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Etiology of Early-Onset Type 2 Diabetes in Indians: Islet Autoimmunity and Mutations in Hepatocyte Nuclear Factor 1α and Mitochondrial Gene

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Context: Indians are at high risk of developing type 2 diabetes mellitus (T2DM) at an early age, despite their lower body mass index. Studies on the etiology of patients presenting as early-onset T2DM in this racial group are not available.

Objective: The objective was to delineate the clinical features in young Indian patients with T2DM and to determine the role of mutations in the hepatocyte nuclear factor 1α (HNF1α) gene [MODY3 (maturity-onset diabetes of the young, type 3)], mitochondrial A3243G mutation, and islet autoimmunity in its etiology.

Design: This was an observational cohort study.

Setting: The setting was an outpatient diabetes clinic in a teaching hospital.

Patients: Ninety-six consecutive young patients with T2DM (onset, ≤30 yr) were included in the study.

Interventions: Glutamic acid decarboxylase and insulinoma antigen 2 antibodies, mitochondrial A3243G mutation, and the common HNF1α mutation P291fsinsC were measured in all patients. The entire HNF1α gene was studied for mutations in 32 subjects with onset less than 25 yr or with normal weight. The common HNF1α A98V polymorphism was studied in 91 patients.

Results: The patients were clinically heterogeneous, with 42% having a normal body mass index. Glutamic acid decarboxylase antibodies were present in three (3%) subjects and mitochondrial A3243G mutation in one (1%) subject. The P291fsinsC mutation was not detected in any patient. A MODY3 mutation (R200W) was detected in one patient (3%). In this family, diabetes cosegregated with the R200W mutation in the proband and his youngest brother but not in three paternal uncles. The Val 98 allele was associated with T2DM (allele frequency, 0.14 *vs.* 0.03 in controls; odds ratio, 5.2; $P < 0.001$).

Conclusions: Despite a significant proportion of young Indian patients with T2DM having normal weight, islet autoimmunity, A3243G mitochondrial, and HNF1α gene mutations were infrequent. **(*J Clin Endocrinol Metab* 92: 2462–2467, 2007)**

THE PREVALENCE OF type 2 diabetes mellitus (T2DM) is increasing rapidly in India (1). According to a nationwide survey, nearly 12% of urban adults have T2DM (2). There are clinical differences between T2DM in Indians and their western counterparts, including a lower body mass index (BMI) and an earlier age at onset of diabetes (3). Previous studies on Indians settled in South Africa have reported a high prevalence of young patients with T2DM (4). In a study from the United Kingdom, the prevalence of T2DM in children and young adults was higher in Indians compared with white subjects (5). Recently, an increasing number of adolescents and young adults with T2DM have been reported from India (6–8).

Previous studies have shown that patients presenting as early-onset T2DM may be etiologically heterogeneous (9–12). In contrast to reports from other racial groups, in which most subjects with early T2DM are obese (13), studies in Indians have reported that up to 40% of subjects can be of normal weight (8). A high frequency (27%) of clinically suspected MODY (maturity-onset diabetes of the young) has also been reported among patients with T2DM younger than 25 yr of age from Chennai, south India (14). It is therefore relevant to seek etiologies of diabetes characterized by impaired insulin secretion in young Indian patients. Some etiologies that need to be considered are MODY (especially MODY3) (15), slowly progressive type 1 diabetes mellitus (T1DM) (16), and mitochondrial diabetes (17). However, etiological studies on young patients with T2DM are yet to be performed in Indians.

The aim of the current study was to investigate the role of mutations in the hepatocyte nuclear factor 1α (HNF1α) gene (MODY3), mitochondrial A3243G mutation, and islet autoimmunity in north Indian patients presenting as early-onset T2DM.

Subjects and Methods

Subjects

We studied 96 young patients presenting with T2DM, who were attending the diabetes clinic at Sanjay Gandhi Postgraduate Institute of

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Abbreviations: BMI, Body mass index; CI, confidence interval; GAD, glutamic acid decarboxylase; HNF1α, hepatocyte nuclear factor 1α; HNF4α, hepatocyte nuclear factor 4α; IA2, insulinoma antigen-2; RFLP, restriction-fragments length polymorphism; SSCP, single-strand conformation polymorphism; STR, short tandem repeat; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; WHR, waist-hip ratio.

Medical Sciences (Lucknow, India). Our hospital is a referral center for the large north Indian state of Uttar Pradesh, as well as adjoining states. Consecutive patients were selected on the basis of onset younger than 30 yr, absence of ketoacidosis, and adequate control of plasma glucose without insulin for at least 1 yr after diagnosis. Fibrocalcific pancreatic diabetes was ruled out by abdominal ultrasonography. We also studied 100 healthy control subjects (78 males) from the same region, mainly hospital volunteers, for the mitochondrial A3243G mutation. Written informed consent was obtained from all subjects, and the study was approved by the institutional ethics committee.

All patients were subjected to a thorough clinical evaluation. Fasting and 2-h postglucose plasma C-peptide were measured in 26 patients who consented for the testing. All 96 subjects were tested for antibodies against glutamic acid decarboxylase (GAD) and tyrosine phosphatase [insulinoma antigen 2 (IA2)], mitochondrial A3243G mutation, and the common HNF1α gene mutation P291fsinsC. The entire HNF1α gene (10 exons and promoter) was studied in 32 patients most likely to have MODY3 mutations. This included 17 patients with onset younger than 25 yr (12 males, family history of diabetes in 14 patients, 7 patients with clinical suspicion of MODY) and 15 other patients with a normal BMI (19.6 ± 2.7 kg/m^2). These studies yielded an etiological diagnosis in five patients (see *Results*). In the remaining 91 subjects (and in 100 controls of the same ethnic background), we studied the common A98V polymorphism of the HNF1α gene.

All available family members of a patient detected with the MODY3 gene mutation (R200W) were further evaluated. A 75-g oral glucose tolerance test or fasting glucose, GAD and IA2 antibodies, A3243G mitochondrial gene mutation, and sequencing of exon 3 of the HNF1α gene were conducted in all family members. In addition, the entire HNF1α, hepatocyte nuclear factor 4α (HNF4α), and glucokinase genes were sequenced in the three paternal uncles with diabetes. Paternity testing was performed in family members with diabetes by using four Y-chromosome short tandem repeat (STR) markers.

Methods

Serum glucose was measured by the glucose oxidase method (Merck, Mumbai, India). Total serum cholesterol, triglycerides, and high-density lipoprotein cholesterol were estimated by enzymatic techniques using an auto analyzer (Technicon RX-XT; Bayer, Tarrytown, NY). Hemoglobin A1c was measured using low-pressure liquid chromatography (Bio-Rad, Hercules, CA) (normal range, 4–6%). Plasma C-peptide was measured by RIA (Diagnostics Products, Los Angeles, CA). The limit of detection of C-peptide was 0.2 ng/ml.

Antibodies to GAD and IA2 were measured by immunoprecipitation of radiolabeled human recombinant GAD65 and IA2 antigens (18) in all patients. Plasmids containing human GAD65 and IA2ic were provided by Dr. A. Lernmark (University of Washington, Seattle, WA) and Dr. E. Bonifacio (San Raffaele Scientific Institute, Milan, Italy), respectively (19, 20). The specificity and sensitivity of the assay were 98 and 74% for GAD antibodies and 98 and 70% for IA2 antibodies, respectively, as determined by the results of Diabetes Autoantibody Standardization Proficiency Program, 2005. Among healthy Indian control subjects, the prevalence of GAD antibodies was 2% (2 of 200) and of IA2 antibodies was 1% (1 of 103).

Histocompatibility leukocyte antigen typing for DRB1 and DQB1 alleles was performed on islet antibody-positive subjects. Genomic DNA was isolated from peripheral blood by the standard phenol chloroform extraction. Using sequence-specific primers, 13 DRB1 and 5 DQB1 alleles were determined in PCR-amplified products. Mitochondrial A3243G mutation was analyzed by PCR-restriction-fragments length polymorphism (RFLP) using the technique described by Ohkubo *et al.* (21). Amplified PCR products were digested with restriction enzyme *Apa*I (MBI Fermentas, Hanover, MD) at 37 C for 4 h, run on a 5% polyacrylamide gel, and detected by staining with silver nitrate.

HNF1α mutation analysis was done by direct sequencing in 17 subjects and by single-strand conformational polymorphism (SSCP) analysis in 15 subjects. Because of multiple commonly present polymorphisms in exons 1 and 7, these were directly sequenced in all subjects. The promoter and all 10 exons with flanking introns were amplified by PCR using the sequence-specific primers and conditions as described by Kaisaki *et al.* (22), with the following exceptions. Primers for exons 2 and 9 were newly designed as follows: exon 2 forward primer, 5′ GAGAGACAGCCCTTGCTGAG 3′; exon 2 reverse primer, 5′ GAGAGACAGCCCTTGCTGAG 3′; exon 9 forward primer, 5′ CCTGTGACAGAGCCCCTCACC 3′; and exon 9 reverse primer, 5′TAGGCCTGCTGCATGCACAGC 3′. DNA sequencing was performed on PCR-amplified fragments on an ABI Prism 310 automated DNA sequencer (PerkinElmer, Foster City, CA), using the Big Dye Terminator kit version 3.0 (PerkinElmer). SSCP analysis was performed using the protocol of Orita *et al.* (23). The gel was stained with silver nitrate, and all samples with the characteristic band shift were subjected to sequencing. Control DNA samples containing mutations for each of the exons [a gift from Dr. M. Vaxillaire (Institute of Biology of Lille, Pasteur Institute, Lille, France) and Dr. A. Hattersley (University of Exeter, Exeter, UK)] were run with each assay. Glucokinase and HNF4α genes were sequenced using sequence-specific primers and conditions (12). The A98V polymorphism of the HNF1α gene was studied by PCR-RFLP. PCR was performed using the following primers: forward primer, 5′ TACCTCCTGGCTGGAGAA 3′; and reverse primer, 5′ TCTAGGCTCTCCTGGGAG 3′. The amplified fragment was cut with 5 U (10 U/μl) restriction enzyme *Hae*III (New England Biolabs, Beverly, MA) for 4 h at 37 C. The bands were detected by electrophoresis on 2% agarose gel after staining with ethidium bromide (0.5 μg/ml).

Paternity testing in diabetic family members was performed by analyzing Y-chromosome STR markers (Dys 394, Dys 393, Dys 390, and Dys 19) (24).

Statistical analysis

Clinical data are presented as mean ± SD or median (interquartile range). Differences in clinical features are compared using the Mann-Whitney *U* test for continuous variables and χ^2 test or Fisher's exact test for categorical variables. A two-tailed P value <0.05 was considered significant.

Results

Clinical features

The clinical data are presented in Table 1. The patients' median age at onset of diabetes was 26 yr (interquartile range, 23–28 yr); 10 subjects were diagnosed at 18 yr of age or younger. At diagnosis, fasting plasma glucose was 227 ± 76 mg/100 ml, and postprandial glucose was 301 ± 87 mg/100 ml. A family history of diabetes was present in 66% of patients, and MODY was clinically diagnosed in eight (8%) subjects. Fifty six (58%) patients were overweight or obese (BMI of >23 kg/m^2 or its equivalent in children) (25, 26), whereas 42% were of normal weight. On comparison of normal-weight and overweight patients, the latter had a significantly higher waist-hip ratio (WHR), serum triglycerides, and acanthosis nigricans (63 *vs.* 12%). Both groups had a high prevalence of diabetes in first-degree relatives, but a history of biparental diabetes was significantly higher in overweight patients (26 *vs.* 3%; $P < 0.01$).

Islet antibodies and mitochondrial A3243G mutation

Three (3%) patients had GAD antibodies, whereas IA2 antibodies were absent in all patients (Table 2). GAD antibodies were present in low titers. DRB1*03, the high-risk allele in Indian patients with T1DM, was present in one of the three patients. Two patients were obese, and only one patient required insulin injections (2 yr after detection of diabetes).

The mitochondrial A3243G mutation was detected in one (1%) patient by PCR-RFLP and was confirmed by DNA sequencing. The mutation was absent in 100 control subjects. This patient was a 37-yr-old female, with onset of diabetes at 29 yr and a BMI of 20 kg/m^2. She required insulin 2 yr after diagnosis. The patient gave no history of hearing loss and

TABLE 1. Clinical features of patients presenting as early-onset T2DM

	All patients (n = 96)	Normal-weight patients (n = 37)[a]	Overweight patients (n = 54)[a]
Age at onset (yr)	26 (23–28)	25 (20–28)	26 (24–29)
Sex (males/females)	66/30	27/10	36/18
Duration (yr)	6.5 (2–14.7)	7 (1.4–14)	6 (2.4–15)
Insulin treatment	30 (31%)	13 (35%)	14 (26%)
BMI (kg/m^2)	24.1 ± 4.8	19.5 ± 2.4	27 ± 3.5
WHR	0.90 ± 0.08	0.85 ± 0.06	0.93 ± 0.08[b]
Acanthosis nigricans	41 (43%)	4 (11%)	34 (63%)[b]
T2DM in first-degree relatives	63 (66%)	23 (62%)	36 (67%)
T2DM in both parents	15 (16%)	1 (3%)	14 (26%)[c]
Hypertension	32 (33%)	10 (27%)	21 (39%)
Triglyceride (mg/dl)	144 (101–204)	105 (87–158)	153 (117–209)[d]
High-density lipoprotein cholesterol (mg/dl)	32 (30–36)	33 (29–36)	31 (30–37)
Hemoglobin A1c (%)	8.4 (6.7–10)	8.2 (7.0–11.8)	8.4 (6.7–9.8)
Plasma C-peptide (ng/ml)[e]			
Fasting	0.33 (0.14–1.39)	0.27 (0.19–0.79)	0.77 (0.13–2.11)
Postglucose	0.60 (0.30–2.81)	0.56 (0.29–2.60)	1.47 (0.36–4.60)

Mean ± SD; median (interquartile range). To convert from metric to SI units: for serum triglyceride (mg/dl to mmol/liter) multiply by 0.01129; high-density lipoprotein cholesterol (mg/dl to mmol/liter) multiply by 0.02586; C-peptide (ng/ml to nmol/liter) multiply by 0.33.

[a] Comparison is between normal-weight (BMI of <23 kg/m^2) and overweight (BMI of >23 kg/m^2) patients after excluding five patients in whom a specific etiology was found.

[b] $P < 0.001$.

[c] $P < 0.01$.

[d] $P < 0.05$.

[e] Fasting and postglucose plasma C-peptide in 26 patients.

was unaware of whether her mother had diabetes. Unfortunately, the patient and family members were not available for additional investigations.

MODY3 gene mutations

The P291fsinsC MODY3 mutation was absent in all 96 subjects. SSCP analysis or direct sequencing of the HNF1α gene in 32 subjects (including seven subjects with MODY) detected a missense mutation, R200W, in one patient (3%). This patient had onset of diabetes at 17 yr, a three-generation family history of diabetes, BMI of 19.1 kg/m^2, and low plasma C-peptide levels (Table 3). He required insulin 15 yr after diagnosis and has severe microvascular complications. In addition to the R200W mutation, we detected seven previously described exonic polymorphisms (L17L, I27L, A98V, G288G, L459L, S487N, and T515T) in different patients.

Family study

In addition to the above-mentioned proband, his father (I:1, expired), eldest sibling (II:1, expired), youngest brother (II:6), and three paternal uncles (I:3, I:5, I:7) had T2DM (Fig. 1). However, the clinical characteristics of the family members with diabetes differed greatly (Table 3). The proband, father, eldest brother, and youngest brother all had onset at younger than 30 yr; the proband and youngest brother had normal BMI and WHR and low plasma C-peptide levels. In contrast, the three paternal uncles had an onset at older than 40 yr, elevated WHR, and higher C-peptide values. The R200W mutation was detected in the proband and his youngest (diabetic) brother. However, the three paternal uncles did not have any mutation in the HNF1α gene or other MODY genes (HNF4α and glucokinase). All available family members were also negative for GAD/IA2 antibodies and the A3243G mutation. Analysis of STR markers confirmed that all males with diabetes had similar paternity.

HNF1α A98V polymorphism

The genotype frequencies of both patients and controls were in Hardy-Weinberg equilibrium. The combined frequency of V/V and A/V genotypes was significantly higher in patients than that in control subjects [odds ratio, 5.3; 95% confidence interval (CI), 2.0–13.2; $P < 0.0001$] (Table 4). Similarly, the frequency of the valine allele was significantly greater in T2DM subjects compared with healthy controls (odds ratio, 5.2; 95% CI, 2.1–12.9; $P < 0.001$). When patients with V/V or A/V genotypes were compared with those with A/A genotype, no significant differences in their clinical characteristics were noted.

Discussion

Our study shows that young Indian patients with T2DM were clinically heterogeneous, with a large proportion (40%)

TABLE 2. Characteristics of islet antibody-positive patients with T2DM

Antibody (titer)	Onset (yr)	Duration (yr)	BMI (kg/m^2)	Family history T2DM	Insulin treatment (duration)	DRB1	DQB1
GAD (150)	27	18	29.4	Yes	Yes (16 yr)	0701/15	02/0501
GAD (70)	29	2.6	18.9	Yes	No	03/11	02/03
GAD (53)	29	4	29.2	No	No	04/11	03/06

Titer in WHO (World Health Organization) units (positive >47 U)

TABLE 3. Clinical features of subjects with diabetes in pedigree with R200W (MODY3) mutation

Subject	R200W mutation	Age at diagnosis (yr)	BMI (kg/m^2)	WHR	Fasting C-peptide (ng/ml)	Postglucose C-peptide (ng/ml)
Father (I:1) (deceased)		28				
Eldest brother (II:1) (deceased)		22				
Proband (II:2)	Yes	17	19.1	0.80	0.18	0.28
Youngest brother (II:6)	Yes	18	23.5	0.77	0.29	0.72
Paternal uncle (I:3)	No	52	30.8	0.90	0.40	0.92
Paternal uncle (I:5)	No	47	25.3	1.05	0.68	1.45
Paternal uncle (I:7)	No	43	25	0.97	0.35	1.04

Subject numbers are from pedigree chart (Fig. 1).

having a normal BMI, infrequent evidence of insulin resistance, low prevalence of biparental diabetes, and low/normal C-peptide. However, specific etiologies that could lead to reduced insulin secretion were uncommon. Thus, islet autoimmunity was present in only 3% and mitochondrial A3243G mutation in 1%; HNF1α gene mutations were detected in only 1 of 32 (3%) patients tested who were most likely to harbor such mutations.

MODY3 is the commonest form of MODY worldwide (15). These patients are of normal BMI, often present with severe hyperglycemia, and have microvascular complications. The prevalence of MODY3 in patients with early-onset T2DM varies from 2.5 to 36% (9–12, 22, 27). However, MODY3 mutations have not been described in Indian patients. The mutation P291fsinsC is found frequently in Caucasian MODY3 subjects and is known as a mutational "hotspot" (22). However, we were unable to detect this mutation in any patient. The prevalence of this mutation has not been reported previously in Indian subjects. Among the 32 patients in whom the HNF1α gene was sequenced, a single patient (3%) carried a MODY3 mutation. This missense mutation, R200W, has been described previously only in patients of European and Japanese origin (28–30). The mutation is located in the DNA binding domain of the HNF1α protein, in the region that constitutes the nuclear localization signal (amino acids 197–205).

We identified two separate etiologies of diabetes in this MODY3 family: the R200W mutation and another, as yet unidentified, cause. Among the subjects with diabetes, those with the R200W mutation had onset at younger than 30 yr, normal BMI, and low C-peptide. In contrast, the diabetic relatives (three paternal uncles) without the mutation were older at onset of diabetes and had central obesity and higher C-peptide levels. Other common etiologies of diabetes were

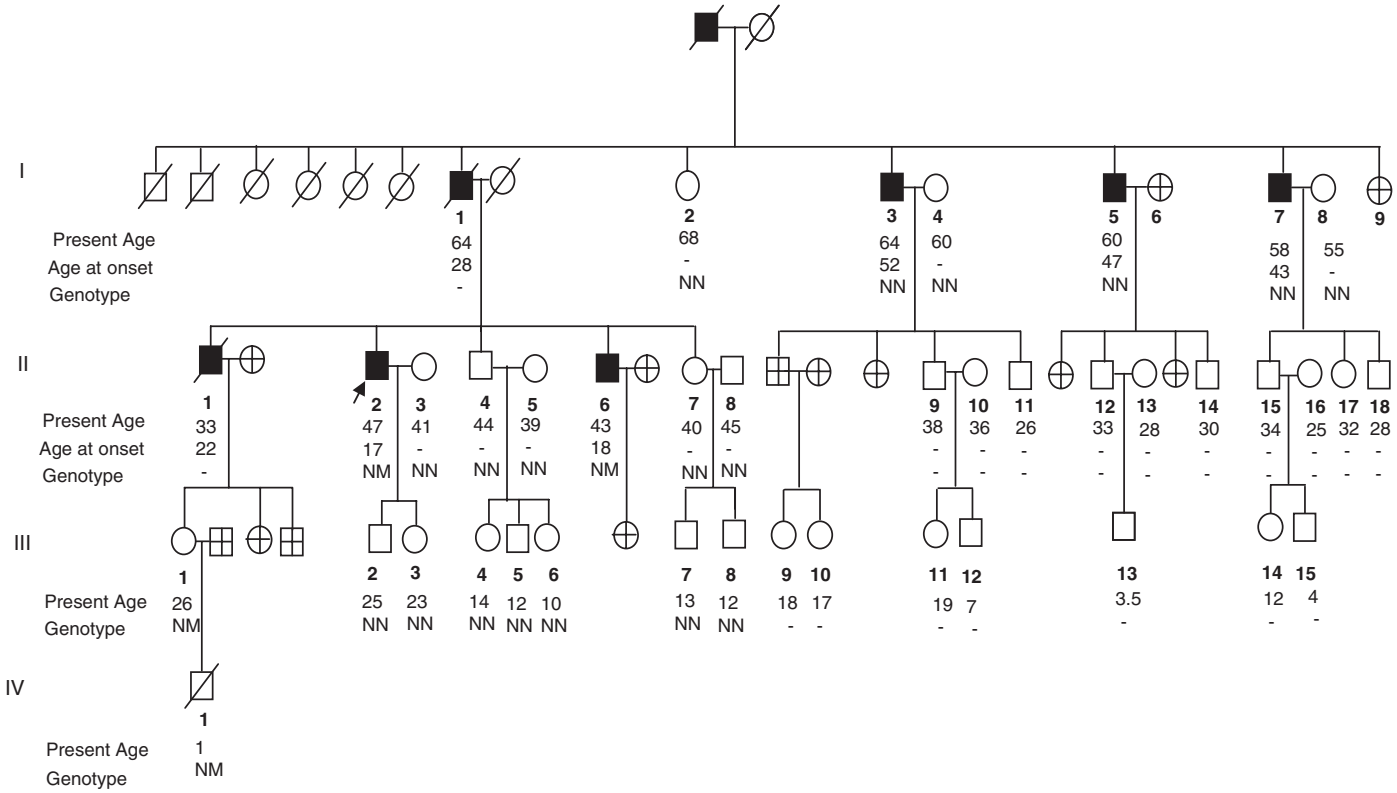

FIG. 1. Pedigree of the patient with R200W (MODY3) mutation. *Filled squares* and *circles*, Diabetes; *open squares* and *circles*, normal glucose tolerance; *crossed squares* and *circle*, not tested. N denotes wild-type allele, and M denotes R200W allele. Age of subject and age at onset of diabetes in years is written below each *symbol*. Proband is depicted with an *arrow*.

TABLE 4. Frequency of HNF1α polymorphism A98V in early-onset T2DM

	Genotype frequencies			Allelic frequencies	
	A/A	A/V	V/V	A	V
T2DM (n = 91)	0.75 (68)	0.23 (21)	0.02 (2)	0.86	0.14
Control subjects (n = 100)	0.94 (94)	0.06 (6)	0	0.97	0.03

Numbers in parentheses indicate number of subjects. Genotype frequencies, odds ratio (A/V and V/V *vs.* A/A), 5.3; 95% CI, 2.0–13.7; $P < 0.001$. Allele frequencies, odds ratio, 5.2; 95% CI, 2.1–12.9; $P < 0.001$.

ruled out, including mutations in other common MODY genes. In view of the late onset and central obesity, it is likely that diabetes in the uncles may be attributable to polygenic inheritance. Because the clinical significance of the two etiologies will differ, such coexistence needs to be kept in mind while screening MODY families. It has been reported previously that MODY3 may coexist with the mitochondrial A3243G mutation (31), MODY4 gene mutation (32), and the small heterodimer partner gene mutation (an obesity-related gene) (27).

A significant association of the A98V polymorphism of the HNF1α gene with early-onset T2DM was noted. A previous study in Scandinavian subjects has suggested an association of valine 98 allele with early-onset familial T2DM (12). The variant may influence transcriptional activity and insulin secretion *in vivo*, although data from Caucasians suggests that the association is likely to only modestly increase risk of adult T2DM (33). Data from other racial groups are scanty. An association of Val 98 allele with south Indian patients presenting with MODY phenotype has been reported recently by Shekhar *et al.* (34). In this study, the allele was associated with an earlier age at onset of T2DM.

Patients with the mitochondrial A3243G mutation present with diabetes and deafness (15). The clinical severity of diabetes is highly variable and may mimic either T1DM or T2DM (35). The mutation is the cause of adult-onset T2DM in 0.3–3% of subjects (15, 21, 36). In two previous reports on south Indian adult patients with T2DM, this mutation was not detected (37, 38), whereas in the current study, the mutation was found in only one subject (1%). Thus, mitochondrial diabetes is a rare cause of either early-onset or adult T2DM in Indians.

Latent autoimmune diabetes in adults is present in approximately 10% of adult Caucasian subjects with T2DM (39). However, its prevalence may be lower in other racial groups (10). GAD and IA2 antibodies have also been reported among children and younger subjects presenting as T2DM (9–12, 16). Islet antibodies in T2DM predict an early insulin requirement (39). However, in a study of 128 Caucasian children with T2DM, antibody-positive and -negative patients were clinically similar (16). In the present study, GAD antibodies were present in only three (3%) patients. Of these, two were obese, and only one required insulin within 2 yr. Thus, the clinical significance of the antibodies in this situation is unclear. In a previous study from India, GAD antibodies were absent in children with T2DM (8). In south Indian T2DM patients between 20 and 40 yr of age, GAD antibodies were an insensitive predictor of insulin requirement (40).

The young T2DM patients in this study were clinically heterogeneous. Approximately 60% of the subjects were overweight or obese, with a high prevalence of biparental diabetes and acanthosis nigricans. Interestingly, nearly 40% were of normal BMI, with a low frequency of acanthosis and low or normal plasma C-peptide. Early-onset T2DM with normal BMI (mean of 22.9 kg/m^2) and low C-peptide levels (mean of 0.26 nmol/liter) has been reported among Mexicans. This is in contrast to reports of early-onset T2DM in other ethnic groups, such as Pima Indians and Black and Hispanic Americans, in whom obesity and insulin resistance are very frequent (13).

Our study has certain limitations. Complete screening for HNF1α mutations was performed only in the 32 subjects who were most likely to carry MODY3 mutations, and hence we cannot provide an exact frequency for these mutations in early-onset T2DM. More detailed and complete metabolic characterization of our subjects may provide information on subgroups in which additional genetic studies would be useful. Finally, the association with Val 98 needs to be confirmed in a larger cohort of Indian patients with early-onset T2DM.

In conclusion, our study shows that north Indian patients with early-onset T2DM are heterogeneous in their clinical presentation and etiology. Islet autoimmunity, A3243G mitochondrial, and HNF1α gene mutations are likely to account for only a small proportion of such patients.

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