ORIGINAL ARTICLE

Genetic testing for monogenic diabetes using targeted next-generation sequencing in patients with maturity-onset diabetes of the young

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KEY WORDS

monogenic diabetes, sequencing

ABSTRACT

INTRODUCTION Molecular diagnosis of monogenic diabetes mellitus is important for individualized patient care. Next-generation sequencing (NGS) enables a simultaneous analysis of multiple genes in a single test.

OBJECTIVES We aimed to assess the feasibility of using NGS for detecting mutations in a set of known monogenic diabetes gene mutations in a cohort of Polish patients with maturity-onset diabetes of the young (MODY) with earlier negative Sanger sequencing results for HNF1A-MODY or GCK-MODY.

PATIENTS AND METHODS We selected a panel of 28 chromosomal genes in which mutations have been reported to cause monogenic diabetes. The MiSeq platform was used for NGS. An exon-capture assay was designed to include coding regions and splice sites. A total of 54 patients with existing negative Sanger sequencing screening results for *HNF1A* or *GCK* gene mutations were selected for the study.

RESULTS NGS results were generated for all 54 patients and 9 positive controls with previously identified *HNF1A* or *GCK* gene mutation. All selected positive controls were confirmed by NGS. Among 28 genes, mutations were detected in 16. The type of the analyzed genetic changes was described in the NGS study as high (n = 3) or moderate (n = 76). Among the detected mutations, there were 4 known *GCK* gene mutations that had been previously missed in Sanger sequencing. So far, Sanger sequencing allowed us to confirm 21 gene mutations detected by NGS, and segregation with diabetes in 14 pedigrees.

CONCLUSIONS Our pilot study using NGS for monogenic diabetes screening in the MODY cohort confirmed that it improves the detection of diabetes-related sequence differences. The screening with NGS should also include diabetic patients for whom Sanger-based screening for particular subtypes of MODY provided negative results.

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INTRODUCTION Monogenic diabetes constitutes a heterogeneous group of single-gene disorders.[1-3] Its diagnosis helps understand the pathogenesis of the disease, defines the risk of diabetes within a family, and enables to modify treatment in a substantial number of patients. Optimal management of diabetes is essential to avoid chronic complications.[4] Maturity-onset diabetes of the young (MODY) is the most common type of monogenic diabetes. It constitutes a group of early-onset autosomal dominant forms of diabetes that together account for up to 2% of all

diabetic cases. Thus, we estimate that in Poland the number of patients with MODY is around 20 000 to 30 000. Most cases are usually diagnosed before the age of 25 years; however, many patients are identified in the fourth or fifth decade of life or even later.[1,2] Some patients with MODY may present with obesity, although it is not a typical feature. Consequently, the overlap of clinical characteristics between MODY, type 1 diabetes, and type 2 diabetes represents a challenge for differential diagnosis.[3] Moreover, the clinical phenotype in individuals with MODY may vary within the same pedigree.[1]

So far around a dozen genes have been reported to be responsible for MODY. Genetic testing has frequently relied on phenotype-guided screening for the most common MODY genes such as *HNF1A* and *GCK*. Therefore, molecular screening in many countries, particularly in those where genetic testing is not reimbursed by a national health care system, has been limited only to the 2 or 3 most common MODY genes. Owing to limitations both in funding and access to genetic diagnostic facilities, patients who test negative for mutations in these genes have been underdiagnosed and consequently have often received inappropriate therapy.[5] With the advent of high--throughput next-generation sequencing (NGS) technology, there has been an improvement in screening strategies enabling a simultaneous analysis of a panel of genes at a comparable cost to testing a few genes by Sanger sequencing.[6,7]

In this study, we aimed to assess the utility of NGS for detecting mutations in a set of known monogenic diabetes genes, using a cohort of Polish patients with negative results for *HNF1A*--MODY or *GCK*-MODY in Sanger sequencing screening.

PATIENTS AND METHODS A contact database of MODY families is maintained at the Department of Metabolic Diseases, Kraków, Poland, based on the following criteria: 1) autosomal dominant inheritance pattern of diabetes mellitus; 2) presence of the disease in at least 3 consecutive generations; 3) at least 2 diabetic family members diagnosed at the age of 30 years or earlier and treated for at least 2 years with diet, oral medication, or insulin at a dose lower than 0.5 U/kg. Details of the ascertainment protocol were described in previous papers.[8,9]

The current study group consisted of 54 probands who had been screened for mutations in the *HNF1A* and *GCK* genes by Sanger sequencing and showed negative results. Additionally, 9 positive controls in whom mutations in the *GCK* or *HNF1A* genes were previously identified by Sanger sequencing were included. The study was performed according to the Declaration of Helsinki, and was approved by the Bioethical Committee of the Jagiellonian University.

We performed basic clinical laboratory analyses, including the measurement of hemoglobin A_{1c} (HbA_{1c}), fasting glucose, and C-peptide levels. HbA_{1c} was measured by high-performance liquid chromatography (Bio-Rad, Hercules, California, United States). Fasting glucose levels were determined with the oxidase method. C-peptide levels were determined by an enzyme immunoassay. A diagnosis of diabetic microvascular complications—retinopathy and nephropathy—was established as described previously.[9] Genomic DNA was extracted from peripheral blood with a Maxwell Instrument (Promega, Madison, Wisconsin, United States). Libraries were prepared according to an established protocol.[7,10]

For target enrichment, we used a custom Agilent Sure Select exon-capture assay with baits designed to target exons and splice sites of 28 genes known to be associated with monogenic forms of diabetes such as MODY, neonatal diabetes, and lipodystrophy. Target genes were as follows: *ABCC8, BLK, CEL, EIF2AK3, FOXP3, GATA4, GATA6, GCK, GLIS3, HNF1A, HNF1B, HNF4A, IER3IP1, INS, KCNJ11, KLF11, LMNA, NEUROD1, NEUROG3, PAX4, PDX1, PPARG, PTF1A, RFX6, SLC19A2, SLC2A2, WFS1,* and *ZFP57*. The final captured library was sequenced on the MiSeq platform (Illumina, San Diego, California, United States) using 75bp paired--end reads.

Data were processed as follows: reads were aligned to the 1000 Genomes Project reference genome (GRCh37-derived reference sequence) using the BWA-MEM 0.7.5a algorithm. Duplicates were removed using SAMTools 0.1.19.[11] Realignment across indels and base quality recalibration were performed with GATK 3.1.[12] Variants were called using a Unified Genotyper and filtered using recommended hard filtering parameters (GATK Best Practices v4).[13,14] The SnpEff tool was used for variant annotation.[15]

Variants were annotated with chromosome location, genomic coordinate, reference and variant nucleotide, absolute number and percentage of reads containing variant nucleotide, reference and variant amino acid, coding frame, gene name, and overlap with a known single nucleotide polymorphism (SNP) from dbSNP 135.[16] The final variant filtering and prioritization was performed with GEMINI based on alternative allele frequency lower than 0.001 in the European American population in the Exome Sequencing Project and lower than 0.3 in the studied cohort, which was a lower threshold than the one typically used (0.2), owing to the small size of the group and the fact that there was more than 1 examined patient per family.[17] Only variants covered by 8 or more reads and with genotype quality over 19 were included in the final analysis. Variant impacts were divided into severity classes according to GEMINI mapping (https://gemini.readthedocs.org). Next, the results were sent for confirmation by the Sanger method. Direct sequencing was performed using labeled dideoxy-terminated nucleotides and 1 oligonucleotide primer. A computer analysis of the chromatograms obtained was conducted using the DNA Sequencing Analysis Software (Applied Biosystems, Foster City, California,

TABLE 1 Mutations identified by NGS and confirmed by Sanger sequencing in patients with diabetes

Proband's ID number	Sex	Age at diagnosis, y	Previous negative gene screening	Gene with currently identified mutation	Nucleotide change / three letter amino-acid description	Reference
M007-1	F	29	*HNF1A* and *GCK*	*GCK*	c.703A>G/p.(Met235Val)	Garcia-Herrero et al[27]
M013-1	F	23	*HNF1A*	*HNF4A*	c.724G>A/p.(Val242Met)	rs202105574, unpublished, HGMD®
				NEUROD1	c.308G>C/p.(Arg103Pro)	rs149703259, unpublished HGMD®
M038-1	F	23	*GCK*	*GCK*	c.491T>C/p.(Leu164Pro)	rs193922296 likely pathogenic (submitted to ClinVar NCBI, August 18, 2011)
M043-1	M	18	*HNF1A*	*GCK*	c.952G>A/p.(Gly318Arg)	Dusatkova et al[28]
M048-1	M		*HNF1A*	*GCK*	c.781G>A/p.(Gly261Arg)	Beer et al[29]
M050-1	F	33	*HNF1A*	*HNF1B*	c.1127C>T/p.(Thr376Ile)	not reported previously
				HNF4A	c.124G>A/p.(Gly42Arg)	Colclough et al[18]
M069-1	F	18	*HNF1A* and *GCK*	*GCK*	c.944T>A/p.(Leu315His)	Dusatkova et al[28]
M076-1	F	20	*HNF1A*	*GCK*	c.952G>A/p.(Gly318Arg)	Dusatkova et al[28]
M080-1	M	36	*HNF1A*	*GCK*	c.660C>A/p.(Cys220Term)	Ziemssen et al[30]
M095-1	F	27	*HNF1A*	*GCK*	c.1313T>C/p.(Phe438Ser)	Lorini et al[31]
M103-1	M	36	*HNF1A*	*GLIS3*	c.745G>A/p.(Gly249Arg)	not reported previously
M104-1	M	26	*HNF1A*	*PTF1A*	c.75C>A/p.(Asp25Glu)	rs376887768, unpublished, HGMD®
				HNF4A	c.1022T>G/p.(Leu341Arg)	Pearson et al[32]
M127-1	F	36	*GCK*	*GCK*	c.523G>A/p.(Gly175Arg)	Gloyn et al[33]
M137-1	F	45	*HNF1A*	*HNF4A*	c.299G>A/p.(Arg100Gln)	rs371557531, unpublished, HGMD®
M140-1	M	9	*HNF1A*	*GCK*	c.904G>C/p.(Val302Leu)	Osbak et al[34]
M144-1	F	15	*HNF1A* and *GCK*	*HNF4A*	c.401G>A/p.(Arg134Gln)	Harries et al[35]
M158-1	F	45	*HNF1A*	*GCK*	c.781G>A/p.(Gly261Arg)	Dusatkova et al[28]
M206-1	F	37	*GCK*	*HNF1A*	c.1594G>A/p.(Ala532Thr)	COSM935976, unpublished, HGMD®

Abbreviations: ID, identification; F, female; HGMD®, The Human Gene Mutation Database; M, male

United States). The Sequencer software version 4.1.4 and bioinformatics tools available online at http://genome.ucsc.edu were used for the submission and comparative analysis.

RESULTS Sequencing results were generated for all 54 patients and 9 positive controls with a previously identified *HNF1A* or *GCK* gene mutation. All previously identified sequence differences (Met57Thr, Leu315His, and Gly318Arg [in 2 patients] in the *GCK* gene and Gln109* [in 2 patients], Arg238Thr [in 2 patients], and Gly292Argfs*25 in the *HNF1A* gene) were found by NGS in the control group. High (n = 3) or moderate (n = 76) impact genetic changes in 16 genes were found in the study group.

So far, 21 mutations in 18 patients have been confirmed using the Sanger method (TABLE 1). None of the sequence differences selected for Sanger verification was a false positive finding. Among them, there were 11 mutations in the *GCK* gene, 1 in *HNF1A*, 5 in *HNF4A*, 1 in *NEUROD1*, 1 in *HNF1B*, 1 in *GLIS3,* and 1 in *PTF1A* (TABLE 1). The segregation of these variants with diabetes has been confirmed in 14 pedigrees.

First, we confirmed the segregation of 11 mutations detected in the *GCK* gene. Among them, there were 4 known amino-acid substitutions (Met235Val, Leu164Pro, Leu315His, and Gly175Arg) previously missed in Sanger sequencing and 5 earlier reported sequence differences (Gly318Arg, Gly261Arg, Cys220Term, Phe438Ser, and Val302Leu) identified in 7 patients screened earlier for the *HNF1A* mutation. At the time of the examination, 6 of these 11 probands were on diet, 1 was treated with insulin, and 4 were on oral drugs.

The mutation identified in the *HNF1A* (Ala532Thr) gene was previously described. The proband from this family was earlier diagnosed with gestational diabetes at the age of 37 years along with a relatively mild manifestation of hyperglycemia, which was treated only with diet. She was screened for the *GCK* mutation at the age of 41 years.

We also positively verified the segregation of the *NEUROD1* Arg103Pro mutation. The pedigree, consisting of 17 family members and 11 mutation carriers, has been reported in a separate paper (under review).

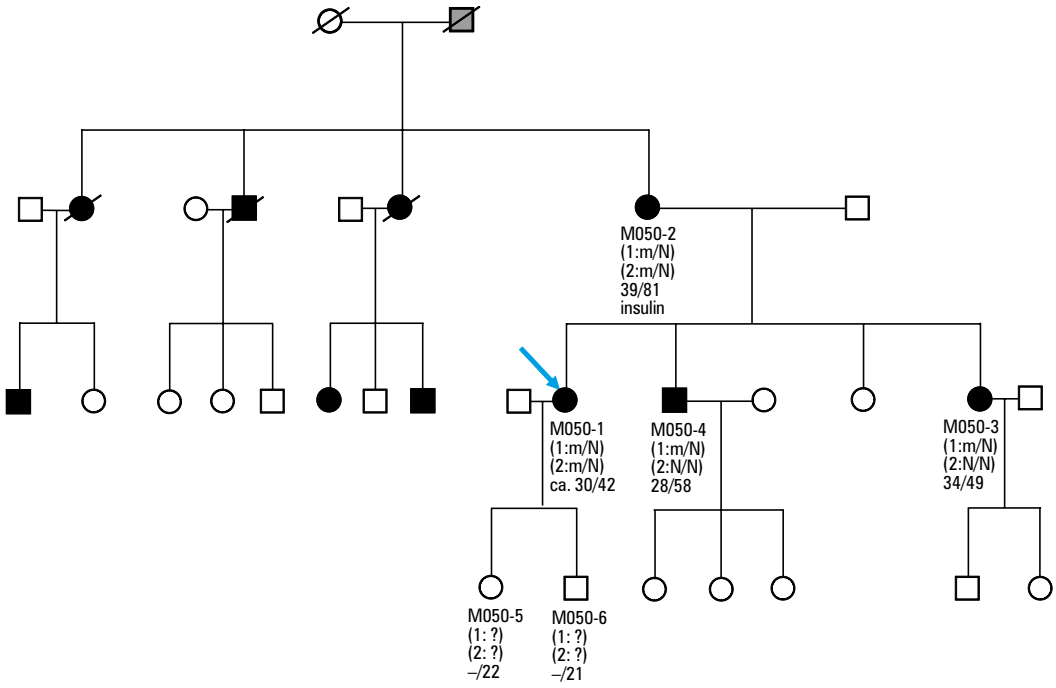

FIGURE 1 Pedigree and genotypes are shown. Segregation of the Gly42Arg in the *HNF4A* gene (1) and Thr376Ile in the *HNF1B* gene (2) mutations: black, grey, and open symbols represent subjects with diabetes, unknown status, and normal glucose, respectively. The numbers under the symbols are the identification numbers. Below is the genotype at codon 1 and 2: N, normal allele; m, mutant allele. Below the genotype is the age at diagnosis of diabetes for affected members and age at examination (years). An arrow indicates the index case.

The last mutation identified by NGS and verified by Sanger sequencing, and for which segregation has been confirmed, is the Gly42Arg substitution in the *HNF4A* gene in family M050 (FIGURE 1). Interestingly, the heterozygous mutation Thr376Ile in *HNF1B* was also identified by both methods in the proband of this family. This patient had been diagnosed with diabetes and added into our database at the age of 33 years. At the time of diagnosis in 2006, her body mass index was 23 kg/m^2 with no diabetic complications, and sulfonylurea treatment was introduced. The patient's DNA was scanned for *HNF1A* mutation with negative results. There were 3 more diabetic family members (a mother and 2 siblings) available for examination. Both siblings carried the *HNF4A*, but not the *HNF1B*, variant, whereas her mother carried both mutations (FIGURE 1). Clinical characteristics of the family members are provided in TABLE 2. The Gly42Arg substitution is localized between the dimerization domain and DNA-biding domain of the polypeptide. The most likely functional mechanism of this variant is related to a modified charge for this part of the transcription factor: glycine belongs to neutral amino acids while arginine is basic.[18,19] This mutation and the consequent sequence alteration have been reported to cause diabetes.[17] The Thr376Ile variant is localized in a transactivation domain of the transcription factor *HNF1B*.[20] This variant has not been reported so far and its pathogenic role is uncertain. Interestingly, a polar amino acid is substituted by a hydrophobic one.

DISCUSSION In the current study, we performed the NGS screening to detect mutations in a set of known monogenic diabetes genes using a cohort of Polish patients with previous negative Sanger sequencing screening results for *HNF1A*-MODY or *GCK*-MODY. To our knowledge, this study is the first of this type to investigate the clinical application of NGS in the diagnosis of diabetes in Poland.

Since an accurate etiological diagnosis of monogenic forms of diabetes has been proved to lead to a marked improvement both in patient care and family counseling, the use of cost-efficient, fast, and high-throughput methods for an accurate DNA sequencing is of major medical interest for contemporary diabetology. All mutations previously found by Sanger in our 9 positive controls were also confirmed by the NGS method, which demonstrated the diagnostic accuracy of the latter tool. Furthermore, 4 *GCK* mutations that had been previously missed by Sanger sequencing were subsequently identified by NGS in our study.

It has been reported that NGS is helpful in identifying variants that were earlier missed by traditional sequencing.[6] Our MODY database was established almost 20 years ago,[21] and over this period, our samples were screened in several collaborating laboratories in which quality control procedures and certification process have been rigorously introduced. Additionally, our screening with NGS resulted in improved diagnosis of MODY by detecting mutations in genes other

TABLE 2 Clinical characteristics of the examined M050 family members

ID number	Clinical status	Diabetes treatment	BMI, kg/m^2	Fasting glucose, mmol/l	C-peptide, ng/ml	HbA_{1c}, %
M050-1	diabetes	oral	28.27	11.78	2	7.6
M050-2	diabetes	insulin	31.25	6.94	1.5	7.2
M050-3	diabetes	oral	33.87	8.95	3	7.3
M050-4	diabetes	insulin	24.77	10.36	1.3	7.1
M050-5	no diabetes	NA	18.18	NA	NA	5.5
M050-6	no diabetes	NA	25	NA	NA	5.0

Abbreviations: BMI, body mass index; HbA_{1c}, hemoglobin A_{1c}; NA; not available; others, see **TABLE 1**

than *HNF1A* and *GCK*, which had previously been beyond our screening capabilities.[8]

A similar study conducted in England, which used NGS-based genetic screening of a 29-gene panel implicated in monogenic diabetes, has also shown an increase of 17% in the detection of mutations.[7] In the same study, 6 of 14 newly identified mutations were in rare monogenic diabetes genes that have not been screened owing to the absence of characteristic features of their subtypes. It is noteworthy that the number of patients presenting with monogenic diabetes is currently underestimated.[7] Shields et al[22] reported that more than 80% of MODY cases were undiagnosed. The use of NGS methodology will likely reduce this estimate rapidly. It is expected that this will be associated by a reduction in the financial cost of sequencing. We estimate that the cost of mutation screening in the current set of genes for 1 sample in this study was similar or even lower than the price for 1 gene (consisting of approximately 10 exons) search by the Sanger method. Based on the results assessed by the simulated model, it is expected that generalized screening for monogenic diabetes will become more cost-effective with advances in sequencing technology.[23]

While it is generally believed that findings from NGS should be confirmed by Sanger sequencing, this confirmation involves only specific exons with suspected sequence differences. Moreover, it is expected that NGS technology will become increasingly reliable with time. For example, the US Food and Drug Administration has already approved the MiSeq platform for cystic fibrosis mutation screening (http://www.illumina.com/clinical/diagnostics/miseqdx-instrument.html). There are also reports on other monogenic diseases, which show that targeted NGS sequencing is currently as reliable as the Sanger method.[24] In line with this, none of the variants selected by us for Sanger verification were false positive, unlike some earlier data generated by NGS, which contained such errors.[25] However, those previous data came from whole-genome sequencing research and included low-coverage data. Our research involved targeted sequencing in a relatively low number of genes with higher coverage. Additionally, we used a newer platform with high reliability. We also focused on the search for rare mutations in a limited cohort of probands from highly selected families. Finally, by using whole-exome search, new methodology will likely accelerate the identification of new genes that have not yet been identified as implicated in monogenic diabetes.

Interestingly, we found more than 1 highly or moderately suspected variant of the MODY gene in some probands, such as the one from pedigree M050. This will be explored in future studies to better understand the range of phenotypes found in affected pedigrees, including the vast majority with only a single mutation confirmed previously as segregating with supposedly monogenic diabetes. The ongoing progress in sequencing has made it clear that the phenotypes associated with so called monogenic diseases can be due to a set of penetrant mutations in several causative genes.[26] This could be the case in family M050 as a pathogenic role of the Thr376Ile in the *HNF1B* gene cannot be excluded. It is nevertheless challenging to interpret NGS results to define which detected mutation is clinically responsible for diabetes and other potential accompanying abnormalities. We believe that the assessment of all known susceptibility genes for monogenic diabetes by NGS will bring an improved and less biased analysis of the causes of the disease in the near future. As the final outcome should benefit the patient and possibly his or her family, the obvious challenge is to detect all putatively deleterious mutations that may be present in the same pedigree.

Our study has a few limitations. First, its novelty is limited as there were some earlier reports on the use of NGS in monogenic diabetes.[6,7,26] Additionally, it should be considered as a pilot study that includes mutations in the most common MODY genes and their segregation in the families that were available for prompt examination and lacked the Sanger confirmation of some mutations detected by NGS. We are currently performing this verification together with NGS screening for the rest of probands from our cohort. Further research is also needed to explore the pedigrees of all index cases.

In conclusion, we demonstrated the feasibility of using NGS for monogenic diabetes screening in routine genetic testing of our MODY cohort in

this pilot study. The screening should include patients with suspected MODY that had not been previously confirmed by Sanger-based screening.

Contribution statement MS was responsible for study design, protocol development, acquisition and interpretation of data, drafting the article, and writing the manuscript; ALG—for study design, acquisition, interpretation of data; PR—for acquisition and interpretation of data; JS—for study design and protocol development; BZ and TP—for acquisition and interpretation of data; TK—for acquisition of data; BKW, MB, and WM—for interpretation of data; PW—for revising the manuscript for important intellectual content and interpretation of data; MTM—for study design, project coordination, writing the manuscript, and approval of the final version of the manuscript.

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ARTYKUŁ ORYGINALNY

Badania genetyczne w kierunku cukrzycy monogenowej za pomocą metody sekwencjonowania nowej generacji u pacjentów z cukrzycą typu MODY

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SŁOWA KLUCZOWE

cukrzyca monogenowa, sekwencjonowanie

STRESZCZENIE

WPROWADZENIE Diagnostyka molekularna cukrzycy monogenowej jest istotna w ramach zindywidualizowanej opieki nad pacjentem. Sekwencjonowanie nowej generacji (*next-generation sequencing* – NGS) umożliwia jednoczesną analizę wielu genów w jednym teście.

CELE Celem badania była ocena możliwości wykorzystania metody NGS do wykrywania znanych mutacji genów związanych z cukrzycą monogenową w grupie polskich pacjentów z cukrzycą typu MODY (*maturity-onset diabetes of the young*) z uzyskanym wcześniej za pomocą sekwencjonowania sangerowskiego negatywnym wynikiem w kierunku *HNF1A*-MODY lub *GCK*-MODY.

PACJENCI I METODY Wybrano panel 28 genów chromosomalnych, w których opisano mutacje będące przyczyną cukrzyc monogenowych. NGS wykonano przy użyciu platformy MiSeq. Zaprojektowano zestaw sond, które pokrywały całe regiony eksomowe i regiony *splice site*. Do badania wybrano 54 pacjentów z wcześniejszym negatywnym wynikiem w kierunku mutacji w genie *HNF1A* lub *GCK* uzyskanym metodą sangerowską.

WYNIKI Otrzymano wyniki NGS u wszystkich 54 pacjentów i 9 pozytywnych kontroli z wcześniej zidentyfikowanymi mutacjami w genach *HNF1A* lub *GCK*. Wszystkie kontrole pozytywne zostały potwierdzone przez NGS. Wśród 28 analizowanych genów mutacje wykryto w 16. Charakter zmian genetycznych włączonych do analizy został opisany w badaniu NGS jako wysoki (n = 3) lub umiarkowany (n = 76). Wśród wykrytych mutacji zidentyfikowano też 4 znane mutacje w genie *GCK*, które nie zostały wcześniej wykryte w badaniu metodą Sangera. Jak dotąd potwierdzono sekwencjonowaniem sangerowskim 21 mutacji wykrytych za pomocą NGS, a segregację z cukrzycą w 14 rodzinach.

WNIOSKI Nasze badanie pilotażowe z wykorzystaniem NGS, jako metody przesiewowej w diagnostyce cukrzycy monogenowej w kohorcie MODY potwierdziło jej przydatność w lepszym wykrywaniu różnic w sekwencjach genetycznych związanych z cukrzycą. Badania przesiewowe z wykorzystaniem NGS powinny także obejmować pacjentów z cukrzycą, u których wcześniejsze sekwencjonowanie sangerowskie w kierunku pojedynczych podtypów MODY dały negatywne wyniki.

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