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Research article

Screening of Mutations in Maturity-onset Diabetes of the Young-related Genes and *RFX6* in Children with Autoantibody-negative Type 1 Diabetes Mellitus

Şimşek E et al. MODY and Autoantibody-negative Type 1 Diabetes Mellitus in Turkish Children

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What is already known on this topic?
The MODY subtypes differ in terms of the age of diabetes onset, the pattern of hyperglycemic presentation, the response to treatment, and the association with extra pancreatic manifestations. Although genetically identifying the MODY subtype has benefits, a large number of patients have not been tested. Mutations in *GCK*, *HNF1A*, and *HNF4A* are the most common causes of MODY.

What this study adds?
This study established three additional novel mutations in different MODY genes. The etiology has not yet been elucidated in 71% patients diagnosed with autoantibodies-negative type 1 diabetes mellitus.

Abstract
***Objective*:** Maturity-onset diabetes of the young (MODY) is the most common type of monogenic diabetes. To date, mutations have been identified in 14 different genes of patients with a clinical diagnosis of MODY. This study screened mutations in 14 MODY-related genes and the regulator factor X6 (*RFX6*) gene in children
Materials and Methods: The presence of clinical features of MODY and negative results for three autoantibody markers of T1DM in children and adolescents were used as inclusion criteria for genetic testing. The screening panel for next-generation sequencing included 14 MODY-related genes (*GCK*, *HNF4A*, *HNF1A*, *HNF1B*, *PDX1*, *NEUROD1*, *KLF11*, *CEL*, *PAX4*, *INS*, *BLK*, *ABCC8*, *KCNJ11*, and *APPL1*) and the *RFX6* gene.
Results: Twenty-four different variants in MODY-related genes were identified in 49 children diagnosed with autoantibody-negative type 1 diabetes mellitus (T1DM). A 12 variants were classified as P/LP while 12 were interpreted as variant of unknown significance (VUS). Nine of the pathogenic or likely pathogenic variants were found in *GCK*, two in *HNF1B*, and one in *ABCC8*. Three variants were novel, and one was a *de novo* variant. All of the variants, except one, showed heterozygotic inheritance.
Conclusion: This study screened mutations in the 14 MODY-related genes and the regulatory factor X6 (*RFX6*) gene in Turkish children diagnosed with autoantibody-negative type 1 diabetes mellitus (T1DM). The frequencies of the MODY subtypes differed from previous reports. Although GCK-MODY was the most frequent mutation in Turkish children, similar to previous studies, the second most prevalent MODY subtype was HNF1B-MODY. This study also established three additional novel mutations in different MODY genes.
Keywords: Diabetes mellitus, mutation, MODY, *RFX6,* Turkish children

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Introduction
Maturity-onset diabetes of the young (MODY) is characterized by an autosomal dominant genetic defect in beta-cell function with at least two consecutively affected generations of diabetes typically before the age of 25-years, and the absence of beta-cell autoimmunity (1). Heterozygous mutations in various transcription factors that play a role in development and maturation of pancreatic beta-cells and mutations in enzymes acting on glucose sensing of the beta-cell result in MODY. MODY is currently categorized into 14 subtypes, each caused by mutations in different genes (2).
The MODY subtypes differ in terms of the age of diabetes onset, the pattern of hyperglycemic presentation, the response to treatment, and the association with extra pancreatic manifestations. Management of MODY is subtype-specific and includes diet, oral antidiabetic drugs (OADs), or insulin. Therefore, the correct genetic diagnosis is important for lifelong treatment and the patient's prognosis. Although genetically identifying the MODY subtype has benefits, a large number of patients have not been tested (3).
This study screened mutations in the 14 MODY-related genes and the regulatory factor X6 (*RFX6*) gene in Turkish children diagnosed with autoantibody-negative type 1 diabetes mellitus (T1DM).

Materials and Methods
This was a prospective cross-sectional study performed between January 2005 and January 2022. The following criteria were used to diagnose diabetes mellitus (4): fasting plasma glucose or random plasma glucose levels ≥ 126 mg/dL or ≥ 200 mg/dL, respectively, glycated hemoglobin (HBA1C) ≥ 6.5%, and C-peptide < 0.6 ng/mL. The clinical diagnosis of MODY was made using the classical criteria of impaired fasting glucose or the development of diabetes before the age of 25-years, negative results for T1DM markers (islet cell antibodies [ICAs], glutamic acid decarboxylase antibodies [GADAs], and insulin autoantibodies [IAAs]) and a family history of diabetes mellitus for at least two consecutive generations. The 14 MODY-related genes and *RFX6* were screened in all patients with a clinical diagnosis of MODY. Blood samples were collected to obtain fasting or random glucose measurements with concomitant C-peptide, HBA1C%, and three antibody tests (ICA, GADA, and IAA) at the time of the T1DM diagnosis.
Peripheral blood samples were collected with EDTA for genetic testing. Genomic DNA was isolated from peripheral blood samples using the MagPurix robotic system (Zinexts, New Taipei City, Taiwan). Primary quality control of isolated DNA samples was performed using a NanoDrop spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany) and samples with an A260/280 value between 1.8 and 2 were included in the study.
Twenty-seven patients with clinically diagnosed MODY were included in next-generation sequencing (NGS) analyses. A panel consisting of the 14 MODY-related genes (*GCK*, *HNF1A*, *HNF4A*, *HNF1B*, *ABCC8*, *KCNJ11*, *INS*, *NEURD1*, *CEL*, *APPL1*, *PDX1*, *KLF11*, *PAX4*, and

BLK), and the *RFX6* gene was designed using Ion Ampliseq Designer software. The MODY panel contained 384 amplicons and was optimized as two pools by Thermo Fisher Scientific (Waltham, MA, USA). Amplicons prepared using the Ion Ampliseq Library v2.0 commercial kit were loaded into the Ion Chef (Ion torrent, Thermo Fisher Scientific) instrument. Template creation, enrichment, and chip loading stages were performed automatically by the Ion Chef device. The sequencing reaction step was performed on the Ion S5 (Ion Torrent, Thermo Fisher Scientific) NGS device. As a result of sequencing, "single-end array" raw data (*fast or UBAM) were used in the NGS platform within the scope of a bioinformatics analysis.

Variant analyses

Sequenced reads were aligned to the reference genome (GRCh37/hg19) using the Ion Torrent platform-specific pipeline software Torrent Suite 4.2. The Ion Reporter 4.0 (Thermo Fisher Scientific), Integrative Genomics Viewer, and Varsome (http://www.varsome.com) (5) software were used to analyze the data. Variants with a minor allele frequency > 0.1% in the Genome Aggregation Database (http://gnomad.broadinstitute.org/) were filtered out. Mutation Taster (http://www.mutationtaster.org/), prediction of effects of human nsSNPs (http://genetics.bwh.harvard.edu/pph2/), and scale-invariant feature transform (http://sift.jcvi.org/) were used to evaluate the effect of nonsynonymous variants on protein function and structure. Human Splicing Finder (http://www.umd.be/HSF/) was used to predict the effect of the mutations on splicing. We interpreted the variants using The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/), ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/) and a literature search. The variants were named according to Human Genome Variants Society (http://www.hgvs.org) nomenclature. The variants were classified as "pathogenic," "likely pathogenic," "uncertain significance," "likely benign," or "benign" following the American College of Medical Genetics and Genomics Standards and Guidelines (ACMG) (6).

Statistical Analysis

The Statistical Package for the Social Sciences software (version 18.0, SPSS Inc., Chicago, IL, USA) was used for descriptive data analysis. Descriptive statistics of the clinical and laboratory findings are expressed as mean ± standard deviation, numbers, or percentages. Comparative statistics were not performed because the number of patients in the MODY subgroups was limited and not homogeneously distributed.

Informed consent was obtained from all subjects or their parents. This study was performed according to the Declaration of Helsinki and approved by the Ethical Comittee of the Eskisehir Osmangazi University (approval number: E-2022-263).

Results

The three antibodies (GADA, ICA, and IAA) were negative in 49 (12%) of 408 patients diagnosed with T1DM between January 2005 and January 2022. These 49 patients were screened for the 14 MODY-related genes and *RFX6*. The MODY diagnosis was confirmed by NGS in 14 of 49 (29%) patients, as pathogenic (P) or likely pathogenic (LP). The clinical and genetic characteristics of the MODY patients are given in Table 1 and Table 2, respectively. The mean age of the MODY patients was 9.5 ± 4.3 years (range, 3.5 –17.8 years). The mean HBA1C level of MODY patients was 6.9 ± 1.1 % (range, 6.2 – 9.8 %). The lowest mean HBA1C level was seen in patients with GCK-MODY (mean, 7.01 ± 0.34%, [range, 6.6–7.8%]). The clinical and genetic characteristics of 13 patients diagnosed with autoantibody-negative type 1 diabetes mellitus and genetic identification with variants of uncertain significance (VUS) are given in Table 3 and Table 4, respectively. The mean age of the children with VUS variants in MODY related genes and in *RFX-6* was 6.4 ± 4.0 years (range, 2.5 –14 years). The mean HBA1C level in these patients was 8.1 ± 1.1 % (range, 6.9 – 10.2 %). A heterogeneous clinical presentation was observed according to the MODY genes. Patients with GCK and ABCC8-MODY presented with hyperglycemia, whereas, HNF1B-MODY presented with diabetic ketoacidosis and multicystic kidneys. A in diabetic patients, whose genetic study revealed VUS variants in MODY related genes and in *RFX-6*, were also observed heterogeneous clinical presentation. Patients with VUS variations in *HNF1A*-, *HNF4A*-, *BLK*-, and *ABCC8* presented with hyperglycemia, in *KLF11* and *RFX6* with diabetic ketosis, whereas in *HNF1B*-, *PAX4*-, and in *INS* genes with diabetic ketoacidosis.

A family history revealed that 23 of 27 patients (including MODY patients and patients with VUS) had one or two consecutive affected generations. Overall, 11 of the 27 diabetic patients whose family history revealed only mild hyperglycemia in two generations and who had not required insulin therapy, were presumptively diagnosed with GCK-MODY and the diagnosis was confirmed by genetic testing. GCK-MODY patients were followed up with diet treatment only, ABCC8-MODY patients with oral sulfonylurea treatment only, whereas HNF1B-MODY were followed up with insulin treatment. In patients with VUS in MODY related genes; HNF1A- and ABCC8-MODY were followed up by sulfonylurea only, whereas all remaining patients with VUS in MODY genes were followed up by insulin treatment. All MODY patients, except one, were heterozygotes, and one was a *de novo* mutation. The distribution of mutations according to the MODY related genes was as follows: nine different pathogenic or likely pathogenic variants in the *GCK* gene, and one likely pathogenic in *ABCC8* (Table 2). Seven of the nine mutations in the *GCK* gene were missense and two were novel splice site and insertion mutations. One of two likely pathogenic mutations in *HNF1B* was a missense mutation, and the other one was a novel splice-site mutation. The distribution of VUS variants according to genes was shown in Table 4. Two of the 12 different VUS variants in MODY related genes and *RFX6* were novel variants, and the other 10 variants were reported previously (Table 4).

Discussion

MODY is the most common type of monogenic diabetes, accounting for 1–6% of all pediatric diabetic cases (2). Although there are several clinical predictors for diagnosing MODY, such as a positive family history of diabetes mellitus before the age of 25–30 years, negative results for antibodies associated with T1DM, high C-peptide concentrations (12), and high levels of C-reactive protein (13) and lipids, its diagnosis is relatively difficult and cases are often misdiagnosed as T1DM (3). A genetic test is the most accurate and cost-effective option for diagnosing the MODY subtypes (14). However, selecting pediatric and adolescent diabetic patients for genetic testing is still controversial. A clinical diagnosis of MODY should be considered in patients who have atypical features of diabetes based on age < 25 years, negative results for antibodies associated with T1DM, the presence of neonatal hypoglycemia, and/or multiple family members with diabetes not characteristic of T1DM or T2DM (1–3). Epidemiological studies have shown that 10–15% of children and adolescents diagnosed with T1DM are negative for the three autoantibodies (GADA, IAA, and ICA). In the present study, 49 (12%) of 408 patients diagnosed with T1DM had negative results for these three antibodies. In our study the MODY diagnosis was confirmed only in 14 of 49 (29%) patients. However, in the remaining 35 (71%) patients, there was no pathogenic or likely pathogenic mutations in any of the 14 MODY genes. This finding revealed that the etiology has not yet been elucidated in a significant group of patients diagnosed with type 1 diabetes mellitus whose autoantibodies are negative. This group of patients may be candidates for genetically inherited diabetes groups that are likely to be identified in the near future. Determining the MODY subtype is important, as the subtypes differ in terms of age of onset, clinical presentation and progression, and response to treatment (2). Based on the current data, the prevalence and frequency of MODY subgroups vary by country. Mutations in *GCK*, *HNF1A*, and *HNF4A* are the most common causes of MODY (15-18). The most common subtype in European countries (UK, Germany, The Netherlands, Norway, and Poland) is HNF1A-MODY, followed by GCK-, HNF4A-, and HNF1B-MODY (19). The most common subtypes in the UK are HNF1A-, GCK-, HNF4A-, and HNF1B-MODY (3,20). However, Chakera *et al.* (21) reported that the estimated prevalence of GCK-MODY is about 1 in 1,000 individuals in the UK. GCK-MODY is the most prevalent MODY in Japan (22). In Korea, only 10% of clinical MODY of childhood-onset type 2 diabetes cases harbor known MODY-related genetic defects (*HNF1A*, 5%; *GCK*, 2.5%, and *HNF1B*, 2.5%) (23). The prevalence of MODY in Middle Eastern, Asian, and African populations is unknown. Agladıoglu *et al.* (24) and Goksen *et al.* (25) reported that the most prevalent MODY subtypes in Turkish children are GCK-MODY and HNF1A-MODY. Yalcıntepe *et al.* (26) reported 31 cases of pathogenic/likely pathogenic variants (*GCK*, n=24; *ABCC8*, n=3; *KCNJ11*, n=2; *HNF1A*, n=1; and *HNF4A*, n=1) in 61 unrelated cases with clinical diagnosed with MODY in Turkish children. Our study revealed that the most prevalent MODY subgroup in Turkish children was GCK-MODY, as reported previously (24-26). Unfortunately, the number of our patients diagnosed with MODY is not sufficient to make a realistic comment about the regional MODY frequency or the distribution of MODY subgroups. It can only be possible after a national-scale MODY study or studies.

The GCK-MODY phenotype is characterized by lifelong nonprogressive fasting hyperglycemia (2,27,28). Patients are often asymptomatic and diagnosed incidentally during pregnancy or routine examinations, and the majority of patients do not require pharmacotherapy. Although patients with GCK-MODY have long-standing hyperglycemia, they have a low prevalence of micro-and macrovascular complications (29). However, in Kawakita *et al.* (30), 7 of 55 patients with GCK-MODY were treated with OADs, and the authors concluded that those 7 patients required OADs because they consumed carbohydrate-rich foods and had sedentary lifestyles. Insulin treatment is also required in pregnant patients with GCK-MODY to prevent maternal hyperglycemia and reduce the risk for the development of overweight fetuses (31). In this study, 11 cases from nine families had nine different mutations in *GCK*. Two cases had novel mutations; one was a splice site type mutation (c45+1G>C) and the other was an insertional type and a *de novo* mutation (c.1090_1091insGCTGCGACCCTCGACCACCG, p.Asp364GlyfsTer6). All cases presented with mild hyperglycemia and were followed up with only a low carbohydrate diet.

Heterozygous mutations in *HNF1B* cause HNF1B-MODY (formerly referred to as MODY-5), which is characterized by early-onset diabetes, pancreas hypoplasia, and multicystic kidney dysplasia (32). In patients with diabetes mellitus, the presence of cystic kidneys and elevated liver enzymes may be used as predictors of an *HNF1B* mutation. In our study, two cases had *HNF1B* mutations and presented with diabetic ketoacidosis. One of these two mutations was previously reported as missense mutation (c.1024T>C), and the other one was a novel splice site mutation (c.1045+1G>A). Both cases were diagnosed with cystic renal disease in the first decade of life. In one of the patients, cystic renal disease was revealed by ultrasonography performed in the intensive care unit, where she presented with diabetic ketoacidosis. The other case was followed up by the pediatric nephrology department for polycystic renal disease. The pediatric nephrology department consulted the pediatric endocrinology department when she was diagnosed with fasting hyperglycemia and developed a high HbA1c level. Both patients were followed up with an intensive insulin regimen protocol. Unlike previous studies (11–13, 19, 20), our study revealed that the *HNF1B* mutations were more prevalent in Turkish children than the *HNF1A* or *HNF4A* mutations. To confirm this hypothesis, more prospective and comprehensive studies are needed with larger cohorts. On the other hand, the presence of renal abnormalities in young patients with diabetes mellitus, initially *HNF1B* mutations, may be screened before the investigation of other MODY subgroups. The major complications in HNF1B-MODY patients have been related to the kidneys, such as chronic kidney disease (33).

The diagnosis of ABCC8-MODY was confirmed in one of the patients diagnosed with type 1 diabetes mellitus whose autoantibodies are negative. The ATP-binding cassette transporter subfamily C member 8 (*ABCC8*) gene is expressed in the pancreas where it controls the expression of the sulfonylurea receptor 1 subunit of the ATP-sensitive potassium channel found on the beta cell membrane (34). Heterozygous mutations in the *ABCC8* gene damage the normal function of potassium channels leading to impaired insulin secretion. ABCC8-MODY (formerly referred to as MODY-12) is characterized by congenital hyperinsulinemic hypoglycemia, a transient or permanent form of neonatal diabetes mellitus, or adulthood-onset diabetes mellitus (2). Patients with ABCC8-MODY respond to sulfonylurea treatment (2,35). Our patient presented with hyperglycemia at 3.5 years old and was regulated by sulfonylurea treatment. As seen in this case, by identifying MODY subgroups, correct treatment options can be chosen from younger ages.

In this study, genetic testing identified 12 different VUS variants in MODY-related genes in 13 of 49 patients diagnosed with autoantibody-negative type 1 diabetes mellitus (T1DM), however all patients had clinical diagnosis of MODY. Two of 12 VUS variants were novel, and the others were reported previously (7-11). A clearer interpretation can be made by comparing the results obtained after segregation analyzes in our patients and previously reported studies related to these VUS variants. Though computational prediction tools and conservation analyses suggest that these VUS variants are not predictive enough to determine pathogenicity, given the current lack of comprehensive MODY-variant classification expertise, current ACMG-based classification should be interpreted cautiously and these VUS variants may be subject to change in the future.

Study Limitations

This study was conducted with a small number of cases, except GCK-MODY, the frequency order of the rare MODY subgroups may change. Segregation could not be tested in all families for VUS variants. Large-scale prospective studies are needed to allow for a stronger interpretation of the frequencies of rare MODY subgroups in Turkish children.

Conclusion

The clinical evaluation and genetic testing can be used to make the correct MODY diagnosis and subgrouping. The incidence and prevalence of MODY vary between countries. A timely and accurate diagnosis of MODY may prevent some subgroups from unnecessarily taking long-term insulin therapy. Therefore, all autoantibody-negative T1DM cases should be screened for known MODY genes, and the treatment of patients should be individualized following the defined MODY subgroup. This study established three additional novel mutations in different MODY genes. Our study also revealed that the 14 currently known MODY genes or *RFX6* were not found pathogenic or likely-pathogenic in 35 of the 49 (71.5%) autoantibody-negative T1DM cases, indicating that this group may have novel MODY subtypes.

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Author Contributions

E.S. and T.S. conducted the literature review, conceived the study, provided scientific guidance, analyzed the data, and drafted the manuscript. As an ophthalmologist, T.S. has also been following up diabetic children since 2014. O.C., S.K., and E.G. reviewed and interpreted the genetic data, and drafted the manuscript. C.B. and M.D. researched the background material, reviewed the data, and contributed to the discussion. All authors actively participated in the critical review of the manuscript and have read and approved the final version. E.S. and O.C. are the guarantors of this study and take full responsibility for the integrity of the data and the accuracy of the data analysis.

Disclosure Statement. The authors have no financial disclosure and no conflicts of interest to declare.

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Table 1. Clinical characteristics of 14 patients diagnosed with confirmed of the maturity-onset diabetes of the young (MODY).

No	MODY gene	Age at diagnosis (Year)	Gender	Family history for MODY/ generations	Clinical presentation	HbA1c % level at diagnosis	Follow-up treatment
1	*GCK*	14.2	Male	+/3	Hyperglycaemia	6.3	Diet
2	*GCK*	6.9	Male	+/3	Hyperglycaemia	6.6	Diet
3	*GCK*	7.5	Male	+/2	Hyperglycaemia	6.4	Diet
4	*GCK*	14.3	Male	+/3	Hyperglycaemia	6.3	Diet
5	*GCK*	7.1	Male	+/3	Hyperglycaemia	6.5	Diet
6	*GCK*	8.4	Female	+/3	Hyperglycaemia	6.3	Diet
7	*GCK*	6.1	Male	+/3	Hyperglycaemia	6.2	Diet
8	*GCK*	10.4	Male	NA	Hyperglycaemia	6.8	Diet
9	*GCK*	17.8	Female	+/3	Hyperglycaemia	6.2	Diet
10	*GCK*	3.8	Female	-/1	Hyperglycaemia	6.3	Diet
11	*GCK*	8.5	Female	+/2	Hyperglycaemia	6.8	Diet
12	*HNF1B*	9.5	Male	+/2	Diabetic ketoacidosis Multicystic kidneys	8.8	Insulin
13	*HNF1B*	13,5	Female	+/3	Diabetic ketoacidosis Multicystic kidneys	9,8	Insulin
14	*ABCC8*	3.5	Female	+/2	Hyperglycaemia	7.6	Sulfonylurea

OADs, oral antidiabetic drugs; NA, not available

Table 2. Mutation analysis screening of the MODY genes in Turkish children diagnosed with autoimmune negative type 1 diabetes mellitus.

Gene OMIM ID/Mody type	cDNA rs ID Types of mutation	Protein	Zygosity	*In-silico* prediction	MAF %	ACMG Class	N
GCK 125851/MODY2	c.109C>T rs762263694 missense	p.(Arg37Trp)	Hetero	PbD/Del/DC	0.00119	P	2
GCK	c.679G>A rs148311934 missense	p.(Val227Met)	Hetero	PbD/Del/DC	0.000796	P	2
GCK	c.116A>C rs1064794268 missense	p.(Gln39Pro)	Hetero	PbD/Del/DC	NA	LP	1
GCK	c.575G>A rs886042610 missense	p.(Arg192Gln)	Hetero	PbD/Del/DC	NA	LP	1
GCK	c.686C>T rs80356655 missense	p.(Thr229Met)	Hetero	PbD/Del/DC	0.000399	P	1
GCK	c.1181T>C missense	p.(Met394Thr)	Hetero	PD/ Del /DC	NA	P	1
GCK	c.45+1G>C novel splice site	p.?	Hetero	NA / NA /DC	NA	LP	1
GCK	c.1090_1091insGCTGCGACCCTCGACCACCG novel insertion	p.(Asp364Glyfs Ter46)	de-novo	NA / NA /DC	NA	LP	1
GCK	c.536G>A rs886039380 missense	p.(Gly179Glu)	Hetero	PbD/ Del /DC	NA	P	1
HNF1B 137920/MODY5	c.1024T>C rs1282596664 missense	p.(Ser342Pro)	Hetero	PD/Tol /DC	0.000402	LP	1
HNF1B	c.1045+1G>A novel splice site	p.?	Hetero	NA/NA/DC	NA	LP	1
ABCC8	c.4369G>A rs72559717 missense	p.(Ala1457Thr)	Hetero	PbD/Del/DC	NA	LP	1

MAF: Minor allele frequency from gnomAD Exomes (The Genome Aggregation Database), ?= unknown effect
In silico prediction: PolyPhen-2, SIFT, Mutation Tester, respectively; Polyphen predictions: B, Bening; PD, Possibly Damaging; PbD, Probably Damaging; SIFT predictions: Del, Deleterious; Tol, Tolerated; Mutation Tester predictions: DC, Disease-causing; Poly, polymorphism; NA, not available; ACMG Class; P,pathogenic; LP, Likely Pathogenic;
Transcripts: GCK(NM_033507.3), HNF1B(NM_000458.4), ABCC8(NM_000352.6)

Table 3. Clinical characteristics of 13 patients diagnosed with autoantibody-negative type 1 diabetes mellitus and genetic identification with variants of uncertain significance (VUS) detected according to According to the American College of Medical Genetics and Genomics (ACMG).

No	MODY gene	Age at diagnosis (Year)	Gender	Family history for MODY/ generations	Clinical presentation	HbA1c % level at diagnosis	Follow-up treatment
1	*HNF1A*	10.1	Male	+/3	Hyperglycaemia	8.4	Diet, sulfonylurea
2	*HNF1A*	14	Female	+/3	Hyperglycaemia	7.1	Diet, sulfonylurea, insulin
3	*HNF1B*	4.0	Male	-/1	Diabetic ketoacidosis	8.9	Insulin
4	*HNF1B*	8.3	Female	NA	Diabetic ketoacidozis	9.4	Insulin
5	*HNF4A*	2.3	Male	+/3	Hyperglycaemia	6.9	Diet, sulfonylurea, insulin
6	*BLK*	1.5	Male	+/2	Hyperglycaemia	7.4	Diet, sulfonylurea, insulin
7	*BLK*	10	Female	No	Hyperglycaemia	10.2	Diet, sulfonylurea, insulin
8	*PAX4*	5.5	Female	+/2	Diabetic ketoacidosis	8.8	Diet, sulfonylurea, insulin
9	*INS*	10.5	Female	+/2	Diabetic ketoacidosis	9.2	Diet, Diet, sulfonylurea, insulin
10	*KLF11*	8.5	Male	+/2	Diabetic ketosis	7.7	Diet, sulfonylurea, insulin
11	*ABCC8*	2.4	Male	+/2	Hyperglycaemia	7.1	Sulfonylurea
12	*ABCC8*	4.2	Male	+/2	Hyperglycaemia	7.3	Sulfonylurea
13	*RFX6*	2.4	Male	No	Diabetic ketosis	7.4	Diet, sulfonylurea, insulin

OADs, oral antidiabetic drugs; NA, not available

Table 4. Genetic identification of the cases with variants of uncertain significance (VUS) detected according to According to the American College of Medical Genetics and Genomics (ACMG).

Gene/ OMIM ID/ Mody type	Variants/ Types of mutation	dsSNP	Zygosity	*In-silico* prediction	Ref
HNF1A 600496 MODY3	c.481G>A p.(Ala161Thr) missense	rs201095611	Heterozygous	pBD/Del/DC	7,8
HNF1A	c.517G>A p.(Val173Met) missense	NA	Heterozygous	PD/Del/DC	9,10
HNF1B 189907 MODY5	c.1006C>G p.(His336Asp) missense	rs138986885	Heterozygous	B/Tol/DC	8
HNF1B	c.1339+5G>A p.? splice site (novel)	NA	Heterozygous	NA/NA/DC	NA
HNF4A 125850 MODY1	c.473C>T p.(Ala158Val) missense	rs754143633	Heterozygous	PbD/Tol/DC	NA
BLK 613375 MODY11	c.497delA p.(Asp166ValfsTer8) deletion (novel)	NA	Heterozygous	NA/NA/DC	NA
BLK	c.569C>G p.(Ser190Cys) missense	rs200875749	Heterozygous	B/Del/DC	NA
PAX4 612225 MODY9	c.521G>T p.(Arg174Leu) missense	rs776151854	Heterozygous	PbD/Del/DC	11
INS 613370 MODY10	c.2455C>G p.(Arg819Gly) missense	rs1555738952	Heterozygous	B/Del/DC	NA
KLF11 610508 MODY7	c.673A>C p.(Ser225Arg) missense	rs200061013	Heterozygous	B/Tol/Poly	NA
ABCC8 600509 MODY12	c.2395A>G p.(Lys799Glu) missense	rs1336775990	Heterozygous	B/Del/DC	NA
RFX6 NA MODY?	c.1782C>G p.(His594Gln) missense	rs4946206	Heterozygous	B/Tol/Poly	NA

dsSNP, the Single Nucleotide Polymorphism Database; In silico prediction: PolyPhen-2, SIFT, Mutation Tester, respectively; Polyphen predictions: B, Bening; PD, Possibly Damaging; PbD, Probably Damaging; SIFT predictions: Del, Deleterious; Tol, Tolerated; Mutation Tester predictions: DC, Disease-causing; Poly, polymorphism; ?= unknown effect; NA, not available.
Transcripts: HNF1A(NM_000545.8), HNF4A(NM_000457.5), HNF1B(NM_000458.4), BLK(NM_001715.3), PAX4(NM_001366110.1), INS(NM_000208.4), KLF11(NM_003597.5), ABCC8(NM_000352.6), RFX6(NM_173560.4)
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