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CASE REPORT

DE NOVO KMT2D HETEROZYGOUS FRAMESHIFT DELETION IN A NEWBORN WITH A CONGENITAL HEART ANOMALY

Stangler Herodež Š[1,2], Marčun Varda N[2,3], Kokalj Vokač N[1,2], Krgović D[1,2,*]

[*]**Corresponding Author:** Assistant Dr. Danijela Krgović, Laboratory for Medical Genetics, University Medical Centre Maribor, Ljubljanska ulica 5, 2000 Maribor, Slovenia. Tel: +386-2-321-27-37. Fax: +386-2-321-27-55. E-mail: danijela.krgovic1@um.si

ABSTRACT

Kabuki syndrome (KS) is characterized by typical facial features and patients are also affected by multiple congenital anomalies, of which congenital heart anomalies (CHAs) are present in 28.0 to 80.0%. In approximately 75.0% of patients, the genetic causes of KS are caused by mutation in the *KMT2D* gene. Although KS is a well-characterized syndrome, reaching the diagnosis in neonates is still challenging. Namely, newborns usually display mild facial features; therefore the diagnosis is mainly based on congenital malformations. In our case, a newborn was referred for next generation sequencing (NGS) testing due to the prenatally observed CHA. After birth, a ventricular septal defect (VSD), vesicoureteral reflux, muscular hypotonia, cleft palate, mild microcephaly, and some dysmorphic features, were noted. The NGS analysis was performed on the proband's genomic DNA using the TruSight One Sequencing Panel, which enriches exons of 4813 genes with clinical relevance to the disease. After variant calling, NGS data analysis was predominantly focused on rare variants in genes involved in VSD, microcephaly, and muscular hypotonia; features observed predominantly in our proband. With the aforementioned protocol, we were able to determine the previously unreported *de novo* frameshift deletion in the *KMT2D* gene resulting in translation termination. Although our proband is a typical representative of KS, his diagnosis was reached only after NGS analysis. Our proband thus represents the importance of genotype-phenotype driven NGS analysis in diagnosis of patients with congenital anomalies.

Keywords: Congenital heart anomalies (CHAs); Genetics; *KMTD2* gene; Kabuki syndrome (KS); Next generation sequencing (NGS) analysis.

INTRODUCTION

Congenital heart anomalies (CHAs) are the most common birth anomalies, and syndromic CHAs represent approximately 25.0% of cases [1,2]. The introduction of molecular karyotyping (molecular chromosome microarrays) and later, next generation sequencing (NGS), in the diagnosis of syndromic CHAs, have enabled the identification of numerous microdeletion and microduplication syndromes as well as single gene disorders as a cause of congenital cardiovascular malformations accompanied by additional features [2]. Representative of the latter is the Kabuki syndrome (KS) with an estimated prevalence of 1/86,000 to 1/32,000 newborns [3]. Kabuki syndrome is a multiple anomaly syndrome characterized by five main features: postnatal growth retardation, dysmorphic facial features, skeletal anomalies, dermatoglyphic abnormalities, and intellectual disabilities (ID) [4]. According to the international consensus diagnostic criteria for KS, a diagnosis of KS can be achieved in probands at any age if the infantile hypotonia, developmental delay (DD) and/or ID was observed, and one or both criteria: molecularly confirmed pathogenic or likely pathogenic variant in causative gene and/or presentation of at least three typical dysmorphic features characteristics for KS at some point in life [4]. The prevalence of CHAs in KS ranges from 28.0 to 80.0% of patients [5].

In approximately 75.0% of patients, the genetic causes of KS are pathogenic or likely pathogenic mutations found in the *KMT2D* gene, which indicates this is the key gene for this syndrome. Additionally, 3.0 to 5.0%

[1] Laboratory of Medical Genetics, University Medical Centre Maribor, Maribor, Slovenia

[2] Medical Faculty, University of Maribor, Maribor, Slovenia

[3] Division of Paediatrics, University Medical Centre Maribor, Maribor, Slovenia

of patients carry causative variants in the *KDM6A* gene [6]. Isolated cases of KS have been reported for *RAP1A*, *RAP1B*, and Kabuki-like phenotype for *HNRNPK* genes [7,8]. Genotype-phenotype correlations showed that carriers of pathogenic or likely pathogenic variants in the *KMT2D* gene display more KS characteristic features and therefore, achieve a statistically significantly higher score using the phenotypic scoring system for KS [9]. Furthermore, the presence of some KS characteristic features, such as the presence of CHAs, differs in *KMT2D* and *KDM6A* mutation-positive patients [4,5]. Although the presence of CHAs ranges from 28.0 to 80.0% of KS patients, approximately 70.0% of patients with *KMT2D* pathogenic variants have some form of CHAs [5].

In newborns, a clinical diagnosis of KS is often challenging due to phenotype evolving over time. Namely, facial features are most evident in children between the ages of 3 and 12 years [4]. Newborns usually display milder facial features, therefore, the diagnosis is based more on congenital malformations [10]. Moreover, some of the observed features in newborns overlap with features in disorders such as the CHARGE syndrome, 22q11 deletion syndrome, IRF6-related disorders, *etc.*, indicating that careful phenotyping along with molecular diagnostics is still crucial in order to reach a proper diagnosis in infants [2,11].

Here, we report of a case of a 2.5-year old boy with CHA observed during pregnancy. Cytogenetic and molecular testing was performed prenatally to exclude aneuploidies and chromosomal structural variations, however, no discrepancies were noted. After birth, the proband was referred for NGS testing, primarily due to the observed CHA. In the first moths, additional features became more prominent in our proband, therefore, an assumption for a single gene genetic disorder was made. After a genotype-phenotype driven analysis, a *de novo* frameshift deletion in the *KMT2D* gene, not previously described, was determined and the proband was diagnosed with KS. To the best of our knowledge, this is also the first Slovenian patient with KS described in the literature.

MATERIALS AND METHODS

Case Presentation. The baby boy was born at 37 weeks' gestation to a healthy 25-year-old mother. The mother's family history revealed a grandmother who was born with duplex kidney, and a father with pyloric stenosis. The father's family history was uneventful.

During pregnancy, CHA was suspected in the fetus. To exclude aneuploidies or microdeletions or microduplication syndromes, the genetic analysis included classic karyotyping, an in-house developed quantitative fluorescent polymerase chain reaction (QF-PCR) method with 20 micro-satellite markers located on chromosomes 13, 18, 21, X and Y, molecular karyotyping with 8 × 60 K BlueGnome CytoChip Oligo (BlueGnome Ltd., Cambridge, Cambridgeshire, UK) and multiplex ligation-dependent probe amplification (MLPA) with the SALSA MLPA P036 probemix (MRC-Holland, Amsterdam, The Netherlands) was used. All the performed prenatal genetic tests were negative. Generally, the mother reported no problems during the pregnancy. The onset of labor was spontaneous and without any complications. The baby's birth weight was 3010 g (50th-90th centile for gestational age), birth length 49 cm (50th-90th centile), head circumference 31.5 cm (10th centile) and Apgar score was 8/9. After birth, perimembranous ventricular septal defect (VSD) and pala-toschisis were diagnosed and some dysmorphic features were noted. The male newborn had a depressed nasal bridge, arched eyebrows with long eyelashes, epicanthus, long palpebral fissures, and somewhat protruding earlobes. The skull had a biparietal narrowing and the occipital part was flat and high. In addition, general muscular hypotonia was present with very scarce spontaneous movements.

The hemodynamically important VSD was surgically treated after birth. Osteomyelitis of the sternum was identified as surgical complication, therefore, prolonged treatment with antibiotics was administered. Retention of the right testis was persistently present, bilateral sensorineural deafness as well as anomalies of spinal vertebrae were also diagnosed. Ultrasound examination revealed renal pelvis dilation on the left and a bilateral vesicoureteral reflux. Despite intensive neurophysiotherapy, the muscular hypotonia persisted and developmental milestones were delayed. In addition, physiological weight gain was constantly impaired. However, no seizures were noted and magnetic resonance imaging (MRI) showed no structural anomalies of the brain.

At 8 months, serious acute pyelonephritis with extended-spectrum β-lactamases (ESBL) producing *E. coli* was diagnosed. After initial treatment, antibiotic prophylaxis with nitrofurantoin was introduced. The boy also experienced frequent infections of the respiratory and gastrointestinal tract.

At the time we reached our diagnosis, the proband was 12 months old. He weighed 6.9 kg (below the 3rd centile), his height was 68 cm (below the 3rd centile), and his head circumference was 42 cm (below the 3rd centile). Dysmorphic features were more prominent, muscular hypotonia was still present and a significant DD became evident. He was able to roll over from his front to his back and *vice versa*, but unable to sit alone. He was also not yet talking. Neurophysiotherapy was implemented. Nephrological re-diagnostics was planned as well as the surgical treatment of palatoschisis. He was also followed-up by an orthopedic surgeon, urologist and otorhinolaryngologist.

Next Generation Sequencing. Genomic DNA was isolated from peripheral blood of the proband with the

QIAamp DNA mini Kit (Qiagen GmbH, Hilden, Germany) after obtaining informed consent from the proband's parents. A library was constructed using the TruSight One Sequencing Panel (Illumina Inc., San Diego, CA, USA), which enriches exons of 4813 genes with clinical relevance to the disease. Sequencing was performed on an Illumina MiSeq platform with pair-end sequencing in 2 × 150 cycles. Data analysis was performed using the on-instrument MiSeq Reporter software 2.5.42.5 (https://www.illu mina.com/systems/sequencing-platforms/miseq/products-services/miseq-reporter.html), according to BWA Enrichment workflow. Variant Studio (Illumina Inc.) software and open-access bioinformatic tools and databases were used for analysis and interpretation of variants obtained in the VCF file.

Sanger Sequencing. A potential causative variant determined by NGS sequencing was confirmed in the proband and excluded in the proband's parents by Sanger sequencing. Briefly, extension products of various lengths were terminated with dideoxynucleotides at the 3' end using GenomeLab DTCS-Quick Start Kit in accordance with the manufacturer's instructions (Beckman Coulter Inc., Indianapolis, IN, USA) and separated by capillary electrophoresis (CEQTM8000 Genetic Analysis System; Beckman Coulter Inc.).

RESULTS

Variants obtained after NGS sequencing were analyzed and interpreted according to the American College of Medical Genetics and Genomics (ACMG) guidelines [12]. First, common variants with an allele frequency of >1.0% according to public databases [Genome Aggregation Database (gnomAD), the 1000 Genomes Project (1KGP) and the Exome Variant Server (EVS)] were excluded from the analysis. The remaining variants were then analyzed, predominantly focusing on variants in genes involved in VSD (HP:0001629), microcephaly (HP: 0000252), and muscular hypotonia (HP: 0001252), features described as predominant in the proband. With the aforementioned protocol, we were able to determine the previously unreported frameshift deletion NM_003482.3: c.11093delG in the *KMT2D* gene resulting in translation termination. Using Sanger sequencing, we confirmed the variant in the proband (Figure 1) and determined that neither of the parents had this deletion (data not shown), thus, the variant was a *de novo* occurrence.

After reaching the diagnosis, the proband was reevaluated according to the international consensus diagnostic criteria for KS at age 10 months and 2.5 years [4]. Figure 2 presents mild facial feature of KS observed in our proband during his follow-up at age 2.5 years.

Figure 2. Mild facial features of KS were observed in our patient during his follow-up at age 2.5 years. A written informed consent was obtained from the patient's parents for publication of proband's pictures.

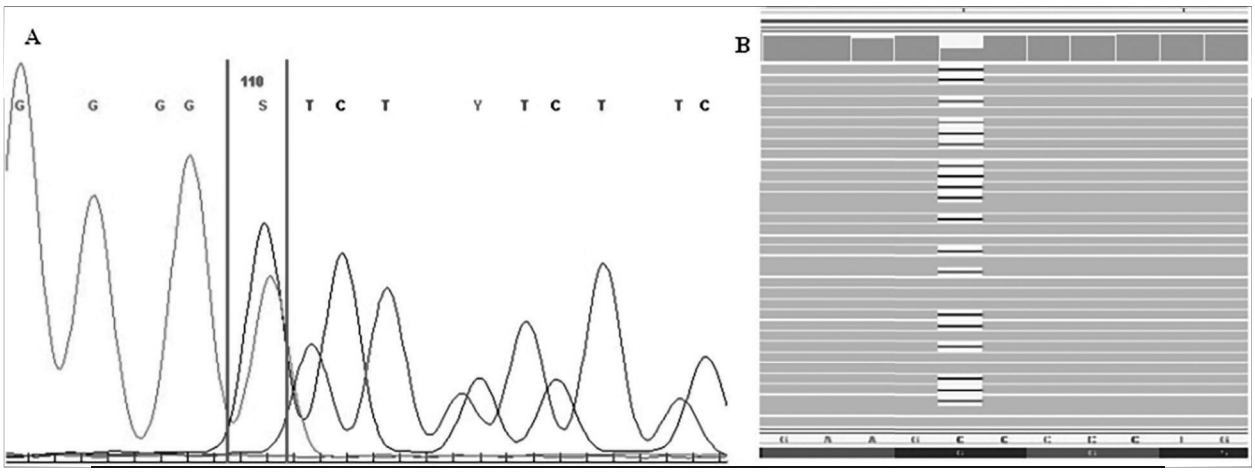

Figure 1. The detected *de novo* heterozygous frameshift deletion in the *KMT2D* gene. (A) Sanger sequencing confirmed a heterozygous frameshift deletion at NM_003482.3: c.11093delG in the *KMT2D* gene in the patient and excluded this deletion in the patient's parents (data not shown). (B) Presentation of the *de novo* heterozygous frameshift deletion in the *KMT2D* gene at position chr12: g.49427394GC>G (hg19). The deletion in our patient after running the NGS analysis is shown as black lines in empty space using Integrative Genomics Viewer (IGV).

Table 1 represents the most common phenotypic features observed in our proband compared to individuals with reported heterozygous pathogenic variant in the *KMT2D* gene. Table 2 summarizes the features observed in our proband at birth and after his follow-up, according to the KS phenotypic scoring system developed by Makrythanasis *et al.* [13], and Adam *et al.* [4].

Table 1. Comparison of features observed in our proband with the most commonly observed features in individuals with heterozygous pathogenic mutation in the *KMT2D* gene.

Phenotypic Features in Patients with *KMT2D* Mutations	**Features Present in Our Proband**
Intellectual disability (IQ <70)	not tested
Fetal fingertip pads	no
Congenital heart defect	yes
Long palpebral fissures	yes
Large, prominent or cupped ears	yes
Hypotonia	yes
Eversion of the lower eyelid	no
Arched or broad eybrows	yes
Cleft palate	yes
Brachydactyly	yes
Short columella with depressed nasal tip	yes
Short stature	yes
Microcephaly	yes
Oligodontia and/or abnormal incisors	no
Feeding difficulties	yes
Developmental delay	yes
Latent eyebrows, sparse or notched	yes
Hearing loss	yes
Non traumatic joint dislocation	no
Hypogammaglobulin or low serum IgA	yes
Hyperinsulinemic hypoglycemia in infancy	no
Lip pits	no
Malpositioned kidneys	no
Idiopathic thrombocytopenia purpura (ITP)	no
Hypospadias in males	no

DISCUSSION

Kabuki syndrome is a rare heterogeneous genetic disorder characterized by typical facial features, of which the most recognizable are elongated palpebral fissures and arched eyebrows giving the children the resemblance to actors in Kabuki theater [14,15]. These features are probably still the most prominent features upon which the clinical diagnosis is reached. Additionally, well defined guidelines were established through past years for better recognition of this rare syndrome [4,13]. Moreover, various combinations of five cardinal manifestations previously mentioned should be taken in consideration by the clinicians when assessing the diagnosis of these patients, because a large number of additional congenital anomalies, together with functional differences, has been reported in individuals pointing to a KS diagnosis [9]. Furthermore, reaching the KS diagnosis in neonates is extremely difficult, as the phenotype seems to evolve during the growth of the child, and typical facial features are not prominent before the age of 3 [4,10]. Therefore, the clinical diagnosis is more focused on the presence of malformations associated with KS, and reevaluations are usually needed before reaching the proper diagnosis, as evidenced in our case [10]. As the KS is a heterogeneous disorder there is phenotypic diversity between the patients, which was observed when phenotypic scoring was applied. Patients carrying the heterozygous pathogenic variant in the *KMT2D* gene have more prominent features and reach higher scores compared with patients with mutations in the other three genes [4].

Although our proband is a typical representative of KS (Table 1, Table 2, Figure 2); his diagnosis was reached only after NGS analysis. Due to prenatally observed CHA in our proband, classic karyotyping, QF-PCR, MLPA, and molecular karyotyping was performed in order to exclude aneuploidies, mosaicism, and pathogenic copy number variations (CVNs) during the pregnancy. All the performed test were negative. Additional clinical signs were observed after birth, which indicated the possibility of monogenic disease in our proband, therefore panel NGS sequencing was performed. The described diagnostic pathway is a standard procedure in our laboratory in prenatally and postnatal for observing cases of congenital anomalies.

As mentioned previously, reaching the diagnosis in neonates is still difficult. Our proband thus represents the importance of genotype-phenotype driven NGS analysis in the diagnostic of patients with congenital anomalies.

Congenital heart anomalies, microcephaly, and muscular hypotonia were the most prominent features observed in our proband after birth. Due to the negative results of molecular karyotyping during pregnancy because of observed CHA, an NGS analysis in phenotype-associated genes was performed. A frameshift deletion in exon 39 in the *KMT2D* gene (NM_003482.3: c.11093delG) was identified causing its loss-of-function (LoF) (Figure 1). After determining the genetical cause of the disorder, reevaluation of the proband was performed by a pediatrician, according to the international consensus diagnostic criteria for KS reaching the score of 7 points (Table 2). Additional features have evolved over the course of development; frequent infections of respiratory and gastrointestinal tract were present, growth retardation and DD were more evident,

Table 2. Phenotypic scoring system for KS proposed by the international consensus diagnostic criteria. This table was adapted from the studies of Makrythanasis *et al.* [13] and Adam *et al.* [4].

Clinical Findings	Possible Score	Scored Features	Feature Present/ Number of Points
Facial features	0-5 points[a]	abnormal dentition	[–]
		arched eyebrows, sparse lateral one-third	[+]
		blue sclerae	[–]
		broad nalal root	[+]
		averted lower eyelids	[–]
		flat nasal tip	[+]
		high or cleft palate	[+]
		large dysplastic ears	[+]
		lip nodules	[–]
		long palpebral fissures	[+]
		micrognathia	[–]
		oligodontia	[–]
		ptosis	[–]
		strabismus	[–]
		thin vermillion of the upper lip and full lower lip	[+]
[a] 0-3 features = 1 point; 4-6 features = 2 points; 7-9 features = 3 points; 10-12 features = 4 points; 13-15 features = 5 points			**3 points**
Limb/extremity features	up to 1 point[b]	brachydactyly or clinodactyly	[+]
		hip dislocation	[–]
		lax joints	[–]
		persistent fetal pads	[–]
[b] 0-1 feature = 0 point; 2-4 features = 1 point			**0 points**
Heart	1 point		1
Kidney	1 point		1
Microcephaly	1 point		1
Short stature	1 point		1[c]
Summary	1-10 points		**3 + 0 + 4 = 7**

[c] Noticed during development.

and dysmorphic facial features became more prominent (Figure 2). Moreover, *KMT2D* pathogenic variant carriers are prone to have renal anomalies, feeding problems, premature thelarche in females, joint dislocations, and palatal anomalies than are those without the *KMT2D* mutations [16,17]. A comparison of our proband with the carriers of heterozygous pathogenic mutation in the *KMT2D* gene has shown that our proband expresses most of the KS-features observed in these individuals (Table 1).

Pathogenic mutations in the *KMT2D* gene represent the leading mechanism of KS [18]. The *KMT2D* gene encodes the histone-lysine N-methyltransferase 2D enzyme, responsible for methylation of lysine 4 of histone H3 (H3K4), and consequently, regulates the activity of the genes involved in early embryonic development [19]. According to Ansari *et al.* [20], the KMT2D enzyme regulates the expression of *HOX* genes, interacts with nuclear receptors and has an important role in activation and signaling of hormone-dependent genes involved in reproduction and organogenesis. Namely, *KMT2D* knockdown showed suppression of the estradiol-mediated-HOXC6 regulation [20]. An *in vivo* study also reported that HOXC6 regulates the expression of the bone morphogenetic protein 7 (BMP7), fibroblast growth factor receptor 2 (FGFR2), and insulin-like growth factor-binding protein 3 (IGFBP3) [21]. It was proposed that the characteristics of patients with a *KMT2D* mutation, such as skull anomalies, cleft palate, and short stature, are the result of down regulation of the *HOXC6* gene [22].

The *KMT2D* gene codes the protein product composed of 5537 amino acids coded in 54 exons. In the pre-

sent case, a not previously described *de novo* heterozygous frameshift deletion in the *KMT2D* gene (NM_003482.3: c.11093delG; p.Gly3698AlafsTer51) was detected by NGS analysis in exon 39 causing the LoF mutation in amino acid position 3698 for its protein product. Sanger sequencing confirmed the deletion detected in the proband and excluded it in the proband's parents (Figure 1).

According to the ACMG guidelines [12], heterozygous frameshift deletion in the *KMT2D* gene at position chr12: g.49427394GC>G [hg19; University of California Santa Cruz (UCSC) Genome Browser] is classified as a pathogenic variant (PVS1, PS3, and PM2 rules) and was previously not described in KS patients. The frameshift variants are classified as a known molecular mechanism associated with the KS (PVS1 rule). Additionally, the detected variant has not been reported in the general population according to the gnomAD (PM2 rule), and was determined to be *de novo* (PS3 rule). Moreover, there is a high conservation of the variant site according to computational evidence.

According to Xin *et al.* [3], 589 variants in the *KMT2D* gene have so far been reported in the literature. Frameshift variants are the leading mechanism of LoF of the gene, as they have been reported in 36.67% of cases. Although mutations seem to be distributed through all exons, the top tree hotspots are exons 39, 48 and 31 carrying the mutations in 23.31, 13.16 and 9.96%, respectively [3]. Increased frequency of mutations in exon 39 could be due to the presence of several long polyglutamine tracts encoded in this exon. Studies on KS patients showed that truncating variants are spread throughout all coding areas (exons) of the gene, whereas missense variants tend to accumulate in or nearby the functional domains of protein KMT2D [9]. Genotype-phenotype studies also showed that patients with a deletion of the whole gene and pathogenic truncating variants that occur in the first half of the gene may have more severe ID [23].

In our proband, the CHA observed prenatally is manifested as a consequence of the detected *KMT2D* pathogenic variant. This feature, of which the most frequently reported are left-side obstructive lesions, septal defects, and conotruncal defects, is present in approximately 70.0% of patients with pathogenic or likely pathogenic variants found in *KMT2D* [5]. Namely, functional studies of KMT2S knockdown showed that decreased expression of *KMT2D* ortholog in zebrafishes causes abnormalities in craniofacial, heart and brain development [23]. This is in concordance with VSD, palatoschisis and dysmorphic features observed in our proband and could be attributed to HOXC6 down regulation, as stated above [20,21].

Postnatal growth deficiency is also common in KS patients and its cause is uncertain [22]. Schott *et al.* (in two different studies) [24,25], established that 27.8% of KS patients with the *KMT2D* mutation, had a growth hormone deficiency. The prevalence of growth hormone deficiency in patients with KS is considerably higher than the prevalence of growth hormone deficiency in general population with only 1.0% [26]. Authors also reported that the treatment with growth hormone leads to a significant increase in height standard deviation score (SDS) after the first year [23]. Our proband had a height of 68 cm (below the 3rd centile) at age of 12 months. In the future, it is of great importance to determine whether this is due to his growth hormone deficiency, making the proband a possible candidate for growth hormone therapy.

Despite the genetic diagnosis achieved, the limitation of this study should be emphasized. The use of panel NGS sequencing and then phenotype driven analysis, unable us to discover aberrations in potentially previously unknown Kabuki-associated genes as well as identification other pathogenic variants in genes that were not included in panel but could contribute to proband's phenotype. However, this type of analysis reduces the possibility of identification of incidental findings as well as identifications of single nucleotide variants in biologically poorly studied genes. However, in this study, a panel that enriches exons of 4813 genes with clinical relevance to the disease was used, leading to the identification of a *de novo* variant in the gene related to the proband's phenotype, indicates that the panel based NGS sequencing was sufficient for reaching of proper genetic diagnosis.

In conclusion, while KS is a well-characterized genetic disorder, placing a diagnosis in infants is still challenging. Our proband demonstrated that careful phenotyping of the patients that undergo NGS testing is needed for the diagnostics to be successful. It also emphasizes the benefit of NGS analysis in early diagnostics, resulting in prospect of target therapies and improving the ability to monitor disease progression [2,4,9].

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Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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