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ORIGINAL ARTICLE

Identification of causative gene variants for patients with known monogenic diabetes using a targeted next-generation sequencing panel in a single-center study

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Abstract

Aims We aimed to verify the usefulness of targeted next-generation sequencing (NGS) technology for diagnosing monogenic diabetes in a single center.

Methods We designed an amplicon-based NGS panel targeting 34 genes associated with known monogenic diabetes and performed resequencing in 56 patients with autoantibody-negative diabetes mellitus diagnosed at < 50 years who had not been highly obese. By bioinformatic analysis, we filtered significant variants based on allele frequency (< 0.005 in East Asians) and functional prediction. We estimated the pathogenicity of each variant upon considering the family history.

Results Overall, 16 candidate causative variants were identified in 16 patients. Among them, two previously known heterozygous nonsynonymous single-nucleotide variants associated with monogenic diabetes were confirmed as causative variants: one each in the *GCK* and *WFS1* genes. The former was found in two independent diabetes-affected families. Two novel putatively deleterious heterozygous variants were also assumed to be causative from the family history: one frameshift and one nonsynonymous single-nucleotide variant in the *HNF4A* gene. Twelve variants remained as candidates associated with the development of diabetes.

Conclusion Targeted NGS panel testing was useful to diagnose various forms of monogenic diabetes in combination with familial analysis, but additional ingenuity would be needed for practice.

Keywords Monogenic diabetes · Genetic analysis · Next-generation sequencing · Targeted panel sequencing · Childhood onset diabetes

Introduction

Monogenic diabetes is an uncommon form of diabetes mellitus caused by one or more defects in a single gene. It consists of many diseases that vary depending on the responsible gene, which have been classified into certain disease groups such as maturity-onset diabetes of the young (MODY), transient or permanent neonatal diabetes (TNDM or PNDM), and diabetes-associated syndromes [1, 2]. Many genes causative of monogenic diabetes have been identified so far; however, monogenic diabetes is not diagnosed in many cases because genetic testing for diabetes has not been commonly performed. Genetic analysis may help understand the pathological basis and provide prognostic or therapeutic suggestion based on previous findings on patients with monogenic diabetes.

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To detect causative gene variants of monogenic diseases, Sanger sequencing has conventionally been used. However, this approach has the weakness of limited throughput capacity, besides being time-consuming and laborious for sequencing a large number of targets. In recent years, massively parallel DNA sequencing has become possible via the development of next-generation sequencing (NGS). Among the NGS methods, targeted resequencing as a multigene panel testing is the most cost-effective tool for simultaneous and rapid analyses of multiple target genes of interest. These advantages of multigene panel testing make it an efficient option for comprehensive DNA sequencing for the genetic diagnosis of known monogenic diseases.

To date, a few studies have used a targeted NGS approach as an initial genetic screening for known monogenic diabetes [3–8]. In many of those studies, multigene panel tests contributed to diagnosing mainly common types of MODY, including MODY3 and MODY2. Meanwhile, the other rare types of MODY, PNDM, or monogenic syndromic diabetes were hardly targeted and rarely diagnosed by methods relying on in silico analysis. In addition, the age of onset of monogenic diabetes varies from the neonatal period to middle age, so diagnostic tests may be valuable for both childhood-onset and adult-onset patients with diabetes. However, most of the previous studies did not document the age of onset about patients who had been diagnosed as any monogenic diabetes and the usefulness of the testing for each age group remains unclear. Against this background, this study was established to use an NGS panel in practice and verify its clinical usefulness for diagnosing various monogenic diabetes in a single center.

Materials and methods

Patients

In this study, 56 unrelated patients with diabetes mellitus who visited Yamagata University Hospital between June 6th, 2017, and March 31st, 2020, were enrolled based on the following criteria: diagnosed with diabetes at < 50 years, and adults with body mass index (BMI) < 28 kg/m^2 or non-obese children. Patients with type 1 diabetes, pregnant women with diabetes, patients previously diagnosed with any form of monogenic diabetes, and patients with secondary diabetes whose cause was assumed not to be a specific genetic factor were excluded. Patients lacking a family history were included in this study because monogenic diabetes could be caused by de novo mutation or biallelic recessive variants.

The following clinical data were obtained from each patient: age at diagnosis of diabetes, age at genetic testing and diabetes duration, sex, BMI, treatment, symptoms, and diabetic microvascular and macrovascular complications. Biochemical parameters at genetic testing were measured in patients, such as fasting or postprandial plasma glucose, glycated hemoglobin (HbA1c), fasting serum C-peptide, and 24-h urinary C-peptide excretion. Oral glucose tolerance test or glucagon test was also performed on patients, if available.

Panel design and sequencing

The targeted NGS approach was performed using Ion AmpliSeq™ technology (Thermo Fisher Scientific, Cleveland, OH, USA) via amplicon-based sequencing. Pools of primer pairs as an NGS panel targeting 34 genes known to cause monogenic diabetes were created using the Ion AmpliSeq™ Custom DNA Panel (Thermo Fisher Scientific). Primers for all exons of the target genes and their flanking regions were designed using the Ion AmpliSeq™ Designer (Thermo Fisher Scientific). The target genes were as follows: 15 MODY genes (*HNF4A*, *GCK*, *HNF1A*, *PDX1*, *HNF1B*, *NEUROD1*, *KLF11*, *CEL*, *PAX4*, *INS*, *BLK*, *ABCC8*, *KCNJ11*, *APPL1*, *RFX6*), 14 non-MODY genes causing neonatal diabetes (*EIF2AK3*, *FOXP3*, *GATA4*, *GATA6*, *GLIS3*, *IER3IP1*, *MNX1*, *NEUROG3*, *NKX2-2*, *PAX6*, *PLAGL1*, *PTF1A*, *SLC19A2*, *ZFP57*), the *PCBD1* gene causing recessively inherited diabetes with *HNF1A*-like phenotypes, and 4 genes associated with some genetic syndromes with diabetes (*WFS1*, *INSR*, *TRMT10A*, *PPP1R15B*). The target size was 129,265 bases and total amplicon number was 927, theoretically covering 95.0% of the target region (Table 1).

Genomic DNA was isolated from patients' blood leukocytes. Target enrichment and NGS library construction were performed using Ion AmpliSeq™ Kit for Chef DL8 (Thermo Fisher Scientific), in accordance with the manufacturer's instructions. Sequencing was performed using the NGS platform of the Ion PGM™ System (Thermo Fisher Scientific).

Bioinformatic analysis

Sequencing reads were run through two independent variant calling pipelines: BWA-SW (http://bio-bwa.sourceforge.net/)–Platypus (https://www.rdm.ox.ac.uk/research/lunter-group/lunter-group/platypus-a-haplotype-based-variant-caller-for-next-generation-sequence-data) and Bowtie 2 (http://bowtie-bio.sourceforge.net/bowtie2/)–GATK UnifiedGenotyper (https://gatk.broadinstitute.org/hc/en-us). In this procedure, reads were aligned to the reference genome GRCh38.p2 (Genome Reference Consortium Human Build 38 patch release 2, https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.28) and variants such as single-nucleotide variants (SNVs) and insertions and/or deletions (indels) of single or a few nucleotides were called. Then concordant variants detected by the two pipelines were annotated using the ANNOVAR program (http://annovar.openbioinformatics.org/).

Table 1 Selected 34 genes for targeted NGS panel and overall coverage of targeted regions

Gene name	Chr	Number of exons	Targeted bases (bp)	Number of amplicons	Overall coverage (%)
MODY1-15					
HNF4A	20	15	5600	40	100
GCK	7	12	3193	26	91.3
HNF1A	12	11	3537	27	99.5
PDX1	13	2	2593	15	86.3
HNF1B	17	10	2899	21	100
NEUROD1	2	2	3022	20	100
KLF11	2	6	4356	27	95
CEL	9	11	2496	20	75.7
PAX4	7	9	2100	18	96.9
INS	11	6	664	7	100
BLK	8	13	2720	25	99
ABCC8	11	40	5314	57	99.5
KCNJ11	11	3	3589	20	80.9
APPL1	3	22	6280	49	90
RFX6	6	19	3697	32	100
Neonatal diabetes					
EIF2AK3	2	18	5013	37	98.9
FOXP3	X	12	2502	23	100
GATA4	8	10	3973	27	87
GATA6	18	7	3846	27	99
GLIS3	9	12	7887	49	99.7
IER3IP1	18	3	1534	10	91.9
MNX1	7	4	2654	15	81.6
NEUROG3	10	2	1275	8	89.6
NKX2-2	20	2	2100	13	96
PAX6	11	23	9627	65	97.1
PLAGL1	6	22	5816	40	98.2
PTF1A	10	2	1353	9	98.7
SLC19A2	1	6	3696	24	96
ZFP57	6	4	1910	13	100
Recessively inherited *HNF1A*-like diabetes					
PCBD1	10	5	1462	11	100
Other genetic syndromes with diabetes					
WFS1	4	9	3720	26	96.1
INSR	19	22	9581	64	98.8
TRMT10A	4	10	3960	32	94.8
PPP1R15B	1	2	5296	30	92.7

Chr chromosome, *MODY* maturity-onset diabetes of the young

Candidate causative variants were selected based on the following criteria: (1) variant allele frequency (VAF) lower than 0.005 in the East Asian population in the 1000 Genomes Database (http://www.internationalgenome.org/) and the Genome Aggregation Database (gnomAD) v2 or v3 (https://gnomad.broadinstitute.org/), and similarly in the Japanese genome reference panel Tohoku University Tohoku Medical Megabank Organization (ToMMo) 14KJPN (https://jmorp.megabank.tohoku.ac.jp/202112/variants); (2) non-synonymous SNVs predicted to be functionally deleterious by a Combined Annotation Dependent Depletion (CADD, https://cadd.gs.washington.edu/) phred-like score of ≥ 20 or null variants such as nonsense or frameshift variants in a gene where loss of function (LOF) is a known mechanism of disease.

Variant confirmation

Visualized sequence reads were counted using Integrative Genomics Viewer (https://software.broadinstitute.org/software/igv/) and it was confirmed that the variant was in a region with a read depth of at least 20×. To search for information about genomic variation and its relationship to phenotype/diseases, we referred to databases such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) or the Human Gene Mutation Database (HGMD®, http://www.hgmd.cf.ac.uk/). To confirm domains and important sites of proteins and predict the functional impact of each variant, we referred to databases such as UniProtKB (https://www.uniprot.org/help/uniprotkb) or InterPro (https://www.ebi.ac.uk/interpro/).

For segregation analysis, pedigree information was obtained from the patient's family, if available. NGS panel testing was also performed on the proband's relatives who had given informed consent and agreed.

The pathogenicity of each candidate causative variant was estimated in accordance with the American College of Medical Genetics and Genomics (ACMG)/Association for Molecular Pathology (AMP) clinical variant interpretation guidelines [9]. A known variant that had previously been reported to cause monogenic diabetes was determined to be the causative variant. A novel variant classified as "pathogenic" or "likely pathogenic" by ACMG/AMP guidelines was also assumed to be causative if the patient's clinical manifestations and segregation data were consistent with the LOF of the relevant gene. Sanger sequencing was performed to confirm the causative variants of patients and their relatives.

The overall workflow of the study pipeline is shown in supplementary Fig. S1.

Results

Clinical characteristics of the 56 participants are shown in Table 2. The median (range) age at genetic testing was 47 (4–75) years, and the age at the diagnosis of diabetes was 31 (3–49) years. Approximately one-quarter of all patients had been diagnosed with diabetes in their childhood or adolescence. The median BMI at genetic testing was 23 kg/m^2. Over 80% of the patients had a family history of diabetes.

Table 2 Clinical characteristics of 56 participants

Variables	n/56 (%) or median (range)
Male:female	31:25
Age at genetic testing (years)	47 (4–75)
Age at diagnosis of diabetes (years)	31 (3–49)
Patients diagnosed at < 20 years	14/56 (25.0)
Diabetes duration (years)	16 (0–40)
Family history of diabetes	47/56 (83.9)
BMI at genetic testing (kg/m^2)	23 (15–27)
FPG at genetic testing (mg/dL)	144 (81–329)
HbA1c at genetic testing (%)	6.8 (5.1–9.4)
Oral hypoglycemic agent use	36/56 (64.3)
Insulin use	29/56 (51.8)

BMI body mass index, *FPG* fasting plasma glucose, *HbA1c* glycated hemoglobin

All patients resided in Yamagata Prefecture, Japan; however, this was not necessarily their birthplace.

Overall, 82 synonymous SNVs, 89 nonsynonymous SNVs, and 1 indel were detected in the target regions for the 56 patients. After filtering by VAF and predicted deleteriousness, 15 nonsynonymous SNVs and 1 frameshift indel were selected as candidate causative variants.

Among these variants, 11 nonsynonymous SNVs identified in 11 patients had already existed in dbSNP (https://www.ncbi.nlm.nih.gov/snp/) regardless of whether they were clinically significant or not (Table 3). Regarding the phenotype–genotype relationships, Yorifuji et al. reported that a heterozygous nonsynonymous variant (NM_000162: c.1142 T > G [p.M381R]) in exon 9 of the *GCK* gene was supposed to cause MODY2 in Japanese patients based on in silico pathogenicity prediction and family segregation analysis [10]. In addition, Khanim et al. summarized various variants associated with Wolfram syndrome, including a heterozygous nonsynonymous variant (NM_001145853: c.2020G > A [p.G674R]) in exon 8 of the *WFS1* gene, based on previous case reports [11]. The other nine known nonsynonymous SNVs were supposed to have the possibility of affecting gene function, but there was no reported evidence of an association with diabetes mellitus.

The remaining four nonsynonymous SNVs and one frameshift indel found in five patients were novel variants (Table 4). None of these variants was found in any databases and their effects on phenotypes were unknown. However, these four nonsynonymous SNVs were in functionally important residues/domains of the proteins, so there was the possibility that the amino acid substitutions affected protein function. A novel heterozygous complex deletion-insertion (delins) variant [NM_000457: c.474_477delinsTGTCCT GCAGGACAGCAGGCTCCT (p.A158Afs)] in exon 4 of the *HNF4A* gene was identified, in which 4 nucleotides were substituted along with another 24 nucleotides showing modification of the reading frame. This frameshift variant resulted in gross protein rearrangements that might lead to protein dysfunction.

Clinical information of 16 cases with candidate causative variants is shown in supplementary Table S1 and pedigree trees of the cases are shown in supplementary Fig. S2.

After assessing the pathogenicity of each candidate causative variant considering the annotated information, and each patient's clinical manifestations and family history, four variants in five patients were classified as causative variants. These five patients were consequently diagnosed as having monogenic diabetes (cases 2, 3, 5, 12, and 15), as shown in Table 5.

Case 2 was a 4-year-old boy diagnosed with diabetes at the age of 3, and case 3 was a 14-year-old boy diagnosed with diabetes at the age of 14. Both of these cases were suspected to involve diabetes inherited in an autosomally dominant manner on the paternal side. They similarly showed mild fasting hyperglycemia with slightly low levels of C-peptide, whereas postprandial blood glucose levels were near-normal and C-peptide showed secretory responses in the glucagon test. These findings were consistent with typical clinical features of MODY2. A heterozygous *GCK* p.M381R variant identically detected in both cases was previously reported to be associated with MODY2, as mentioned above. Therefore, we diagnosed both case 2 and case 3 with MODY2. These two patients resided fairly close to each other, but we could not find any clear familial relationship between them.

Case 5 was a 52-year-old woman who had suffered from diabetes since she was 12 years old, and she had received multiple daily insulin injections for type 1 diabetes mellitus. She had no family history of diabetes. She was referred to the neurology department for evaluation of dysarthria, lightheadedness, cognitive impairment, and nocturia that had occurred around the age of 50. At that time, the previous diagnosis of type 1 diabetes mellitus was negated because the patient was negative for pancreatic autoantibodies. She had suffered from poor vision from the age of 42, the cause of which was found to be bilateral optic nerve atrophy, whereas she had no diabetic retinopathy. She was also diagnosed with sensorineural deafness, ataxia, and dementia associated with atrophy of brainstem and cerebellum, along with neurogenic bladder. Given the significantly reduced C-peptide, she was diagnosed as being insulin-dependent. These various symptoms generally match Wolfram syndrome. A heterozygous *WFS1* p.G674R variant was previously reported in several patients and families affected by Wolfram syndrome, in compound heterozygotes combined with another *WFS1* gene variant [12–14]. We could not find other candidate causative *WFS1* gene variants in this case,

Table 3 Patients with existing variants in dbSNP

Case	Gene	RefSeq Nucleotide Amino acid	dbSNP ID	1000G EAS VAF	gnomAD EAS VAF	ToMMo 14KJPN VAF	CADD Phred score	Clinvar value	Zygosity
1	ABCC8	NM_000352 c.2500C > T p.R834C	rs140068774	0.001	0	0.00166	35	LB	Het
2	ABCC8	NM_000352 c.2434G > A p.D812N	rs146916682	0.001	0.0008	0.00318	28.1	NA	Het
2, 3	GCK	NM_000162 c.1142 T > G p.M381R	rs193922266	NA	NA	NA	28.7	LP	Het (case 2) Het (case 3)
4	APPL1	NM_012096 c.1235G > A p.R412Q	rs199533180	0.001	0.0001	0.00428	23.8	NA	Het
5	WFS1	NM_001145853 c.2020G > A p.G674R	rs200672755	NA	0.0002	0.00011	24.4	P/LP	Het
6	GLIS3	NM_152629 c.1060G > A p.A354T	rs559065074	NA	NA	NA	23.9	NA	Het
7	NEUROD1	NM_002500 c.232G > C p.D78H	rs748959606	NA	0.00005	0.0005	25.1	NA	Het
8	NEUROG3	NM_020999 c.221A > T p.E74V	rs750686014	NA	0.0004	0.00173	25.2	NA	Het
9	MNX1	NM_005515 c.521C > T p.A174V	rs1268262063	NA	NA	0.00011	23.7	NA	Het
10	PCBD1	NM_000281 c.114 T > A p.F38L	rs1846573144	NA	NA	0.00028	23.6	NA	Hom
11	ABCC8	NM_000352 c.182A > G p.H61R	rs1848682183	NA	NA	0.00007	22.5	NA	Het

dbSNP the single nucleotide polymorphism database, *1000G* 1000 genomes project, *EAS* east asian, *VAF* variant allele frequency, *NA* not available, *gnomAD* the genome aggregation database, *ToMMo* tohoku university tohoku medical megabank organization, *CADD* Combined annotation dependent depletion, *LB* likely benign, *LP* likely pathogenic, *P* pathogenic, *Het* heterozygous, *Hom* homozygous

but the possibility remained that we missed some variants present in a region not covered by the NGS panel. Considering these findings, we diagnosed case 5 with Wolfram syndrome. We needed genetic analysis of the relatives including her parents and children of case 5 to distinguish whether the true genetic cause was any types of compound heterozygous *WFS1* mutations or aforesaid heterozygous *WFS1* p.G674R alone; however, because her father was dead and the mother and children had few relationships with her, we could not perform genetic testing with them.

Meanwhile, case 12 was a 12-year-old girl diagnosed with diabetes at the age of 12. She had a family history of diabetes on the maternal side over three generations. She showed significant postprandial hyperglycemia, although fasting blood glucose and C-peptide levels were almost normal. These findings resembled typical clinical features of MODY1 due to *HNF4A* gene mutation. A novel heterozygous *HNF4A* p.A158Afs variant was identified in this case, the clinical significance of which was unknown. This frameshift variant might disrupt the transcription factor hepatocyte nuclear factor 4 alpha (HNF-4-alpha) over a length of 316 amino acids including a ligand-binding domain, so it was expected to damage protein function. We also performed segregation analysis for case 12 and her parents and confirmed linkage of genotype and phenotype. We consequently diagnosed the proband with MODY1.

Case 15 was a 13-year-old girl diagnosed with diabetes at the age of 13. Her mother had been diagnosed with MODY1 at another facility when she was 18 years old. We could not obtain detailed information on the diagnosis of her mother,

Table 4 Patients with novel variants

Case	Gene	RefSeq Nucleotide Amino acid	CADD Phred score	Polyphen-2	SIFT	GERP + + RS score	Zygosity
12	HNF4A	NM_000457 c.474_477delinsTGTCCTGCAGGA CAGCAGGCTCCT p.A158Afs	NA	NA	NA	NA	Het
13	EIF2AK3	NM_001313915 c.1458G > A p.M486I	33	D	D	5.48	Het
14	GLIS3	NM_152629 c.590 T > G p.L197R	24.8	D	D	5.83	Het
15	HNF4A	NM_000457 c.391C > G p.Q131E	23.2	P	D	5.16	Het
16	PDX1	NM_000209 c.544A > G p.M182V	22.8	P	D	3.58	Het

CADD combined annotation dependent depletion, *NA* not available, *PolyPhen-2* polymorphism phenotyping v2 humDiv model, *D (Polyphen-2)* probably damaging, *P* possibly damaging, *SIFT* sorting intolerant from tolerant, *D (SIFT)* deleterious, *GERP + +* genomic evolutionary rate profiling, *Het* heterozygous

including of the *HNF4A* genotype. In biochemical analysis, plasma glucose and C-peptide levels were like those in case 12. A novel heterozygous *HNF4A* p.Q131E variant was identified, but predicted to be located in the DNA-binding domain of the nuclear receptor HNF-4-alpha and to change the acidity of the residue. Support for its predicted pathogenicity was also provided by several in silico algorithms based on conservation metrics or protein-level scores, such as Polymorphism Phenotyping v2 (PolyPhen-2, http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (SIFT, https://sift.bii.a-star.edu.sg/), and Genomic Evolutionary Rate Profiling (GERP + +, https://bio.tools/gerp). Considering these findings, we diagnosed case 15 as having MODY1 although it was unknown whether she and her mother had the identical *HNF4A* variant. To verify this problem, we planned to obtain informed consent for the NGS panel testing from the patient's parents; however, had not accomplished it.

Discussion

According to the American Diabetes Association's Standards of Medical Care in Diabetes, monogenic diabetes is estimated to account for up to 5% of all patients with diabetes [15]. Identification of each causative gene for patients with monogenic diabetes by genetic testing can provide insights into the pathophysiology of the disease and a reasonable therapeutic approach. Therefore, various in-house genetic testing methods to detect known causative gene variants for monogenic diabetes have been developed worldwide in recent years. However, NGS approaches can pick up massive numbers of variants regardless of their association with disease, and many of those variants generally have no definitive clinical significance. This sometimes makes NGS-based diagnosis of monogenic disorders difficult and may help explain why genetic testing for monogenic diabetes has not become common globally.

In previous studies, cases of monogenic diabetes diagnosed using NGS panel testing accounted for 6–25% of the total patients with non-autoimmune diabetes mellitus whose disease onset had occurred at infancy to young adulthood [3–8]. In these studies, targeted NGS panel testing mainly aimed at diagnosing MODYs, and the subjects were usually patients with diabetes with a strong suspicion of having MODY. However, in recent years, late-diagnosed cases such as those of some forms of MODY with middle-age onset [16–18] or insulin resistance syndrome of type A [19] were often reported. Although rare, pathogenic variants associated with neonatal diabetes could also be detected in cases with late-onset diabetes [20]. Thus, we anticipated that a certain number of adult-onset cases of monogenic diabetes would be identified from among the study participants. We detected 16 candidate mutations in the gene, but a definitive diagnosis could be reached in only 5 cases. Among most of these five cases, diabetes had developed via a dominant form of inheritance in their close relatives, so genealogical research could easily be conducted. NGS-based genetic diagnosis has an advantage that it enables estimation of the pathogenicity and clinical impact of genetic variants without in vitro/in vivo functional analysis or familial study. In some previous studies related to monogenic diabetes,

Table 5 Five cases with a confirmed diagnosis of monogenic diabetes with four causative variants

Case No	2	3	5	12	15
Sex	Male	Male	Female	Female	Female
Age at genetic testing (years)	4	14	52	12	13
Age at diagnosis of diabetes (years)	3	14	12	12	13
BMI (kg/m^2)	17	19	21	23	19
BMI-SDS	1.21	− 0.2	NA	1.39	− 0.2
Pharmacological treatment	None	None	Basal-bolus treatment	None	None
Complications of diabetes	None	None	None	None	None
Other complications/symptoms	None	None	Optic atrophy, deafness, ataxia, dementia, and dysuria	None	None
HbA1c (%)	6.6	7.1	8.4	7.6	9.4
FPG (mg/dL)	120	123	144	120	109
PPG (mg/dL)	117	147	450	218	NA
2-h PG during OGTT (mg/dL)	85	NA	NA	293	372
Fasting serum CPR (ng/mL)	0.45	0.78	0.2	1.88	1.9
Urinary CPR (μg/day)	31.1	37.6	1.2	145.7	156
CPR after glucagon load (ng/mL)	2.27	7.58	NA	5.33	4.9
Insulinogenic index	0.25	NA	NA	0.11	0.05
Variant	Heterozygous *GCK* p.M381R	Heterozygous *GCK* p.M381R	Heterozygous *WFS1* p.G674R	Heterozygous *HNF4A* p.A158Afs	Heterozygous *HNF4A* p.Q131E
Family history of diabetes (genotype compared with proband)	Father (Het), paternal grandmother (Het)	Father (Het), paternal grandfather (NA), paternal aunt (Het), paternal grandaunt (Het)	No	Mother (Het), maternal grandmother (NA)	Mother (NA), maternal grandfather (NA)
Parental consanguinity	No	No	No	No	No
Diagnosis	MODY2	MODY2	Wolfram syndrome	MODY1	MODY1

BMI body mass index, *BMI-SDS* body mass index standard deviation score (for < 18 years of age), *NA* not available, *HbA1c* glycated hemoglobin, *FPG* fasting plasma glucose, *PPG* postprandial plasma glucose, *2-h PG during OGTT* 2-h plasma glucose during an oral glucose tolerance test, *CPR* C-peptide immunoreactivity, *Het* heterozygote, *MODY* maturity-onset diabetes of the young

patients were successfully diagnosed by only in silico functional analysis and information on the clinical features [3, 4]. However, this approach leads to many variants whose association with phenotypes is unclear (variants of uncertain significance, VUSs) remaining and makes variant interpretation difficult. To solve this problem, familial segregation analysis provides additional important information to assess the genotype–phenotype association. In the current study, we were encouraged to examine the clinical presentation and pedigree information of each patient, taking advantage of the small scale and single-center nature of this study. Consequently, we could identify two novel *HNF4A* gene variants causative of MODY1 with the help of segregation analysis.

In our study, most of the mutations were family-specific or sporadic; in contrast, the *GCK* p.M381R variant was detected in two unrelated families, all of the members of whom lived in Yamagata. The mutation was identified in the proband by sequencing and confirmed in other family members (Supplementary Fig. S1). All patients had classic clinical parameters of MODY2 with no unusual findings. Interestingly, this mutation was reported several years ago [10] and was found in another family in the same area. Nevertheless, as in other MODY2 studies, almost all patients had a private family mutation. In all of our cases, the patients were from the local region, suggesting a founder effect, although haplotype analysis was not performed. Indeed, several reports of a founder effect in MODY2 have been published. The particular isolation of local communities in our study could have led to a narrow, population-specific spectrum of mutations. MODY2 is characterized by mild, stable, and asymptomatic hyperglycemia due to an elevated threshold for glucose-stimulating insulin secretion. Because patients with MODY2 rarely have microvascular or macrovascular diabetes complications, there is a consensus that

medical therapy is unnecessary for them, except during pregnancy, and it is not a major obstacle in terms of offspring preservation [21, 22]. Therefore, if a patient with MODY2 is found, it may be necessary to investigate other individuals in the region to consider whether a founder effect has occurred.

There are certain limitations to this study. First, for many selected candidate causative variants, it was difficult to determine their pathogenicity and they remained as VUSs because of an inability to demonstrate familial segregation. Segregation analysis would help in interpreting the genotype–phenotype correlation; however, particularly for adult participants, it was often difficult to contact the parents, resulting in a lack of family information. To solve this problem, we attempted to obtained missing data on patients and their families. Second, causative variants that were theoretically confirmed by a computational method do not always affect the pathogenesis of diabetes mellitus. The pathogenic effect of each candidate causative variant needs to be substantiated as much as possible by experimental biotechnology. As mentioned in ACMG/AMP clinical variant interpretation guidelines, variants that are well established by in vivo or in vitro functional studies supportive of a damaging effect on the gene are preferred evidence for NGS-based diagnosis. Third, we might miss some true pathogenic variants in undiagnosed patients because of technical limitations of the targeted NGS panel. Because GC-rich regions and repeats are difficult to enrich by PCR, sequencing coverage of our NGS panel could not reach 100% for all targeted regions. In addition, we have not analyzed other types of genomic alterations such as copy number variations or mitochondrial DNA mutations. We consider performing additional extensive genome analyses including whole-genome sequencing, especially for patients highly suspected of having a certain monogenic form of diabetes whose causative variant was negative in this study.

At the beginning of this study, we had expected to detect monogenic diabetes cases with various onset ages. However, contrary to our expectations, all of five diagnosed patients with monogenic diabetes had onset age of < 15 years as a result. Because recent investigations had shown that onset ages of some monogenic diabetes might be older than previously thought, we chose age at diabetes diagnosis < 50 years as inclusion criteria in this study; however, it might lead to lower pretest probability for monogenic diabetes of the participants. We will consider the optimal inclusion and exclusion criteria for participants to maximize diagnostic usefulness of our NGS panel testing.

In conclusion, we distinguished some monogenic diabetes cases from patients with childhood- and adolescent-onset non-autoimmune, non-highly overweight diabetes mellitus by resequencing 34 known causative genes using an NGS panel. Although it was sometimes difficult to confirm the pathogenicity of candidate variants only by computational prediction and database information, it was possible to extract some candidate genes in late-onset diabetes patients under 50 years old; in addition, segregation analyses supported by the interpretation of genotype–phenotype relationships resulted in the diagnosis of monogenic diabetes. Targeted NGS panel testing enabled us to efficiently perform genetic screening for various forms of monogenic diabetes and appeared to be useful to obtain additional genetic information.

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Author contributions All authors contributed to the study conception and design. The idea of the study was conceived by SS. Data collection was performed by YH, KN, NT, SK, WK, and CN. Data analysis was performed by HS. The first draft of the manuscript was written by KT and all authors commented on previous versions of the manuscript. KI supervised the project. All authors read and approved the final manuscript.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Human rights statement Name of institutional or national ethical committee on human experimentation. The Ethical Review Committee of Yamagata University Faculty of Medicine.

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Informed consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and/or with the Helsinki Declaration of 1964 and later versions. Informed consent or substitute for it was obtained from all patients for being included in the study. For patients between the ages of 5 and 18, written informed consent was obtained from each patient or their parents/legal guardians and informed assent was given for themselves.

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