each assay. Aliquots (10 μL) of the released reaction products were counted in 5 mL scintillation fluid using a 1214 Rackbeta Liquid Scintillation Counter (Pharmacia, Piscataway, NJ). After subtraction of the background counts, the ratio of mutant (^{3}H-dGTP) to wild-type (^{3}H-dATP) counts was calculated. In the minisequencing reaction, two molecules of dGTP were incorporated in the mutant allele, versus one molecule of dATP in the wild-type allele. However, since the specific activity of ^{3}H-dATP was twice that of ^{3}H-dGTP, no further correction was necessary to calculate the mutant to wild-type ratio.

RESULTS

The patient population had an onset of diabetes between 4 months and 22 years, with a median age of 7.6 years. There were 265 unrelated families, including five with affected sibling pairs. There was no family history available on five adopted children. Of 265 children whose biologic parents were available, eight (3.0%) had mothers who had diabetes (five with IDDM and three with NIDDM) and 15 children (5.7%) from 13 pedigrees had fathers who had diabetes (nine with IDDM and four with NIDDM). Both parents of two patients (0.8%) were diabetic (both NIDDM in one case and a mother with IDDM and a father with NIDDM in the second case). Of 114 patients (of 270) with IAA titers measured at the time of diagnosis, 48 (42.1%) were positive. Of 111 patients with ICSA titers measured at the time of diagnosis, 52 (46.8%) were positive. In our study population, there was one child with mild mental retardation, another who was hypotonic at birth and had developmental delay, and one with congenitally impaired vision. However, these conditions were not progressive. None of the patients had deafness, muscle weakness, stroke-like episodes, or other symptoms suggestive of a mtDNA abnormality.

The PCR product of a positive-control DNA sample that was heteroplasmic for the A3243G mutation showed partial digestion resulting in fragments of 429 bp (normal) and fragments of 312 and 117 bp (A3243G; Fig 1). Because of the possible low-level heteroplasmy for the mtDNA A3243G mutation, the sensitivity of the assay was determined in serial dilutions of mixtures of a normal control DNA and DNA heteroplasmic for the A3243G mutation. The lowest level at which the A3243G mutation was clearly detected was at 20% heteroplasmy for the mutant allele (Fig 1). None of 270 pediatric IDDM patients were positive for the mtDNA A3243G mutation by PCR amplification and *Apa*I restriction analysis.

To exclude the possibility of low levels of heteroplasmy, patients were analyzed by the more sensitive and quantitative minisequencing method. A standard curve was obtained from five separate PCR and minisequencing reactions of DNA samples known to be heteroplasmic for the mtDNA A3243G mutation. The minisequencing method detected heteroplasmy to a minimum level of 5% mutant (Fig 2). At less than 5% heteroplasmy, the distinction between increasing dilutions of the positive control with normal DNA was unreliable because of nonspecific background signals. The ratio of mutant to wild-type radioactive counts obtained by the minisequencing approach excluded the possibility of 5% or greater heteroplasmy in each of 270 patient samples studied.

DISCUSSION

The majority of patients previously reported with the mtDNA A3243G mutation were classified as having NIDDM, which was controlled by diet or oral hypoglycemic agents. Notably, and in contrast to patients with classic NIDDM, patients with the A3243G mutation were not overweight and had a delayed insulin response to glucose. Consistent with heteroplasmy and the replicative segregation of mtDNA, many of these patients eventually developed IDDM. However, unlike classic IDDM patients, who have an acute onset, most of the adult patients with the A3243G mutation had a slowly progressive form.[16,20,21,28] The term "mitochondrial diabetes mellitus" has been proposed for this "intermediate" form of diabetes associated with mtDNA mutations.[24]

The pediatric population studied here was composed entirely

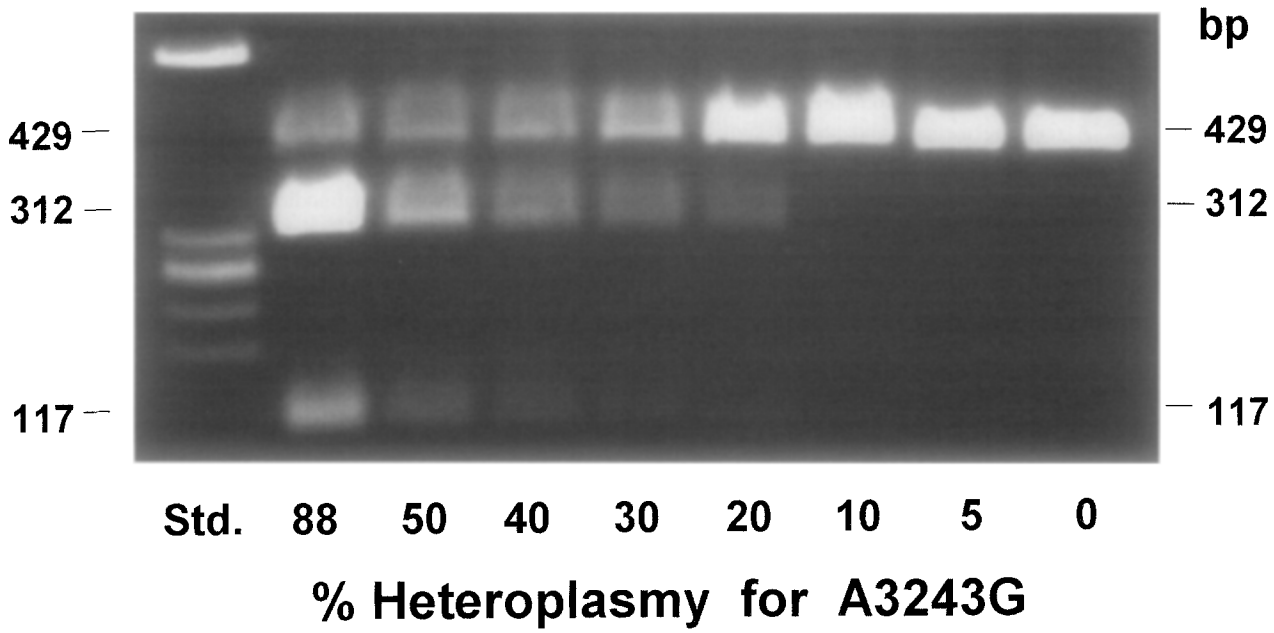

Fig 1. *Apa*I restriction of PCR products from serial dilutions of DNA from an individual heteroplasmic for the mtDNA mutation. The normal PCR product (429 bp) remains uncut. PCR product containing the A3243G mutation was restricted with *Apa*I and resulted in bands of 312 and 117 bp. The Std. lane contained the molecular weight marker ϕX174 *Hae*III (New England Biolabs, Beverly, MA). The remaining lanes contained varying levels of heteroplasmy for the A3243G mutation, as indicated.

of individuals diagnosed with an acute onset of IDDM in childhood or adolescence (mean age, 7.6 years). To determine if the A3243G mutation could be present in the leukocyte mtDNA of this young IDDM population, initially a restriction digestion assay was used with a sensitivity to detect approximately 20% heteroplasmy. Restriction analysis does not provide accurate quantitation, since it is influenced by possible heteroduplex formation during PCR that can result in incomplete cleavage and therefore underestimate the amount of mutant PCR product.[35] The A3243G mutation was not detected among 270

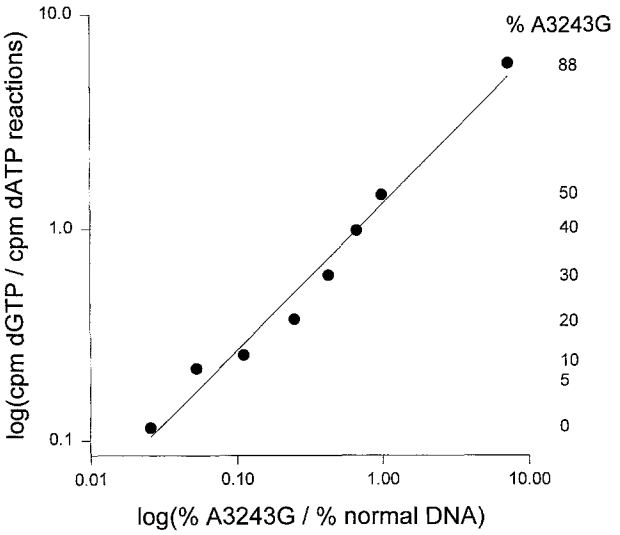

Fig 2. Standard curve for quantitative analysis of the mtDNA A3243G mutation by solid-phase minisequencing. The ratios were derived from samples of known heteroplasmy.

IDDM patients from 265 unrelated families. However, since heteroplasmy less than 20% could still have clinical relevance, we used the more sensitive solid-phase minisequencing method developed by Syvänen et al[36] to screen our population. The minisequencing approach was applied to quantitation of mtDNA point mutations,[32,37,38] and we were able to identify 5% heteroplasmy for the A3243G mutation (Fig 2). However, in this population of 265 unrelated families with 270 affected individuals, the A3243G mtDNA point mutation was not detected. Although unlikely, it is possible that the A3243G mutation is present in some of these patients at low or undetectable levels in leukocytes, but has reached a pathologic frequency in the pancreatic islet cells, due to the replicative segregation genetics of mtDNA. Because of the possibility of tissue variability, PCR/restriction analysis or minisequencing from leukocytes should not be considered a definitive test, but rather an effective screening procedure.

Our results are in agreement with those of Vionnet et al[22] and Odawara et al,[24] who did not detect the A3243G mutation in a total of 206 French and Japanese adult patients with IDDM. Kadowaki et al[23] divided a population of 140 Japanese IDDM patients into those with and those without a family history of diabetes. The A3243G mutation was detected in three (6%) of 55 IDDM patients with a history of diabetes, but in none of 85 patients with no history of diabetes.[23] Interestingly, none of 90 IDDM patients studied by Vionnet et al[22] had the mutation, despite a positive history of diabetes in all the families. Similarly, we did not detect any association between familial IDDM and the A3243G mtDNA mutation in 26 families with an affected first-degree relative.

It is notable that all of the patients in this study had diabetes without any other symptoms suggestive of a mitochondrial

disease. In contrast, the majority of patients with the A3243G mutation had diabetes associated with other symptoms, including progressive hearing loss, cardiomyopathy, or neurological involvement.[10-16,20,23,25,26,28] These symptoms are typical clinical findings in mtDNA rearrangements[39] and should signal the diabetologist to screen for mtDNA mutations.

Autoantibodies to insulin and other β-cell antigens are well-recognized immunologic markers that are present in the majority of IDDM patients at diagnosis.[2] Oka et al[21] found the A3243G mutation in three of 27 Japanese NIDDM patients who were islet cell antibody (ICA)-positive. All three patients progressed to insulin dependency. Unlike typical IDDM patients who have initially high ICA titers that usually disappear 6 months to 3 years after the onset of diabetes,[2] ICA titers in the patients described by Oka et al[21] were low and constantly positive. They postulated that a continuously positive, low-titer ICA reflected gradual islet cell destruction due to the mitochondrial metabolite defects.[21] Among our patients who were tested for autoantibodies at the time of diagnosis, 42.1% were positive for IAA and 46.8% for ICSA, yet none expressed the A3243G mutation.

Our findings, in the context of other recent studies,[10-29] suggest that the A3243G mutation should be evaluated in patients with slowly progressive forms of diabetes, maternal inheritance, negative autoantibodies, and involvement of other systems. However, it should be noted that the A3243G mutation can also be found, albeit rarely, in patients without a family history of diabetes and/or positive antibodies.

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