Original Article

Identification of *PCDH19* Gene Mutations/Deletions in Patients with Early Onset Epilepsy

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Abstract

Background and Aims: *PCDH19* gene, which encodes protocadherin 19, is associated with epilepsy and intellectual disability, mainly in affected females. The clinical manifestations are heterogeneous and the main features include early onset seizure, generalized or focal seizures sensitive to fever, and brief seizures occurring in clusters. The disorders exhibit a unique and unusual X-linked pattern of expression. We aimed to investigate *PCDH19* mutations/deletions in patients with epilepsy and describe the clinical/molecular features. **Methods:** *PCDH19* gene was analyzed in 35 Turkish female patients from 34 families with early-onset epilepsy via direct sequencing and multiplex ligation-dependent probe amplification analysis. Additionally, array comparative genomic hybridization analysis was performed in patients with whole gene deletion. **Results:** We identified 2 different heterozygous mutations in 2 unrelated probands (5.7%) which were located in exon 1. Additionally, whole gene deletions were detected in dizygotic twin girls (5.7%), who had distinct clinical features and the deletion was inherited from the unaffected father. The second twin suffered more severe clinical manifestations including autistic features, behavioral problems, mild-moderate mental retardation and seizures, which were under control with multidrug regimen when compared with the first twin. **Conclusion:** *PCDH19* is a major causative gene in patients with epilepsy and further data is required to gain a better understanding of phenotype-genotype correlation. In addition to gene sequencing, deletion/duplication analysis will improve the molecular diagnosis in patients with clinical findings.

Keywords: Early onset epilepsy, MLPA, *PCDH19* gene

Introduction

Protocadherins are the largest mammalian subgroup of the cadherin superfamily of homophilic cell-adhesion protein. *PCDH19* (Protocadherin 19) gene, which encodes protocadherin-19, is located on Xq22.1 chromosome. This locus has been described as a cause of epilepsy and intellectual disability in 1997 in which *EFMR* (Epilepsy and mental retardation limited to female disease locus) was used instead of *PCDH19*.[1] Afterwards, *PCDH19* was identified as a responsible gene by Dibbens *et al*. in 2008. Protocadherin 19 has a potential role in neuronal migration and formation of synaptic connections during brain development.[2] The disorders related with *PCDH19* gene exhibit a unique X-linked pattern of expression. This mode of inheritance might be explained by 'cellular interference'. According to this hypothesis, the coexistence of wild-type and mutant cell populations may result in an abnormal cell-cell interactions. Consequently, heterozygous females and mosaic males show clinical features, whereas hemizygous and non-mosaic males are also typically unaffected.[3,4]

The clinical manifestations of *PCDH19*-related epilepsy are heterogeneous. The main features include early seizure onset, generalized or focal seizures sensitive to fever, brief seizures occurring in clusters.[5] Epilepsy and mental retardation limited to female (EFMR) named as early infantile epileptic encephalopathy type 9 is a genetic disorder, which occurs in females due to mutations in *PCDH19* gene.[2] *PCDH19* mutations is also identified in patients with Dravet Syndrome who do not have any detectable *SCN1A* mutations. Furthermore, mutations are detected in patients with different types of epilepsy, including Ohtahara syndrome as well as in nonepileptic patients with autism or Asperger syndrome.[6] The most frequent seizure types include generalized tonic-clonic seizures (GTCS) and/or focal seizures in clusters. Other seizure types such as myoclonic seizures, atypical absences and atonic seizures are rarely observed. Cognitive development has been reported to range from normal through mild to severe intellectual disability with

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or without behavioral and psychiatric problems.[7] Recently, sleep disorders including disturbances in sleep pattern have also been described.[8] Additionally, several neuropsychiatric features have been reported in carrier males.[9]

Most *PCDH19* mutations are clustered in exon 1 probably because it is the biggest exon, corresponding to the extracellular domain, but also other exons may be involved.[10] To date, more than 175 novel variants, including missense, nonsense, splicing, small deletion/insertion in *PCDH19* gene have been reported as disease causing. Whole-gene or intragenic deletions of the gene have also been reported.[11-13]

In this study, we aimed to assess *PCDH19* mutations/deletions in 35 female patients with early-onset epilepsy and describe the clinical features of the mutation/deletion positive patients.

Methods

Patient selection

The study was composed of 35 female patients who suffered from epilepsy including both febrile and afebrile focal or generalized seizures which had started before the age of 36 months. The clinical features and neuroimaging data of the female patients with *PCDH19* mutations/deletions were evaluated. The seizure semiology, family history, seizure frequency, susceptibility to fever, brain imaging, the presence of mental retardation or behavioral disorders and anticonvulsant medications were also recorded. The local Ethics Committee approved the study. Written informed consent was obtained from all individuals involved.

Molecular analysis

DNA isolation and Sanger sequencing

Genomic DNA from the peripheral blood lymphocytes of all individuals were extracted with QIAamp DNA Blood Mini Kit (Qiagen GMBH, Hilden, Germany) using standard procedures. All coding exons and exon-intron boundaries (+/-10bp flanking intronic sequence) of *PCDH19* gene were amplified by polymerase chain reaction (PCR). The sequences were evaluated using CLC Genomics Workbench 3 sequencing program (Qiagen Company). "Ensembl.org" database (GRCh38.p12) with ENST00000373034.8 transcript ID of the *PCDH19* gene was used to compare the individuals and the reference sequence. All variations were checked from mutation and SNP databases (Human Genome Mutation Database, National Center for Biotechnology Information, ensembl.org). Each variation was confirmed by bidirectional sequencing. Variation descriptions were done according to the nomenclature recommended by the Human Genomic Variation Society. Furthermore, in silico programmes, such as SIFT (sorting intolerant from tolerant), PolyPhen 2 (polymorphism phenotyping v2), Mutation Taster, KD4V (Comprehensible Knowledge Discovery System for Missense Variant), were used to describe the pathogenity of novel variations in coding exons and exon-intron boundaries.

Multiplex ligation-dependent probe amplification (MLPA) analysis

MLPA was performed to detect large deletions and duplications using P330 SALSA MLPA Kit (MRC-Holland, The Netherlands). The PCR products were analysed by ABI 3130 capillary electrophoresis system and Coffalyser software. The area under the peak for each amplified fragment was measured and normalized to the peak areas of reference samples.

Array comparative genomic hybridization (array CGH) analysis

For the analysis of the patients, CytoScan 750K Array Kit from Affymetrix was used, which was covering a total number of 750,436 markers with 200,436 SNP markers. Among these probes, 63 are covering *PCDH19* gene. The purified DNA was then fragmented, labelled and hybridised overnight on to an array. The arrays were washed using an Affymetrix® GeneChip® Fluidics Station and scanned using an Affymetrix® GeneChip® scanner. gDNA was digested with Nsp1 and then ligated to a common oligonucleotide adaptor for amplification by PCR. After purification, the PCR products were fragmented and then labelled with a biotinylated deoxynucleotide analogue using the TdT enzyme followed by overnight hybridisation to the array. The arrays were washed using an Affymetrix® GeneChip® Fluidics Station (which included DNA selective staining with a streptadivin conjugated reporter molecule) and then scanned using an Affymetrix® GeneChip® scanner. The data files generated for each sample were analysed using Chromosome Analysis Suite (ChAS) Software (v1.0.1 or v1.2.2). All results were subjected to quality control with regard to SNPQC, MAPD, waviness, sex and signal intensity.

Results

We analyzed 35 female patients from 34 families with epilepsy and identified 2 different heterozygous mutations in 2 unrelated probands (5.7%) and whole gene deletions in dizygotic twin girls (5.7%). At the initial of the disease, febrile/afebrile GTCS was detected in the patients with mutation/deletion. The clinical characteristics of the patients are shown in Table 1.

The first patient had a de novo missense (c.695A > G; p.N232S) mutation in *PCDH19* gene. During follow-up, in addition to GTCS, nonmotor-onset type seizures, especially behavior arrest type, were also observed in Patient 1. Seizures were resistant to medical treatment in this patient and vagus nerve stimulation procedure was performed. After this procedure, behavior arrest type seizures had become more pronounced. Developmental milestones were also normal, but mild mental retardation, anxiety disorder and soft speech (hypophonia) were observed in Patient 1 at the age of 9 years. The second patient who had a novel missense mutation (c.1441G>T; p.D481Y) with a family history of epilepsy, underwent ventriculoperitoneal shunt procedure in the neonatal period for the management of hydrocephalus. In addition to mental retardation, autistic features, especially stereotypical motor movements and depression, were also detected.

Seizures, except Patient 3 (first twin), had started in the first year of life in patients with *PCDH19* gene mutations/deletions and Patient 3 had suffered only one afebrile GTCS with status epilepticus, at the age of 3.5 years. While the first twin had mild mental retardation and no behavioral abnormalities, the second one had autistic features, hyperactivity, obsession and mild-moderate mental retardation. The second twin also needed special education and her seizures were controlled by multidrug regimen.

Both the heterozygous missense mutations were located in exon 1 which is the largest one [Table 2]. Whole gene deletions were detected in dizygotic twin sisters by MLPA analysis. Then, array CGH analysis was performed to determine the size of the deletion and a 1.45 Mb deletion was detected on chromosome Xq21.33q22.1 (98,251,338-99,689,191), which contained only one morbid gene, *PCDH19*. The array CGH analysis of their parents was performed and the same deletion was detected in the asymptomatic father [Figure 1].

Discussion

In this study, we described the clinical features and the genotypes of 4 Turkish female patients with *PCDH19* related epilepsy. While two of them had missense mutations, the other two patients, who were dizygotic twins, had whole gene deletions. The frequency of *PCDH19* mutations/deletions in the current study was 11.4%, whereas the mutation rates was reported as 6.4% and 20.2% in different previous studies.[7,10]

Among the *PCDH19* gene mutated patients, seizures are the cardinal features and about two-thirds may exhibit variable intellectual disabilities. Autistic features are usually observed and psychiatric problems, such as obsession, depression, psychosis and hyperactivity may also develop. Delay in language acquisition is a common feature.[14] There are more than 175 cases of *PCDH19*-related epilepsy which have been reported in the current literature and most of these mutations (>90%) are located in exon 1, which encodes the extracellular cadherin domain of the protein.[6,13] Two of our patients had two different point mutations in exon 1. A de novo missense mutation (c.695A>G; p.N232S) which was previously reported in several studies was identified in a single patient of our study group. Different types of seizures, mental retardation, sleep disorders and autistic features were reported in these patients who were managed with multiple antiepileptic drug regimen.[7,8] In our case, normal developmental milestones were present before the onset of the seizures. While the initial seizures were usually GTCS type, nonmotor-onset type seizures were detected during follow-up. Mild mental retardation, anxiety disorder and soft speech (hypophonia) were also observed. Due to the prolonged and refractory seizures, vagal nerve stimulation procedure was performed in this patient.

The second patient had a novel missense mutation (c. 1441G>T; p.D481Y). Although developmental milestones were normal, mental retardation was detected during follow-up. In a recent study, a missense mutation (c.1441G>A; p.D481N) at the same

Table 1: The clinical features of the four patients with PCDH19 gene mutations and deletions

Patient No	Case 1	Case 2	Case 3 First Twin	Case 4 Second Twin
PCDH19 variation	c.695A>G (p.N232S)	c.1441G>T (p.D481Y)	Whole gene deletion	Whole gene deletion
Methods	Sanger sequencing	Sanger sequencing	MLPA and array CGH	MLPA and array CGH
Age at onset	8 months	11 months	3,5 years	6 months
Age at examination	12 years	8 years	4,5 years	4,5 years
Sex	Female	Female	Female	Female
Type of the first seizure	GTCS	GTCS	GTCS	GTCS
Seizure type during follow up	Different types of seizures	GTCS	GTCS	Different types of seizures
SE	+	+	+	+
Sensitivity to fever	+	+	-	+
Seizure clusters	+	+	-	+
Intellectual disability	Mild	Moderate	Mild	Mild to moderate
Behavioral problems	Anxiety disorder, hypophonia	Depression and autistic features	-	Hyperactivity, obsession, autistic features
MRI/CT	Normal	Hydrocephalus	Normal	Normal
AEDs	VPA, CBZ, LCM, CNZ	VPA, LTG, LEV	LEV	VPA, LEV, CLB

GTCS: Generalized tonic-clonic seizure; SE: Status Epilepticus; EEG: Electroencephalography; MRI/CT: Magnetic resonance imaging/Computed tomography; MLPA: Multiplex ligation-dependent probe amplification analysis; Array CGH: Array comparative genomic hybridization analysis; AED: Antiepileptic drugs; CBZ: Carbamazepine; LCM: Lacosamide; VPA: Sodium Valproate; LTG: Lamotrigine; LEV: Levetiracetam, CLB: Clobazam, CNZ: Clonazepam

Table 2: The genetic data of the patients carrying *PCDH19* gene missense mutations

Patient No	cDNA	Protein	Type	Exon	Transmission	Reported/Novel
Patient 1	c.695A>G	p.N232S	Missense	Exon 1	*De novo*	Reported
Patient 2	c.1441G>T	p.D481Y	Missense	Exon 1	NA	Novel

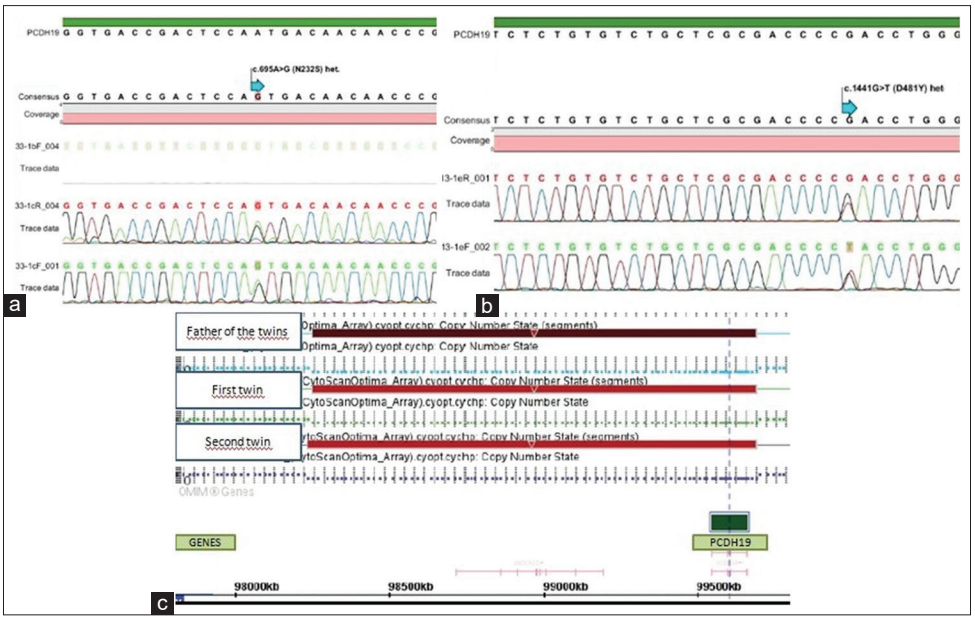

Figure 1: (a) Heterozygous de novo missense variant which was detected in patient 1. (b) Heterozygous novel missense variant which was detected in patient 2. (c) Array CGH results of the twins and their father who had the same deletion

codon was reported in a patient who had sleep and behavioral problems and suffered from uncontrolled seizures.[8] In our study, the patient had behavioral abnormalities including depression and autistic features. Stereotypical motor movements were also identified in the patient. Furthermore, the seizures were controlled with triple antiepileptics.

Previous studies have claimed that chromosomal rearrangements may occur and MLPA analysis should be performed to detect *PCDH19* rearrangements in addition to direct sequencing.[7] In 2017, Smith *et al*. reported 3 different patients with whole gene deletions who had uncontrolled seizures, autistic features and developmental delay. They described the whole gene deletions as the most severely affected variant type.[8] Additionally, copy number variations are important in the genetic etiology of the patients with early-onset epilepsy, and in the literature, these variations were reported in 15 patients with *PCDH19*-related epilepsy.[15] In the present study, MLPA analysis of the gene revealed a whole gene deletion in a patient and her dizygotic twin sister for the first time who had different clinical features. We confirmed the deletion with array CGH analysis and detected a 1.45 Mb deletion on chromosome X which included only one morbid gene, *PCDH19*. While the seizures were under control with a single antiepileptic drug in the first twin, the second twin had to take multidrugs for management of the seizures. The first twin also had mild clinical characteristics, but the second one had mild to moderate mental retardation, autistic features and some behavioral problems (hyperactivity, obsession).

The disorder shows an unusual X-linked inheritance affecting heterozygous females, but sparing hemizygous males who pass the variant to each daughter. However, males with mosaic mutations can also be affected.[4] Consequently, heterozygous variants in *PCDH19* gene have been found to be associated with loss of function and development of the disease.[16] In the present study, parental array CGH analysis showed that the deletion was inherited from the unaffected father. As far as we know, this is the first case in the literature that describes the paternal inheritance of X chromosome deletion, which includes only *PCDH19* gene.

Phenotypic variability of *PCDH19* mutations is an unsolved mystery yet to be elucidated.[8] Partial or completely skewed X-inactivation may play a great role in females with a mild phenotype or asymptomatic carrier. Other modifier genes or environmental factors may also be involved in the phenotypes associated with *PCDH19* mutations.[17,18] In our study, while both twins had the same deletion on their X chromosome, they had different clinical features and seizure frequency. This variable phenotypic spectrum can be explained by skewed X-inactivation and the contribution of other genetic modifiers.

The study has several limitations. The number of patients is relatively small in this retrospective study but the mutation rate in our study was not very different from the current literature.

Conclusion

PCDH19 gene is an increasingly recognized cause of genetic epilepsy in females with a wide clinical spectrum. Patients with early-onset, fever-sensitive and cluster seizures and behavioral problems should be tested for the *PCDH19* mutations and deletions. Future studies will allow better understanding of the phenotype-genotype correlation. The genetic diagnosis is important for the management of the disease, making prognostic predictions and genetic counseling.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient (s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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