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ORIGINAL PAPER/PRACA ORYGINALNA

Endokrynologia Polska
DOI: 10.5603/EP.a2018.0064
Tom/Volume 70; Numer/Number 1/2019
ISSN 0423–104X

The importance of combined NGS and MLPA genetic tests for differential diagnosis of maturity onset diabetes of the young

Znaczenie skojarzonych badań genetycznych metodami NGS i MLPA w diagnostyce różnicowej cukrzycy MODY

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Abstract

Introduction: Maturity onset diabetes of the young (MODY) is a rare form of monogenic diabetes. Being clinically and genetically heterogeneous, it is often misdiagnosed as type 1 or type 2 diabetes, leading to inappropriate therapy. MODY is caused by a single gene mutation. Thirteen genes, defining 13 subtypes, have been identified to cause MODY. A correct diagnosis is important for the right therapy, prognosis, and genetic counselling.
Material and methods: Twenty-nine unrelated paediatric patients clinically suspected of having MODY diabetes were analysed using TruSight One panel for next-generation sequencing (NGS) and multiplex ligation-dependent probe amplification (MLPA) assay.
Results: In this study we identified variants in MODY genes in 22 out of 29 patients (75.9%). Using two genetic tests, NGS and MLPA, we detected both single nucleotide variants and large deletions in patients. Most of the patients harboured a variant in the *GCK* gene (11/22), followed by *HNF1B* (5/22). The rest of the variants were found in the *NEUROD1* and *HNF1A* genes. We identified one novel variant in the *GCK* gene: c.596T>C, p.Val199Ala. The applied genetic tests excluded the suspected diagnosis of MODY in two patients and revealed variants in other genes possibly associated with the patient's clinical phenotype.
Conclusions: In our group of MODY patients most variants were found in the *GCK* gene, followed by variants in *HNF1B*, *NEUROD1*, and *HNF1A* genes. The combined NGS and MLPA-based genetic tests presented a comprehensive approach for analysing patients with suspected MODY diabetes and provided a successful differential diagnosis of MODY subtypes. **(Endokrynol Pol 2019; 70 (1): 28–36)**

Key words*: MODY; NGS; MLPA, differential diagnosis*

Streszczenie

Wstęp: Cukrzyca MODY to rzadka forma cukrzycy monogenowej. Z uwagi na jej zróżnicowanie kliniczne i genetyczne często jest błędnie diagnozowana jako cukrzyca typu 1 lub 2, co prowadzi do zastosowania niewłaściwego leczenia. Cukrzyca MODY jest spowodowana mutacją pojedynczego genu. Zidentyfikowano 13 genów, których mutacje powodują 13 podtypów cukrzycy MODY. Postawienie prawidłowej diagnozy jest ważne, ponieważ umożliwia zastosowanie właściwego leczenia, określenie rokowania i poradnictwo genetyczne.
Materiał i metody: Używając panelu genów TruSight One do sekwencjonowania nowej generacji (NGS) i zależnej od ligacji multipleksowej amplifikacji sond (MLPA), zbadano 29 niespokrewnionych pacjentów pediatrycznych z podejrzeniem klinicznym cukrzycy MODY.
Wyniki: Warianty genów związanych z cukrzycą MODY zidentyfikowano u 22 spośród 29 chorych (75,9%). Za pomocą dwóch badań genetycznych, NGS i MLPA, wykryto u chorych zarówno warianty pojedynczego nukleotydu, jak i duże delecje. U większości chorych występowały warianty genu *GCK* (11/22) i (nieco rzadziej) genu *HNF1B* (5/22). Pozostałe warianty dotyczyły genów *NEUROD1* i *HNF1A*. Zidentyfikowano jeden nowy wariant w genie *GCK*: c.596T>C, p.Val199Ala. Zastosowane badania genetyczne pozwoliły wykluczyć cukrzycę MODY u dwóch chorych i wykazały warianty w innych genach prawdopodobnie związane z fenotypem klinicznym pacjentów.
Wnioski: W badanej grupie chorych z cukrzycą MODY większość wariantów wykryto w genie *GCK*. Warianty występowały również w genach *HNF1B*, *NEUROD1* i *HNF1A*. Połączenie badań genetycznych NGS i MLPA umożliwia kompleksową analizę u chorych z podejrzeniem cukrzycy MODY i jest skuteczną metodą w diagnostyce różnicowej podtypów cukrzycy MODY. **(Endokrynol Pol 2019; 70 (1): 28–36)**

Słowa kluczowe*: MODY; NGS; MLPA; rozpoznanie różnicowe*

Introduction

Maturity onset diabetes of the young (MODY) is a rare, monogenic type of diabetes that results from dysfunction of pancreatic β-cells. It is characterised by an onset of hyperglycaemia before 25 years of age, autosomal dominant inheritance, and some forms are insulin independent upon diagnosis. MODY is found in 1–2% of

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all patients with diabetes. Due to overlapping clinical features with the common types of diabetes, type 1 and type 2, MODY could be misdiagnosed [1–3].

MODY arises from changes in single genes important for function, regulation and development of β-cells, glucose sensing or interaction with insulin [4]. Until now genetic variants of 13 genes have been associated with MODY. The most affected MODY genes are *HNF1A*, *GCK*, and *HNF4A* followed by *HNF1B* [1, 4]. Each gene manifests a distinct clinical subtype. GCK-MODY subtype is unique because usually it does not require therapy and is not associated with the risk of microvascular complications [5]. In the case of HNF1A/4A-MODY subtypes, low doses of sulphonylurea provide better glycaemic control than treatment with insulin [6]. Renal impairment is a dominant clinical feature for HNF1B-MODY subtype, where insulin therapy is usually required [7, 8].

Distinguishing MODY diabetes from type 1 and type 2 diabetes, as well as determining the exact MODY subtype, has a great therapeutic and prognostic value for the patients. Patients with undiagnosed MODY diabetes are treated as type 1 or type 2 diabetes [9]. Consequently, they may receive unnecessary therapy or have difficulties in achieving a good glycaemic control [6, 9]. The clinical criteria, according to which MODY patients are recognised or distinguished from other diabetic patients, are not sufficiently sensitive and are overlooked in half of MODY cases [3]. Genetic testing can determine if a patient's diabetes has a genetic cause, but it can also provide a correct sub-classification of MODY patients and the best management option [10].

Here we aimed to genetically characterise clinically suspected MODY patients, evaluate the relative frequency of MODY subtypes and to examine the nature of variants in MODY genes of Serbian paediatric patients. We used two different methods to analyse genes relevant for MODY: next-generation sequencing (NGS) and MLPA (multiplex ligation-dependent probe amplification) assay. This comprehensive approach for detection of both single nucleotide variants and large deletion in MODY genes enables a successful differential diagnosis of MODY subtypes. Additionally, it provided the first steps for implementing genetic diagnosis of MODY in Serbia.

Material and methods

Subjects and clinical characteristics

Twenty-nine unrelated paediatric patients and 38 family members were included in this study. Patients were treated at the Children's University Hospital in Belgrade, Serbia. They all had atypical diabetes that did not fully satisfy criteria for diabetes type 1 or type 2. They met the criteria for a clinical diagnosis of monogenic diabetes: hyperglycaemia detected before the age of 25 years, presence of diabetes in the family, with autosomal dominant mode of inheritance, and in most cases insulin independence upon diagnosis of diabetes. Some patients had additional renal abnormalities. Clinical characteristics of patients included in the study are presented in Table I.

Table I. *Clinical characteristics of patients included in the study*

Tabela I. *Charakterystyka kliniczna chorych włączonych do badania*

Patients	**29**
Male/female	**23/6**
Diabetes	
Age [years, M ± SD (range)]	15.76 ± 3.96 (8.0–25.0)
Age at diagnosis [years, M ± SD (range)]	12.57 ± 3.74 (7.0–20.0)
Fasting glucose level [mmol/l]	6.69 ± 1.47
HbA_{1c} (%)	6.12 ± 0.84
BMI [kg/m^2]	20.21 ± 3.67
Family history of diabetes [yes]	21
Therapy	
Yes [Insulin/OHA]	6 (2/4)
No*	23
Other clinical features	
Renal malformations	9

Clinical parameters are presented as mean ± SD; OHA — oral hypoglycaemic agents; *patients without therapy or on diet management

The study was approved by the Ethics Committee of the Children's University Hospital, Belgrade and was performed in accordance with the ethical standards of the Declaration of Helsinki. Informed consent was obtained from all participants included in the study.

DNA Extraction and next-generation sequencing

The DNA was extracted from whole blood samples using a QIAamp DNA Blood Mini Kit (Qigen, Germany) following the manufacturer's protocol.

Sequencing of genes was performed using Illumina's TruSight One Sequencing Panel (Illumina, San Diego, CA) containing 4813 clinically relevant genes. This panel contains all reagents necessary for tagmentation, indexing, amplification, and enrichment of the samples (library preparation). Each step was carried out according to the manufacturer's instructions. The library was quantified on the Qubit 2.0 Fluorometer and sequenced on an Illumina MiSeq instrument (Illumina, San Diego, CA) using 150bp paired-end reads. The generated sequencing data were submitted for analysis if the data

passed the acceptance criteria for analytical runs according to the manufacturer's instructions.

Processing sequencing data

Sequencing data were aligned to the hg19 genome within Variant Studio Software (Version 2.2, Illumina Inc.) and filtered under conditions of heterozygous and homozygous mutations and frequency below 5%. The following genes were analysed: *HNF4A, GCK, HNF1A, PDX1, HNF1B, NEUROD1, KLF11, CEL, PAX4, INS, BLK, ABCC8,* and *KCNJ11.* Variants reported by the software as deleterious, damaging/probably damaging, or without any prediction were further inspected. They were checked in literature and public databases: HGMD database (public version), Ensemble (https://www.ensembl.org/), ExAc (http://exac.broadinstitute.org), dbSNP (http://www.ncbi.nlm.nih.gov/snp), and ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). Novel variants were evaluated *in silico* using online predicting algorithms: Mutation Taster (http://www.mutationtaster.org/), SIFT (http://sift.jcvi.org/), and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/). Additionally, variants were searched for in our population-specific TruSight One-derived variant database. Variants classified as tolerated or benign by the software were excluded from further analysis.

Validation of pathogenic variants

Variants recognised as pathogenic were validated by a standardised Sanger sequencing method (BigDye® Terminator v3.1 Cycle Sequencing Kit [Applied Biosystems®]). Primers were designed for each variant using the Primer Blast online tool (www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences and PCR conditions are presented in Tables II and III.

Table II. *Primers sequences and annealing temperatures for each PCR reaction*
Tabela II. *Sekwencje primerów i temperatura hybrydyzacji (annealing) każdej reakcji*

Gene	Exon		Primer sequence (5′ to 3′)	PCR product (bp)	Annealing Temp.* (°C)
GCK	3	F	TAG TCC CTT GTG CCT TCC CT	380	60
		R	CTC CCC ACC CCT GGT AGA CA		
	4	F	CAT TCA GTG GCC AGG TGT TG	320	59
		R	GGG GGC TAC ATT TGA AGG CA		
	5	F	GGG ACT CAG CCC TGC AGA AAT A	380	60
		R	TGG AAG CCA AGG AGA AAG GCA		
	6	F	AGG GCA TCC TTC TCA ACT GG	434	60
		R	ACC AGG CTC TGC TCT GAC ATC		
	7	F	TGA AGC AAC CCA GGT CTT CC	506	60
		R	GAG CAG AAG GGA TGG AGC TT		
	8	F	TGA ACC AGC TGG GGG AGT G	374	60
		R	GAG ACC AAG TCT GCA GTG CC		
	9	F	ATC GCC CCC ATT TCT CCA GAG	489	58
		R	ATC TTG GAG CTT GGG AAC CGC		
HNF1B	1	F	CGG GGA GTA ACA GGT GTC TG	596	58
		R	GGG ACT TCT CTG GTG GGA AAC		
	2	F	CCT CAT GTC TAC CCC AAA GTT G	484	58
		R	GGC CAA ATC TAC TTG CCA CC		
	4	F	TCT TCT CCT CGA GAG CCA CA	576	59
		R	AGA TCC GTG GCA AGA ACC AG		
HNF1A	1	F	GAG TTT GGT TTG TGT CTG CCG	521	60
		R	GGG GAC TCA ACT CAG AAG GG		
	4	F	TGC TCA CCC AAT TCG ATT CTC T	605	60
		R	GCA TGA ATG GAA TGG AAC CAA ACT		
NEUROD1	2	F	CGC AAG GTG GTG CCT TGC TAT TC	596	60
		R	GCA GCG GTG CCT GAG AAG ATT G		

Primer sequences and PCR temperature profile for Sanger validation of detected variants. PCR reactions were performed using QIAGEN Hot Start Taq® DNA polymerase kit (Germany) according to manufacturer's recommendations.

Table III. *Temperature profile for all reactions*
Tabela III. *Profil temperaturowy wszystkich reakcji*

Reaction	Temperaure	Time
Initial denaturation	95°C	15 min
Denaturation	95°C	30 s
Annealing	*°C	30 s
Extension	72°C	45–60 s
Final extension	72°C	10 min

Multiplex ligation-dependent probe amplification

SALSA P241-B MODY kit (MRC-Holland, Amsterdam, The Netherlands) for multiplex ligation-dependent probe amplification assay (MLPA) was used to detect genomic rearrangements in genes for most common subtypes of MODY diabetes: *HNF4A*, *GCK, HNF1A*, and *HNF1B*. MLPA was carried out using 120 ng of genomic DNA according to the manufacturer's instructions. Coffalyser® software was used for graphic and statistical analysis.

Results

In order to provide a genetic diagnosis for 29 clinically suspected MODY patients, two methods were used. Using the TruSight One panel for next-generation sequencing, all genes confidently likened to MODY diabetes were analysed. When there was a suspicion of a HNF1B-MODY phenotype MLPA assay was additionally used in order to identify large deletions as a potential cause of this subtype of MODY diabetes. MLPA assay was also used if no changes in MODY genes were found in patients by sequencing. By this approach we detected variants in MODY genes in 22 out of 29 patients (75.9%). Detected variants are summarised in Table IV. Sanger sequencing was used to validate detected variants, and false positives were not detected.

Table IV. *Variants in MODY genes identified by NGS and MLPA assay*
Tabela IV. *Warianty genów związanych z cukrzycą MODY wykryte za pomocą badań NGS i MLPA*

No. P.	NGS	MLPA	MODY gene	Exon	Type of sequence change	cDNA change	Protein change	References
1	✓		*GCK*	3	Missense	c.214G>A	p.Gly72Arg	[45, 46]
1	✓		*GCK*	4	Missense	c.446C>T	p.Thr149Ile	[16]
1	✓		*GCK*	5	Missense	c.533G>A	pGly178Glu	[47]
1	✓		*GCK*	5	Missense	c. 572G>A	p.Arg191Gln	[18, 48]
1	✓		*GCK*	6	Missense	c.596T>C	p.Val199Ala	Novel
1	✓		*GCK*	7	Missense	c.745G>C	p.Gly249Arg	[49]
1	✓		*GCK*	7	Missense	c.763A>G	p.Thr255Ala	[19]
1	✓		*GCK*	7	Missense	c.781G>A	p.Gly261Arg	[19, 50–52]
1	✓		*GCK*	7	Missense	c.812T>C	p.Leu271Pro	ClinVar database
1	✓		*GCK*	8	Missense	c.908G>T	p.Arg303Leu	[53]
1	✓		*GCK*	9	Missense	c.1148C>T	p.Ser383Leu	[45, 54]
1	✓	✓	*HNF1A*	1	Missense	c.293C>T	p.Ala98Val	[36, 55]
1	✓		*HNF1A*	4	Insertion	c.872dupC	p.Pro291fs	[32, 56–58]
1	✓		*HNF1B*	1	Missense	c.182T>G	p.Val61Gly	[59]
1	✓	✓	*HNF1B*	2	Deletion	c.477delT	p.Met160Terfs	[60]
1	✓		*HNF1B*	4	Missense	c.1006C>G	p.His336Asp	[61]
1	–	✓	*HNF1B*	1, 2, 3	Exon deletion	c.1-?_809+?del	p.Met1_Arg270del	[62]
1	✓	✓	*HNF1B*	3, 4	Exon deletion	c.545-?_1045+?del	p.Gln182_Ser384del	[62][63]
2	✓	✓	*NEUROD1*	2	Missense	c.590C>A	p.Pro197His	[43]
1	✓		*NEUROD1*	2	Missense	c.590C>A	p.Pro197His (hom*)	
1	✓		*NEUROD1*	2	Missense	c.750C>A	p.Ser250Arg	ExAc, dbSNP databases#

Sequence information is based on GeneBank reference sequences GCK: NM_000162.3; HNF1A: NM_000454.5; HNF1B: NM_0004585.2; NEURD1: NM_002500.4; #variants were found only in sequencing databases; No.P. — number of patients; hom* — variant in homozygous state; NGS — DNA samples analysed by Next generation sequencing method and TruSight One Panel; MLPA — DNA samples analysed by Multiplex Ligation-dependent Probe Amplification using SALSA P241-B probemi

Variants in GCK gene

In the *GCK* gene we detected 11 different variants, one in each of the patients. This presents 50% of all detected variants in different MODY genes in 22 patients. All variants in GCK gene were heterozygous missense variants dispersed in almost all exons. Most of them were found in exon 7. Variants were confirmed by Sanger sequencing in parents and in family members where samples were available.

One novel variant was found in the *GCK* gene p.Val199Ala (c.596T>C) in one patient. Levels of FBG and HbA_{1c} for this patient were 6.7 mmol/l and 6.2%, respectively. The variant was confirmed by Sanger sequencing in the mother who had mild hyperglycaemia. Prediction algorithms reported a damaging effect of the amino acid substitution on protein function for the variant p.Val199Ala. This variant was searched for in ExAc and dbSNP databases as well as in our population-specific TruSight One variant database (in-house database) and was not found in either one.

Variants in HNF1B gene

Heterozygous alterations in *HNF1B* gene were observed in 5/22 patients. Both nucleotide variants and large deletions were detected. All variants have been previously reported. The variant p.His336Asp was also found in our in-house database.

In this group, the average levels of FBG and HbA1c were almost normal, and body mass index was in the normal range. All patients except one (data unavailable) had renal abnormalities. Family history of diabetes was reported for three patients. One patient needed insulin therapy because of high blood glucose, two were on diet management, and two were without therapy.

Variants in HNF1A gene

Two heterozygous variants, one missense and one insertion (c.293C>T, p.Ala98Val, and c.GLy292ArgfsTer25, p.Pro291fs) were detected in *HNF1A* gene by NGS. The detected insertion was confirmed in other family members: mother and sister. The variant p.Ala98Val was detected in a patient who had kidney transplantation before the genetic test was performed. The patient was initially suspected to have a HNF1B-MODY phenotype, but no variants or large deletions were found in *HNF1B* gene. Variant p.Ala98Val was also detected in one sample of our in-house database.

Variants in NEUROD1 gene

In the remaining 4/22 patients two different variants were found, p.Pro197His, and p. Ser250Arg. Variant p.Pro197His was detected in three unrelated patients, and in one of them this variant was found in a homozygous state. The variant was also present in four samples of our in-house database. The variant p.Ser250Arg was reported only as sequence data in ExAc and dbSNP databases, and no data on the effect of this variant on clinical features in patients were reported. The variant is predicted to be probably benign by all predicting algorithms. Among analysed patients, it was found in one patient with a BMI = 27.8 kg/m^2 and FBG/HbA_{1c} level 6.0 mmol/l/6.1% and without therapy. No other variants in MODY genes were found in this patient.

Discussion

In patients with diagnosed diabetes but with clinical features atypical for diabetes type 1 or type 2, other rare types of diabetes should be considered, MODY being one of them. In patients with clinical characteristics suggestive of MODY diabetes, a genetic test could confirm or disprove the presumed diagnosis.

Next-generation sequencing technologies present an adequate method when there are larger numbers of genes to be analysed, as in the case of MODY diabetes [11, 12]. Here, using the TruSight One panel comprising of 4813 genes, we have created a "virtual" gene panel of 13 genes that were associated with MODY diabetes and screened patients with a suspected diagnosis of MODY diabetes. [13]. Additionally, introduction of the MLPA assay made it possible to detect large deletion in most common MODY genes in patients with specific phenotypes. This primarily refers to HNF1B-MODY subtype, where whole or partial deletions of the *HNF1B* gene were reported in half of all cases, while for other MODY types they are very rare [14, 15].

A change in the MODY gene was found in 22/29 patients (75%). Twenty different variants were found in common MODY genes, *GCK*, *HNF1A*, and *HNF1B*, and in *NEUROD1* gene. The dominant subtype in our group was GCK-MODY (50%), followed by HNF1B-MODY (22.7%).

GCK-MODY is caused by at least 600 different variants that are distributed across the gene [16]. In Italy, the Czech Republic, and Spain, where paediatric patients are routinely tested for blood glucose levels, GCK-MODY is more frequently detected than other types of MODY [17–19]. In our group, 11 patients carried a different variant in the *GCK* gene, of which 10 had been previously reported. Most patients carrying a variant in the *GCK* gene are asymptomatic and their mild hyperglycaemia is incidentally detected [17, 20]. Patients in our GCK group developed diabetes in early adolescence, at around 11 years of age. Their clinical phenotype corresponds to the phenotype of previously described patients with variants in *GCK* gene [20–22]. If not genetically confirmed, GCK-MODY diabetes can

be misdiagnosed and may be treated with unnecessary glucose lowering medications [21–23]. Confirmation of having GCK diabetes brings a relief to patients by knowing that their diabetes will not progress during life, and that they are at very low risk of developing microvascular complications [5, 16, 21, 23]. Also, awareness of having GCK-MODY diabetes may be important for women when planning a family [21]. During pregnancy, due to the risk of foetal macrosomia in unaffected babies, mothers may be treated with insulin to avoid possible complications [21, 24].

We have identified one novel variant, p.Val199Ala (c.596T>C), in the *GCK* gene. Based on the *in silico* prediction algorithms, SIFT, PolyPhen-2, and Mutation taster, the variant, p.Val199Ala (c.596T>C) is considered likely to be pathogenic. Additional facts support the *in silico* prediction: (I) low frequency of this variant because it was not found in the population databases ExAc and dbSNP nor in our population-specific TruSight One variant database; (II) the patient's phenotype matches the phenotype of GCK-MODY diabetes; (III) the variant confirmed in the mother with mild hyperglycaemia was inherited in an autosomal dominant manner, which is consistent with the inheritance mode of the disease; and (IV) a different amino acid substitution on the same codon, p.Val199Leu, was previously reported in GCK-MODY patients [16].

Alterations in the *HNF1B* gene were found in 5/22 patients. Single nucleotide variants as well as whole exon deletions were detected, which confirms the necessity of applying two methods when there is a suspicion of an HNF1B-MODY phenotype [25]. Patients may present with similar clinical features regardless of whether they carry a large deletion or single nucleotide variation in the *HNF1B* gene [25, 26].

Family history of diabetes and renal anomalies were observed in our patients. The average glucose levels were close to normal. This scenario, that renal malformations can occur prior to diabetes, has already been observed, especially in young patients, such as the ones in our HNF1B group, with an average age at diagnosis of 10 years [8, 15]. Results from a systematic review have reported that while renal malformations were observed in almost all *HNF1B* carriers under 25 years old, only 21% of them developed diabetes. These patients should be monitored for blood glucose levels because they are at high risk of developing diabetes later in life [27]. It is possible for *HNF1B* variant carriers to be free from insulin therapy or only use insulin sensitisers, as long as beta-cell function is preserved [28].

Patients with kidney malformations are candidates for the analysis of the *HNF1B* gene [29]. In our two patients with kidney malformations no changes in the *HNF1B* gene were found by sequencing or by MLPA assay. By using the TruSight One panel we were able to expand the list of analysed genes for these two patients. This enabled us to exclude HNF1B-MODY diabetes and to explore other genes that could be associated with the phenotype of these patients. A homozygous variant in *BB1* gene (NM_024649.4: c.951+1G>A) responsible for Bradet-Biedl syndrome [30] was detected in one patient, while in the other patient two heterozygous variants in *PKHD1* gene (NM_138694.3: c.2414C>T, p.Pro805Leu and c.9530T>C p.Ile3177Thr) were found. These *PKHD1* gene variants are responsible for the autosomal recessive polycystic kidney disease (ARPKD) [31]. Variants in both genes have been previously reported and associated with the related diseases [30, 31].

HNF1A-MODY is considered to be the most frequent type of MODY diabetes in the Netherlands and the United Kingdom [9]. According to a large study comprising more than 2000 probands with diagnosed diabetes, half of the detected variants were found in the *HNF1A* gene [7]. In our group we found two patients with variants in the *HNF1A* gene. The low number of these patients may be because of the difficulty recognising them, which leaves them misdiagnosed. HNF1A-MODY is usually misdiagnosed with diabetes type 1 [6, 32–34]. Additional tests for IA2 and GAD antibodies, or highly sensitive C-reactive protein (hsCRP), could help differentiate HNF1A-MODY from type 1 diabetes, but are not routinely performed [6, 33]. For these patients, the right diagnosis is crucial because it alters therapy. *HNF1A* variant carriers should be switched from insulin injections to oral sulphonylurea tablets because they are very sensitive to low doses of sulphonylurea. It is the first choice of therapy because it provides a better glycaemic control and reduces the risk of hypoglycaemia compared with insulin treatment [6].

One of the detected variants in the *HNF1A* gene was the most common variant, p.Pro291fs, present in 25% of all HNF1A variant carriers [20]. The other variant, p.Ala98Val, was reported previously and was not associated with monogenic diabetes [35, 36]. The striking clinical feature of the patient was that he had kidney transplantation. It has been previously reported that variants in the *HNF1A* gene may produce a phenotype with renal abnormalities, but very rarely [37]. Without functional studies or clinical presentation of other patients carrying this variant, it is difficult to evaluate whether this variant in the *HNF1A* gene was associated with the renal phenotype and/or monogenic diabetes, or it was a coincidental finding.

Variants in the *NEUROD1* gene associated with monogenic diabetes are very rare and have been reported in few families. Carriers of reported variants had diverse phenotypes. For most of them a higher

body mass index and later onset of diabetes was a common feature [38–42]. We detected two variants in the *NEUROD1* gene: p.Pro197His and p.Ser250Arg. The first variant, p.Pro197His, was detected in three patients both in homozygous and heterozygous state, but the state of zygosity of the allele did not manifest a difference in the clinical phenotype. Functional studies showed that p.Pro197His variant does not alter the transcription of the insulin gene and therefore its role in the pathogenesis of diabetes was excluded [43]. Variant p.Ser250Arg was reported only as sequence data and was predicted to be benign. Here it was detected in a slightly overweight patient with elevated glycaemia and without therapy. There are some indications that obesity may be the factor that triggers the development of diabetes in *NEUROD1* variant carriers [40]. Given that detected changes were not likely to be associated with diabetes, and that disease-causing variants were not found in other MODY genes, the exact cause of diabetes in these patients remains unknown.

This type of study was the first one conducted in our country, and it set the roots for implementing genetic testing of MODY diabetes into routine genetic practice. Accurate diagnosis will lead to improvement of patient care and management. Benefits of implementing this methodology can be seen on different levels. By sequencing all genes simultaneously, the rate of molecular confirmation of the diagnosis is higher than when requesting an analysis for a specific gene. The genetic test for a specific MODY gene is requested according to the observed phenotype and available clinical characteristics in patients [10, 44]. Due to clinical variability, the selection of an appropriate gene to test can be challenging, and if the presumption is wrong, it could lead to a false negative result. The consequence is not only the failure to identify the possible causative disease variant, but also additional costs for further analysis. As the cost of NGS sequencing continues to drop, it will become more available. This type of analysis does not only provide information for 13 MODY genes selected for the study, but for the entire clinical exome per patient, where in some cases it could be used to extend the initial gene list without further costs.

Conclusions

This study is the first to report molecular the genetic basis of MODY of Serbian paediatric patients. The results of this study provided two major conclusions. First, there is a need for a genetic test when clinical parameters indicate GCK-MODY diabetes. Conformation of this type of diabetes will provide appropriate care to patients and reduce healthcare costs due to unnecessary medications. Secondly, poor recognition of HNF1A-MODY indicates the necessity for additional non-genetic testing prior to genetic, in order to better distinguish from other patients with common types of diabetes.

Describing more patients and providing clinical characteristic will contribute to our present knowledge of MODY diabetes. Implementation of genetic testing in clinical practice will improve the care of MODY patients because it will be based on an individualised approach, save costs of unnecessary therapy, and offer genetic counselling for family members.

Acknowledgements

This work has been funded by grants from the Ministry of Education, Science, and Technological Development, Serbia (III 41004) given to S.P.

Conflict of interest

The authors have nothing to declare.

Authors contribution

J.K. — experimental: molecular analysis, data analysis, preparation of the manuscript; V.Z. — samples, clinical data and management of patients, critical review of the manuscript; S.S. — samples, clinical data and management of patients; M.J. — samples, clinical data; M.A. — experimental: molecular analysis, data analysis; S.P. — design of the study, coordination of the research, preparation of the manuscript; M.U. — design of the study, coordination of the research, preparation of the manuscript.

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