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PPP3CA truncating variants clustered in the regulatory domain cause early-onset refractory epilepsy

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Conflict of Interest: Baylor College of Medicine and Miraca Holdings Inc. have formed a joint venture with shared ownership and governance of Baylor Genetics (BG), formerly the Baylor Miraca Genetics Laboratories (BMGL), which performs chromosomal microarray analysis and clinical exome sequencing.

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Abstract

PPP3CA encodes the catalytic subunit of calcineurin, a calcium-calmodulin-regulated serine-threonine phosphatase. Loss-of-function (LoF) variants in the catalytic domain have been associated with epilepsy, while gain-of-function (GoF) variants in the auto-inhibitory domain cause multiple congenital abnormalities. We herein report five new patients with *de novo PPP3CA* variants. Interestingly, the two frameshift variants in this study and the six truncating variants reported previously are all located within a 26-amino acid region in the regulatory domain (RD). Patients with a truncating variant had more severe earlier onset seizures compared to patients with a LoF missense variant, while autism spectrum disorder was a more frequent feature in the latter. Expression studies of a truncating variant showed apparent RNA expression from the mutant allele, but no detectable mutant protein. Our data suggest that *PPP3CA* truncating variants clustered in the RD, causing more severe early-onset refractory epilepsy and representing a type of variants distinct from LoF or GoF missense variants.

Keywords

calcineurin; epileptic syndromes; truncating variants; loss-of-function; gain-of-function; constitutively activation

Introduction

Calcineurin, the Ca^{2+}/calmodulin-regulated protein phosphatase, is a heterodimer consisting of a catalytic subunit calcineurin A and a protein regulatory subunit calcineurin B. *PPP3CA* (protein phosphatase 3, catalytic subunit, alpha isozyme) encodes a major isoform of calcineurin A that is highly abundant and widely distributed in the mammalian brain, enriched at synapses[1–3]. In addition to a catalytic domain (CD), calcineurin A contains a binding site for calcineurin regulatory subunit - calcineurin B (CnBB), the regulatory domain (RD) that contains a calmodulin binding domain (CaMB), and an autoinhibitory domain (AID)[4]. According to the calmodulin-dependent activation model, upon calmodulin

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binding, a conformational change and displacement of AID enables protein substrate access and leads to full phosphatase activation[5].

Heterozygous *PPP3CA* deleterious variants were reported in sixteen patients[6–11] (Supplementary Table 1). The variants in the CD led to decreased calcineurin signaling in yeast models, suggesting a mechanism of loss-of-function (LoF), whereas the variants in the AID caused increased calcineurin signaling suggesting gain-of-function (GoF)[7]. Apparent genotype-phenotype correlation was observed; patients with missense or frameshift variants outside of the AID had epileptic encephalopathy (MIM 617711), whereas variants within the AID are associated with congenital abnormalities (MIM 618265)[7]. However, with only two patients with variants in the AID and six patients with truncating variants among all previously published works, identification of new patients and further genotype-phenotype studies are necessary to better characterize PPP3CA-related disorders.

We identified two frameshift and three missense *de novo PPP3CA* variants in five unrelated patients. The addition of these cases to the existing literature further supports that *PPP3CA* defects cause multiple distinct disorders.

Ethical approval

This study was performed in accordance with a research protocol that was approved by the Institutional Review Board at Baylor College of Medicine (BCM) (H-22769). Informed consent to participate in this study was obtained for Patients 2–5 according to the protocol at BCM and Patient 1 according to a research protocol approved by the Research Ethics Board at The Hospital for Sick Children (REB#1000055520). Additional studies on Patient 2 were performed according to the Undiagnosed Diseases Network (UDN) research protocol approved by the National Human Genome Research Institute IRB (#15HG0130).

Results

Pathogenic variants in *PPP3CA* were identified in five new patients (Fig 1, Table 1, Supplementary Data). Proband-only exome sequencing (ES) was performed for Patient 1 who had refractory epilepsy, and no definite causative variant was identified at the time of reporting. At the time of re-analysis in 2016, the heterozygous c.1299dupC (p.Ser434Glnfs*17) frameshift variant in *PPP3CA* was suspected to be the causative variant, and therefore, parental Sanger sequencing was performed, which revealed that the variant was *de novo*. Searching our internal ES database revealed a heterozygous c.1308_1311dupACTT (p.Ser438Thrfs*14) frameshift variant in Patient 2 affected by refractory epilepsy and subsequent Sanger sequencing showed that the variant was *de novo*.

More recently, a *de novo* heterozygous c.1417G>T (p.Ala473Ser) variant was detected in Patient 3 by ES . Patient 4 had a *de novo* heterozygous c.760A>G (p.Arg254Gly) variant identified by trio ES through the DDD project. The pathogenic variants in Patients 3 and 4 are in highly conserved amino acids and are predicted to be deleterious by SIFT, probably damaging by Polyphen-2 and disease causing by MutationTaster. Patient 5 had a *de novo* heterozygous c.844G>A (p.Glu282Lys) pathogenic variant identified by trio ES. The same change has been previously reported in two patients[6].

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To determine whether the mutant allele was expressed, RT-PCR and qRT-PCR on total RNA from whole blood was performed on Patient 2. Sanger sequencing analysis of the RT-PCR product, which spans exons 11 and 12, revealed that the mutant allele was expressed, and the level of the mutant allele was slightly reduced compared with the wild-type allele based on peak sizes (Fig 2A). qRT-PCR showed that total *PPP3CA* RNA, including mutant and wild-type transcripts, was 18–22% less than that in his mother (Fig 2B).

Slightly reduced expression of *PPP3CA* was also observed in RNA sequencing analysis of cultured skin fibroblasts in Patient 2. Mutant *PPP3CA* transcript was detected with a mildly skewed ratio (69 vs. 49) at the allele level for the read counts of wild-type and mutant, respectively (Fig 2C). The major transcript detected in RNA-seq was consistent with the transcript variant 2 (NM_001130691.1), which is known to be the major transcript in blood cells and not in the brain. No abnormal splicing pattern was observed around the exon containing the c.1308_1311dupACTT variant. In addition, total *PPP3CA* transcript showed a moderate reduction compared to the control group (relative ratio=0.70, P-value=0.01) (Fig 2D). These data indicate that a significant amount of the mutant transcript was present; therefore, nonsense medicated decay (NMD) did not play an apparent role in the expression level of the mutant.

PPP3CA protein levels in lymphoblasts of Patient 2 were measured by Western blot. The PPP3CA monoclonal antibody was raised against the N-terminal region with a resulting band at ~53 kDa for the normal protein while the truncated protein is predicted to be at ~48 kDa. Only the band for the wild-type protein was observed while no band corresponding to the truncated protein was observed, indicating that the mutant protein was undetectable by this analysis (Fig 2E). In addition, the expression of the wild-type PPP3CA protein was reduced, although not significantly (p-value = 0.06 by student t-test), compared to his mother (Fig 2F).

Discussion

We presented molecular and clinical findings of five new patients with pathogenic variants in the *PPP3CA* gene. Genotype-phenotype correlation of these patients and the 16 previously reported patients demonstrated that *PPP3CA*-associated neurodevelopmental disorders are diverse in both clinical features and disease-causing mechanisms.

All patients had developmental delay, cognitive dysfunction, and abnormal electrical activity in the brain. The other common findings are clinical epilepsy (81%), brain abnormalities (57%), hypotonia (62%), and autistic features (55%), (Supplementary Table 2). Our data suggest that the truncating variants in the RD represent a new type of mutations, distinct from missense variants in the CD and missense variants in the AID (Fig 1). All these truncating variants are clustered within a short 26-amino acid segment between CaMB and AID, while no truncating variants were reported outside of the RD in the literature or public databases. The truncating variants cause more severe epilepsy than the other two types of variants. All eight patients with a truncating variant invariably had severe intractable epilepsy. The age of seizure onset ranged between 6 weeks to 2 years with 63% having seizures in the first 6 months for the patients with truncating variants, whereas of the seven

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patients with mutations in the CD and clinical seizures, the onset time ranged from 3 months to 13 years and only 29% had seizures before 6 months. The major features in each type of mutation are shown in Fig 1. Since the number of patients is small, reporting of more patients is needed to confirm our observations.

Understanding the cellular consequences of truncating variants in the RD is important to provide guidance for an effective treatment of intractable epilepsy since immunosuppressants cyclosporin A and tacrolimus (FK506) are potent inhibitors of calcineurin. Currently it is inconclusive how truncating variants cause diseases. A LoF effect through reduced expression of calcineurin may contribute to the etiology. Although this study and previous studies demonstrated that the mutant transcripts escaped NMD[7,8], the truncating protein was undetectable[9] (Fig 2E), or expression level was very low[8] (Supplementary Table 3). Additionally, patients with truncating variants all had epilepsy as seen in most of the patients with LoF variants in the CD, but often lacked the skeletal phenotypes seen in the GoF patients. However, some observations cannot be explained by simple LoF. First, truncating variants have been only reported in the RD and have not been reported in the much larger region of the CD. In addition to the eight patients with truncating variants in the RD (Fig 1), four new patients in the ClinVAR database were reported to have truncating variants, including c.1394del (p.H465fs), c.1311_1315del (p.S438fs), c.1283dup (p.T429fs) and c.1271_1274dup (p.L426fs), all of which are in the RD. Second, patients with truncating variants had more severe early onset refractory epilepsy comparing with those with LoF missense variants. Third, the truncated protein without the AID was shown to have constitutive activity similar to missense variants in the AID[8]. Future research remains needed to precisely determine the underlying mechanism of truncating *PPP3CA* variants.

Only one missense variant p.Ala447Thr in the RD was previously reported in a patient with intractable seizure but no skeletal abnormalities (Supplementary Table 1). Interestingly, the variant is at the last nucleotide in exon 12 of *PPP3CA*, multiple splicing prediction programs predicted that this change affects mRNA splice donor site. Thus, this change may cause an out-of-frame deletion of exon 12 by exon skipping leading to a truncating protein. None of the other nine missense variants in Fig 1 were predicted to have an impact on splicing.

In summary, we report five new patients with *de novo PPP3CA* variants, expanding the knowledge of the PPP3CA associated disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement:

The data supporting the findings of this study are available within the article and its supplementary materials. The four non-DECIPHER variants identified in this study have been submitted to ClinVar (accession numbers SCV000746566.1, SCV001571658.1-SCV001571660.1).

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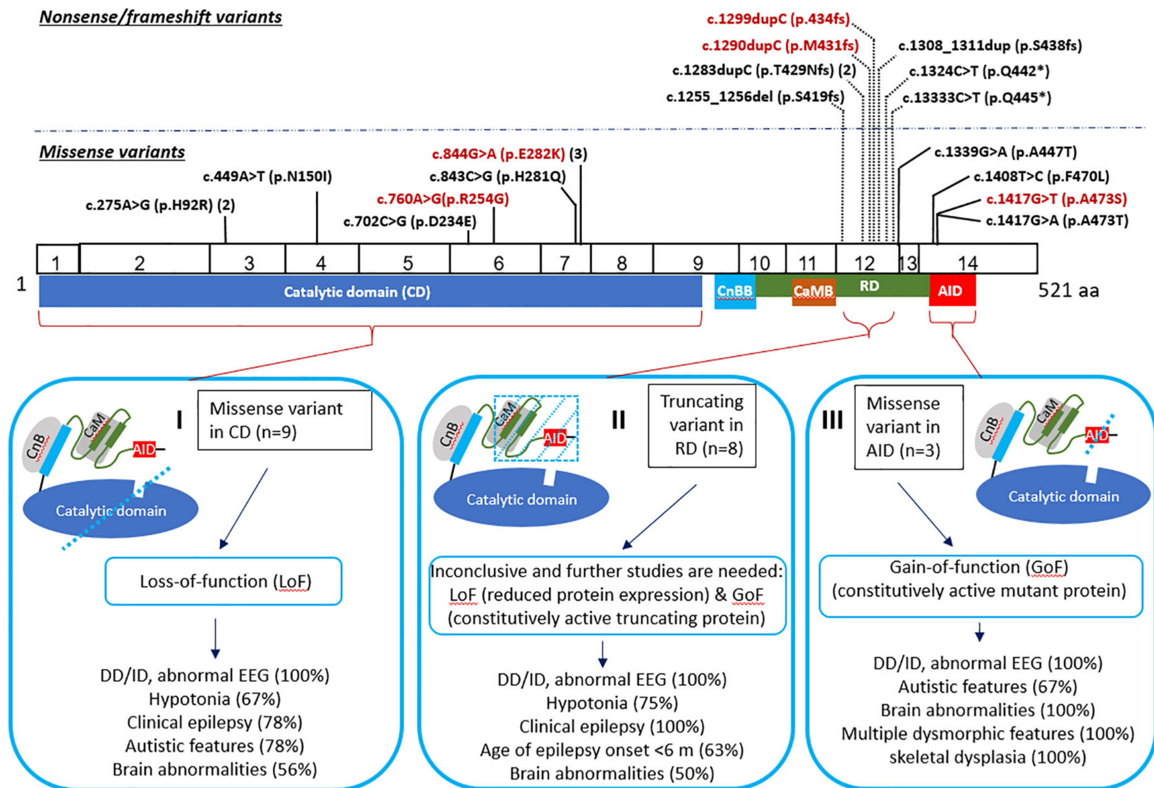

Fig 1. *PPP3CA* pathogenic variants reported in 19 patients.

The five variants in the current study were highlighted in red. The variants are presented according to their location in *PPP3CA* (NM_000944.4, NP_000935.1). White boxes represent exons. Colored boxes represent domains. The variants, except for p.A447T in the RD, were categorized into three types. For each type, the number of cases, etiology and major clinical findings in 50% patients were shown. Developmental delay (DD), intellectual disability (ID), electroencephalogram (EEG).

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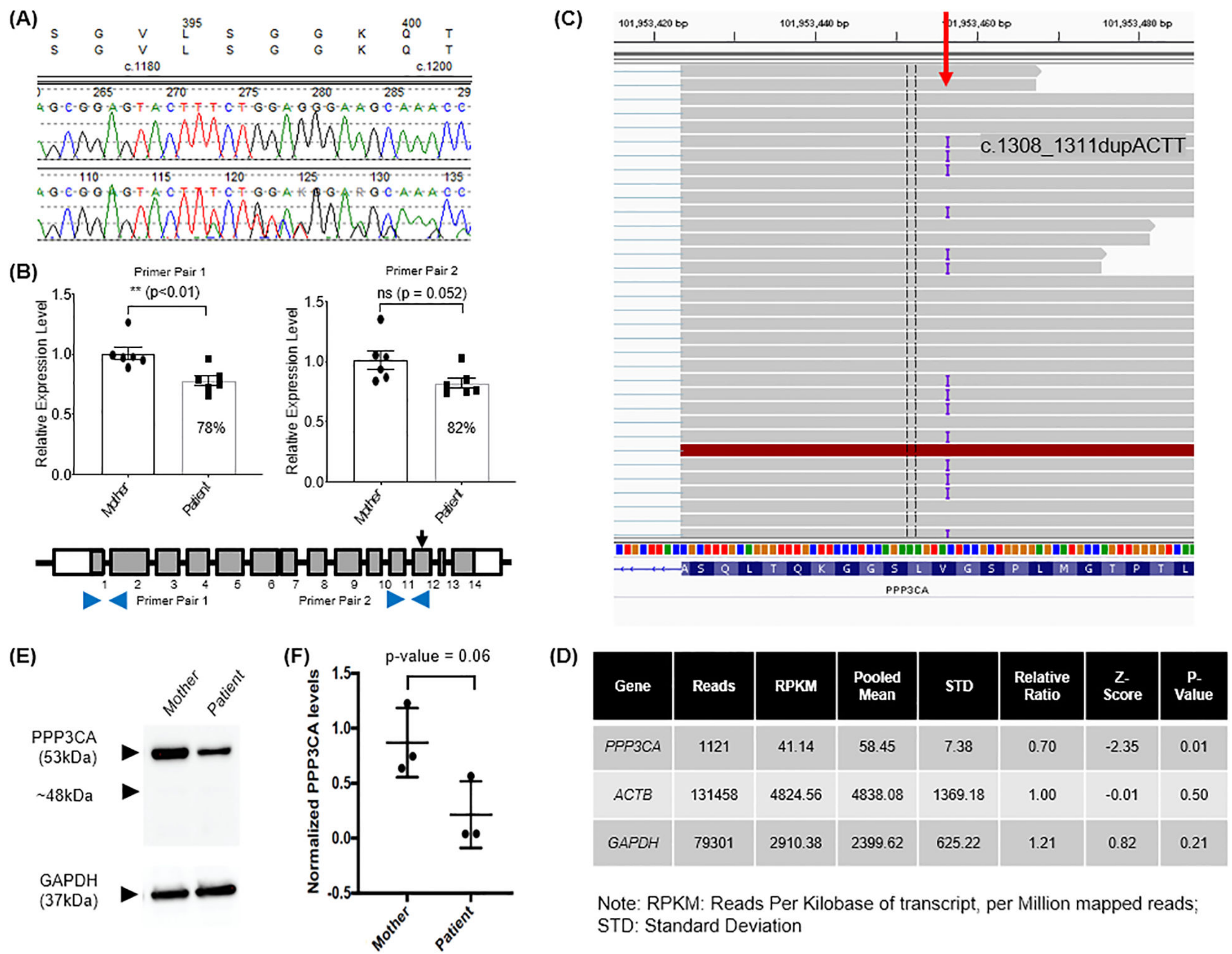

Gene	Reads	RPKM	Pooled Mean	STD	Relative Ratio	Z-Score	P-Value
PPP3CA	1121	41.14	58.45	7.38	0.70	-2.35	0.01
ACTB	131458	4824.56	4838.08	1369.18	1.00	-0.01	0.50
GAPDH	79301	2910.38	2399.62	625.22	1.21	0.82	0.21

Note: RPKM: Reads Per Kilobase of transcript, per Million mapped reads; STD: Standard Deviation

Fig 2. *PPP3CA* expression in Patient 2.

Expression in whole blood (A-B) or cultured fibroblast cells (C-D). (A) The mutant allele was detected by Sanger sequencing of RT-PCR products. (B) qRT-PCR showed that total *PPP3CA* RNA expression in blood was slightly decreased compared to his mother. (C) RNA-seq detected both the wild-type and mutant alleles at a ratio of 69:49. (D) RNA-seq showed moderate decrease in RNA level for *PPP3CA* but not the control genes *ACTB* and *GAPDH*, as evidenced by the reduced relative ratios in comparison with the pooled means. (E) Western blot of lymphoblastoid cell lines. The mutant PPP3CA, predicted to be at ~48 kDa, was not observed. (F) Quantification of PPP3CA levels normalized by GAPDH levels in each of the three replicates.

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Table 1

Clinical findings of the five patients with *de novo PPP3CA* pathogenic variants in this study

Patient	1	2	3	4	5
Ethnicity	European	East Asian	Caucasian	Caucasian	Ashkenazi Jewish
Sex	M	M	F	F	F
Age of last evaluation	3.5 y	10 y	4 y	14 y	5 y
Mutations	c.1299dupC (p.Ser434Glnfs*17)	c.1308_1311dupACTT (p.Ser438Thrfs*14)	c.1417G>T (p.Ala473Ser)	c.760A>G (p.Arg254Gly)	c.844G>A (p.Glu282Lys)
Gestation	Full term	Full term	39 wk	37 wk	Full term
Birth weight (g)	3260, 43%ile	3600, 86%ile	2580, 6.4%ile	4280, 96%ile	3033, 28%ile
Birth length (cm)	NA	NA	44.45, 0.2%ile	NA	49.53, 34%ile
FOC at birth (cm)	NA	NA	31, 0.8%ile	35, 64%ile	NA
Height (cm)	88.7, 10–25%ile, 2.5 y	107, 84%ile, 4 y	94, 1.33%ile, 4 y	141, 36%ile, 11 y	NA
Weight (cm)	12.45, 25%ile, 2.5 y	NA	13.6, 4.05%ile, 4 y	40, 70%ile, 11 y	NA
OFC (cm)	48, 2–50%ile, 2.5 y	52, 89%ile, 4 y	45.8, <1 %ile, 4 y	53.3, 12 y	NA
Development	Sitting at 9 m, walking at 2.5 y, not able to pronounce syllables	Speech delay, saying 100–200 words at 5 y	Sitting at 13 m, walking at 30 m, 1st word at 18 m, combining words at 3 y	Sitting at 8–9 m, walking at 20 m, 1st word at 3–4 y	Sitting at 6–7 m, walking at 19.5 m, 1st word at >2 y
Hypotonia	P	P	P	P	NP
Cognitive Dysfunction	Non-verbal, global delays	Able to follow simple commands	Needs special education, poor language development	Non-verbal, needs special education	Needs special education
Autistic Features	NP	P	P	P	P
Age of seizure onset	4 m	18 m	NP	13 y	NP
Seizure Types	4 m: GTCS; 6 m: infantile spasms	18 m: grand mal seizure, myoclonic seizure; GTCS, M	NP	13 y: GTCS	NP
EEG	6 m: generalized interictal discharges consistent with hypsarrhythmia. 3.5 y: slow awake background	4 y: multifocal epileptic discharges	4 y: Generalized background slowing	14 y: slow waves in the awake background with superimposed fast activities	4 y: Abundant small to medium amplitude spikes in sleep; 5 y: Frequent right temporal spike and slow waves in sleep
Brain MRI findings	6 m: small subdural collection. 2.5 y: Resolved subdural hematoma, prominent pericerebral spaces and asymmetric ventricles	18 m: Normal	4 y: T1 & T2 hyperintensities in periventricular white matter, volume loss of cerebral white matter, thinning corpus callosum	13 y: Normal	3 y: Two small foci of susceptibility in the left cerebellar hemisphere
Dysmorphic Features	Inverted nipples, increased abdominal girth, small penis, and B/L tapering of all digits	Inverted nipples	Microcephaly, low set ears, midface hypoplasia, cleft palate, micrognathia, wide/webbed neck,	Depressed nose tip	NP

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Patient	1	2	3	4	5
			B/L 5th digit clinodactyly, simian crease, B/L absent plantar crease		
Skeletal Abnormalities	NP	NP	Oblique talus, pelvic obliquity, short metacarpals, thin proximal radius, digit contracture, shortened/bowed femurs	Femoral anteversion, tibial torsion with foot drop unilateral	NP
Other findings	Constipation, light skin pigmentation, light hair color and thin texture	Constipation, frequent gagging, hyper-flexibility	Spasticity in lower extremities, unsteady gait	None	None

Note: Abbreviations used are as follows: B/L, bilateral; EEG, electroencephalogram; GTCS, generalized tonic-clonic seizure; M, myoclonic seizure; NP, not present; P, present; NA, not available; y, years; m, months; wk, weeks.