Institut für Medizinische Genetik und Angewandte Genomik

Identifying the genetic basis of rare neurological disorders

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TABLE OF CONTENTS

1 ABSTRACT

Background: Advances in sequencing technology have made genetic diagnostics more efficient and accurate. The number of genes and pathogenic variants associated with rare neurological disorders has dramatically expanded in the past two decades, broadening our knowledge on genetic-based neurological disorders.

Methods: Patients were referred by neurologists from different centers for genetic testing. Whole exome sequencing (WES) was performed from patients' and their parents' DNA isolated from EDTA-blood for diagnostic purposes. Functional consequences of identified variants in the genes *KCNC1* and *SLC12A6* were studied in *Xenopus* oocytes.

Results: WES identified previously unreported *de novo* variants in *HIVEP2*, *KCNC1,* and *SLC12A6*. Two patients with different truncating variants in *HIVEP2* presented with mild intellectual disability without any dysmorphic features, congenital malformations or behavioral difficulties. Three new *de novo* missense variants in *KCNC1* were found in five unrelated individuals causing different phenotypes such as isolated non-progressive myoclonus, intellectual disability or epilepsy with ataxia and developmental delay. Autosomal dominant *de novo* variants in *SLC12A6* caused progressive early-onset neuropathy without MRI abnormalities. All identified missense variants in the *KCNC1* and *SLC12A6* led to a significant reduction of the protein function in the oocytes.

Conclusion: To date, only few cases were reported who carried pathogenic variants in *HIVEP2*, *KCNC1,* and *SLC12A6*. Clinical characteristics of the patients reported in this study were different from the previously published patients. Thus, we report new *de novo* variants and new phenotypes thereby expanding the clinical spectrum of the genes and underlining the importance of *de novo* genetic variation and the complexity of genotype-phenotype correlations in these genes.

2 INTRODUCTION

In the last two decades, tremendous progress has been made in our understanding of the genetic causes underlying neurological disorders (Zoghbi and Warren, 2010). The number of genes and mutations associated with rare neurological disorders is constantly increasing (Rauch et al., 2012).

Genetic studies in neurology help to understand the role of genes and proteins in the development and function of the central and peripheral nervous system, not only expanding our knowledge on the mechanisms of brain-mediated disorders but also providing prospects for new and emerging therapies (Steinlein, 2008). Advanced sequencing technology with efficient analytic methods and skills enables rapid identification of the rare genetic causes that are involved in a large number of neurological disorders helping clinicians to diagnose their patients and in some cases, treat them accordingly based on the molecular mechanism (Zoghbi and Warren, 2010, Steinlein, 2008, Oyrer et al., 2018).

2.1 NEXT GENERATION SEQUENCING

Next generation sequencing (NGS) is a parallel sequencing technology that sequences millions of DNA fragments simultaneously (Voelkerding et al., 2009, Tucker et al., 2009). Whole exome sequencing (WES) is a sequencing method in which protein coding (exonic) regions are enriched for subsequent sequencing. Currently, it is a faster and more cost-effective method than whole genome sequencing (WGS) which sequences the complete genome (Helbig et al., 2016a, Ostrander et al., 2018).

Similar to Sanger sequencing, NGS uses fluorescent nucleotides to identify each nucleotide (Voelkerding et al., 2009). Genomic DNA is sheared into fragments and special adapters are ligated to both ends of each fragment (Tan et al., 2019). This enables sequencing from both ends (pair-end sequencing) producing a

forward and a reverse read. One read has approximately 100 – 150 base pairs (bp) (Tan et al., 2019). The reads are processed through a bioinformatics pipeline and aligned to a reference genome sequence (Oliver et al., 2015). After alignment, differences between the read sequences and the reference genome can be detected (Oliver et al., 2015).

For data analyses, genomic variants are usually filtered for rare variants with a minor allele frequency (MAF) of <0.1% in the population (Fritzen et al., 2018). There are several public databases that provide exome and genome data sets of control healthy population such as ExAC (http://exac.broadinstitute.org/), gnomAD (https://gnomad.broadinstitute.org/), and 1000 genomes project (http://browser.1000genomes.org/index.html). The filtered rare variants are prioritized according to the patient's phenotype (Park et al., 2019a). In silico tools such as CADD (http://cadd.gs.washington.edu/), SIFT (http://sift.jcvi.org/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) help predict the degree of possible pathogenicity of a variant based on an algorithm considering evolutionary conservation, mutation type and protein function (Yu, 2009). The large number of genomic variants needs to be filtered strategically for an efficient analysis and also to avoid discarding relevant candidates in an earlier filtering step.

2.1.1 Types of Genetic Variation

Chromosomal aberration is a structural alteration of chromosomal DNA where a section of chromosomal DNA is missing, added or dislocated (Genetic-Alliance, 2009). In a point mutation, a single nucleotide is substituted with another. If the substitution codes a different amino acid, the mutation is called missense mutation and if it codes the same original amino acid, it is a synonymous mutation. A nonsense mutation occurs when the substitution leads to a stop codon terminating the protein synthesis (Nordheim et al., 2015).

A frameshift mutation leads to a different amino acid sequence creating an altered protein or a truncated shortened protein due to a premature stop codon (Kurosaki and Maquat, 2016, Hug et al., 2016). In order to reduce the production of dysfunctional shortened proteins, nonsense-mediated mRNA decay (NMD) is activated to eliminate shortened mRNAs (Hug et al., 2016). NMD activation depends on the size of the mRNA product (Hug et al., 2016). Deletion or duplication of a considerable number of nucleotides, e.g. more than one exon, is considered as a copy number variant (CNV), which can also result in an altered or truncated protein.

Since the introduction of the standard human sequence variant nomenclature by the committee of the Human Genome Variation Society (HGVS), the term variant is preferably used to describe a difference in a DNA sequence than mutation (Richards et al., 2015). Both terms are considered as synonyms for genetic alterations; a variant as well as a mutation might be frequent or rare in a population and its consequence can be benign or deleterious (Richards et al., 2015). The term mutation is additionally used to describe the DNA changing event.

2.1.2 *De novo* Variants

Monogenetic disorders have a variety of inheritance patterns that can be autosomal dominant, autosomal recessive, X-linked or mitochondrial (Ropers, 2008, Deciphering Developmental Disorders, 2015). Since genetic disorders were believed to be inherited, genetic studies in the past primarily concentrated on large families with multiple affected family members, preferably consanguineous families (Hu et al., 2019). However, recent studies have indicated that spontaneously occurring variants, referred to as *de novo* variants, are increasingly found to cause monogenetic and sporadic severe neurological disorders in childhood (Deciphering Developmental Disorders, 2015, Veltman and Brunner, 2012, Vissers et al., 2010).

Spontaneous mutation rate in humans are estimated to be approximately between 1.1 and 1.7 × 10^{-8} per base pair in a generation with approximately 50-100 *de novo* variants in a genome of an average newborn (Lynch, 2010, Roach et al., 2010, Narasimhan et al., 2017, Conrad et al., 2011). 1.5% of these variants will affect the coding regions in DNA leading to approximately 0.86 non-synonymous variants in coding DNA per newborn (Lynch, 2010). When deletions, insertions, inversions and splice variants close to the coding DNA are considered as well, the numbers add up to 0.9 to 4.5 possibly deleterious *de novo* variants per person (Lynch, 2010). While fetal chromosomal abnormalities have a direct association with increasing maternal age, recent observations reported that the rate of *de novo* variants are higher in advanced paternal age with more than two additional *de novo* variants per year (Kong et al., 2012). If a *de novo* variant is non-synonymous, approximately 5% will result in nonsense variant and the remaining 95% will be missense variants (Lynch, 2010).

The application of trio-WES simplified the identification *de novo* variants in affected patients, rapidly increasing the diagnostic yield of genetic testing, expanding the knowledge on rare genetic disorders which has been scarce so far and establishing further variants and genes as disease causing (Heyne et al., 2018, Wang et al., 2019).

2.2 Clinical Genetics

Identifying the underlying genetic cause of a rare neurogenetic disorder is a complex and time-consuming process. Apart from basic clinical encounter that includes medical examination and patient history taking, standard diagnostics such as EEG, EMG, MRI and blood/cerebral fluid analyses are important for accurate phenotyping (Zoghbi and Warren, 2010). In particular, patients suspected of having neurogenetic disorders should be asked for family history and their early developmental phase. Alongside this information, molecular genetic diagnostics enables clinicians to diagnose their patients and also end the

diagnostic journey, which is of great value to patients and families, in rare cases even allowing precision medicine (Rauch et al., 2012, Ropers, 2010).

2.2.1 Genetic Background of Intellectual Disability

Intellectual disability (ID) is a common neuropediatric disorder with a prevalence estimated at around 1-3% depending on the socioeconomic environment (Polder et al., 2002, Maulik et al., 2011). The term ID is used in patients with reduced learning ability and limited adaptive behaviors with an intellectual quotient (IQ) lower than 70 (Ropers, 2008).

ID can be caused by a variety of non-genetic factors such as toxic causes, birth complications, accidents, malnutrition, infections, environmental burdens and poor social care (Ropers, 2010, Kaufman et al., 2010). Yet, genetic defects remain as one of the main causes for ID (Ropers, 2008). A large number of cases are affected by chromosomal abnormalities or CNVs containing multiple genes. The most common ID disorder is trisomy 21 (1 in approximately 700 live births), also known as Down syndrome, a chromosomal disorder where a third copy of chromosome 21 is present (Ropers, 2010, Reeves et al., 2001).

Syndromic ID disorders are severe forms of ID where patients present additional neurologic symptoms and/or congenital malformations (Kaufman et al., 2010). In non-syndromic ID, patients have mild cognitive deficits without any other medical signs or symptoms (Kaufman et al., 2010). Family members are often deeply concerned about the social abilities, medical presentations as well as prognosis of the patients, for which genetic diagnosis plays an important role. Although ID is a notably common inherited disorder, the genetic causes in individual cases are very rare. In most cases the pathogenic variants occurred *de novo* (Hu et al., 2019, Rauch et al., 2012, Veltman and Brunner, 2012, Vissers et al., 2010).

2.2.2 Genetic Background of Epilepsy

Epilepsy is a common neurological condition affecting approximately 0,4-1% of the population (World-Health-Organization, 2019). Less common are epilepsies and epilepsy syndromes that are believed to have a genetic origin. They were initially classified as idiopathic epilepsy. Epileptic encephalopathy patients have a higher rate of positive findings in genetic testing than other epilepsy patients (Hamdan et al., 2017).

The number of genes associated with epilepsy is continuously growing, therefore, when using a selected gene panel, a constant update of panels is needed to incorporate newly discovered disease genes. Meanwhile, there are more than 500 genes associated with epilepsy and seizures (Ortega-Moreno et al., 2017). There are several genes such as *SCN1A*, *KCNQ2*, *STXBP1*, *TPP1*, *PCDH19*, *CACNA1A*, *GABRA1*, *GRIN2A*, *SLC2A1*, *CDKL5*, *ARX* and *TSC2* where pathogenic variants are more frequently identified (Hamdan et al., 2017, Heyne et al., 2019, Heyne et al., 2018, Parrini et al., 2017). A targeted diagnostic panel of about 100 genes would only solve about 20% of cases, while WES and WGS identifies pathogenic variants in about 32-45% of epilepsy patients (Butler et al., 2017, Hamdan et al., 2017, Helbig et al., 2016a, Parrini et al., 2017, Ortega-Moreno et al., 2017). Therefore, WES significantly increases the diagnostic yield in epilepsy patients.

Early identification of the underlying genetic cause in epilepsy syndromes can be of paramount importance for clinicians to initiate effective antiepileptic medication. The majority of disease causing genes in epilepsy syndromes encodes voltage- or ligand-gated ion channels (Steinlein, 2008, Oyrer et al., 2018). The trend towards targeted molecular therapy directed against the specific gene defects is rapidly increasing. Especially in severe, refractory and monogenetic disorders, clinicians and scientists are striving to find targeted treatment options to alleviate seizure symptoms. In addition, discovery of the genes underlying genetic

epilepsies also contributed to our knowledge about the cellular pathomechanisms that are involved in epileptogenesis. Therefore, understanding the genetic bases of epilepsy may provide new insights and possibilities for therapy.

2.2.3 Genetic Background of Charcot-Marie-Tooth disease

Hereditary peripheral neuropathy is a clinically and genetically heterogeneous disorder that is classified based on the clinical characteristics, electrophysiological measurements, inheritance patterns and gene defects (Tazir et al., 2014). The most common inherited disorder of the peripheral nervous system is hereditary sensory and motor neuropathy (HSMN), also known as Charcot-Marie-Tooth (CMT) disease (Klein et al., 2013, Tazir et al., 2014). Prior to genetic diagnostics, other possible causes for peripheral neuropathy need to be considered. These include inflammatory neuropathies, vascular diseases, metabolic disorders, toxic causes or nutritional deficiencies (Baets et al., 2011, Wilmshurst et al., 2019).

The eponym CMT and its classification are more frequently used than the Dyck classification using the term HSMN (Tazir et al., 2014). Hereditary demyelinating neuropathies are referred to as CMT1 and axonal neuropathies as CMT2. Severe demyelinating neuropathy with onset in early infancy was first described as Dejerine-Sottas syndrome and later classified as CMT3. Rare autosomal recessive demyelinating neuropathy was labeled as CMT4 (Tazir et al., 2014). In addition, the term dominant intermediate CMT (DI-CMT) was introduced for autosomal dominant inherited CMT with both axonal and demyelinating features and X-linked CMT disorder was named CMTX (Berciano et al., 2017, Nicholson and Myers, 2006, Nicholson and Nash, 1993).

However, the number of CMT-related genes almost doubled in the past 10 years expanding the clinical spectrum of CMT genes (Rossor et al., 2013). It became apparent that many genes could not be assigned to one phenotype or one

classification (Baets et al., 2011, Berciano et al., 2017, Tazir et al., 2014). Some genes had both dominant and recessive mutations and caused overlapping clinical features such as hereditary spastic paraplegia (Toft et al., 2019). Peripheral neuropathy was also often overshadowed by other clinical features in severe syndromic disorders, where both central and peripheral nervous system were affected (Baets et al., 2011, Wilmshurst and Ouvrier, 2011, Wilmshurst et al., 2019). Patients with severe early onset neuropathy presented first with muscular hypotonia, weakness, delayed motor development and distal sensory loss, often followed by foot deformities (Baets et al., 2011, Wilmshurst and Ouvrier, 2011).

2.3 Study Design and Aims

Patients were referred for genetic testing by different neurologists. Patients' exome data were evaluated for diagnostic purposes. This led to identification of new variants in known disease genes (*HIVEP2*, *KCNC1* and *SLC12A6*). So far, very few patient cases were published who harbored pathogenic variants in these three genes. Our patients showed different clinical features than the previously published patients, thus, we expanded the phenotypic spectrum of *HIVEP2*, *KCNC1* and *SLC12A6* related disorders. The new findings (new variants and new phenotypes) were published in the journals *Molecular Syndromology*, *Annals of Clinical and Translational Neurology* and *Journal of Medical Genetics*.

3 RESULTS

The results section consists of three first author publications. The first article has been published in *Molecular Syndromology* as an original article providing detailed clinical course of two patients harboring pathogenic variants in *HIVEP2* gene and a review of the literature. The second article has been published in *Annals of Clinical and Translational Neurology* in which we report three new *de novo* missense variants in *KCNC1* identified in five unrelated individuals and their consequences functionally confirmed in *Xenopus* oocytes. The third article, published in *Journal of Medical Genetics*, describes dominant-acting *de novo* variants in *SLC12A6* causing a milder phenotype than the previously known *SLC12A6*-related recessive disorder, Andermann syndrome.

3.1 Novel *HIVEP2* Variants in Patients with Intellectual Disability

Original Article

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Novel *HIVEP2* Variants in Patients with Intellectual Disability

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Keywords
Exome sequencing · *HIVEP2* · Intellectual disability · MRD43

Abstract
Intellectual disability (ID) occurs in approximately 1% of the population. Over the last years, broad sequencing approaches such as whole exome sequencing (WES) substantially contributed to the definition of the molecular defects underlying nonsyndromic ID. Pathogenic variants in *HIVEP2*, which encodes the human immunodeficiency virus type I enhancer binding protein 2, have recently been reported as a cause of ID, developmental delay, behavioral disorders, and dysmorphic features. HIVEP2 serves as a transcriptional factor regulating NF-κB and diverse genes that are essential in neural development. To date, only 8 patients with pathogenic de novo nonsense or frameshift variants and 1 patient with a pathogenic missense variant in *HIVEP2* have been reported. By WES, we identified 2 novel truncating *HIVEP2* variants, c.6609_6616delTGAGGGTC (p.Glu2204*) and c.6667C>T (p.Arg2223*), in 2 young adults presenting with developmental delay and mild ID without any dysmorphic features, systemic malformations, or behavioral issues.

Intellectual disability (ID) occurs in approximately 1% of the population [Polder et al., 2002; Maulik et al., 2011] and represents a major socioeconomic problem. Family members can be burdened by a variety of factors that are highly dependent on the social abilities and medical presentation of the patients. Advances in sequencing technologies enabled clinicians to identify rare genetic causes for ID in isolated cases [Ropers, 2010; Rauch et al., 2012]. Especially the application of trio-whole exome sequencing (WES) led to the identification of numerous nonsynonymous de novo mutations that have been associated with autism, ID, and schizophrenia. These observations lend support to the "de novo hypothesis," i.e., that under consideration of evolutionary conservation, protein function and mutation type, these unique events may play a significant role in the pathogenesis of a number of rare developmental disorders [Vissers et al., 2010; Kong et al., 2012; Veltman and Brunner, 2012].

HIVEP2 (OMIM 143054) encodes the human immunodeficiency virus type I enhancer binding protein 2, a member of the large zinc-finger transcriptional proteins family (ZnF-C2H2 type) that is involved in immunological responses, adipogenesis, bone formations, and brain development [Dörflinger et al., 1999; Hong et al., 2003;

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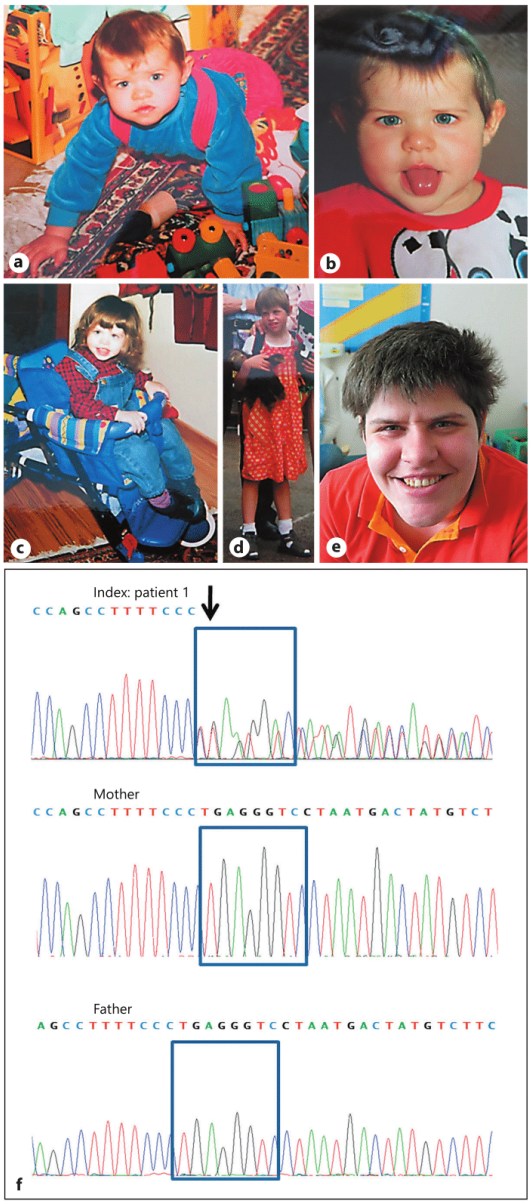

Fig. 1. Patient 1. **a** 15 months. **b** 18 months. **c** 2 years. **d** 7 years. **e** 27 years. **f** The *HIVEP2* frameshift variant c.6609_6616delTGAGGGTC was confirmed in patient 1 by Sanger sequencing (arrow) but was absent in both parents.

Takagi et al., 2006; Imamura et al., 2014]. Recently, de novo variants in *HIVEP2* have been suggested to cause autosomal dominant mental retardation type 43 (MRD43, OMIM 616977) in a small number of patients. This is consistent with evidence that *HIVEP2* is expressed in several brain regions and plays an important role in neural maturation and brain development [Takao et al., 2013; Srivastava et al., 2016]. *Hivep2* knock-out mice displayed anxiety and hyperactivity that have also been observed in the majority of the reported patients [Takagi et al., 2006]. Here, we provide detailed information on the clinical presentation of 2 individuals carrying predictively truncating de novo *HIVEP2* variants over a disease course of 23 and 27 years, respectively.

Clinical Reports

Patient 1 is the first child of healthy unrelated German parents. The girl was born after a normal pregnancy at 40 weeks of gestation by spontaneous vaginal delivery with normal length (53 cm; 72nd centile) and weight (3,570 g; 59th centile). The first clinical presentation was orofacial hypotonia with an open mouth posture in infancy. Considerable global developmental delay became apparent approximately at the age of 2 years. Motor development was slightly delayed, but she walked independently at 18 months of age. Some neurological signs such as general muscular hypotonia, poor posture, and notable difficulties in gross and fine motor coordination were noted. Language development was severely affected as she spoke first words at the age of 4 years. A nonverbal intelligence test at 9 years of age revealed mild ID with an IQ of 58. Her behavior has generally been friendly, amiable, and cooperative. Her medical history is unremarkable; there were neither organic malformations, complicated hospitalizations nor any evidence of seizures. She does not show any distinctive dysmorphic features, but a marginally broader nose, narrow chin, and thin upper lip could be conceived (Fig. 1). Her height, weight, and head circumference have always been within the normal range. Currently, at the age of 27 years, she works at a sheltered workshop and does not have any difficulties socializing with other workmates. Prior to WES, conventional chromosomal and SNP array analyses revealed normal results, and Fragile X syndrome was excluded via trinucleotide repeat expansion analysis. Screening for congenital metabolic diseases and congenital disorders of glycosylation yielded negative results.

Patient 2, a female, was the only child of a healthy 29-year-old woman. There is no family medical history regarding ID or any other neurological disorders. Pregnancy was unremarkable and prenatal sonographic scans were normal. She was born at 37 weeks of gestation by uncomplicated vaginal delivery with a birthweight of 2,450 g (19th centile), birth length of 49 cm (72nd centile), and head circumference of 31 cm (9th centile). The patient demonstrated mild motor developmental delay: she started rolling over at 7 months, could sit up and stand with support at 11 months, and learned to walk independently at approximately 20–24 months. She spoke first words at the age of 24 months. She has an elongat-

ed and mildly narrow face as well as small ears, but no specific dysmorphic features. Neurological examination was unremarkable without signs of muscular hypotonia, seizures, or autism. EEG was normal. She was diagnosed with mild ID with an IQ of 52. Until the age of 16 years, she attended special schools for ID students. Now, she is 23 years old and has slow speech and limited vocabulary. She tends to use short and repetitive sentences and is often difficult to understand for others, with the exception of her parents. Although she presents irritability and impulsivity, she rarely shows aggressive behavior. Repetitive interests and limited food preference were seen as characteristic features within normal range for a patient with ID. Predominant autistic features were excluded. Regarding medical problems, the patient frequently suffered from upper airway infections, constipation, and oligomenorrhea. Otherwise, no other complicated medical issues have occurred so far. Chromosomal abnormalities, including microdeletions, microduplications, and other copy-number variants (CNVs) have been excluded by conventional karyotyping and array CGH.

Methods and Results

For patient 1, diagnostic WES was performed on genomic DNA extracted from peripheral blood. Coding regions were enriched using a SureSelect XT Human All Exon kit v6 (Agilent Technologies, Santa Clara, CA, USA) for subsequent sequencing as 2 × 125-bp paired-end reads on a HiSeq2500 system (Illumina, San Diego, CA, USA) as published previously [Fritzen et al., 2018]. Generated sequences were analyzed using the megSAP pipeline (https://github.com/imgag/megSAP). Clinical variant prioritization included different filtering steps including a search for rare (MAF <0.1% in ExAC, gnomAD, 3,000 in-house exome datasets) variants in genes that have been associated with the patient's phenotype according to an in-house standard operating procedure.

For patient 2, WES was performed on genomic DNA isolated from the proband's EDTA blood using a TruSeq Exome Enrichment kit (Illumina) on an Illumina HiSeq 2500 sequencer. Approximately 100 million paired reads per sample were generated. The average sequencing depth was 70.2× with over 80% of the target sequence covered at ≥20×. Genomic variants were filtered and prioritized according to the following criteria: the gene reported is to be involved in neurological or mental disorders, its frequency in publicly available variant databases <0.01%, and in silico predicted pathogenic effect of the variant. Bidirectional Sanger sequencing was performed to confirm the variants in both patients and their de novo status.

Mutational Analysis

WES revealed a heterozygous frameshift variant NM_006734.3:c.6609_6616delTGAGGGTC (p.Glu2204*) in patient 1 and a heterozygous nonsense variant c.6667C>T (p.Arg2223*) in patient 2. Both variants are located in the last exon of *HIVEP2*. Sanger sequencing confirmed that the variants were absent from either of the patients' parental DNAs extracted from peripheral blood, suggesting a de novo status of the variants in both subjects. These variants were absent from the 1000 Genomes database, the Genome Aggregation Database (gnomAD), and the Exome Aggregation Consortium (ExAC) database (last consultation: 1 September 2018).

Discussion

Six years ago, in a cohort study of 51 patients, Rauch et al. [2012] reported 1 de novo heterozygous truncating variant in *HIVEP2* as a possible cause of nonsyndromic ID in a female patient. Only limited clinical information of the patient was presented. Srivastava et al. [2016] reported further clinical details for this patient who exhibited not only severe ID, but also dysmorphic features, congenital malformations, ataxic gait, and aggressive behavior. In addition, the same authors identified 2 further unrelated children with de novo heterozygous *HIVEP2* truncating variants presenting with moderate ID, developmental delay, mild facial dysmorphism, and, in one of them, behavioral problems, suggesting loss of function and haploinsufficiency as the pathomechanism [Rauch et al., 2012]. More recently, Steinfeld et al. [2016] identified 5 de novo truncating variants and 1 de novo missense variant in *HIVEP2* in 6 unrelated children with ID. Last, in 2 cohort studies, 1 de novo missense variant and 1 de novo nonsense variant have been identified among patients affected by bipolar disorder and ID, respectively [Kataoka et al., 2016; Hamdan et al., 2017]. However, both publications did not provide any clinical phenotype details.

HIVEP2 variants identified so far in ID patients are shown in Figure 2. Clinical details are given in Table 1. Overall, only 8 patients with pathogenic nonsense or frameshift variants and 1 patient with a pathogenic missense variant in *HIVEP2* have been reported together with clinical information. All of them, 5 females and 4 males, showed mild to moderate ID. Motor development was markedly delayed in all. Seven out of 9 patients were able to walk independently at the age of ~2–3 years and 1 of them needed a walker until the age of 4–5 years. Apart from general muscular hypotonia which was present in 7 out of 9 patients, other overlapping neurological features such as ataxia, dystonia, dyspraxia, dysphagia, gait difficulties, spasticity, and progressive Parkinsonism and quadriplegia have been registered in 7 cases. All of them had remarkable language development delay and 7 showed pronounced behavioral abnormalities, such as hyperactivity, impulsivity, lack of concentration, but also anxiety, aggressive and/or hand flapping behavior. Less frequently reported clinical features include microcephaly (3 cases) or seizures (2 cases). Some of them also presented with nonspecific dysmorphic features, which were not predominant in our patients. The majority of reported cases suffered from minor medical problems, mostly gastrointestinal symptoms, such as abdominal

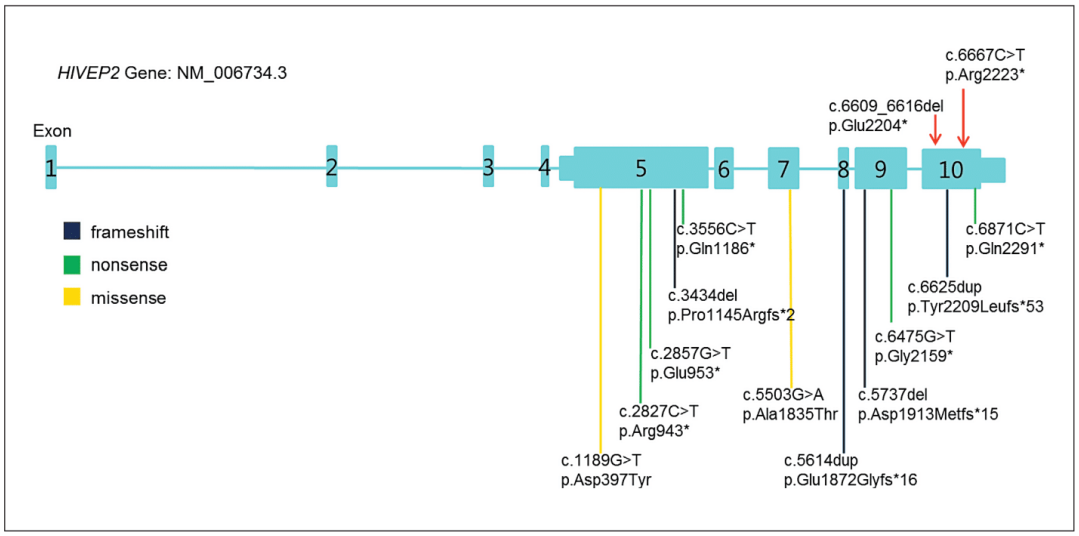

Fig. 2. Previously reported de novo variants in *HIVEP2* identified in ID patients and predicted to be pathogenic. Patient 1 had a frameshift truncating variant c.6609_6616delTGAGGGTC (p.Glu2204*), and patient 2 had a nonsense variant c.6667C>T (p.Arg2223*), both located in the last exon of *HIVEP2*.

pain, constipation or reflux. Congenital anomalies are uncommon. Brain MRI did not reveal any specific pathological findings. In comparison to the previously reported cases, our 2 patients did not show any complex neurological findings and had an uncomplicated medical history without predominant behavioral abnormalities. The variants identified in our patients are located in the last exon of *HIVEP2* and are predicted to result in the generation of a truncated protein. This could possibly lead to some residual function or a dominant-negative effect and might also explain the milder phenotype of these patients.

HIVEP2 operates as a transcription factor for various genes involved in brain development, such as the somatostatin receptor type II (*SSTR-2*) that is known to show expression in the frontal cortex and hippocampus during embryonic brain and nerve formation [Takagi et al., 2006]. HIVEP2 also binds to nuclear factor-κB (NF-κB) reducing the transcription of NF-κB-dependent genes that play an important role in immune response. *Hivep2* knock-out mice displayed chronic inflammation signs in several brain areas due to upregulation of NF-κB target gene proposing a paradigm that alterations in immune response can influence neurodevelopmental disorders [Takao et al., 2013; Choi et al., 2015]. RNA expression analysis in hippocampus and amygdala illustrated a downregulation of some early genes that are involved in stress response and neuroplasticity [Takagi et al., 2006]. *Hivep2* knock-out mice showed not only severe cognitive impairments but also behavioral abnormalities, which could be compared with those of 7 of the reported patients. No remarkable deficits in health condition, physical appearance, or motor functions were observed in the mutant mice. Administration of haloperidol and anti-inflammatory drugs to murine models reduced inflammation markers in the brain and improved social interactions and memory abilities [Takao et al., 2013]. Although behavioral problems have not been observed in our patients, this type of pharmacological therapy has been suggested as a possible treatment for such symptoms [Takao et al., 2013].

This report supports the growing evidence that WES can be a useful approach to elucidate the underlying molecular cause of ID [Helsmoortel et al., 2015; Vrijenhoek et al., 2018]. Patients with heterozygous pathogenic variants in *HIVEP2* share a nonspecific phenotype of devel-

Table 1. Patients with *HIVEP2* variants

	This publication		Srivastava et al. [2016]		
	patient 1	patient 2	patient 1	patient 2	patient 3
Gender	Female	Female	Female	Male	Female
Age at the time of publication	27 ys	23 ys	4 ys 7 mo	3 ys 9 mo	21 ys
Mutation	Frameshift	Nonsense	Nonsense	Nonsense	Frameshift
Variant	c.6609_6616delTGAGGGTC, p.Glu2204*	c.6667C>T, p.Arg2223*	c.2827C>T, p.Arg943*	c.3556C>T, p.Gln1186*	c.5737delG, p.Asp1913Metfs*15
Inherital status	De novo	De novo	De novo	De novo	De novo
Birth					
Head circumference	Unknown	31 cm (9th)	(10th)	(50th)	(3rd)
Height	53 cm (72th)	49 cm (72th)	(70th)	(25th)	(25–50th)
Weight	3,570 g (59th)	2,450 g (19th)	(85th)	(25–50th)	(25–50th)
Current measurements					
Head circumference	54.5 cm; Z = –0.6	48 cm; Z = –1	48 cm; Z = –1.5	48 cm; Z = –1.4	52.2 cm; Z = –2
Height	Unknown	111 cm (75th)	100 cm (70th)	80 cm (25th)	164 cm (25–50th)
Weight	Unknown	15.3 kg (15th)	17.1 kg (85th)	11 kg (25–50 th)	59 kg (25–50th)
Developmental delay					
Age at walking	18 mo	20–24 mo	36 mo	30 mo	36 mo
First words	4 ys	24 mo	24 mo	24 mo	7.5 ys
Language capability at the time of publication	Short sentences, limited vocabulary, partly inadequate to situation	Slow speech, difficult to understand, short and repetitive sentences, limited vocabulary	Language age equivalent of 15 mo	Unknown	Partially slurred articulation
Neurological features					
Intellectual disability	Mild delay	Mild delay	Severe delay	Moderate delay	Severe delay
IQ	58	52	Unknown	Unknown	Unknown
Behavioral disorders	No	No	Hyperactivity, impulsivity, distractibility	No	Aggressivity, impulsivity, self-stimulation, hyperactivity
Muscular hypotonia	Yes	No	No	Yes	Yes
Other neurological features	No	No	Dysphagia, frequent head tilt and leftward eye movements with preserved ability to fix/follow objects	Clumsy gait	Ataxia, dystonia
Brain CT/MRI	Not done	Not done	Slightly thin corpus callosum	Hypoplasia of corpus callosum	Mild frontal atrophy
Seizures	No	No	No	No	No
Other features					
Dysmorphic features	Slightly broader nose, narrow chin, thin upper lip	Elongated and mildly narrow face, small ears	Widely set eyes, broad nasal root, slightly upturned nose, high-arched palate	High forehead, medial eyebrow flare, widely set eyes, broad nasal root, small mouth, slightly tapering fingers, flat feet	Upslanting palpebral fissures, mild synophrys, small ears with attached earlobes, prominent nose with high nasal bridge and columella extending below the alae nasi, short philtrum, thin upper lip, overbite, hypertrophic gingiva, tapering fingers, radial deviation of 4th fingers, wide feet with short toes, hirsutism
Other medical issues	No	Constipation, oligo-menorrhea, frequent upper airways infections	Asymmetric cry, gastroparesis and projectile vomiting, unilateral hip dysplasia	Significant salivation, hypermetropia	Chronic constipation, gingival swelling and bleeding, high TSH levels, umbilical hernia, minor pulmonary stenosis

opmental delay/ID and other variable clinical features. Our 2 cases are the oldest reported patients with pathogenic *HIVEP2* variants that have been clinically characterized so far. Both reached maturity with mild ID but without predominant behavioral difficulties or other serious medical problems. Clinical follow-up of additional patients will outline the development of the mental phenotype and further define the clinical spectrum of *HIVEP2*-associated ID. Functional studies are needed to shed light on the molecular mechanisms underlying the pathological effect of the variants on the central nervous system.

Table 1 (continued)

	Steinfeld et al. [2016]					
	patient 1	patient 2	patient 3	patient 4	patient 5	patient 6
Gender	Female	Female	Male	Male	Female	Male
Age at the time of publication	7 ys	14 ys	10 ys	2 ys	11 ys	6 ys
Mutation	Nonsense	Nonsense	Frameshift	Missense	Frameshift	Frameshift
Variant	c.6475G>T, p.Gly2159*	c.2857G>T, p.Glu953*	c.5614dupG, p.Glu1872Glyfs*16	c.1189G>T, p.Asp397Tyr	c.6625dupT, p.Tyr2209Leufs*53	c.3434delC, p.Pro1145Argfs*2
Inherital status	De novo	De novo	De novo	De novo	De novo	De novo
Birth						
Head circumference	Unknown	32.4 cm	Unknown	Unknown	Unknown	Unknown
Height	53.34 cm (90th)	48.26 cm (25–50th)	53.3 cm (90th)	Unknown	Unknown	Unknown
Weight	3,997 g (90th)	3,062 g (25th)	3,856 g (75th)	3,770 g (50–75th)	3,880 g (75–90th)	4,100 g (90th)
Current measurements						
Head circumference	48.5 cm; $Z = -2.4$	51 cm; $Z = -2.6$	48.7 cm; $Z = -3.2$	46.7 cm; $Z = -1.7$	50.8 cm; $Z = 0.7$	52 cm; $Z = 0.3$
Height	125 cm (75th)	149.5 cm (5th)	140.9 cm (50–75th)	80.8 cm (<3rd)	152 cm (50–75th)	120 cm (50th)
Weight	22.8 kg (50th)	47.5 kg (25–50th)	31.4 kg (25–50th)	10.6 kg (<5th)	44 kg (50–75th)	24 kg (50–75th)
Developmental delay						
Age at walking	24 mo	36 mo	With walker, 30–36 mo; without walker 4–5 ys	Not yet	30 mo	22 mo
First words	18 mo	Unknown	Unknown	24 mo	10 mo	18 mo
Language capability at the time of publication	Speaks in short sentences; had 100 words by 3 ys	No verbal speech	No verbal speech	3 words	Full sentences and imitation; difficult to understand	Sentences with 3–4 words
Neurological features						
Intellectual disability	Mild delay	Delayed	Delayed	Unknown	Mild delay	Mild delay
IQ	75	Unknown	Unknown	Unknown	50	50–70
Behavioral disorders	Hand-wringing, oppositional/defiant behaviors	Hands in hair, hands up like puppet, wringing of hands	Tic-like head jerking at one point, impulsive, distractible	No	Sensitive to stimuli, requires structure	Hyperactivity, concentration problems, anxiety
Muscular hypotonia	Yes	Yes	Yes	Yes	Yes	No
Other neurological features	Static encephalopathy, dystonia, exercise intolerance/easily fatigued	Ataxia, spasticity, cerebral palsy, quadriplegia, muscle weakness, dystonia	Spasticity, muscle weakness, tremors, progressive parkinsonism, right hemiparesis, tongue fasciculations	No	Dyspraxia	No
Brain CT/MRI	Normal	Mild volume loss	Normal MRI abnormal MR spectroscopy	Normal	Incomplete myelination at age 4 ys	Normal
Seizures	Possible	Yes	Yes	No	No	No
Other Features						
Dysmorphic features	Mild retrognathia	Elongated and narrow face	Low anterior hairline, hirsute, prominent eyebrows, synophrys, epicanthal folds, mildly thickened helices and simple antihelices in ears, mild dental crowding of lower jaw	Small hands and feet	Somewhat high nasal bridge, broad mouth, rather flat philtrum, mild bifrontal narrowing, mildly broad halluces, sacral dimple, broad thorax, mild finger webbing	Square face, high/broad forehead, unilateral strabismus, high nasal bridge, columella under alae nasi, small square ears with transverse crease, small square teeth, microretro-gnathia
Other medical issues	Temperature instability, abdominal pain, GERD	Iron deficiency anemia, nephrocalcinosis on renal US, hip dysplasia, irregular menstrual periods, syncope, dyspnea, constipation, GERD, strabismus	GERD, milk protein intolerance as infant, frequent tonsillitis, strabismus (esotropia)	Reactive airway disease; AFOs primarily for ankles rolling due to hypotonia, short stature	Hypermobility of fingers, increased inversion of feet and decreased eversion	Bronchial hyperreactivity, breath holding spells, amblyopia and strabismus, high hyper-metropia

AFO, ankle-foot orthoses; GERD, gastroesophageal reflux disease; mo, months; TSH, thyroid-stimulating hormone; US, ultrasound; ys, years; *Z*, *Z*-score. Percentiles are given in parentheses.

Statement of Ethics

Informed consent was obtained prior to investigation.

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

J. Park collected and analyzed the data and drafted the manuscript. R. Colombo and L. Janiri kindly provided the clinical and molecular information of the second patient. In addition, R. Colombo contributed to the conception of the work, reviewed for accurate interpretation of molecular data, and revised the work thoroughly for scientific content. K. Schäferhoff and M. Grimmel were involved in WES data analysis and provided the molecular information. M. Sturm developed and applied the bioinformatics data analysis pipeline. T. Haack reviewed the work critically for appropriate content and supervised execution of the project. M. Kehrer, U. Grasshoff, and A. Dufke contributed as clinicians to the phenotypic analysis and cared for close communication with patient 1. M. Kehrer initiated and supervised the project.

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3.2 *KCNC1*-RELATED DISORDERS: NEW *DE NOVO* VARIANTS EXPAND THE PHENOTYPIC SPECTRUM

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BRIEF COMMUNICATION

KCNC1-related disorders: new de novo variants expand the phenotypic spectrum

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Abstract

A recurrent de novo missense variant in *KCNC1*, encoding a voltage-gated potassium channel expressed in inhibitory neurons, causes progressive myoclonus epilepsy and ataxia, and a nonsense variant is associated with intellectual disability. We identified three new de novo missense variants in *KCNC1* in five unrelated individuals causing different phenotypes featuring either isolated non-progressive myoclonus (p.Cys208Tyr), intellectual disability (p.Thr399Met), or epilepsy with myoclonic, absence and generalized tonic-clonic seizures, ataxia, and developmental delay (p.Ala421Val, three patients). Functional analyses demonstrated no measurable currents for all identified variants and dominant-negative effects for p.Thr399Met and p.Ala421Val predicting neuronal disinhibition as the underlying disease mechanism.

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Introduction

Epilepsy and intellectual disability (ID) are common neuropsychiatric disorders with an approximate prevalence of 0.3 to 2%.[1–4] A subgroup of cases is due to pathogenic variants in potassium channels, which, however, might also present with a range of additional neurological features, such as ataxia.[5–9] The potassium channel subfamily K_V3 consists of four subunits ($K_V3.1$, $K_V3.2$, $K_V3.3$, and $K_V3.4$) which are encoded by *KCNC1*, *KCNC2*, *KCNC3*, and *KCNC4*.[10] Mutations in *KCNC3* are a well-established cause of spinocerebellar ataxia type 13, whereas *KCNC2* and *KCNC4* have so far not been associated with human disease.[8,9]

The evolutionarily highly conserved voltage-gated potassium channel $K_V3.1$ is predominantly expressed in fast-spiking neurons to enable high-frequency firing by fast channel activation and membrane repolarization.[10] Fast-spiking neurons include GABAergic interneurons in the neocortex and hippocampus, Purkinje cells in cerebellum, and neurons in central auditory nuclei.[10,11] To date, only one recurrent de novo missense variant in *KCNC1* (c.959G > A, p.Arg320His) has been reported as a cause of progressive myoclonus epilepsy and ataxia (MEAK; OMIM #616187). The respective phenotype is similar to Unverricht-Lundborg disease.[12–15] Subsequently, one nonsense variant (c.1015C > T, p.Arg339*) has been identified in three affected members of single family with ID without seizures.[16] Here, we report three new pathogenic de novo missense variants in *KCNC1* in five unrelated patients. The provided clinical information adds to the phenotypic delineation of *KCNC1*-related disease.

Patients, Materials, and Methods

Clinical and genetic investigations

Patients were evaluated by neurologists and referred for diagnostic whole exome sequencing (WES) at different centers. The methods for WES and Sanger sequencing have been previously described.[17,18] Written informed consent to participate in this study was obtained from all patients or their parents.

Functional analysis

The functional evaluation of identified *KCNC1* variants was performed using two-electrode voltage-clamp recordings as previously described.[13] Briefly, the three missense variants were introduced in the human *KCNC1* cDNA (NM_004976) cloned in a pCMV Entry Vector (OriGene Technologies, USA) using the Quick Change Method (Stratagene, USA). The plasmids were linearized and in vitro transcription was performed using T7 RNA Polymerase (Roche Diagnostics GmbH, Germany). *Xenopus laevis* oocytes (EcoCyte Bioscience, Germany) were washed in OR2 and incubated in Barth solution with gentamycin. Fifty-nanoliters of cRNA (2 $\mu g/\mu L$) was injected using Robooinject® (Multichannel Systems, Germany) and stored at 16°C. Potassium currents were recorded after 2–3 days at room temperature (21–23°C) on Roboocyte2® (Multichannel Systems, Germany). Data analysis and graphical illustrations were achieved using Roboocyte2+ (Multichannel Systems, Germany), Excel (Microsoft, USA), and Graphpad Software (GraphPad Software, USA). Statistical evaluation for multiple comparisons ($P < 0.05$) was conducted using one-way ANOVA on ranks with Dunn's post hoc test.

Results

Genetic testing

WES revealed three different heterozygous missense variants in *KCNC1* (NM_001112741.1) in five unrelated patients. Patient 1 carries c.623G > A, p.Cys208Tyr, patient 2 c.1196C > T, p.Thr399Met, and patients 3, 4, and 5 c.1262C > T, p.Ala421Val. All variants are absent from public databases [1000 Genomes project, Genome Aggregation Database (gnomAD 2.0.2), Exome Aggregation Consortium (ExAC 0.3.1)]. In line with a postulated de novo status, none of the variants was detected in DNA extracted from parental whole blood (Fig. 1A). Additional rare variants identified in patients 1, 2, and 3 are provided in Table S1. We cannot entirely rule out a potential contribution of these changes to the observed phenotypes. However, to the best of our knowledge there is currently no evidence supporting a functional relevance and putative disease association of these additional changes.

Clinical phenotypes

Patient 1 (Cys208Tyr)

A 23-year-old German woman reported that mild tremor-like symptoms began on both hands at the age of 2 years.

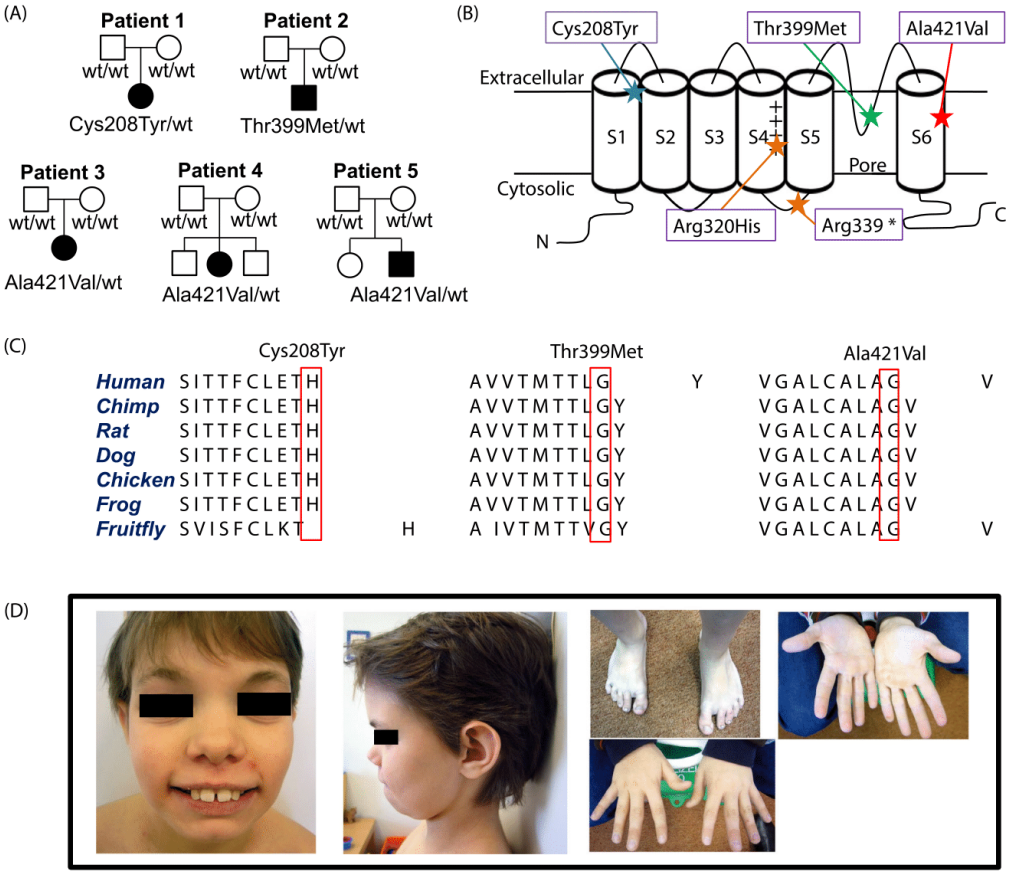

Figure 1. (A) Pedigrees of the five unrelated affected individuals (closed symbols) with de novo *KCNC1* variants and status of healthy family members (open symbols). wt indicates for wild type. (B) Graphical illustration of the $K_V3.1$ channel demonstrates the domain structures. The positions of the identified variants (Cys208Tyr, Thr399Met, Ala421Val) and the previously published variants (Arg320His and Arg339*) are highlighted with stars. The plus sign illustrates the positively charged arginine in the voltage-sensing S4 segment.[21] (C) Amino acid sequences across different species indicate that the variants are localized in highly conserved regions. (D) Images of patient 2 at 11 years of age show hypertelorism, long palpebral fissures, broad nose, large ears, diastema, small chin, and sandal gap. The hands of patient 2 do not have any dysmorphic features.

Her psychomotor development was normal. Neurological examination revealed mild and nonprogressive constant high-frequency action and postural myoclonus (or irregular tremor) on both arms with dystonic features on the right hand and arm with constant hyperextension of the fourth and fifth finger and reduced arm swing during gait (Video S1). The patient initially received diagnostics and various treatments for tremor without success (see below), and the quite jerky aspect of the irregular movements is more reminiscent of myoclonus in our opinion. An electromyographical (EMG) recording which might have helped to distinguish between tremor and myoclonus has not been performed. Copper metabolism, F-dopa PET, and MRI scans of the brain and multiple EEG recordings were normal. Treatment trials with beta blockers, levodopa, and primidone were unsuccessful. Epileptic seizures were not reported.

Patient 2 (Thr399Met)

An 18-year-old German male works in a sheltered workshop and shows some articulation difficulties. When he

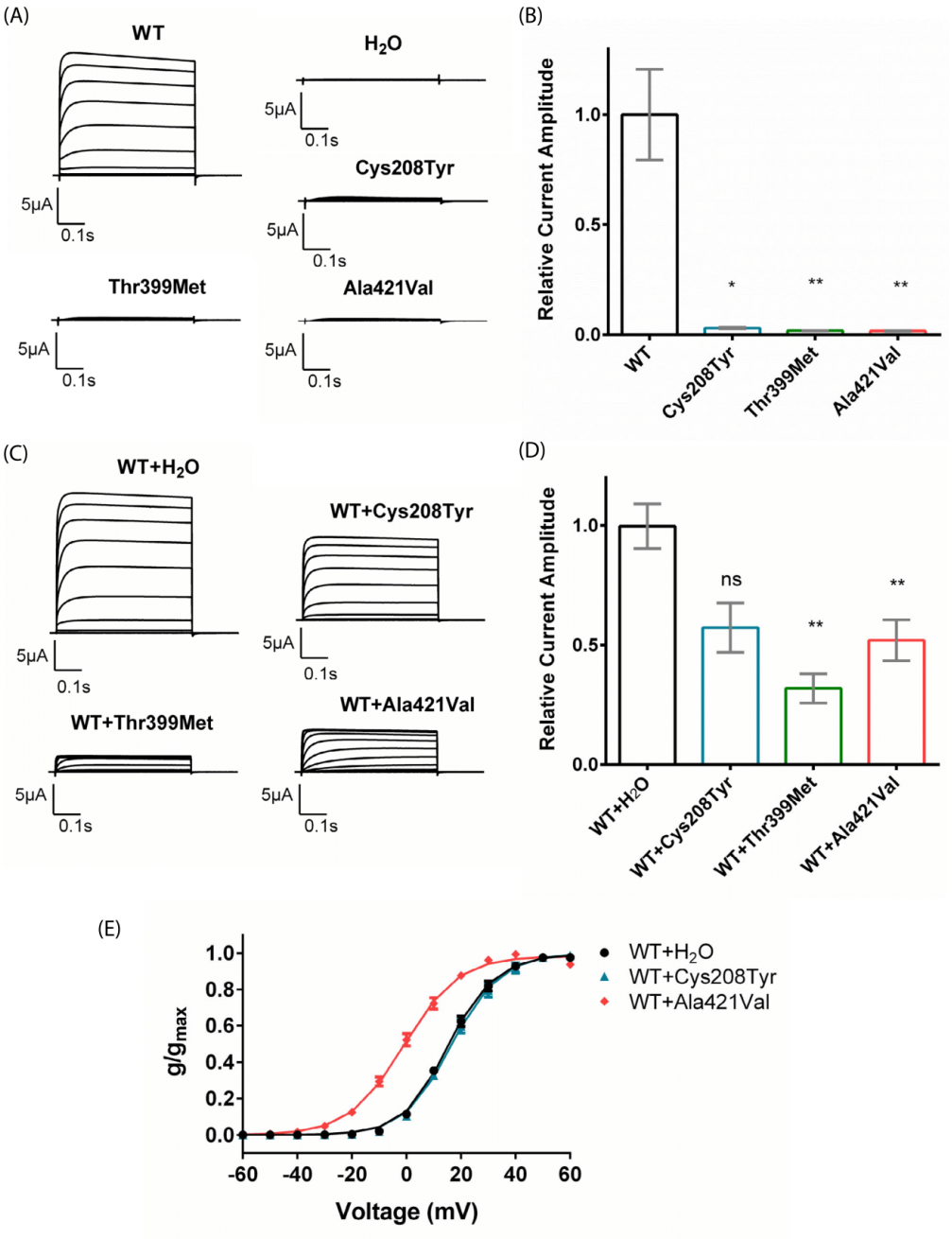

Figure 2. Functional consequences of the identified *KCNC1* variants. (A) Representative traces of $K_V3.1$ currents recorded in *Xenopus laevis* oocytes expressing the wild type (WT) and the single-site variants (Cys208Tyr, Thr399Met, Ala421Val) in response to the voltage steps from − 60 mV to + 60 mV. (B) Relative current amplitudes of oocytes injected with the WT (n = 23), Cys208Tyr (n = 8), Thr399Met (n = 14), and Ala421Val (n = 8) mutant channels (Dunn's test, $P < 0.05$). Mean current amplitudes of currents elicited by a + 40 mV voltage step were analyzed between 0.4 and 0.5 msec and normalized to the mean value of WT channels recorded on the same day. (C) Representative current traces recorded in oocytes that were coinjected with WT cRNA and either water or a mutant cRNA in a 1:1 ratio. (D) Relative current amplitudes recorded from oocytes coexpressing WT and mutant channels (WT + H_2O (n = 36), WT + Cys208Tyr (n = 8), WT + Thr399Met (n = 6), WT + Ala421Val (n = 27)) were normalized to the mean current amplitude of oocytes coinjected with the WT channel and water recorded on the same day (Dunn's test, $P < 0.05$). (E) Mean voltage-dependent activation of $K_V3.1$ channel for WT (n = 20), WT + Cys208Tyr (n = 5) and WT + Ala421Val (n = 10) channels. Lines illustrate Boltzmann Function fit to the data points. The activation curve of WT + Ala421Val channels showed a significant shift to more hyperpolarized potentials in comparison to WT channels alone. All data are shown as means ± SEM. The following symbols were used for statistical differences: $^{*}P < 0.05$, $^{**}P < 0.01$ and ns for not significant.

was last examined at the age of 11 years, he was attending special school. He was diagnosed with mild to moderate intellectual disability and showed behavioral abnormalities, for example, difficulties socializing with other children. Motor development was slightly delayed, but his language was severely affected with first words by the age of 5 years. Dysmorphic features are shown in Figure 1D. There were neither congenital malformations nor any reported seizures. Neurological examination was unremarkable. EEG and MRI scan of the brain were normal.

Patient 3 (Ala421Val)

Patient 3 is a 5-year-old Croatian female in whom seizures (focal onset impaired awareness seizures, tonic-clonic and myoclonic seizures) were first noted at 5 months of age and occurred up to 40 times per day. First generalized tonic-clonic seizures started at the age of 2 years. Treatments with levetiracetam, zonisamide, and carbamazepine were unsuccessful. Finally, a combination therapy with clobazam and topiramate reduced her seizure frequency to 1–2 per month. She had global developmental delay and mild gait ataxia, which was so far nonprogressive. The EEG showed multifocal epileptic discharges and irregular spike-wave complexes with polyspikes followed by bilateral synchronic 2/s spike-wave activities. Cerebral MRI was normal.

Patient 4 (Ala421Val)

Patient 4, a 2-year-old female of Turkish origin, first presented with febrile seizures 3 weeks after birth. Seizures then occurred 15–35 times per day and lasted for 5–35 sec with even higher seizure frequencies during episodes with high fever, which did not respond to levetiracetam or valproate. She mostly had myoclonic absence seizures (Video S1), and less frequently myoclonic seizures without impaired awareness or absence seizures without myoclonus. During the myoclonic absence seizure, her eyes rolled upwards and both her proximal arms twitched for a few seconds. Her global development was delayed. She is now able to walk and vocalize but does not speak. Twenty-four-hour-EEG showed normal background activity and multiple seizure episodes with rhythmic bifrontal 2–3/s spike-wave discharges for 6–20 sec. Cerebral MRI was unremarkable (Fig. S2).

Patient 5 (Ala421Val)

This 2-year-old Chinese-French male showed first myoclonic seizures at 5 months of age. The initial seizure frequency was 1–2 per day, which increased to 60–70 per day after 4 months. Treatment with valproate, levetiracetam, and clonazepam reduced his seizure frequency dramatically to once per month. He had predominantly myoclonic absence seizures (Video S1 with face covered) presenting with rapid eyelid myoclonia accompanied by twitching of proximal arms. The myoclonus was mostly on the left side, but occasionally occurred on both arms. In addition, he also had absences without myoclonus lasting for approximately 10 sec. At 8 months, he had two generalized tonic-clonic seizures. The EEG showed episodes with generalized rhythmic discharges of 2–4 Hz, sometimes only as waves, sometimes as spike-waves, lasting up to 15 sec and often accompanied by myoclonic movements visible in the electromyographic trace (Fig. S3). His development was delayed; he is now able to speak ten simple words, but cannot stand alone without any help. Cerebral MRI did not show any abnormalities.

Functional consequences of *KCNC1* variants

Current amplitudes recorded in oocytes expressing either of the three mutant channels were barely detectable and similar to water-injected controls (Fig. 2A and 2). Coexpression of wild type (WT) with mutant channels indicated dominant-negative loss-of-function effects with a significant decrease in K^+ current amplitudes of approximately 68% and 48% for Thr399Met and Ala421Val mutant channels compared to WT alone (Fig. 2C and D),

Table 1. Clinical features of *KCNC1* patients

	This publication	This publication	This publication	This publication	This publication	MEAK patients[13–15]	Poirier et al. 2017
Patient	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	22 cases	3 cases
Variant	**c.623G > A, p.Cys208Tyr**	**c.1196C > T, p.Thr399Met**	**c.1262C > T, p.Ala421Val**	**c.1262C > T, p.Ala421Val**	**c.1262C > T, p.Ala421Val**	**c.959G > A, p.Arg320His**	**c.1015C > T, p.Arg339***
Inheritance	De novo	De novo	De novo	De novo	De novo	De novo (14), 3 families (8)	Paternal
Age at onset (current age)	2 years (23 years)	1–2 years (18 years)	5 months (5 years)	3 weeks (2 years)	5 months (2 years)	3–15 years	1–2 years
First sign	Myoclonus or "tremor"	Developmental delay	Myoclonic seizures	Febrile seizures	Myoclonic seizures	Myoclonus or "tremor"	Developmental delay
Seizures	No	No	Tonic-clonic, focal onset impaired awareness, myoclonic, generalized	Myoclonic absence, myoclonic, absence	Myoclonic absence, absence, generalized	Tonic-clonic, myoclonic, generalized	No
Action-induced Myoclonus	Mild, nonprogressive	No	No	No	No	Severe, progressive	No
EEG	Normal	Normal	Normal background, irregular spike-wave activity with polyspikes and rhythmic generalized 2 Hz spike-waves	Normal background activity, generalized 2–3 Hz spike-wave discharges	Normal background, generalized 2–4 Hz rhythmic slow waves and sometimes spike-waves	Normal background, generalized polyspike, polyspike-wave and spike-wave (13), unknown (9)	Normal
Brain MRI	Normal	Normal	Normal	Normal	Normal	Global symmetrical cerebellar atrophy (13) unknown (9)	Normal
Ataxia	No	No	Mild, so far nonprogressive	Balancing difficulties possible	No	Progressive	No
Developmental delay	No	Yes	Yes	Yes	Yes	Mild (2), no (20)	Yes
Cognitive Decline	Possible memory deficits (MOCA 28/30)	No	No	No	No	Yes (11), possible (2), no (7)	No
Dysmorphism	No	Hypertelorism, long palpebral fissures, broad nose, large ears, diastema, small chin	No	No	No	No	Prognathism, protruding ears, short philtrum, fetal pads, epicanthal folds, ptosis
Others	Dystonia, scoliosis	Frequent diarrhea and vomiting	Mild muscular hypotonia	Mild muscular hypotonia	Cannot walk yet	Wheelchair-dependent (11)	Clinodactyly of the fifth finger (1)

whereas coexpression of Cys208Tyr mutant and WT channels did not cause a significant amplitude reduction. The activation curve showed a hyperpolarizing shift when WT channels were coexpressed with Ala421Val mutant channels in comparison to WT channels alone (Fig. 2E), whereas Cys208Tyr channels did not show any significant

difference. Thr399Met showed a strong dominant-negative effect on the WT which impeded the evaluation of further gating parameters.

Discussion

We here identified three new de novo missense variants in *KCNC1* in five unrelated individuals presenting with different clinical phenotypes compared to previously reported *KCNC1* patients. Patient 1 (Cys208Tyr) exhibited nonprogressive, relatively mild, action-induced myoclonus (or irregular tremor) as the only clinical sign without any cerebellar, epileptic, or cognitive symptoms. In contrast, MEAK patients (Arg320His) had a more severe and progressive action-induced myoclonus, epilepsy, and ataxia leading to wheelchair dependency in 11 of 22 published patients by the age of approximately 17 years.[13–15] The phenotype of patient 2 (Thr399Met), who showed ID and dysmorphic features, is more similar to the family reported by Poirier et al. in which three affected members carried the nonsense mutation Arg339*.[16] All three had similar dysmorphic features, which however differed from those observed in patient 2 (Fig. 1D, Table 1). Compared to MEAK patients, also the three unrelated patients (3, 4, and 5) carrying the Ala421Val variant presented with different symptoms, neither showing myoclonus, but myoclonic and absence seizures and developmental delay. The presence of ataxia is difficult to judge as all three are still very young. While this study was underway, the change Ala421Val has been submitted to ClinVar by another group, indicating that it might represent a more frequent recurrent cause of ID and seizures.[19]

Functional studies demonstrated a complete loss-of-function for all three variants with a significant dominant-negative effect on WT channels for Thr399Met and Ala421Val (Fig. 2A–D). Similar to the previously published variant Arg320His, Ala421Val caused a hyperpolarizing shift of the activation curve when coexpressed with WT, which was not observed for Cys208Tyr (Fig. 2E).[13] In contrast to the haploinsufficiency of the truncating variant in the previously described family with ID, the variant Thr399Met, also causing ID alone, showed a pronounced dominant-negative effect.[16] Our current data do thus not reveal a clear correlation between the electrophysiological properties of mutant channels and clinical phenotypes. It is striking, however, that both recurring variants (Arg320His and Ala421Val) cause a different but homogeneous phenotype each, indicating specific effects of the variants themselves, despite their similar biophysical properties. Further functional characterizations in neuronal cells are needed to shed more light on the cellular and network mechanisms underlying the pathological effect of the variants on the nervous system.

$K_V3.1$ is prominently expressed in inhibitory GABAergic interneurons in which these channels enable high-frequency firing by a rapid membrane repolarization.[10] The identified variants thus probably lead to impaired firing of GABAergic interneurons predicting neuronal disinhibition as the underlying disease mechanism. Patients 3 and 5 were both treated with benzodiazepines. Their effect on GABA neurotransmitters, enhancing the inhibitory effect on neurons might have played a critical role in reducing the patients' seizure frequencies. Also Oliver et al described that clonazepam beside valproate was most effective in MEAK patients.[14] Another more specific therapeutic strategy might be to directly activate mutant heterotetrameric K_V3 channels. The feasibility of such an approach with a compound called RE01 has been recently reported in vitro.[20]

In conclusion, we provide evidence that de novo variants in *KCNC1* cause more diverse phenotypes than described so far, such as nonprogressive myoclonus (or tremor) alone, intellectual disability, or epilepsy with myoclonic, absence and generalized tonic-clonic seizures with developmental delay.

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Author Contributions

J. P. was responsible for the conception and design of the study, collecting and analyzing the data, and drafting the manuscript. J. P., M. K, U. B. S. H., M. G, S. B. W, M. H., M. S, and T. B. H. contributed to analysis and interpretation of data. A. H., K. C., E. H., B. A., D. K., L. K., A. T., L. C. M., T. M. S., E. B. R., H. E., and M. W. contributed to phenotyping, acquisition, and analysis of data. T.B.H. and H.L. were responsible for the conception, design and supervision of the study, and writing of the manuscript. All authors revised the manuscript for intellectual content.

Conflict of Interest

Nothing to report.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Brain imaging, X-ray and EEG of patient 1 with a Cys208Tyr variant.
Figure S2. Brain imaging and EEG of patient 4 with a Ala421Val variant.
Figure S3. EEG-electromyographic (EMG) recording of patient 5 with a Ala421Val variant.
Table S1. Identified rare variants in patients 1, 2, and 3.
Video S1. The supplementary video shows action myoclonus in patient 1 and myoclonus absence seizures in patients 4 and 5.

3.3 *DE NOVO* VARIANTS IN *SLC12A6* CAUSE EARLY-ONSET PROGRESSIVE SENSORIMOTOR NEUROPATHY

SHORT REPORT

De novo variants in *SLC12A6* cause sporadic early-onset progressive sensorimotor neuropathy

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ABSTRACT

Background Charcot-Marie-Tooth disease (CMT) is a clinically and genetically heterogeneous disorder of the peripheral nervous system. Biallelic variants in *SLC12A6* have been associated with autosomal-recessive hereditary motor and sensory neuropathy with agenesis of the corpus callosum (HMSN/ACC). We identified heterozygous de novo variants in *SLC12A6* in three unrelated patients with intermediate CMT.

Methods We evaluated the clinical reports and electrophysiological data of three patients carrying de novo variants in *SLC12A6* identified by diagnostic trio exome sequencing. For functional characterisation of the identified variants, potassium influx of mutated KCC3 cotransporters was measured in *Xenopus* oocytes.

Results We identified two different de novo missense changes (p.Arg207His and p.Tyr679Cys) in *SLC12A6* in three unrelated individuals with early-onset progressive CMT. All presented with axonal/demyelinating sensorimotor neuropathy accompanied by spasticity in one patient. Cognition and brain MRI were normal. Modelling of the mutant KCC3 cotransporter in *Xenopus* oocytes showed a significant reduction in potassium influx for both changes.

Conclusion Our findings expand the genotypic and phenotypic spectrum associated with *SLC12A6* variants from autosomal-recessive HMSN/ACC to dominant-acting de novo variants causing a milder clinical presentation with early-onset neuropathy.

INTRODUCTION

Charcot-Marie-Tooth disease (CMT), also known as hereditary sensory and motor neuropathy (HSMN), is the most common inherited disorder of the peripheral nervous system with a variety of inheritance pattern that can be autosomal-dominant, autosomal-recessive or X linked.[1] Demyelinating CMT with median motor nerve conduction velocity (MNCV) below 38 m/s is classified as CMT1 and axonal CMT with MNCV above 45 m/s as CMT2. For CMT with axonal and demyelinating features with MNCV between 25 and 45 m/s, the term intermediate CMT has been introduced.[1–3] To our knowledge, six genes (*DNM2*, *YARS*, *MPZ*, *INF2*, *GNB4* and *NEFL*) have been associated with autosomal-dominant intermediate CMT (DI-CMT) and assigned with phenotype *Mendelian Inheritance in Man* numbers.[2] Due to advances in sequencing technologies, the number of CMT-associated genes almost doubled in the past 10 years.[4] The contribution of de novo sequence variation in the pathogenesis of a number of sporadic early-onset neurological phenotypes is increasingly recognised.[5 6] In particular, the application of trio exome sequencing led to the identification of numerous disease-causal de novo mutations, thereby expanding the phenotypic spectrum associated with many disease loci and linking dominant phenotypes to genes that have originally been reported in the context of recessive disorders.[4 7]

Potassium chloride cotransporters (KCC1, KCC2, KCC3 and KCC4) are encoded by the *SLC12A* gene family (*SLC12A4*, *SLC12A5*, *SLC12A6* and *SLC12A7*) and are involved in regulation of the intracellular ionic milieu by mediating electroneutral potassium and chloride efflux in response to osmotic changes.[8] By modulating intracellular chloride concentrations, KCC plays an important role in the maintenance of cell volume, neural excitability and epithelial transport.[9] KCC3, which is encoded by *SLC12A6*, is predominantly expressed in the brain and spinal cord (neurons and glial cells) of the central nervous system and in the dorsal root ganglion of the peripheral nervous system.[10 11] Dysregulation of chloride concentrations in neurons may influence the neuronal activity and their response to GABA.[8] However, the specific pathophysiology of peripheral neuropathy caused by KCC3 defects is yet unclear.

Biallelic loss-of-function variants in *SLC12A6* are associated with hereditary motor and sensory neuropathy with agenesis of the corpus callosum (HMSN/ACC), also known as Andermann syndrome (OMIM #218000), which is characterised by severe progressive motor and sensory neuropathy, developmental delay, intellectual disability and variable degrees of agenesis of the corpus callosum.[12–14] A single heterozygous de novo missense variant (p.Thr991Ala) has been postulated to act as a gain-of-function variant causing severe motor neuropathy without agenesis of the corpus callosum in a 10-year-old patient.[15] Using diagnostic trio exome sequencing, we identified two new de novo variants in *SLC12A6*, affecting evolutionarily highly conserved amino acid residues, in three unrelated patients with early-onset, severe and progressive CMT without intellectual disability. Our results further support the hypothesis of dominant-acting heterozygous de novo SLC12A6 variants as the underlying disease mechanism in a subset of CMT patients in addition to the well-established

recessive-type *SLC12A6* variants causing a more severe clinical presentation.

MATERIALS AND METHODS

Patients

Patients were examined by neuropaediatricians and referred for diagnostic genetic testing to different centres. The investigated patients or their guardians provided informed written consent for diagnostic exome sequencing and data analysis in the context of the respective disease phenotypes in a routine diagnostic setting. In a second step, clinical and genetic data were retrospectively evaluated in more detail after receiving additional informed consents from the patients' families including also the permission for data publication.

Diagnostic exome sequencing

Diagnostic exome sequencing was performed on DNA isolated from patients' and parental EDTA-blood as described previously.[16]

Functional analysis

KCC3-mediated K^+ transport was measured using unidirectional ^{86}Rb uptakes in groups of 20–25 *Xenopus laevis* oocytes injected with 5 ng c-myc-tagged wild-type or mutant KCC3 cRNA.[17 18] Oocytes were incubated with 1 mL isosmotic or hyposmotic flux solution containing 5 µCi ^{86}Rb for 1 hour then washed four times with ice-cold solution. ^{86}Rb uptake in single oocyte was measured by β-scintillation counting, and K^+ influx was expressed in pmoles K^+ per oocyte per hour. For Western blot analysis, groups of 8 oocytes were homogenised in CHAPS-containing lysis buffer, subjected to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, incubated with mouse monoclonal anti-cmyc antibody (clone 9E10, Thermo Fisher Scientific) followed by horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody.

RESULTS

Clinical presentation

Detailed clinical and electrophysiological data are summarised in table 1.

Patient 1 (Arg207His)

This 14-year-old boy is the second child of healthy German parents (figure 1A). He learnt to walk freely at 18 months and showed bilateral muscle weakness in the lower limbs with frequent falls. He did not show any learning difficulties and he now has normal speech and cognition. On examination, he presented with an intermediate type sensorimotor predominantly demyelinating neuropathy and diminished patellar and deep tendon reflexes. He had distal more than proximal muscle atrophy and weakness, but clinically intact proprioception and nociception. Mean MNCV was determined between 23 and 33 m/s, distal motor latencies were prolonged and sensory nerve conduction velocity was reduced. Nerve hypertrophy was not observed in the nerve sonography of the roots, the large upper and lower limb nerves. He required a right ankle foot orthosis due to contractures at the age of 9 years. Mild haemolytic anaemia was observed since the age of 4 years, which could not be explained so far. MRI scans of the brain were normal. Epileptic seizures were not reported.

Patient 2 (Arg207His)

This 11-year-old boy was born by vaginal delivery; he is the fourth of five siblings (figure 1A). His early motor milestones were normal. Walking was delayed and his gait remained impaired. He never achieved running. MRI of the brain and lumbar spine were normal. He developed spells of shaking which did not have an EEG correlate at age 9 years and were diagnosed as anxiety. One EEG showed a 2 s burst of generalised spike and wave activity in sleep. He never had a clinical seizure and his cognition was normal. EMG at age 5 years showed a severe motor and sensory peripheral neuropathy. Repeat EMG at age 11 years showed progression of a severe mixed axonal/demyelinating (predominantly demyelinating) chronic neuropathy. His gait and fine motor skills progressively worsened. On examination, he has length-dependent sensory loss, and distal more than proximal muscle weakness, unobtainable tendon reflexes and muscle atrophy (figure 1B and online supplementary file 1). He ambulates with braces and falls frequently. He was recently diagnosed with obstructive sleep apnoea.

Patient 3 (Tyr679Cys)

Patient 3 (figure 1A) is a 15-year-old girl who demonstrated mild motor developmental delay. She learnt to walk independently at 24 months. At 13 years, she was small for her age with 38 kg (10th centile) and 141 cm (3rd centile). Head circumference has always been within normal range (52.2 cm, 10th centile). She showed marked distal lower limb weakness and hand weakness. Apart from ankle contractures and pes planovalgus, other deformities such as scoliosis or dysmorphic features were not noted. Neurological examination revealed spastic gait with brisk patellar and deep tendon reflexes. Vibration sense and pinprick sensation was normal in the limbs, but reduced in the periumbilical region. Electrophysiological examinations over the course of 3 years revealed a progressive axonal/demyelinating neuropathy with MNCV between 33 and 45 m/s. MRI of the brain and spine were normal. She suffered from celiac disease and occasional migraine attacks. There were neither organic malformations, complicated hospitalisations nor any evidence of seizures.

Genetic evaluation

Prior to diagnostic trio exome, mutations in CMT-related genes, including copy number variants in *PMP22* gene were excluded in patients 1 and 2. Diagnostic trio whole exome sequencing revealed de novo missense variants in *SLC12A6* (NM_133647.1) in patients 1, 2 and 3. Patients 1 and 2 carry the same missense variant c.620G>A, p.Arg207His and patient 3 carries the missense variant c.2036A>G, p.Tyr679Cys (figure 1A, table 1). Both variants are absent from public databases (1000 Genomes project, Genome Aggregation Database (gnomAD r2.0.2), Exome Aggregation Consortium (ExAC r0.3), 04/2019) as well as an in-house database.

Functional analyses

To assess the functional consequence of single amino acid substitutions in KCC3, we performed standard K^+ influx measurements in *Xenopus laevis* oocytes injected with wild-type or mutant mouse KCC3 RNA. The mouse sequence is highly conserved and all mutated and neighbouring residues are identical between the two species. In fact, these residues are also conserved with all members of the SLC12A family of transporters (figure 1C) and within KCC3 are conserved from *C. elegans* to *H. sapiens* (figure 1D). As seen in figure 1E, the level of K^+ influx under isosmotic conditions (grey boxes) in KCC3-injected oocytes is

Table 1 Clinical and genetic findings in patients with *SLC12A6 de novo* variants

	Patient 1	Patient 2	Patient 3	Kahle *et al* (2016)[15]	Andermann syndrome[13 14]
Age at onset (current age)	1–2 years (11 years)	1–2 years (11 years)	1–2 years (15 years)	1–2 years	Infancy
Sex	Male	Male	Female	Male	Male and female
Mutation	c.620G>A, p.Arg207His	c.620G>A, p.Arg207His	c.2036A>G, p.Tyr679Cys	c.2971A>G, p.Thr991Ala	Missense, splice and truncating
Inheritance	De novo (dominant)	De novo (dominant)	De novo (dominant)	De novo (dominant)	Autosomal recessive
Main diagnosis	Polyneuropathy	Polyneuropathy	Spastic paraparesis, neuropathy	Polyneuropathy	Complex neurodevelopmental disorder, Intellectual disability, neuropathy
First sign	Frequent falls and tripping	Delayed walking	Spastic gait and delayed walking	Foot dragging at 9 months, foot drops at 15 months	Developmental delay, generalised hypotonia, areflexia
Motor development	Delayed, walking at 18 months	Delayed	Delayed, first steps at 24 months	Delayed	Delayed, standing and walking at 4–6 years
Neuropathy					
Motor or sensory	Both, predominant motor	Both	Both	Both, predominant motor	Both
Axonal or demyelinating	Both, predominant demyelinating with intermediate type	Both, predominant demyelinating	Both, predominant axonal	Both, predominant axonal, Secondary demyelinating	Both
Sensory loss	No	Absent vibration sense and reduced perception of touch	Periumbilical hypaesthesia	No	Yes
Reflexes	Diminished BR, PR and AR	Absent BR, PR and AR	Normal BR, brisk and extended reflex zones in PR and AR	Absent AR	Absent
Spasticity	No	No	Yes	No	No
UL weakness prox. (MRC Scale)	Shoulder and elbow extension (5-/5), otherwise normal (5/5)	Yes (4/5)	No	No	Yes, severe
UL weakness dist. (MRC Scale)	Handwriting problems, otherwise stable	Yes (3/5) hand intrinsics	Hands (5-/5), otherwise normal	Wrist and finger extension (2/5)	Yes, severe
LL weakness prox. (MRC Scale)	Hip abduction (4+/5), hip flexion (5-/5)	Minimal (4+/5)	No	Yes	Yes, severe
LL weakness dist. (MRC Scale)	Foot dorsiflexion (4-/5), di-fficulties standing on heels	Yes (3/5)	Diminished (4/5)	Severe (1-2/5)	Yes, severe
UL muscle atrophy prox./dist.	No/no	No/yes	No/no	Yes/yes	Yes/yes
LL muscle atrophy prox./dist.	No/yes	No/yes	No/yes	Yes/yes	Yes/yes
UL motor nerve					
DML	Prolonged median: 5.5 ms	Prolonged, median: 2.1 ms, ulnar: 3.8–5.9 ms	Normal	Prolonged, median: 4.8–5.5 ms, ulnar: 4.3–6.3 mV	Prolonged
NCV	Reduced, median: 33 m/s	Reduced, median: 32–35 m/s, ulnar 25–27 m/s	Normal	Reduced, median: 14–31 m/s, ulnar: NR-14 m/s	Variably reduced
CMAP	Reduced, median: 1.2 mV	Reduced-normal, median: 3.7–7.3 mV, ulnar: 0.8–1.4 mV	Normal	Reduced, median: 0.2 mV, ulnar: 0.1 mV	Reduced
UL sensory nerve	Reduced, median; SNAP (3.5 mV), NCV (21 m/s)	NR	Normal	Reduced, median: SNAP (9 mV), NCV (44 m/s)	Reduced or NR
LL motor nerve					
DML	Prolonged, tibial: 5.2 ms	Prolonged, tibial: NR, peroneal: 9.2–44 ms	Prolonged, tibial: 3.9–5.5 ms, peroneal: 4.6–5.4 ms	Prolonged, tibial: 6.1 ms, peroneal: NR	Prolonged
NCV	Reduced, tibial: 23 m/s	Tibial: NR, peroneal: normal 90 m/s	Reduced, tibial: 33–40 m/s, peroneal 37–44 m/s	NR, tibial and peroneal	Reduced
CMAP	Reduced, tibial: 3.5 mV	Reduced, tibial: NR, peroneal: 0.2–2.3 mV	Reduced, tibial: 1–1.9 mV, peroneal: 0.5–0.8 mV	Reduced, tibial: 0.4 mV, peroneal: NR	Reduced
LL sensory nerve	NR	NR	NR	Normal SNAP, reduced sural NCV: 27 m/s	Reduced or NR

Continued

Table 1 Continued

	Patient 1	Patient 2	Patient 3	Kahle *et al* (2016)[15]	Andermann syndrome[13 14]
Brain and spinal MRI	Normal	Normal	Normal, non-specific minimal white matter lesions periventricular, stable over 2 years	Normal	Variable degrees of agenesis of the corpus callosum (complete, partial and also normal)
Seizures, EEG	No	No, bursts of generalised spine and polyspike and wave discharges in sleep EEG	No	No	Yes (>15%)
Intellectual disability	No	No	No	No	Yes
Dysmorphism	No	No	No	No	Yes
Deformities (foot, scoliosis and so on)	Achilles tendon retraction	Scoliosis, hand and foot deformities	Achilles tendon retraction, planovalgus foot	Unknown	Scoliosis and achilles tendon retraction
Other	Recurrent mild haemolytic anaemia, no hypertrophic Nerves in nervesonography	None	Celiac disease, short stature, migraine, bladder and bowel incontinence	Lost the ability to independently ambulate at 9 years	Wheelchair or bedridden by adolescence, average age of death 33 years
Disease progression	Yes	Yes	Yes	Yes	Yes

AR, achilles reflex;BR, biceps reflex; CMAP, compound muscle action potential; DML, distal motor latency;LL, lower limb;MRC Scale, Medical Research Council scale for muscle strength; NCV, nerve conductance velocity; NR, no response; PR, patellar reflex;SNAP, sensory nerve action potential; UL, upper limb; dist., distal; prox., proximal.

not different from that of water-injected oocytes. This reflects the known absence of KCC3 function under isotonic conditions. When oocytes are subjected to a hypotonic shock (black boxes), K^+ influx is significantly higher in KCC3-injected oocytes than basal flux in water injected oocytes. This KCC3-mediated activity is absent in oocytes injected with either Arg270His or Arg270Cys mutant transporters ($p<0.001$, one way analysis of variance (ANOVA)). We also tested function of the Tyr679Cys mutant and although while its function was not completely eliminated like in the Arg270 mutants, the level of flux was significantly reduced compared with wild-type KCC3 ($p<0.05$, one way ANOVA).

DISCUSSION

Clinical features observed in the three patients reported in this manuscript are milder than the previously reported patients with Andermann syndrome with biallelic truncating or missense variants in *SLC12A6*, but similar to the patient reported by Kahle *et al* (2016)[15] with a heterozygous de novo variant, p.Thr991Ala.[15] All our patients presented with early motor developmental delay, muscle weakness and progressive intermediate, mixed mostly predominantly demyelinating neuropathy, particularly affecting the lower limbs. Nerve enlargement has not been found in patient 1. All three patients had normal cognition and brain MRI. No seizures, learning difficulties, hearing problems or dysmorphic features were observed, although patient 2 (p.Arg207His) had pathological EEG findings without any reported seizures. Patient 3 with p.Tyr679Cys exhibited spasticity of the lower limbs in addition to sensorimotor neuropathy. Foot and spine deformities such as scoliosis, pes cavus and planovalgus foot were also observed. In contrast, patients with Andermann syndrome present with a more severe and complex neurodevelopmental disorder with progressive sensorimotor neuropathy, dysmorphic features, developmental delay, intellectual disability, seizures and agenesis of the corpus callosum.[12 13] Similarly, *Slc12a6*$^{-/-}$ mice exhibit a severe phenotype with impaired motor function and significant axonal swelling accompanied by hypomyelination while *Slc12a6*$^{-/+}$ mice are asymptomatic.[19] Although p.Thr991Ala was found in a heterozygous state in one patient, the heterozygous KCC3 Thr991Ala mice do not display any abnormalities while homozygous KCC3 Thr991Ala mice demonstrate a severe phenotype comparable to the knockout mice.[15] However, not significant, a slightly decreased sciatic nerve amplitude could be observed in the heterozygous mice compared with that of the wild-type mice.[15] While the heterozygote mice do not display an overt phenotype, their nerve fibres showed intermediate shrinkage when compared with wild-type or homozygote mice.[20]

To date, mostly truncating variants (nonsense, splice and frameshift) have been reported to cause the recessive disorder. One missense variant, p.Gly539Asp, has been identified in a compound-heterozygous state with a truncating variant and a homozygous missense variant p.Arg207Cys was reported in a patient affected by the Andermann syndrome.[13 14] The missense variant p.Arg207His, affecting the same codon, was found recurrent in a heterozygous state in two of our patients. Functional studies demonstrated a complete loss-of-function for the variants p.Arg207His and p.Arg207Cys, while p.Tyr679Cys only partially reduced the influx of potassium ions in *Xenopus* oocytes. In contrast, the previously published de novo variant p.Thr991Ala showed increased potassium influx indicating a gain-of-function. This was demonstrated in mouse fibroblasts natively expressing the mutant transporter,[15] as well as in HEK293 cells overexpressing the mutated threonine residue.[8 21] It is striking that p.Arg207His and p.Arg207Cys show different inheritance patterns, although they affect the same amino acid codon and show similar biophysical properties. Although the heterozygous de novo variants cause a homogeneous phenotype, our experimental data assessed in *Xenopus* oocytes displayed different functional features in all identified variants. Thus, our current data do not reveal a distinct correlation between the biophysical properties of mutant cotransporters and the difference in inheritance pattern, which have to be caused by other, so far unknown factors.

CONCLUSION

Diagnostic trio exome sequencing has proven an efficient tool to establish new disease genes and/or variants that are involved in early-onset neuropathies. We provide evidence that heterozygous de novo variants in *SLC12A6* cause early-onset progressive CMT with or without spasticity, which is milder than the previously reported recessive phenotype. Thus, autosomal-dominant inheritance of *SLC12A6* variants also needs to be considered in patients with early-onset neuropathies. Further functional

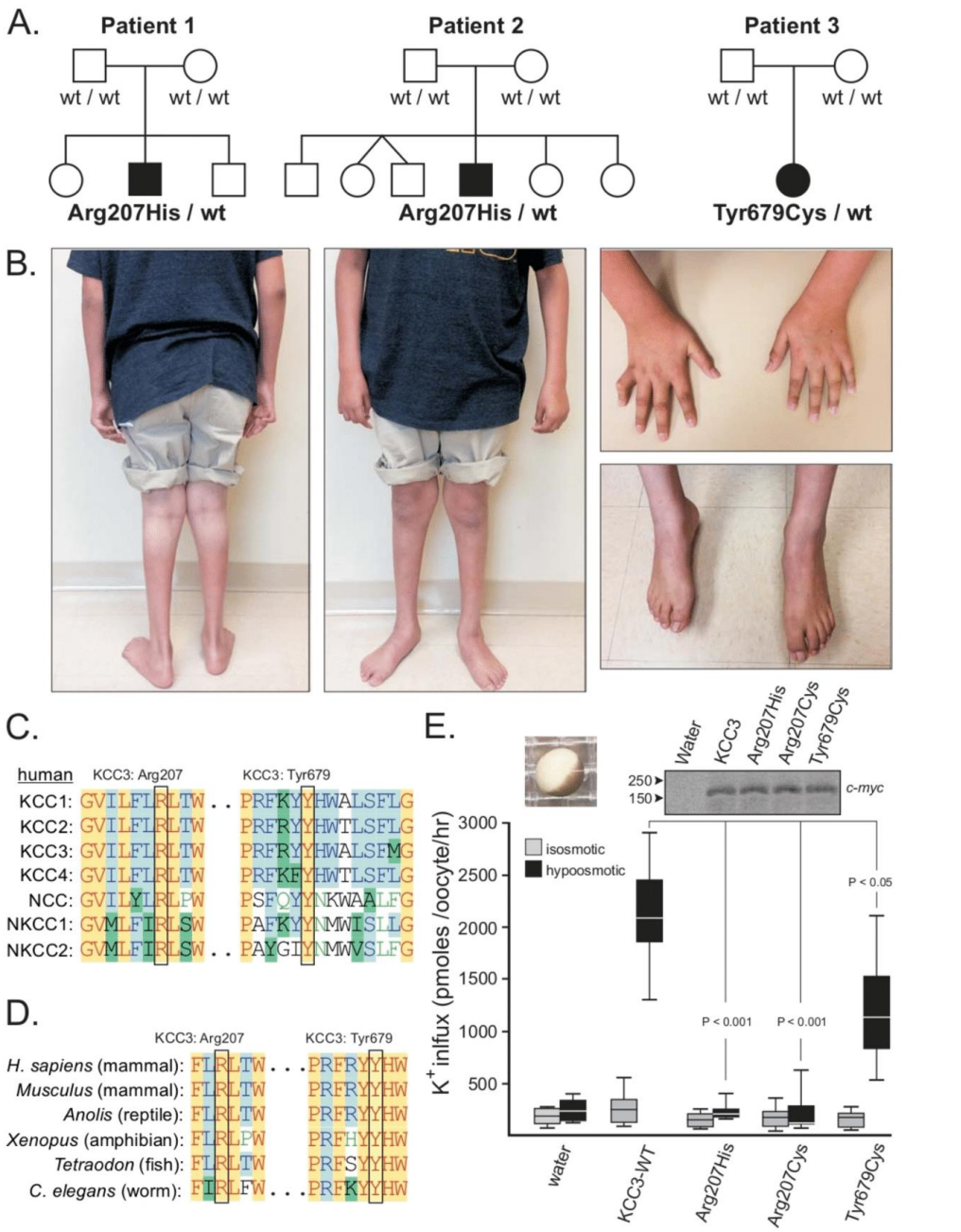

Figure 1 (A) Pedigrees of the three unrelated affected patients (closed symbols) with de novo variants in *SLC12A6* and healthy family members (open symbols). Wildtype is depicted as wt. (B) Images of patient 2 at 14 years show muscle atrophy predominantly in distal lower limbs, contractures of fourth and fifth fingers and flat feet. (C) Conservation of KCC3 Arg207 and Tyr679 among the different members of the SLC12A (cation-chloride) cotransporters. The arginine and tyrosine residues are boxed. (D) Conservations of same residues within KCC3 transporters from different species. (E) K+influx measured in oocytes injected with water, KCC3-WT, Arg270His, Arg270Cys and Tyr679Cys. Oocytes were incubated under isotonic (grey bars) or hypertonic (black bars) conditions. Statistical analysis was done using one way analysis of variance, groups of 20–25 oocytes. Top inset: Western blot showing expression of all transporters in oocytes and absence of expression in water-injected oocytes. Antibody was mouse monoclonal anti-cmyc.

studies are needed to determine the exact mechanism by which different variants in *SCL12A6* that might even affect the same amino acid residue exert their detrimental effect either in an autosomal-recessive or autosomal-dominant fashion.

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Contributors JP collected and analysed clinical and genetic data and was responsible for drafting the manuscript. BRF conducted functional experiments, collected and analysed the functional data and contributed to manuscript. KS, HK, KR, AW, AG and UG contributed to phenotyping, acquisition and analysis of clinical and electrophysiological data. MR, MR, ND and MS contributed to analysis and interpretation of genetic data. TBH and ED were responsible for the conception, design and supervision of the study and writing of the manuscript. All authors revised the manuscript for intellectual content.

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4 DISCUSSION

Diagnostic WES revealed two novel *de novo* variants in *HIVEP2*, three in *KCNC1,* and two in *SLC12A6*. In total, these seven new variants were found in ten unrelated patients. In 8 patients, the *de novo* variants were found using trio WES and the variants from the other two patients were tested in parental DNA per Sanger sequencing which confirmed the *de novo* status. Our patients presented different clinical phenotypes than the previously published patients with pathogenic variants in these three genes (Park et al., 2019a, Park et al., 2019b, Park et al., 2019c).

4.1 HIVEP2

Loss-of-function (LoF) variants in *HIVEP2* have been suggested to cause autosomal-dominant mental retardation type 43 (MRD43, OMIM 616977) (Srivastava et al., 2016, Steinfeld et al., 2016). Our two patients showed mild ID without any dysmorphic features, neurological symptoms or behavioral abnormalities (Park et al., 2019a). In contrast, nine previously published patients exhibited mild to severe ID, dysmorphic features and behavioral difficulties. 8 of 9 patients showed motor development delay, muscular hypotonia as well as other overlapping neurological symptoms such as dystonia, ataxia, dysphagia, spasticity, progressive Parkinsonism or quadriplegia (Srivastava et al., 2016, Steinfeld et al., 2016). Subsequently, Jain *et al.* reported another ID patient with a nonsense variant in *HIVEP2* gene and Goldsmith *et al.* introduced two additional patients with ID, hyperphagia, variable dysmorphic features and Angelman-like syndromes (figure 1) (Goldsmith et al., 2019, Jain and Atwal, 2019).

In total, 13 of 14 published ID patients had frameshift or nonsense variants, while one patient had a missense variant (Goldsmith et al., 2019, Jain and Atwal, 2019, Srivastava et al., 2016, Steinfeld et al., 2016, Park et al., 2019a). All variants

occurred *de novo*. In a cohort study with bipolar disorders, one patient had a *de novo* missense variant and another cohort study published an ID patient with a nonsense variant in the last exon in *HIVEP2*, but both publications did not provide any detailed clinical information on their patients (Hamdan et al., 2017, Kataoka et al., 2016).

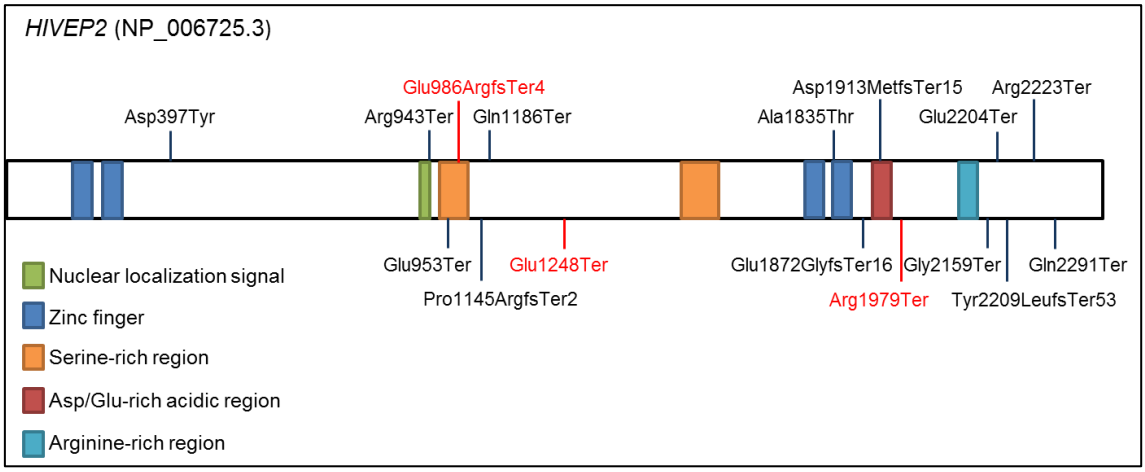

Figure 1: Published *de novo HIVEP2* variants. Complementary to the figure 2 from Park *et al.*, 2019a, the protein domains of *HIVEP2* (NP_006725.3) are illustrated in different colors. Three new truncating variants that have been reported after the publication of Park *et al.*, 2019a are marked as red.

HIVEP2 serves as a transcription factor that is involved in neural maturation and is expressed in several brain regions (Srivastava et al., 2016, Takao et al., 2013). *HIVEP2* knock-out mice exhibit severe cognitive impairments, anxiety and hyperactive behaviors that have also been seen in the majority of the reported patients (Takagi et al., 2006). The probability of LoF intolerance (pLI) score is 1.0 in gnomAD and ExaC browers, predicting that heterozygous LoF variants in *HIVEP2* gene are rare and probably not tolerated. All publications so far support the evidence that truncating variants in *HIVEP2* gene cause ID, proposing haploinsufficiency as the main pathomechanism (Goldsmith et al., 2019, Jain and Atwal, 2019, Srivastava et al., 2016, Steinfeld et al., 2016, Park et al., 2019a).

Functional experiments on the variants to confirm LoF theory have not yet been conducted. According to the NMD phenomenon, if the termination codon is downstream of 50 nucleotides of the last exon complex, the mRNA is likely to be translated that could result in a deleterious gain-of-function (GoF) or a dominant-negative acting protein (Brogna and Wen, 2009, Hug et al., 2016). Four patients, including our two patients (Glu2204* and Arg2223*), have a *de novo* variant leading to a stop codon in the last exon predicted to escape the NMD. Since our patients showed a milder phenotype, we discussed that the truncated protein could have some residual or a dominant-negative function. The patient with Tyr2209Leufs*53 also had a slightly milder phenotype than other patients, but this patient additionally showed dyspraxia and delayed myelination compared to our patients (Steinfeld et al., 2016). Unfortunately, detailed clinical information on the patient with Gln2291* has not been provided (Hamdan et al., 2017). Steinfeld *et al.* identified a *de novo* missense variant Asp397Tyr in a 2-year-old patient, who exhibited mild GDD without any other neurological features at the time of publication (Steinfeld et al., 2016). It is possible that the *de novo* missense variant leads to a protein with reduced residual function or dominant-negative effect as we have discussed for our two truncating variants in the last exon. However, the severity of ID, dysmorphic features, neurological features as well as social capabilities varied among other patients regardless of the location of the truncating variants. Therefore, a clear genotype-phenotype distinction could not be drawn.

Further clinical follow-up studies will be needed to evaluate the development and prognosis of *HIVEP2*-associated disorders. Our patients are the oldest reported patients so far who are both fully grown adults with mild ID, but without behavioral difficulties or any other medical issues (Park et al., 2019a).

4.2 KCNC1

Patient 1 (Cys208Tyr) exhibited non-progressive mild action-induced myoclonus without any cerebellar signs or seizures. The Ala421Val variant was found

recurrent in three unrelated patients (Park et al., 2019c). One month after the appearance of our paper, Cameron *et al.* also found the same Ala421Val variant recurrent in six unrelated patients (Cameron et al., 2019). All patients harboring the Ala421Val variant had a homogenous phenotype with early-infantile epileptic encephalopathy with non-progressive ataxia, myoclonic seizures and developmental delay, but no action-induced progressive myoclonus (Cameron et al., 2019, Park et al., 2019c). In addition, we found a *de novo* missense variant (Thr399Met) in patient 2, who showed ID and dysmorphic features without any seizures (Park et al., 2019c).

Initially, *KCNC1* was associated with progressive myoclonus epilepsy. Muona *et al.* found one recurrent missense variant (Arg320His) in *KCNC1* causing progressive myoclonus epilepsy establishing a new syndrome: myoclonus epilepsy and ataxia due to potassium channel mutation (MEAK) (Muona et al., 2015). Phenotypic comparison of MEAK patients demonstrated that they all suffered from severe progressive myoclonus seizure and ataxia leading to wheelchair dependency in 50% of affected patients (Kim et al., 2018, Muona et al., 2015, Oliver et al., 2017). Recently, Poirier *et al.* detected a nonsense variant (Arg339*) in *KCNC1* in a family with three affected members with notable ID and dysmorphic features but no seizures (Poirier et al., 2017). The clinical presentation of our patient 2 was more comparable to the family who had the nonsense variant (Arg339*), but his dysmorphic features were different.

Functional analysis of all identified *de novo* variants showed a complete loss-of-function of Kv3.1 channel. When the mutant channel was injected in 1:1 ratio with the wildtype (WT) channel, the previously published Arg320His reduced the Kv3.1 potassium current amplitude by 80% in a dominant-negative manner (Muona et al., 2015). The Cys208Tyr channel did not have any dominant-negative effect, which might possibly account for the less progressing disease course (Park et al., 2019c). Haploinsufficiency was suggested to cause ID without seizures, since mRNA quantification of Arg339* showed >50% reduced

expression of *KCNC1* (Poirier et al., 2017). However, when the Arg339* mutant Kv3.1 channel was co-expressed with WT channel, there was a dominant-negative reduction of current amplitude. Therefore, Cameron *et al.* proposed that some mutant protein may have escaped NMD causing additional damaging effect. Our patient 2 with Thr399Met had a similar phenotype to the patients with Arg339* and the Thr399Met mutant Kv3.1 channel also displayed a significant dominant-negative effect (Park et al., 2019c). We illustrated here that Ala421Val had similar biophysical properties as the previously published Arg320His, but with less dominant-negative reduction of potassium current amplitude by 50%. Cameron *et al.* did not detect any dominant negative effect or a shift in the voltage-activation curve for Ala421Val measured in *Xenopus* oocytes (Cameron et al., 2019). Nonetheless, electrophysiological data assessed in *Xenopus* oocytes could not explain the clinical differences of the putative pathogenic variants. In vitro, Kv3.1 interacts with other Kv3 subunits: Kv3.2, Kv3.3 and Kv3.4 (Cameron et al., 2019, Rudy and McBain, 2001, Song et al., 2006). Functional consequences of these co-expressed subunits by the mutant variants have not been examined yet.

Kv3.1 channels are predominantly expressed in fast spiking GABAergic interneurons, thus the LoF variants possibly lead to neuronal disinhibition due to impaired firing of the GABAergic interneurons (Sekirnjak et al., 1997, Song et al., 2006, Rudy and McBain, 2001). Valproate was the most effective antiepileptic drug for patients with Arg320His and Ala421Val. Besides valproate, some patients responded to zonisamide and benzodiazepines. Topiramate, levetiracetam and lamotrigine were less effective. Since LoF is the key pathomechanism, direct activation of Kv3.1 channels may be a potential therapeutic intervention for *KCNC1* related disorders (Cameron et al., 2019, Oliver et al., 2017, Park et al., 2019c). Currently, there is no activator of Kv3 channels available, but such approach has been recently trialed *in vitro* with a compound called RE01 (Munch et al., 2018).

The two variants, Arg320His and Ala421Val, seem to be hotspots for *de novo* variants since the variants were found recurrent in multiple unrelated patients internationally (Cameron et al., 2019, Park et al., 2019c). For these two variants, a distinctive genotype-phenotype correlation could be assessed. All patients with Arg320His showed the MEAK syndrome and all patients with Ala421Val had GDD with myoclonic and absence seizures. While the clinical course of MEAK patients is well described, information on clinical development lack in patients with Ala421Val because they are still very young. Therefore, there is a need for further clinical follow-up studies as well as functional analyses of the individual variants to evaluate possible therapeutic options (Cameron et al., 2019, Oliver et al., 2017, Park et al., 2019c).

4.3 SLC12A6

The final paper published in *Journal of Medical Genetics* describes a new inheritance pattern of *SLC12A6* gene showing that dominant-acting *de novo* variants cause a milder phenotype than the recessive disorder, Andermann syndrome, which was the only disorder that had been so far associated with the gene *SLC12A6* (Park et al., 2019b). Our patients had a homogenous phenotype with infantile onset motor development delay, muscle weakness and progressive intermediate neuropathy. All patients had normal cognition and unremarkable brain MRI. Patient 3 had a different *de novo* variant (Tyr679Cys) than the other two patients and showed spastic gait additionally. Kahle *et al*. found a heterozygous *de novo* missense variant (Thr991Ala) in a 10-year-old patient showing the same phenotype as our patients (Kahle et al., 2016). We classified the disorder as dominant intermediate CMT (DI-CMT). To date, only six genes have been associated with DI-CMT (Berciano et al., 2017).

Andermann syndrome is characterized by developmental delay, severe progressive motor and sensory neuropathy and variable degrees of agenesis of the corpus callosum (Howard et al., 2002, Akcakaya et al., 2018, Hauser et al., 1993, Larbrisseau et al., 1984, Rudnik-Schoneborn et al., 2009, Uyanik et al.,

2006). Numerous biallelic LoF variants (nonsense, splice and frameshift) have been associated with Andermann syndrome. One missense variant, Gly539Asp, has been found in a compound-heterozygous state with a splice variant in a patient with Andermann syndrome (Rudnik-Schoneborn et al., 2009). Uyanik *et al.* identified a homozygous missense variant Arg207Cys in a single patient (Uyanik et al., 2006). The recurrent *de novo* variant Arg207His found in two of our patients affects the same amino-acid codon, yet causes an early onset CMT in a heterozygous state (Park et al., 2019b). The parents carrying heterozygous Arg207His variant were described to be asymptomatic (Uyanik et al., 2006). Slc12a6-/- mice exhibit a severe phenotype comparable to the Andermann syndrome with impaired motor function accompanied by hypomyelination and axonal swelling while Slc12a6-/+ mice are asymptomatic (Byun and Delpire, 2007). However, slightly decreased sciatic nerve amplitude was measured in the heterozygous mice (Kahle et al., 2016).

SLC12A6 is predominantly expressed in the central and peripheral nervous system and encodes a potassium chloride cotransporter (Pearson et al., 2001, Shekarabi et al., 2011). Functional analyses demonstrated complete LoF of the variants Arg207His and Arg207Cys (Park et al., 2019b). The Tyr679Cys mutant co-transporter illustrated a slight reduction of potassium influx and the previously published Thr991Ala demonstrated a GoF (Kahle et al., 2016). Although all patients with heterozygous *de novo* variants had a homogenous phenotype, all variants displayed different biophysical properties.

Since LoF variants are associated with Andermann syndrome, it is plausible that homozygous Arg207Cys variant causes Andermann syndrome as a LoF variant. In this context, homozygous Arg207His might also cause Andermann syndrome. The question yet arises, why the substitution to Histidine is deleterious in a heterozygous form. Since Tyr679Cys had residual effect and Thr991Ala was reported as a GoF variant, we speculated that Arg207His might have additional residual function in a dominant-negative manner. Injection of Arg207Cys mutant

co-transporter with WT co-transporter produced a pronounced dominant-negative effect, while Arg207His did not affect the WT co-transporter (unpublished data), which did not support our hypothesis. Continuing functional evaluation is needed to shed light on the importance of this arginine residue in these diseases.

4.4 Loss-of-Function *de novo* Variants

All our new *de novo* variants in *HIVEP2*, *KCNC1* and *SLC12A6* were reported as LoF.

LoF variants in *HIVEP2* cause ID with variable facultative clinical features (Goldsmith et al., 2019, Srivastava et al., 2016, Steinfeld et al., 2016, Jain and Atwal, 2019, Park et al., 2019a). The primary pathomechanism is possibly haploinsufficiency, but our two truncating variants were located in the final exon, that may escape NMD and show residual function. It is yet difficult to conclude that this is the reason for the less severe disease course of our patients, since we lack detailed clinical information on the other two patients who also had truncating variants in the final exon (Hamdan et al., 2017, Steinfeld et al., 2016). Furthermore, the severity of *HIVEP2* related disorder varied among other truncating variants prior to the final exon as well.

All putative pathogenic *de novo* variants in *KCNC1* revealed LoF in *Xenopus* oocytes (Cameron et al., 2019, Muona et al., 2015, Park et al., 2019c). Some variants caused additional reduction of potassium current amplitudes in a dominant negative manner. Truncating variants were associated with ID without seizures, but haploinsufficiency alone may not be causal for ID since Arg339* mutant channel had a dominant-negative effect and Gln492* produced 70% of potassium currents compared to the WT currents (Cameron et al., 2019). Also, we reported an ID patient without seizures harboring a dominant-negative missense variant (Park et al., 2019c).

Biallelic LoF variants in *SCL12A6* were associated with Andermann syndrome, indicating that haploinsufficiency does not cause DI-CMT (Bowerman et al., 2017, Rudnik-Schoneborn et al., 2009, Uyanik et al., 2006). Although the previously published Thr991Ala yielded GoF, the *de novo* variants we identified displayed LoF, but no dominant-negative effect (Kahle et al., 2016, Park et al., 2019b). Heterozygous LoF alone would not explain the dominant phenotype since LoF variants cause the recessive disorder.

Specific molecular mechanisms causing the various disorders by *HIVEP2*, *KCNC1* and *SLC12A6* are not well understood. Measurement of protein expression level e.g. by quantifying mRNA in fibroblasts might contribute to determine whether the LoF variants cause haploinsufficiency or dominant-negative effects. The level of mRNAs subjected to NMD might vary in individuals which could account for the variable severity. For the first characterization of the identified heterozygous variants, we used *Xenopus* oocytes which allow a proper investigation of dominant-negative effects since the amount of mRNA can be well controlled. In a second step, further characterization in different models such as neural line IPS cells or mouse models will be needed to estimate the biophysical properties of the variants *in vivo*. Apart from the individual function of the mutant proteins, the pathogenic variants might also influence other cell regulatory factors or key molecules. Another possibility is that there is a variant in the second allele, that alone would not be pathogenic but combined with one of the *de novo* variants causes various symptoms. But in this case, the inheritance mode would be recessive and the *de novo* variants should be found in some healthy controls as well.

Briefly, this cumulative dissertation states that the phenotypic consequences of heterozygous LoF variants are variable in different genes and their pathomechanism is more complex. LoF variants do not always simply conclude that the variants cause a 50% reduction of protein function or expression in the

cell and it cannot be stated that this mechanism alone is responsible for the clinical outcome.

5 CONCLUSION/SUMMARY

Using diagnostic WES and trio-WES, we found new *de novo* variants in the genes *HIVEP2*, *KCNC1* and *SLC12A6*. Clinical phenotypes of our patients were different from the previously reported *HIVEP2*, *KCNC1* and *SLC12A6* related disorders.

Patients with de *novo* truncating variants in *HIVEP2* had ID with other non-specific variable clinical characteristics (Park et al., 2019a, Srivastava et al., 2016, Steinfeld et al., 2016). A clear genotype-phenotype regarding the severity of ID and neurological features could not be concluded. The *de novo* Arg320His variant in *KCNC1* caused MEAK syndrome and patients with a *de novo* Ala421Val change showed GDD with epilepsy, thus a clear genotype-phenotype correlation could be assessed for these two variants (Cameron et al., 2019, Muona et al., 2015, Oliver et al., 2017, Park et al., 2019c). However, trio-WES revealed other *de novo* variants causing ID without seizures or non-progressive mild myoclonus/tremor. Therefore, we propose that the phenotypic spectrum of *KCNC1* related disorder might be more diverse, and rare *de novo* variants in *KCNC1* should be considered in patients with epilepsy, ID as well as myoclonus/tremor. Heterozygous *de novo* variants in *SLC12A6* have been identified in infantile-onset progressive neuropathy patients; we classified the disorder as DI-CMT. Homozygous or compound-heterozygous truncating variants are known to cause Andermann syndrome (Hauser et al., 1993, Larbrisseau et al., 1984, Rudnik-Schoneborn et al., 2009, Uyanik et al., 2006).

Functional evaluation in *Xenopus* oocytes displayed LoF for all our new identified variants in *KCNC1* and *SLC12A6*, but a clear correlation between the gene

variant function and phenotype could not be deduced. Therefore, it should be reflected that variant-specific pathomechanism leading to a certain phenotype can be far more complex. Clarifying the consequences of pathogenic variants in vivo may play an important role for developing a molecular targeted therapy.

Our publications demonstrate that the phenotypic spectrum of all three genes is wider than first described. Further identification of patients and functional analyses might provide a clear genotype-phenotype correlation and even further extend the phenotypic spectrum, but they might also reveal that the clinical presentation, disease severity and progression will vary between individuals regardless of the specific location or function of a single variant.

6 DEUTSCHE ZUSAMMENFASSUNG

Durch diagnostische Exom-Sequenzierung sowie Trio-Exom-Sequenzierung bei neurogenetisch erkrankten Patienten identifizierten wir neue *de novo* Varianten in den Genen *HIVEP2*, *KCNC1* und *SLC12A6*. Wir berichten hier nicht nur von neuen Varianten, sondern auch von neuen Phänotypen, die bisher noch nicht mit diesen Genen assoziiert worden waren.

Patienten mit trunkierenden *de novo* Varianten im *HIVEP2*-Gen zeigten eine unspezifische Intelligenzminderung mit verschiedenen neurologischen Symptomen. Eine eindeutige Genotyp-Phänotyp-Korrelation bezüglich des Schweregrads der Erkrankung konnte nicht festgestellt werden.

Eine zuvor berichtete heterozygote Arg320His Variante im *KCNC1*-Gen verursachte ein MEAK-Syndrom (Progressive Myoklonusepilepsie und Ataxie durch Kaliumkanaldefekt), und Patienten mit einer heterozygoten *de novo* Ala421Val Variante zeigten eine globale Entwicklungsverzögerung mit Epilepsie, so dass für diese beiden Varianten eine klare Genotyp-Phänotyp-Korrelation festgestellt werden konnte. Es wurden noch zwei weitere *de novo* Varianten im *KCNC1*-Gen gefunden, die jeweils eine Intelligenzminderung ohne Krampfanfälle oder einen nicht progressiven leichten Myoklonus/Tremor verursachten. Daher gehen wir davon aus, dass das phänotypische Spektrum von *KCNC1* noch vielfältiger sein könnte. Zudem möchten wir hervorheben, dass seltene *de novo* Varianten bei Patienten mit Epilepsie, Intelligenzminderung oder auch Myoklonus/Tremor in Betracht gezogen werden sollten.

Heterozygote *de novo* Varianten im *SLC12A6*-Gen wurden bei Patienten mit frühbeginnender progredienter Neuropathie identifiziert. Wir klassifizierten die Erkrankung als DI-CMT (dominant intermediate Charcot-Marie-Tooth). Bisher

wurden homozygote oder compound-heterozygote Nonsense-, Splice- und Frameshift Varianten mit dem Andermann-Syndrom assoziiert.

Die funktionelle Auswertung in den Xenopus Oozyten zeigte für alle neu identifizierten Varianten in den Genen *KCNC1* und *SLC12A6*, eine Loss-of-Function (LoF). Ein eindeutiger Zusammenhang zwischen den Funktionen der Genvarianten und den verschiedenen Phänotypen konnte jedoch nicht abgeleitet werden. Der genaue Pathomechanismus, der zu einem bestimmten Phänotyp führt, ist wahrscheinlich komplexer und wird vermutlich durch andere bisher unbekannte Faktoren verursacht, so dass weitere funktionelle Studien zur Entwicklung einer zielgerichteten Therapie eine wichtige Rolle spielen werden.

Unsere Studien unterstreichen, dass das phänotypische Spektrum von Mutationen in den drei Genen breiter ist als zuvor beschrieben. Eine ausführliche Phänotypisierung durch längere Beobachtung sowie weitere Rekrutierung von Patienten könnten das phänotypische Spektrum sogar noch mehr erweitern und vielleicht in der Zukunft einen klaren Zusammenhang zwischen den verschiedenen Varianten und Phänotypen liefern. Es ist jedoch auch möglich, dass der klinische Verlauf unabhänging von der spezifischen Position oder Funktion einer einzelnen Variante immer etwas unterscheiden wird.

7 ERKLÄRUNGEN ZUM EIGENANTEIL

Die Arbeit wurde im Institut für Medizinische Genetik und angewandte Genomik unter Betreuung von Prof. Olaf Rieß sowie Dr. Tobias Haack durchgeführt.

Die Konzeptionen der drei Studien erfolgten durch Zusammenarbeit mit Prof. Holger Lerche (Ärztlicher Direktor Neurologie mit Schwerpunkt Epileptologie), Dr. Martin Kehrer (Facharzt für Humangenetik) und Prof. Eric Delpire (Professor in Vanderbilt Universität für Anästhesie sowie Molekulare Physiologie und Biophysik). Herr Dr. Haack und Herr Prof. Lerche haben die Arbeiten betreut und alle Koauthoren haben die Manuskripte korrigiert.

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Ich versichere, die Manuskripte mit Unterstützung von allen oben genannten Autoren selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

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