Detection and analysis of glucose metabolism-related genes in childhood diabetes using targeted next-generation sequencing: In pediatric population-a hospital-based study

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Abstract. The aim of the present study was to explore the genetic causes of antibody-negative diabetes and investigate its characteristics. A total of 64 patients with new-onset diabetes (>6 m, <16 y) were identified and their initial clinical characteristics were analyzed. Of which, 32 cases with autoantibody-negative diabetes (male, 16 cases; female, 16 cases) were screened for auto-antibodies, including islet cell antibody, glutamic acid decarboxylase antibody and islet antigen-2, which were negative, and fasting C-peptide was ≥0.3 ng/ml. Peripheral blood DNA was extracted from the subjects and their parents for high-throughput sequencing of glucose metabolism-related genes. The group with the pathogenic variation was used as the experimental group. The control group comprised 32 cases of type 1 diabetes (T1D). Their baseline clinical characteristics were determined and statistically analyzed. Out of the 32 antibody-negative diabetes cases, 21 had possible related mutations. There were 2 HNF1B missense mutations, 1 GCK missense mutation and 1 de novo KCNJ11 missense mutation. GCGR c.118G>A p.G40S was present in patients with type 2 DM (T2DM); the locus is associated with T2DM susceptibility in China. An LIPC frameshift mutation was identified, which had not been previously reported; the gene was found to markedly affect protein function and be associated with glucose and lipid metabolism. It was concluded that children with antibody-negative T1D have monogenic diabetes. The present findings shed light on the etiology and mechanism of antibody-negative diabetes, which will enable the comprehensive analysis of antibody-negative diabetes genotypes and phenotypes and further help improved precision treatment.

Introduction

The number of children with diabetes mellitus (DM) has increased in recent years. DM has become one of the most serious diseases affecting the physical and mental development of children (1). The most common type of diabetes in children is type 1 diabetes (T1D). T1D is an autoimmune disease caused by an immune-mediated destruction of the insulin-producing pancreatic β cells. Autoantibodies directed against islet autoantigens, such as insulin, glutamic acid decarboxylase 65 (GAD65), islet cell antibody (ICA), islet antigen-2 (IA-2) and zinc transporter 8 (ZnT8), are markers of islet autoimmunity, which precedes the clinical onset of T1D (2). Most patients with T1D have multiple detectable islet cell autoantibodies in their blood at diagnosis, with <10% only having one when assessed using a combination of islet cell autoantibodies and antibodies against GAD65, IA-2, ICA and insulin. The addition of ZnT8 antibodies may increase this autoimmunity detection rate to 98% (3).

Insulin deficiency is usually attributed to the autoimmune destruction of islet β cells in childhood diabetes, but monogenic diabetes is also a common cause of insulin deficiency (4). The prevalence of obesity has increased rapidly and type 2 DM (T2DM) incidence in children has also been increasing. In previous years, ~12% of young adults diagnosed with diabetes in the United States have been classified as T2DM (5,6).

The incidence of monogenic diabetes in children is low, but the atypical clinical manifestations, limitations of the detection methods and lack of understanding of such diseases often
renders their diagnosis unclear or wrong (7). In the present study, next generation sequencing (NGS)-based mutation screening of known causative genes for glucose metabolism-related genes in antibody-negative childhood diabetes was conducted and the findings may prove to be helpful for the improvement of precision treatment.

Materials and methods

Cohort. The medical records of Chinese patients from the inpatient or outpatient clinics of the Children's Hospital of Soochow University (Soochow, China) between January 2010 and December 2017 were analyzed. A total of 64 patients with new-onset diabetes (>6 m, <16 y) were identified and their initial clinical characteristics were analyzed. Of which 32 cases with autoantibody-negative diabetes (male, 16 cases; female, 16 cases) were screened for ICA, GAD antibody (GADA), IA-2 and ZnT8 at diagnosis. Having at least one positive antibody and a fasting C peptide of ≥0.3 ng/ml at initial diagnosis was considered positive autoimmunity (8-10). Peripheral blood DNA was extracted from the 32 patients with autoantibody-negative diabetes and their parents for high-throughput sequencing of glucose metabolism-related genes. Pathogenicity analysis of the candidate mutated site has careful consideration of the patient's clinical presentations and sequencing result base on Standards and Guidelines for the Interpretation of Sequence Variants revised by the American College of Medical Genetics (ACMG). Patients with autoantibody-negative diabetes were classified into the pathogenic mutation group, variant of unknown significance (VUS) group and no-mutation group based on the results, and the three groups of data were compared.

The group with autoantibody-negative diabetes was the experimental group. The control group comprised 32 T1D cases (male, 16 cases; female, 16 cases). The only difference between the experimental group and the control group is that the experimental group patients are autoantibody-negative, and the control group patients tested positive for at least two of the islet auto-antibodies. T1D was diagnosed according to the criteria of the American Diabetes Association (11). All children (experimental and control) present with a very early form of diabetes (>6 m, <16 y). No significant differences were observed in age and sex. The control group comprised 32 patients with typical childhood T1D, who had been enrolled during the same period. After half a year follow-up, the control group still relied on insulin and the c-peptide was so low that it could not be detected. The control group patients matched the experimental group in age and body mass index (BMI). They were positive for at least two islet auto-antibodies (ICA, GADA, IA-2 or ZnT8).

The trial exclusion criteria were as follows: i) In addition to diabetes, other diseases with a definite diagnosis; ii) chronic inflammatory disease, such as chronic diarrhea and iii) obesity (95th centile of BMI) and acanthosis nigricans skin.

NGS and validation. A total of 159 glucose metabolism-related genes were screened, including the known monogenic diabetes and T2DM susceptibility-related genes, covering the entire coding region and exon-intron boundaries (±5 bp). The focus was put on screening 13 maturity-onset diabetes of the young (MODY) genes (GCK, HNF1A, HNF4A, HNF1B, NEUROD1, INS, CEL, PD1X, PAX4, BLK, KLF11, KCNJ11 and ABC8) and 20 neonatal DM (NDM) genes (GCK, KCNJ11, ABCC8, INS, PD1X, PTF1A, HNF1B, NEUROD1, NEUROG3, RFX6, EIF2AK3, FOXP3, GLIS3, SLC19A2, SLC2A2, IER3IP1, ZFP57, WFS1, GATA6 and GATA4). DNA samples from the 32 cases were fragmented using a Scientz08-III automated sonicator. DNA samples were extracted using a DNA extraction kit (CWE2100 Blood DNA kit V2; Beijing Kangwei Century Biotechnology Co., Ltd.) on an 96-channel automatic nucleic acid extraction machine (Beijing Kangwei Century Biotechnology Co., Ltd.). DNA samples (750 ng) were fragmented into 150-200 bp via ultrasound treatment for 35 min (running for 3 sec with 1 sec intervals) with 50% ultrasonic intensity at 4°C. High-throughput sequencing was performed on an Illumina HiSeq 2500 system and the data were sequenced using an Illumina sequencer. Data reading and bioinformatics analysis were performed following Control Software assessment (The Genome Analysis Toolkit; http://www.broadinstitute.org/gsa/wiki/index.php/Home_Page). The obtained sequences were separately Basic Local Alignment Search Tool-matched with GenBank (http://www.ncbi.nlm.nih.gov/genbank) sequences to confirm the variant sites; suspected variant sites were searched with MITOMAP (www.mitomap.org/MITOMAP). For polymorphism screening, gene variants were detected and verified by Sanger sequencing.

For the validation of this panel, parents and family members of positive patients were analyzed. The detected pathogenicity of rare variants (minor allele frequency ≥0.01) was evaluated according to the recommendations of the ACMG for variant classification and reporting (12). These guidelines classify variants into 5 categories: Pathogenic, likely pathogenic, VUS, likely benign and benign. The ACMG criteria for variant classification are based on a set of different evaluation fields. Population data was determined from public genomic databases (1000 Genomes Project, GnomAD and dbSNP) (https://www.internationalgenome.org, http://gnomad.broadinstitute.org/about and https://www.ncbi.nlm.nih.gov/SNP/). Other criteria to consider were the type of variant (e.g. frameshift, nonsense or essential splice variants) and clinical, functional and genotype-phenotype data from the literature and disease databases (Human Gene Mutation Database Professional and PubMed) (http://www.hgmd.cf.ac.uk/index.php and https://www.ncbi.nlm.nih.gov/pubmed). If such variants had not been previously reported, they were evaluated to predict their possible functional significance using in silico prediction tools, such as SIFT, PolyPhen2 and Mutation Taster (https://sift.jcvi.org, http://genetics.bwh.harvard.edu/pph2/ and http://www.mutationtaster.org). Rare variants were considered to be a VUS if the available information had limited or contradictory evidence for pathogenicity.

Statistical analysis. Statistical analysis was carried out using SPSS software 21 (IBM Corp.). Data were expressed as numbers with percentages, the mean ± standard deviation or the median and interquartile range as appropriate. Measurement data were used analysis of variance and nonparametric statistics. The results were subsequently adjusted using the Bonferroni method. Qualitative data were used chi-square test. The areas under the receiver operator characteristic (ROC) curves were used to analyze the diagnostic precision of the optimal fasting C-peptide
for the pathogenic variant and no-variant groups. P<0.05 was considered to indicate a statistically significant difference.

The Regional Ethics Committee approved the study protocol, which was carried out in accordance with the World Medical Association Code of Ethics of the Declaration of Helsinki for experiments involving humans. Each participant or responsible adult signed an informed consent form.

**Results**

**Patients.** Out of the 32 cases of antibody-negative diabetes, 21 had possible related variants (Tables I and II), 11 cases presented with no mutation related to diabetes. A total of 11 genes were associated with diabetes, out of which 4 were pathogenic, 3 likely benign and 4 of VUS. A total of 12 genes were associated with susceptibility to diabetes (Table II).

Table III. Genes associated with monogenic diabetes.

| Proband | Gene | Mutation(s) by nucleotide | Mutation(s) by amino acid | ACMG | Disease |
|---------|------|---------------------------|---------------------------|------|---------|
| N2      | HNF4A| c.427-4G>A                | -                         | -    | MODY1   |
| N4      | CEL  | c.115G>A                  | V39I                      | Likely benign | MODY8 |
| N6      | BLK  | c.659G>A                  | C220Y                     | Uncertain significance | MODY11 |
| N6      | INS  | c.217G>T                  | G73C                      | Uncertain significance | MODY10 |
| N8      | KLF11| c.709A>G                  | K220E                     | Uncertain significance | MODY7  |
| N11     | ABCC8| c.1730_1741dup            | -                         | Likely pathogenic | MODY12 |
| N14     | HNF1B| c.1395c>G                 | S465R                     | Pathogenic | MODY5  |
| N15     | CEL  | c.115G>A                  | V39I                      | Likely benign | MODY8 |
| N17     | ALMS1| c.3252 G>T                | Q1084H                    | Likely benign | Alstromsyndrome |
| N17     | ALMS1| c.3601 G>T                | Y1201H                    | -    | -       |
| N18     | HNF1B| c.494G>A                  | R165H                     | Pathogenic | MODY5  |
| N19     | KCNJ11| c.602G>A                  | R201H                     | Pathogenic | NDM    |
| N21     | GCK  | c.593A>T                  | D198V                     | Pathogenic | MODY2  |

ACMG, American College of Medical Genetics and Genomics; MODY, maturity-onset diabetes of the young; NDM, neonatal diabetes mellitus.

Comparison of the clinical data. Table IV shows the genetic screening results. No significant differences were observed in the onset time, age and family history between the group with
pathogenic variants (MODY, 3 cases; NDM, 1 case; T2DM, 3 cases) (male, 4 cases; female, 3 cases) and the control group. The fasting C-peptide, diabetic ketoacidosis (DKA) incidence and insulin dosage were significantly different between the pathogenic variant and control groups (P<0.05). Fasting C‑peptide and glycated hemoglobin (HbA1c) at first visit, as well as insulin dosage, were significantly different between the no-variant and control groups (P<0.05). Fasting C-peptide, DKA incidence and HbA1c at first visit, as well as insulin dosage, were significantly different between the pathogenic variant and no-variant groups (P<0.05).

**ROC analysis.** ROC analysis of the pathogenic variant and no-variant groups revealed that the optimal fasting C -peptide cut-off value for predicting diabetic which need to detect gene was 0.64 ng/ml, with a specificity of 85.7%, sensitivity of 92.7% and AUC of 0.735 (Fig. 1).

**Discussion**

In the present study, fasting C-peptide levels were detected in three groups of patients with childhood diabetes: A pathogenic variant group, a group with no pathogenic variant and an islet autoantibody-positive control group (Table IV). Fasting C-peptide levels were increased in the pathogenic variant (0.89 ng/ml) and no-variant groups (0.48 ng/ml) compared with the control (0.17 ng/ml), indicating that the autoantibody-negative groups had a better islet function than the control, which is consistent with the results of Michels et al (16). The ROC curve indicated that C-peptide was sensitive and specific in the autoantibody-negative group, and can be used as a prognostic indicator for gene detection in such patients.

DKA is considered a typical manifestation of T1D. With a previous report of DKA in MODY and T2DM, it is now believed that DKA can occur in all forms of diabetes (17). In the present study, an NDM case exhibited DKA and hyperglycemic hyperosmolar status, but the pathogenic variant group had a lower DKA incidence than the other groups (14 vs. 75 vs. 83.33%), suggesting that DKA incidence is lower in non-T1D cases. In the present study, there were 2 cases of MODY5 in the pathogenic variant group. The combined number of cases may have affected the results. Some monogenic types of diabetes, such as HNF4A- and HNF1A-MODYs, are associated with macrosomia, and others, such as HNF1B-, INS- or GCK- (when inherited from the father) MODYs are associated with a low birth weight. Birth weight is therefore not a useful marker to distinguish monogenic from other forms of diabetes (18).
Among the three groups, patients in the pathogenic variant group used less insulin (0.14±0.24 U/kg vs. 0.51±0.30 U/kg vs. 0.68±0.21 U/kg). Pörksen et al. (19) also believed that patients with autoantibody-positive diabetes presented with a more severe islet \( \beta \) cell injury and a higher insulin dose than those with autoantibody-negative diabetes. A lower HbA1c was observed in the pathogenic variant group, as compared with the other groups, indicating that these patients had better blood glucose levels than those in the other two groups. This may be linked to poor islet function in T1D and high blood glucose at initial diagnosis.

In recent years, an increasing number of ketoacidosis cases without precipitating cause have been reported in children and adults with T2DM (17). It is difficult to determine the type of diabetes in the initial diagnosis of these people. They were overweight and even had DKA. However, insulin secretion and insulin action were significantly impaired in patients with DKA. After a period of treatment, \( \beta \) cell function and insulin sensitivity were improved, and insulin treatment was stopped within a few months of follow-up (20-22). The present study records 3 cases of this atypical T2D. In the initial diagnosis of these people, it was difficult to determine the type of diabetes they presented with. Through the analysis of gene results and family history, as well as the monitoring of islet function, 3 children were treated with diet and medicine and they were not treated with insulin in the follow-up for several months.

GCGR is a candidate gene for T2DM (23). The Gly40Ser variant of GCGR results in the substitution of serine for the 40th amino acid glycine in the encoded protein, reducing the affinity of the receptor agonist and cyclic adenosine monophosphate synthesis by the target cell. Gly40Ser variants lead to a decline in receptor function, which, in theory may be

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Table IV. Comparison of the clinical data of the pathogenic mutation group, no-mutation group and control group.

| Data                              | Pathogenic mutation group (n=7) | No-mutation group (n=11) | Control group (n=32) | \( \chi^2/F/Z \) value | P-value |
|-----------------------------------|---------------------------------|--------------------------|----------------------|------------------------|---------|
| Onset time (week)\( ^{d,e} \)    | 4.00 (4.00, 5.20)               | 4 (0.88, 13.25)          | 2.00 (2.00, 4.00)    | 2.50                   | 0.29    |
| Birth weight (g)\( ^{d,e} \)     | 2.978.57±522.70                 | 3.195.00±404.45          | 3.403.23±379.46      | 4.20                   | 0.02    |
| Onset age (year)\( ^{d} \)       | 9.86±5.00                      | 8.80±4.13                | 7.29±4.26            | 1.01                   | 0.37    |
| BMI (kg/m\(^2\))\( ^{d} \)       | 18.01±3.50                     | 16.60±2.39               | 16.39±1.44           | 4.70                   | 0.11    |
| Fasting C-peptide (ng/ml)\( ^{d} \) | 0.89 (0.61, 2.90)               | 0.48 (0.38, 0.88)        | 0.17 (0.05, 0.37)    | 24.74                  | <0.01   |
| Daily insulin dose (IU/kg)\( ^{d,e} \) | 0.14±0.24                      | 0.51±0.30               | 0.68±0.21            | 13.77                  | <0.01   |
| DKA, n (%)\( ^{e,g,h} \)         | 1 (14.29)                       | 8 (72.73)                | 25 (78.13)           | 10.90                  | <0.01   |
| Family history, n (%)\( ^{d} \)  | 4 (57.14)                       | 3 (27.28)                | 11 (34.38)           | 1.41                   | 0.49    |

\(^a\)Peripheral blood DNA was extracted from the 32 patients with autoantibody-negative diabetes and their parents for high-throughput sequencing of glucose metabolism-related genes. Pathogenicity analysis of the candidate mutated site has careful consideration of the patient's clinical presentations and sequencing result base on Standards and Guidelines for the Interpretation of Sequence Variants revised by ACMG. A total of 32 Patients with autoantibody-negative diabetes were divided into pathogenic mutation group (n=7), variant of unknown significance group (n=14) and no-mutation group (n=11) based on the results. Variant of unknown significance group is not in Table IV. \(^b\)This category included children with no candidate gene found. \(^c\)The control group comprised 32 patients with typical childhood type 1 diabetes. \(^d\)Onset time means the time elapsed from onset of symptoms to blood collection (expressed in weeks). \(^e\)Data were analyzed by Kruskal-Wallis test followed by Bonferroni's test. \(^f\)Data were compared using analysis of variance. \(^g\)Post hoc tests were performed and the results were adjusted by using the Bonferroni method. \(^h\)DKA indicates diabetic ketoacidosis. \(^i\)P<0.01 vs. the Control group; \(^j\)P<0.01 vs. the Pathogenic mutation group.
associated with insufficient insulin secretion in T2DM, ultimately leading to increased blood glucose. Hansen et al. (24) found that glucagon-mediated insulin secretion was decreased in murine islet cell tumors with GCGR variants, suggesting that the Gly40Ser variant may lead to islet ß cell dysfunction (25). In the present study, a pathogenic variant, i.e., Gly40Ser, was found in the GCGR gene of a girl. She suffered from mental retardation and her mother and grandmother had diabetes. At initial diagnosis, her fasting C-peptide levels were 2.77 ng/ml and her BMI was 20.8 kg/m². The mother, who had T2DM, had the same variant. After the patient had been admitted to hospital, insulin was used to control her blood glucose and she was changed to metformin to control her blood glucose levels and T2DM. This indicated that GCGR is associated with T2DM in the Chinese population.

Variants in transcription factors expressed in pancreatic ß cells are a major cause of MODY. A de novo mutation was detected in HNF1B, namely R165H and kidney ultrasound revealed a renal cyst. This variant has been reported previously and it is pathogenic (14,26,27). The patient's daily dose of insulin was 0.62 U/kg and they had no renal function. Due to the presence of long-term kidney disease in MODY5, patients had a long-term renal function follow-up to ensure prompt intervention in case of problems.

Another example of an HNF1B gene variant is the S465R variant. The S465 site of the HNF1B gene was highly conserved. HNF1B (S465R) has been detected in 2 Japanese patients with diabetes. Furuta et al. (13) verified that the S465R variant decreases HNF1B gene activity and proposed that these 2 patients may have had T2DM instead of MODY5. Several studies have also shown that dyslipidemia and insulin resistance are features of MODY5, suggesting that insulin resistance is not an exclusion criterion for MODY5 (28,29). The patients in this article had the same loci, but the onset age was 12 years old, there was no insulin resistance, the patient was not overweight (BMI of 17.2 kg/m²), kidney function was normal at initial diagnosis, oral medication controlled blood glucose and the fasting C-peptide levels decreased to 0.61 ng/ml in the second year. Continuing deterioration in ß cell function led to diabetes and the need for insulin treatment. Patients with MODY5 may therefore be misdiagnosed as having T2DM.

In addition to screening for common monogenic diabetes genes, the genes involved in glucose metabolism were also examined. The LIPC gene that regulates lipid and lipoprotein metabolism may be a potential candidate gene for T2DM (30). It was reported by Chiu et al. (31) that in mice, LIPC knockout protected against obesity but did not affect glucose homeostasis. González-Navarro et al. (32) induced dyslipidemia in LIPC knockout mice receiving a high-fat diet and demonstrated that LIPC deficiency promoted steatosis and glucose intolerance. In the present study, a frameshift mutation of LIPC was detected in a patient with ketoacidosis. The patient had a random blood glucose of 24 mmol/l, HbA1c of 7.3%, BMI of 17.2 kg/m² and fasting C-peptide of 0.69 ng/ml at first visit, as well as normal triglycerides and low- and high-density lipoprotein cholesterol. Currently (3 years after diagnosis), the patient's fasting C-peptide levels have been reduced to 0.01 ng/ml. She requires insulin to control her blood glucose. LIPC markedly affects protein function and is related to glucose and lipid metabolism. Through association analysis, the literature supported the association between LIPC and T2DM, but the specific mechanism is unknown. Correlation analysis, functional testing and verification of a large sample is required.

The inclusion criteria for the control were selected based on a combination of clinical features and 4 auto-antibody assays. The possibility of misdiagnosing patients with rarer forms of monogenic diabetes cannot be completely excluded, as patients may carry variants in other known DM genes not tested in this study, or in a gene not yet identified as a monogenic cause of diabetes. The authors' next study will focus on genetic testing for the control group. The new genetic variant loci discovered also require further functional verification. VUS verification in the cells and mouse model is currently being performed by the present team.

In conclusion, the present study showed that targeted next-generation sequencing is necessary for identifying antibody-negative diabetes. Monogenic diabetes is uncommon in children with antibody-negative diabetes, but attention should be paid to the presence of monogenic diabetes in antibody-negative diabetes. Long-term, large-scale studies are also required for the evaluation of the clinical value of C-peptide levels.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LC designed the study. XW and FW conducted the data collection. XW formulated the research question. XW and FW analyzed the data and wrote the first draft of the manuscript. TC, RX, HW, XC, DZ and HS contributed to the interpretation and discussion of the results and commented on the drafts. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent was obtained from all study participants and their parents in advance, and all procedures were performed in accordance with the Declaration of Helsinki and approved by the ethics committee of Children's Hospital of Soochow University.

Patient consent for publication

Not applicable.
Competing interests

The authors declare that they have no competing interests.

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