Schinzel—Giedion Syndrome: First Czech Patients Confirmed by Molecular Genetic Analysis

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Abstract

Schinzel–Giedion syndrome (SGS) is a very rare genetic disorder characterized by distinctive facial features, severe developmental delay, seizures, and skeletal abnormalities. Whole exome sequencing, Sanger sequencing, and correlation with already published variants and cases allowed us to identify two different de novo mutations in the *SETBP1* gene: NM_015559.2 (SETBP1): c.2601C > G (p.Ser867Arg) and c. 2608 G > A (p.Gly870Ser) in two Czech patients presenting with SGS features. Both mutations are within exon 4 of *SETBP1*, supporting the notion that exon 4 represents the mutation hotspot of the gene in patients with SGS.

Keywords

- Schinzel–Giedion syndrome
- *SETBP1* gene
- phenotype

Introduction

Schinzel–Giedion syndrome (SGS) is a very rare autosomal dominant disorder with an unknown prevalence. SGS is characterized by postnatal growth retardation, severe developmental delay, unusual facial appearance as well as abnormalities of the hair, kidney, skeleton, heart, and brain. Facial dysmorphisms include prominent forehead, hypertelorism, midface retraction and low-set ears.[1,2] Mutations in *SETBP1* gene encoding the SET binding protein 1 have been identified as the molecular cause of this disease.[3–7] *SETBP1* located on chromosome 18q12.3 (NM_015559.2) contains five coding exons and most of the SGS-associated mutations have been found within the exon 4 of the gene.[8] *SETBP1* expression is ubiquitous and has been reported highly expressed also in cancer cells.[9]

Case Report

We present two patients with SGS in whom we identified two different de novo mutations in the *SETBP1* gene: one with missense variant NM_015559.2 (SETBP1): c.2601C > G (p.Ser867Arg) involving the same amino acid residue substitution as reported in the article by Carvalho et al[3] and one with a previously reported missense variant c. 2608 G > A (p. Gly870Ser).[4] Both variants are in exon 4 of the *SETBP1* gene.

The patients genomic DNA was subjected to masivelly parallel sequencing. First, gene panel examination of 97 selected genes was performed (HaloPlex; Agilent Technologies, Santa Clara, California, United States) with no causal variant found. Subsequently, whole exome sequencing (WES) with exome capture from DNA was performed using SureSelect Human All Exon V5 kit (Agilent Technologies)

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according to the manufacturer's protocol using the HiSeq 4000 platform (Illumina; San Diego, California, United States).

The first patient is now a 20-year-old man with drug-resistant epilepsy (Lennox–Gastaut syndrome), severe intellectual disability, and autism spectrum disorder (ASD). Pregnancy and delivery were uneventful. He was born 2 weeks post term after induction. Psychomotor development was abnormal from the beginning. He was able to walk independently at the age of 3 years. He used syllables at the age of 2 but soon after lost his verbal abilities. Dysmorphic features gradually developed (according to nearly 16 years of follow-up). ASD was diagnosed at the age of 4.

Epilepsy started at the age of 7 years with myoclonic and myoclonic–astatic seizures. Later tonic–clonic and sleep-related tonic seizures occurred and thus Lennox–Gastaut syndrome was diagnosed. Brain magnetic resonance imaging showed diffuse brain atrophy. The electroencephalographic trait is consistent with the diagnosis of epileptic encephalopathy with continuous epileptiform discharges. The whole spectrum of antiepileptic drugs (VPA, CLZ, LEV, SLT, CLB, ETS, DZP, PHB, PRI, RFN, TPM, ZNS, and LCS) and their combinations have been tried without long-lasting results. Ketogenic diet decreased the intensity of seizures. Vagus nerve stimulation was not effective. Cardiologic examination was normal.

At the age of 20, he is nonverbal, with profound intellectual disability, unable to walk except a few steps with support. He is fed via percutaneous endoscopic gastrostomy and cannot control his sphincter.

At the current age, his phenotype includes microcephaly, coarse face, bitemporal narrowing, prominent forehead, thick eyebrows, exophthalmos, strabismus, depressed nasal bridge, full lips, open mouth appearance with protruding tongue (macroglossia), sparse teeth, and delayed dental replacement. The patient has low set, dysplastic ears with anteverted lobuli, asymmetry of the chest, defective posture, scoliosis, genua valga, stand uncertain with bent knees, shorter limbs, pes calcaneovalgi, longer great toe on the feet together with sandal gap, conical fingers with hyperconvex nails on the hands, longer thumbs proximally recessed, and short feet and hands.

Candidate variants from whole exome sequencing were detected through our in-house pipeline (Galaxy). Data was analyzed using DeNovoGear (http://denovogear.weebly.com/). Heterozygous mutation in exon 4 NM_015559.2 (SETBP1): c.2601C > G (p.Ser867Arg) already published as pathogenic and causal for SGS was detected and confirmed by Sanger sequencing. Similar substitution, differing only in alteration of the nucleotide C to A, wherein the amino acid substitution remains unchanged, has already been described as a cause of SGS.[3] After evaluating the phenotype of our patient with the phenotype described in the literature, it is highly probable that the phenotype corresponds to SGS and it is possible to assume that the variant is pathogenic. Prediction tools also evaluate the variant as deleterious (http://sift.bii.a-star.edu.sg/) and disease causing (http://www.mutationtaster.org/). The variant is most likely to be de novo as we did not find it in the healthy parents.

The second patient is a 1-year-old girl with facial dysmorphism, microcephaly, hypertelorism, hirsutism, and exophthalmos (►**Fig. 1**). Correlation and re-evaluating the phenotype of our first patient in his childhood with the phenotype of the 1-year-old girl were key for the exact syndromic diagnosis of SGS by the clinical genetician. Consequently, we performed direct Sanger sequencing analysis of exon 4 of the *SETBP1* gene (the most common cause of the SGS) in the patient. As expected, heterozygous mutation in exon 4 NM_015559.2 (SETBP1): c. 2608 G > A (p.Gly870Ser) was found. This variant was already published as pathogenic.[4] The variant was confirmed de novo, since both healthy parents carry the wild-type allele.

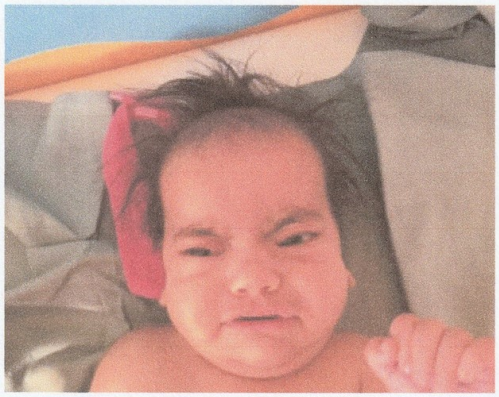

Fig. 1 Female patient with facial dysmorphism.

Maternity and paternity were tested for both patients with a set of six microsatellite markers from three different chromosomes.

Discussion

The phenotype of the patients with SGS is characterized by profound developmental delay, facial dysmorphism including macroglossia, exophthalmos, hirsutism, and multiple congenital anomalies. An heterozygous variant c. 2608 G > A (p.Gly870Ser) in *SETBP1* was first found by exome sequencing in patient with typical SGS phenotype. Hypothesis of a gain-of-function or dominant negative effect[4,6] confirmed previous study finding by other two Caucasian patients with the same variant and similar phenotype. Ko et al[7] published variants in the *SETBP1*-specific highly conserved 11 bp region of exon 4 in SGS patient. Hypothesis that haploinsufficiency or loss-of-function variants in *SETBP1* have a milder phenotype than SGS was also stated.[10] Hishimura[11] reported this variant in a patient with prenatal findings typical for SGS infants. The second variant c. 2601 C > A (p.Ser867Arg) was reported by Carvalho et al[3] describing facial dysmorphism and neurological involvement as diagnostic criteria for targeted molecular analyses of the *SETBP1* in SGS patients. In our case, whole exome sequencing, Sanger sequencing, and correlation with already published variants associated with a particular phenotype helped us to identify the cause of the disease in our patients. Mutational hotspot, located in the fourth coding exon of the *SETBP1* gene, where mutations are clustered, is highly conserved among different species; region is a degron which affects protein degradation. *SETBP1* mutations avoid ubiquitination and affect stability of the

SETBP1 protein, while variants outside the degron cause different form of SGS with milder phenotype.[12] Detecting variants in exon 4 of the *SETBP1* gene in both first Czech SGS patients supports the notion that exon 4 represents the mutation hotspot of the gene.

Conflict of Interest
None.

Acknowledgments
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Massively Parallel Sequencing Detected a Mutation in the *MFN2* Gene Missed by Sanger Sequencing Due to a Primer Mismatch on an SNP Site

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Summary

We describe a patient with early onset severe axonal Charcot-Marie-Tooth disease (CMT2) with dominant inheritance, in whom Sanger sequencing failed to detect a mutation in the *mitofusin 2* (*MFN2*) gene because of a single nucleotide polymorphism (rs2236057) under the PCR primer sequence. The severe early onset phenotype and the family history with severely affected mother (died after delivery) was very suggestive of CMT2A and this suspicion was finally confirmed by a *MFN2* mutation. The mutation p.His361Tyr was later detected in the patient by massively parallel sequencing with a gene panel for hereditary neuropathies. According to this information, new primers for amplification and sequencing were designed which bind away from the polymorphic sites of the patient's DNA. Sanger sequencing with these new primers then confirmed the heterozygous mutation in the *MFN2* gene in this patient. This case report shows that massively parallel sequencing may in some rare cases be more sensitive than Sanger sequencing and highlights the importance of accurate primer design which requires special attention.

Keywords: Massively parallel sequencing (MPS), *mitofusin 2* (*MFN2*), polymerase chain reaction (PCR), primer mismatch, single nucleotide polymorphism (SNP)

Introduction

Charcot-Marie-Tooth (CMT) disease or hereditary motor and sensory neuropathy (HMSN) is the most common inherited neuromuscular disorder with a prevalence of up to 1/1214 in the general population (Braathen, 2012). According to electrophysiological criteria, CMT is subdivided into two main categories: demyelinating neuropathies with upper limb motor conduction velocities (MCVs) of median or ulnar nerve reduced (< 38 m/s) and axonal forms, which affect mostly axons and are characterized by nearly normal MCVs (> 38 m/s) but reduced amplitude (Harding & Thomas, 1980, Reilly et al., 2011). CMT2A is among the most prevalent type of axonal dominant inherited neuropathy in the Czech Republic and the situation might be similar in other populations (Zuchner & Vance, 2006; Cartoni & Martinou, 2009; Brozkova et al., 2013). CMT2A is caused by mutations in the *mitofusin 2 (MFN2)* gene (Zuchner et al., 2004). Typical clinical symptoms are early onset progressive distal limb muscle weakness and/or atrophy, steppage gait, distal sensory loss, and mobility impairment, which frequently leads to wheelchair dependency (Feely et al., 2011; Stuppia et al., 2015).

We report a patient with an autosomal dominant form of CMT2A, who carries the *MFN2* mutation c.1081C>T (p.His361Tyr) in heterozygous state, initially missed by Sanger sequencing due to a PCR primer mismatch. The patient presented with early onset severe CMT2 with a similarly affected mother in whom CMT2A was also suspected. The causal mutation was initially missed by Sanger sequencing because of a single nucleotide polymorphism under the primer sequence.

Molecular genetic diagnosis of CMT2A (and other CMT forms) is typically achieved by detection of *MFN2* mutations by PCR amplification of 17 coding exons and adjacent

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intronic sequences followed by Sanger sequencing. Polymerase chain reaction is based on repeated cycles of heat denaturation of target DNA and oligonucleotide primer annealing and extension (Mullis et al., 1986). Primer mismatches (e.g., due to variation in the target sequence) can lead to failure of PCR amplification. In diploid genomes a variant in the primer binding site on one allele can result in allele dropout. Other variants on this allele escaping amplification will be missed by sequencing of this PCR product. It is a well-known phenomenon, but there have not yet been many studies published. Before the era of massively parallel sequencing (MPS), only a very limited check existed to detect such errors. There are a few publications (Wong et al., 2001; Lam & Mak, 2006; Rossetti et al., 2012) similar to this article. More recently, new DNA sequencing technologies have emerged and are revolutionizing medical genetic research and clinical testing (Mestan et al., 2011; Rabbani et al., 2014). Technologies such as massively parallel sequencing allow for faster genetic diagnosis at comparatively lower costs (Grada & Weinbrecht, 2013). Although generally considered to be less sensitive and more error-prone than Sanger sequencing, these technologies have occasionally led to the identification of variants in previous Sanger mutation-negative samples (Lam et al., 2012; Ratan et al., 2013).

The project was approved by the Ethics Committee of 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol.

Case Report

We report a patient with an autosomal dominant form of Charcot-Marie-Tooth type 2A (CMT2A). The patient was referred to our clinic at the age of four years. The pedigree is shown in Figure 1. He reached early developmental milestones normally. However, from the age of three, the grandparents noticed he had difficulty in walking, especially downstairs. Upon examination at the age of four, he presented with atrophic calves, bilateral pes cavus and abnormal gait. Muscle tone was reduced at the extremities and reflexes were decreased. Nerve conduction studies (NCS) showed that sensory nerve action potential (SNAP) of the suralis nerve was not recordable. Nerve conduction study results are summarized in Table 1. The mother of the patient is deceased. According to clinical reports she had the same type of CMT2 neuropathy. The disease began at the age of four and her ability to walk just before delivery was limited. The overall clinical presentation and the type of inheritance were suggestive of early onset severe CMT2A due to a dominant mutation in the *MFN2* gene (Feely et al., 2011).

All 17 coding exons of the *MFN2* gene were Sanger sequenced in the first instance. PCR primers were based on the

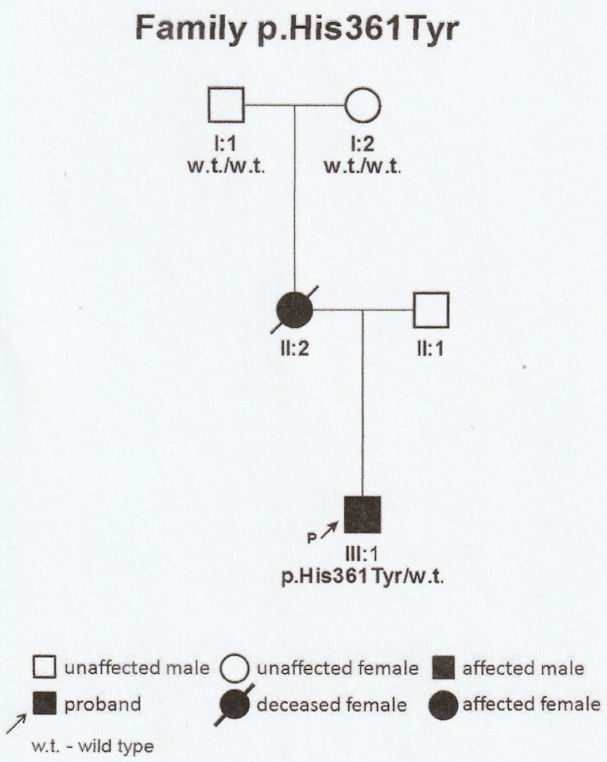

Figure 1 Pedigree of the patient's family.

published sequences (Zuchner et al., 2004), but despite the clinical suspicion no pathogenic mutation was found. Analysis of the *MPZ* gene was continued but no causal mutation was identified. The patient DNA was then subjected to MPS – targeted re-sequencing with a panel of genes currently associated with hereditary neuropathy (78 genes).

The data were analyzed with SureCall (Agilent Technologies, Santa Clara, CA, USA). Six hundred and thirty-one variants were called; VCF files were analyzed with Genome Trax (Biobase, MA, USA) and Annovar (http://annovar.openbioinformatics.org). The quality report for the sample showed that the percentage of analyzable target regions that were covered at least 10 times was 97.33 %.

A heterozygous mutation in the *MFN2* gene (NM_014874.3: c.1081C>T, p.His361Tyr) was identified. This mutation had not been detected by Sanger sequencing performed previously. We wanted to understand why the more precise Sanger sequencing method had missed this mutation, while massively parallel sequencing had succeeded. The reason for this phenomenon was found by comparing BAM files and electropherograms. In the BAM file, we found a single nucleotide polymorphism in the primer site of the reverse primer for exon 11 (9th coding) in the *MFN2* gene (dbSNP: rs2236057). The proband was

Table 1 Neurophysiology in patient III: 1.

Motor nerve conduction velocity (MNCV)		**Conduction velocity (CV) (m/s)**	**Amplitude (mV)**
	Tibial nerve	40.0	5
	Ulnar nerve	44.8	6
Sensory nerve conduction velocity (SNCV)		**Conduction velocity (CV) (m/s)**	**Amplitude (μV)**
	Sural nerve	not recordable	not recordable
	Ulnar nerve	38.6	16

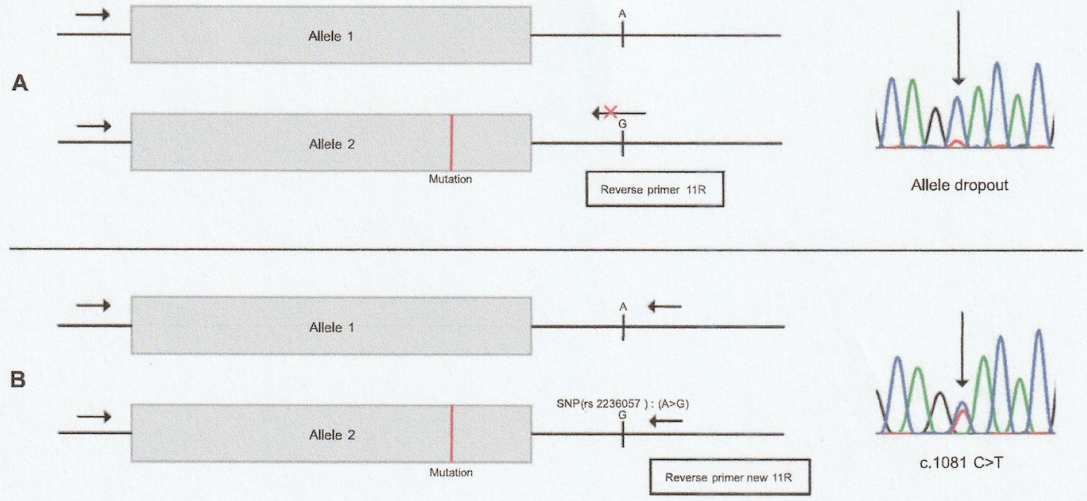

Figure 2 PCR amplification for exon 11 was initially carried out with previously designed primers. Due to the G allele for rs2236057 on the mutated chromosome, we detected only the wild-type allele. A mutant allele was not amplified and only a tiny red dropout peak was visible (A). For new re-designed primers we moved the sequence further away from the polymorphic site to avoid the SNP (rs2236057). New analysis was performed. The alleles were therefore equally amplified and the mutation c.1081C>T (p.His361Tyr) was clearly visible (B).

heterozygous for the A (reference) and the G (alternative) alleles (according to GRCh37/hg19). The G allele is the more frequent allele at this locus (frequency 66.15%-http://exac.broadinstitute.org/), despite the A allele being the reference allele. This SNP caused inadequate annealing and failed amplification of the mutant allele (Fig. 2A).

The original PCR primer (Reverse primer 11R) had been derived from the A allele (reference sequence), suggesting that the missed mutation resided on the G allele which could not be amplified (Fig. 2A). Based on the BAM file we were able to design new primers (Reverse primer new 11R) in such a way, that the new primer would anneal to a part of the DNA sequence, where this patient is not carrying any SNPs (Fig. 2B). PCR was performed again and the mutation c.1081C>T (p.His361Tyr) was confirmed in the heterozygous state. The sequences obtained were compared to reference sequence NM_014874.3. We retrospectively checked the Sanger sequencing chromatograms and found that a small peak is visible at the mutation site and there is minimal decrease of the signal of the wild type peak. Such a picture may have been caused by mosaicism, but the family history was contradictory and further results showed another explanation. This variant has already been reported as pathogenic (Verhoeven et al., 2006). The c.1081C>T (p.His361Tyr) variant was predicted to be deleterious by SIFT (http://sift.jcvi.org/) and to be disease causing by MutationTaster (http://www.mutationtaster.org/). This position is highly conserved. There are no data for minor allele frequency in the Exome Variant Server-EVS (http://evs.gs.washington.edu/EVS/) or the Exome Aggregation Consortium - ExAC (http://exac.broadinstitute.org/).

Segregation of the mutation with the disease in the family was further tested. The unaffected father was not tested as he was not available for DNA testing. The patient's mother, who was similarly affected, is deceased and her DNA was not available. However, we tested the maternal grandparents, who

are without any neurological symptoms, and the mutation was not detected. Therefore, we assume that the mutation arose *de novo* in the affected mother; however, we were unable to prove this by DNA analysis.

Discussion

Allele dropout is a well-known cause of missed mutations due to unequal amplification of heterozygote alleles. The present case illustrates that MPS can enable detection of allele dropout in previous Sanger sequencing mutation-negative samples. This case further highlights the importance of designing primers for successful and correct amplification. As we saw in this case, and as has been described in only a few publications, a single nucleotide polymorphism under the primer binding site may lead to the failure of PCR by allele dropout. Due to allele dropout, the mutation is not visible and misdiagnosis may occur (Mullins et al., 2007).

As reported in the literature, primers should ideally be completely SNP free. However, this standard is in reality very difficult to achieve, because there may be rare new SNPs in individual patients. It seems advisable to compare primer sequences against previously published SNPs, as well as against databases such as EVS, ExAC and dbSNP. Moreover, primers for exon 11 published in the original paper (Zuchner et al., 2004) should not be used for diagnostic testing of the *MFN2* gene in CMT patients since there is a substantial risk of missing the mutation in this exon due to allele dropout of the mutated allele. For CMT patients, we successfully use DNA testing depending on the clinical presentation, type of neuropathy, mode of inheritance and age of onset for the most common causes: CMT1A/HNPP by the MLPA method and Sanger sequencing of genes *GJB1, MPZ* and *MFN2* in the first instance. If these tests do not reveal the cause of the disease, we continue with MPS with a disease-specific gene panel. MPS is a valuable tool for rare, unusual or atypical cases and types of CMT. In contrast to ordinary Sanger sequencing technology, MPS detects SNPs by default. Special care must be taken when selecting PCR primers for clinical testing.

Acknowledgements

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Web Resources

http://annovar.openbioinformatics.org
http://www.ncbi.nlm.nih.gov/snp/
http://evs.gs.washington.edu/EVS/
http://exac.broadinstitute.org/
http://sift.jcvi.org/
http://www.mutationtaster.org/

Conflict of Interest

Authors declare no conflict of interest.

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ORIGINAL ARTICLE

Two Novel Variants Affecting *CDKL5* Transcript Associated with Epileptic Encephalopathy

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Background: Variants in the human X-linked cyclin-dependent kinase-like 5 (*CDKL5*) gene have been reported as being etiologically associated with early infantile epileptic encephalopathy type 2 (EIEE2). We report on two patients, a boy and a girl, with EIEE2 that present with early onset epilepsy, hypotonia, severe intellectual disability, and poor eye contact.
Methods: Massively parallel sequencing (MPS) of a custom-designed gene panel for epilepsy and epileptic encephalopathy containing 112 epilepsy-related genes was performed. Sanger sequencing was used to confirm the novel variants. For confirmation of the functional consequence of an intronic *CDKL5* variant in patient 2, an RNA study was done.
Results: DNA sequencing revealed *de novo* variants in CDKL5, a c.2578C>T (p. Gln860*) present in a hemizygous state in a 3-year-old boy, and a potential splice site variant c.463+5G>A in heterozygous state in a 5-year-old girl. Multiple *in silico* splicing algorithms predicted a highly reduced splice site score for c.463+5G>A. A subsequent mRNA study confirmed an aberrant shorter transcript lacking exon 7.
Conclusions: Our data confirmed that variants in the *CDKL5* are associated with EIEE2. There is credible evidence that the novel identified variants are pathogenic and, therefore, are likely the cause of the disease in the presented patients. In one of the patients a stop codon variant is predicted to produce a truncated protein, and in the other patient an intronic variant results in aberrant splicing.

Keywords: *CDKL5* gene, early onset seizures, infantile epileptic encephalopathy 2, massively parallel sequencing, splice site variant

Introduction

CYCLIN-DEPENDENT KINASE-LIKE 5 (*CDKL5*) gene was first identified as a serine/threonine kinase 9 (*STK9*) with transcriptional mapping (Montini *et al.*, 1998). Severe X-linked infantile spasm (ISSX) caused by *STK9* disruption in two unrelated girls with infantile spasm syndrome was reported (Kalscheuer *et al.*, 2003). Later, neurodevelopmental disorder with infantile spasm, mental retardation, and phenotypic features of atypical Rett syndrome caused by variants in *CDKL5* was published (Tao *et al.*, 2004; Weaving *et al.*, 2004). Archer *et al.*, published in 2006 that *CDKL5* variants are the cause of infantile spasm, early epileptic seizures (later intractable) with onset in the first months of the life in females. Pathogenic variants in *CDKL5* were also noted, which are responsible for early infantile epileptic encephalopathy type 2 (EIEE2) characterized by early onset epilepsy, generalized tonic seizures, severe hypotonia, absent speech, and severe intellectual disability (Bahi-Buisson *et al.*, 2008; Fehr *et al.*, 2013).

The *CDKL5*-related encephalopathy is an X-linked dominant disorder, yet reported predominantly in females, only a few boys have been reported so far. The *CDKL5* gene is localized on chromosome Xp22.13 and contains 21 exons. *CDKL5* is an ubiquitously expressed protein with highest levels in the brain, thymus, and testes, involved in the brain maturation (Lin *et al.*, 2005; Rusconi *et al.*, 2008). The protein product shuttles in the cytoplasm and

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nucleus (Amendola *et al.*, 2014). In the nucleus, *CDKL5* also participates in the control of nuclear speckle morphology. Interestingly, the nuclear speckles in the interchromatin compartment are dynamic parts involved in the activation of a transcription factor by continuous activation of splicing factors. This mechanism affects pre-mRNA splicing (Ricciardi *et al.*, 2009). *CDKL5* and *MECP2* seem to share a common molecular pathway and symptoms from Rett syndrome and can also be found in some females with *CDKL5*-related encephalopathy. But findings from various studies suggest that *CDKL5* disorder is an independent entity with its own specific traits (Evans *et al.*, 2005; Fehr *et al.*, 2013). Variants in *CDKL5* occur mostly *de novo*, but germline mosaicism has also been published (Weaving *et al.*, 2004).

Three stages of *CDKL5*-related epilepsy have been described by Bahi-Buisson *et al.* (2008). At stage I, brief tonic seizures typically occur around the age of 4 weeks, interictal electroencephalography (EEG) is often normal at this period, but neurological examination reveals overall motor hypotonia and poor eye contact. Seizures usually respond to antiepileptic treatment. This "honeymoon" period may last several months, but EEG might already worsen at that time and motor and mental developments show no progression. Stage II presents with epileptic spasms or tonic seizures between the age of 6 months and 3 years. Infantile spasms and hypsarrhythmia are corticosteroid responsive at least in some cases, and some improvement of social contact and behavior is also reported. Severe mental and motor developmental delay, absent speech, and visual interaction are consistent with the diagnosis of epileptic encephalopathy. At stage III (after 3 years of age), patients have multiple types of seizures: epileptic spasms, tonic seizures, absences, and frequent myoclonic seizures. Some patients do not enter stage III and remain seizure free.

Previously, most of the *CDKL5*-related encephalopathies were shown to affect mainly females, probably because the studies included mostly females. Male patients with *CDKL5* variants have been reported later (Elia *et al.*, 2008; Fichou *et al.*, 2009; Sartori *et al.*, 2009). The clinical severity of the *CDKL5* disease for both genders has been described as being very similar (Liang *et al.*, 2011).

Materials and Methods

The study was approved by the Ethics Committee of 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol. Affected individuals were selected by an experienced epileptologist KŠ after referral to University Hospital Motol. We have a large cohort of patients with severe epilepsy of unknown etiology at our Centre for Epilepsies in University Hospital Motol. Patients were first selected by our epileptologist (K.Š.) and geneticist (M.V.) according to the criteria: negative brain magnetic resonance (MR) and availability of DNA samples from probands and both parents to verify variants. Previously, negative array comparative genome hybridization (CGH) testing is not a necessary condition. Patient's legal representatives signed informed consent.

DNA samples were isolated from peripheral blood using automated magnetic beads technique (iPrep™ PureLink™ gDNA Blood Kit; Invitrogen).

Massively parallel sequencing (MPS) of a custom-designed gene panel for epilepsy and epileptic encephalopathy with 112 related epilepsy genes was performed (HaloPlex; Agilent Technologies, Santa Clara, CA). The panel included *ADAR*, *ADCK3*, *ADSL*, *ALDH7A1*, *ALG13*, *AMT*, *AP4S1*, *ARHGEF9*, *ARX*, *ASAH1*, *ATP1A2*, *ATP1A3*, *BRAT1*, *C10ORF2*, *CACNA1A*, *CASK*, *CDKL5*, *CLCN4*, *CPT2*, *DCX*, *DEPDC5*, *DNM1*, *DOCK7*, *EEF1A2*, *FASN*, *FLNA*, *FOLR1*, *FOXG1*, *GABBR2*, *GABRA1*, *GABRB3*, *GABRG2*, *GAMT*, *GCSH*, *GLDC*, *GNAO1*, *GPHN*, *GRIN1*, *GRIN2A*, *GRIN2B*, *GRIN2D*, *HCN1*, *HDAC4*, *HNRNPU*, *CHD2*, *IQSEC2*, *KCNA2*, *KCNB1*, *KCNC1*, *KCNH5*, *KCNJ10*, *KCNQ2*, *KCNQ3*, *KCNT1*, *KCTD7*, *KIAA2022*, *MBD5*, *MECP2*, *MEF2C*, *MFSD8*, *MOCS1*, *MOCS2*, *MTOR*, *NEDD4L*, *NRXN1*, *PANK2*, *PCDH19*, *PHF6*, *PIGA*, *PIGQ*, *PLCB1*, *PNKP*, *PNPO*, *POLG*, *PRIMA1*, *PRRT2*, *PURA*, *QARS*, *RNASEH2A*, *RNASEH2B*, *RNASEH2C*, *ROGDI*, *RYR3*, *SAMHD1*, *SCN1A*, *SCN1B*, *SCN2A*, *SCN8A*, *SLC12A5*, *SLC13A5*, *SLC19A3*, *SLC25A22*, *SLC2A1*, *SLC35A2*, *SLC6A1*, *SLC9A6*, *SPTAN1*, *ST3GAL3*, *STXBP1*, *SYN1*, *SYNGAP1*, *SYNJ1*, *SZT2*, *TBC1D24*, *TDP2*, *TPP1*, *TREX1*, *TSC1*, *TSC2*, *UBE3A*, *WDR45*, and *ZEB2*.

Our designed gene panel targeted coding regions of the genes and adjacent exon–intron boundaries (flanking introns ± 50 bp). Data from MPS were analyzed with SureCall software (Agilent Technologies) and NextGENe Software (SoftGenetics LLC, State College, PA). Average read length was 110 bp. 99.1% of the target region was covered at least 10×. Both methods are based on BWA-MEM alignment (Li and Durbin, 2010)

Sanger sequencing was performed with primers designed for exon 7 and 18 of the *CDKL5* gene to confirm the novel variants. Parentity was tested using six microsatellite markers.

For confirmation of the functional consequence of the intronic *CDKL5* variant in patient 2, a new blood sample for RNA study was taken. Total RNA was extracted from blood mononuclear cells using TRIzol reagent and transcribed into full length cDNA using Cloned AMV First-Strand cDNA Synthesis Kit according to manufacturer's instructions with Oligo (dT)20 as a primer (Thermo Fisher Scientific).

We designed primers for amplifications of cDNA of exons 4–8: forward ACATGAAATTGTGGCGATCA, reverse TACCATCTGGTGGCAACGTA. We expected aberrant splicing, therefore, we amplified exon 7 and adjacent exons to study the splicing changes. PCR products obtained from the cDNA were loaded on 2% agarose gel. The PCR products were sequenced using BigDye Terminator kit v3.1 (Life Technologies, CA). Capillary electrophoresis on the ABI 3130 Genetic Analyzer (Life Technologies) was performed.

Results

Patient 1

The patient is a 3-year-old boy, the first child of healthy, unrelated parents. He was born in the 38th gestational week through caesarean section because of cephalopelvic disproportion. Birth weight was 2750 g. Early postnatal cardiologic examination confirmed Fallot tetralogy. Postnatal adaptation was otherwise normal.

At the age of 4 weeks, he had a few staring spells with jerking of upper limbs and head deviation to the right. EEG was

normal. One month later, he presented with a series of short episodes of behavioral arrest and wide eye opening. Right-sided frontocentral spikes were seen on EEG at that time. At the age of 4 months, hypomotor seizures were frequent. When he was 1 year old, cardiosurgical correction of the Fallot tetralogy was performed. In the postoperative period, a series of epileptic spasms appeared with hypsarrhythmia on the EEG. Later he developed myoclonic seizures and tonic seizures.

Seizures have been intractable: valproic acid, topiramate, phenobarbital, vigabatrin, levetiracetam, phenytoin, clobazam, and their combinations were not permanently effective. Adrenocorticotropic hormone was partially effective. Ketogenic diet was administered from the age of 2 to 3 years with a reduction of seizure frequency, but only for a few months. At the age of 3.5 years, tonic seizures in series predominate but myoclonic seizures are present as well.

Psychomotor development was delayed since the first months. Overall hypotonia and poor social and visual contact were observed, as well. From the age of 3.5 years, relatively short lower limbs became apparent. He has no head control; he is not capable of independent sitting. Speech is absent; he performs only some unintelligible vocalizations and has central visual impairment.

Before performing epilepsy gene panel testing the patient underwent repeated brain MR imaging, showing only mild frontal atrophy. The metabolic screening and skin biopsy showed normal results. Targeted screening for DiGeorge syndrome was also negative. Moreover, array CGH was also done and no causal variants were found. The clinical phenotype is summarized in Table 1.

Patient 2

This female patient was born from the third, normal pregnancy of healthy, unrelated parents. Delivery and early postnatal period were uneventful. Bilateral tonic seizures of upper limbs followed by clonic jerking appeared at the age of 6 weeks. Initial EEG recordings did not show epileptiform activity, later bilateral frontocentral spikes were observed.

Seizures became more complex, usually starting with behavioral arrest "freezing" followed by tonic flexion or extension of the upper limbs and continued as asynchronous clonic jerking of the face, eyelids, and limbs and ended as a series of spasms. As she became older, the tonic phase was less prominent and the jerks were more asynchronous and irregular. Reactivity was disturbed during the seizures.

The seizures were drug resistant: phenobarbital and vigabatrin had partial effect; levetiracetam, clonazepam, valproic acid, topiramate, and phenytoin did not control the seizures, but seizures disappeared for 1 year at the age of 3 years. This happened without any intervention, there was no change in medication with vigabatrin, topiramate, and clobazam. During this period, she improved in walking and acquired some nonverbal communication skills. Seizures reoccurred at the age of 4 years. Therefore, ketogenic diet was implemented with good result: seizure freedom was achieved 3 months after initiation of the diet. The girl remains hypotonic, nonverbal, with severe intellectual disability and motor delay. Repeated brain MR and metabolic screening showed normal results. The clinical phenotype of the patient is summarized in Table 1.

Table 1. Clinical Phenotype of Patients

Variant	*Patient 1—male c.2578C>T (p.Gln860*)*	*Patient 2—female c.463+5G>A*
Age at seizure onset	4th week	6th week
Seizures	Multiple types	Multiple types in series
Drug-resistant seizures	Yes	Yes
Eye contact	Poor	Poor
Speech	Absent	Absent
Hand stereotypies	No	No
Intellectual disability	Profound	Severe
Hypotonia	+	+
Gait	Not achieved	Atactic gait
EEG	Multifocal discharges, hypsarrhythmia	Multifocal spikes
MR	Mild frontal atrophy	Normal

EEG, electroencephalography; MR, magnetic resonance.

A novel nonsense variant c.2578C>T (p. Gln860*) in hemizygous state in exon 18 of *CDKL5* according to the reference sequence NM_003159.2 was found in patient 1. The predicted reading frame is interrupted by a premature STOP codon, leading to a serious effect on the protein and probably nonsense-mediated mRNA decay. The variant was confirmed by Sanger sequencing of exon 18 of the *CDKL5* gene (Fig. 1) and was not found in the healthy parents, so we assume it occurred *de novo*. To our knowledge, this variant has not been previously described, is not listed in HGMD Professional (www.hgmd.cf.ac.uk/ac/index.php), and is absent from global/population databases such as the 1000 Genomes Project (www.1000genomes.org), Exome Aggregation Consortium (http://exac.broadinstitute.org), and Exome Variant Server (http://evs.gs.washington.edu/EVS). According to the ACMG criteria published in Richards *et al.* (2015), the variant has one very strong (PVS1: null variant in a gene wherein loss of function [LOF] is a known mechanism of disease), one strong (PS2: *de novo*: both maternity and paternity confirmed in the patient with the disease and no family history), one moderate (PM2: absent from controls in Exome Sequencing Project, 1000 Genomes Project, or Exome Aggregation Consortium), and ≥2 supporting criteria (PP3: multiple lines of computational evidence support a deleterious effect on the gene or gene product; PP4: the patient's phenotype or family history is highly specific for a disease with a single genetic etiology); therefore, we consider it as pathogenic and causal for EIEE2 in this patient.

In patient 2, MPS identified a heterozygous intronic variant close to the donor splice site of exon 7 of *CDKL5* gene (NM_003159.2). The variant occurs *de novo*, because it was not found in the healthy parents (parentity was tested). According to the ACMG rules, the variant has one strong and ≥2 supporting criteria. Therefore, the variant is evaluated as likely pathogenic.

Computer analysis using Alamut v.2.1. (Interactive Biosoftware, Rouen, France) based on the evaluation by a few

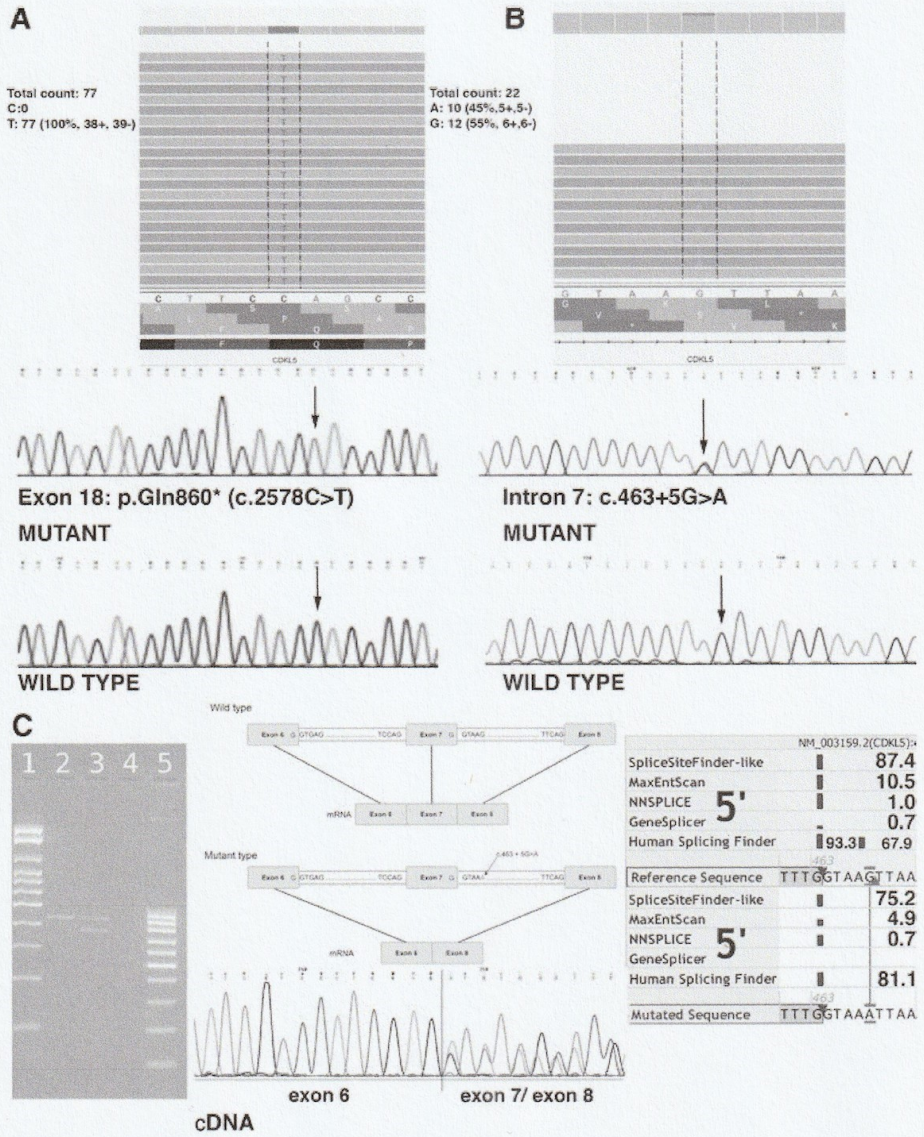

FIG. 1. Schematic view of two novel variants in the *CDKL5* gene and RNA study. (**A**) Variant p. Gln860* in the *CDKL5* gene in patient 1—visualized from IGV (http://software.broadinstitute.org/software/igv/), Sanger sequencing electrophoretogram of mutant and wild type allele. (**B**) Variant c.463+5G>A in the *CDKL5* gene in patient 2. (**C**) Two fragments formed due to the aberrant transcript visible on the 2% agarose gel, from the left side: 1 = LowRanger 100 bp DNA ladder (Norgen Biotek, CA, USA); 2 = control DNA sample; 3 = patient 2; 4 = negative control; 5 = 50 bp DNA ladder (Norgen Biotek, CA). Schematic presentation for mechanism and results of RNA splicing in wild and mutant type for c.463+5G>A variant. Predictions of splice sites in the wild type and mutated *CDKL5* sequences calculated using various predictor programs. A higher score indicates a strong splice site effect. *CDKL5*, cyclin-dependent kinase-like 5.

predictors SpliceSiteFinder-like (www.umd.be/HSF/), MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), NNSPLICE (www.fruitfly.org/seq_tools/splice.html), GeneSplicer (www.cbcb.umd.edu/software/GeneSplicer/gene_spl.shtml), Human Splicing Finder (www.umd.be/HSF/) was performed. These different algorithms predicted high reduction of the splice site score (Fig. 1C). The difference between the sequences higher than 10%, such as in our case, predicts a strong effect in the splicing process (Shapiro and Senapathy, 1987).

PCR product from cDNA of patient 2 with splice site variant was loaded on 2% agarose gel. In comparison with the control sample, two fragments (differing in 60 nucleotides corresponding to exon skipping of exon 7) were observed due to the formation of an aberrant shorter transcript.

Sanger sequencing demonstrated the presence of exons 4, 5, 6, 7, and 8 on the wild type allele and exons 4, 5, 6, and 8 on the mutant allele.

Discussion

Most of the *CDKL5*-related encephalopathies were yet described in females and only rarely in males. This may be caused because variants in *CDKL5* were known as the cause for the so called atypical Rett syndrome. Therefore, *CDKL5* sequencing was frequently the next step after *MECP2* testing, which was performed predominantly in girls.

We present two novel variants in the *CDKL5* gene, one in a female and one in a male. The overall clinical manifestation of the male patient was similar to the phenotype reported in a few boys with rare point variants in *CDKL5* (Elia *et al.*, 2008; Wong and Kwong, 2015). Both children presented with different seizure types, two cases in particular showed very complex seizure types with features of tonic, clonic, and myoclonic convulsions as well as series of spasms. Seizures were drug resistant, but ketogenic diet was at least partially effective in patient 1 and granted

seizure freedom in patient 2. Global hypotonia and poor eye contact since early infantile age were present in both children. We did not observe gastrointestinal problems or scoliosis in our patients, but this can be due to their young age. Both patients had transient sleep problems due to myoclonic jerks; however, their epileptic origin has not been proven in either child. We have not observed any stereotyped hand movements typical for Rett syndrome patients. According to our knowledge, no co-occurrence of Fallot tetralogy or any other congenital cardiac anomaly with only *CDKL5* variants has been described. The only report describing a boy with severe encephalopathy, congenital cataracts, and tetralogy of Fallot attributes the phenotype to the interstitial deletion of Xp22 comprising also *CDKL5* (Van Esch *et al.*, 2007).

There is enough evidence that the variants are pathogenic and, therefore, are the cause of the disease in the presented patients. In patient 1, hemizygous variant p. Gln860* predicting premature stop codon, thus causing a serious effect on the protein, arose *de novo*. According to ACMG guidelines, we consider the variant as pathogenic, whereas in patient 2, the intronic variant c.463+5G>A affects the splicing and leads to the formation of aberrant transcript.

The splice donor site variant: NM_003159.2: c.463+5G>A in the intron 7 of the *CDKL5* gene may cause leaky splicing mechanism and leads to the formation of a 20 amino acids shorter protein without exon 7 (Fig. 1C). We showed that variant *in intron 7 c.463+5G>A changes the 5′ donor splice site of intron* 7. Aberrant splicing has been already described as a mechanism that could lead to EIEE2 (Weaving *et al.*, 2004; Archer *et al.*, 2006).

It has been suggested that splice site variants effect on the phenotype correlates with the proportion of functional CDKL5 due to mutated alleles (Bahi-Buisson *et al.*, 2008). The precise effect on the severity of the phenotype is not known. It is expected that a milder phenotype correlates with less severe mental retardation and the ability to walk even though gait is atactic. The clinical presentation in patient 2 is milder than in patient 1. This may be caused by *leaky splicing. Also, variability of X inactivation in the* different brain regions could lead to various phenotype manifestation (Weaving *et al.*, 2004). The other hypothesis is that exon 7 skipping (deletion of 60 nucleotides) causes in-frame deletion, protein is shortened only by 20 amino acids, and this may cause milder phenotype in the patient. Similar clinical phenotype with splice site variant IVS6-1G>T that also results in exon 7 skipping has been reported as pathogenic (Archer *et al.*, 2006). Our patient, similarly to the patient in Archer *et al.* (2006), had severe mental retardation, hypotonia, epilepsy seizure starting in the first months, and "honeymoon periods" with subsequent return of seizures.

Conclusions

We showed enough evidence that the variants are pathogenic and, therefore, are the cause of the disease in the presented patients. In one patient, stop codon variant causes a serious effect on the protein and the intronic variant in the second patient affects the splicing. Using RNA study, we were able to verify the formation of aberrant transcript in patient 2.

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Author Disclosure Statement

No competing financial interests exist.

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