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FULL-LENGTH ORIGINAL RESEARCH

Epilepsia®

PRRT2 missense mutations cluster near C-terminus and frequently lead to protein mislocalization

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Summary

Objective: Variants in human *PRRT2* cause paroxysmal kinesigenic dyskinesia (PKD) and other neurological disorders. Most reported variants resulting in truncating proteins failed to localize to cytoplasmic membrane. The present study identifies novel *PRRT2* variants in PKD and epilepsy patients and evaluates the functional consequences of *PRRT2* missense variations.

Methods: We investigated two families with PKD and epilepsies using Sanger sequencing and a multiple gene panel. Subcellular localization of mutant proteins was investigated using confocal microscopy and cell surface biotinylation assay in Prrt2-transfected cells.

Results: Two novel *PRRT2* variants, p.His232Glnfs*10 and p.Leu298Pro, were identified, and functional study revealed impaired localization of both mutant proteins to the plasma membrane. Further investigation of other reported missense variants revealed decreased protein targeting to the plasma membrane in eight of the 13 missense variants examined (p.Trp281Arg, p.Ala287Thr, p.Ala291Val, p.Arg295Gln, p.Leu298Pro, p.Ala306Asp, p.Gly324Glu, and p.Gly324Arg). In contrast, all benign variants we tested exhibited predominant localization to the plasma membrane similar to wild-type Prrt2. Most likely pathogenic variants were located at conserved amino acid residues near the C-terminus, whereas truncating variants spread throughout the gene.

Meng-Han Tsai and Fang-Shin Nian contributed equally to this article.

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Significance: *PRRT2* missense variants clustering at the C-terminus often lead to protein mislocalization. Failure in protein targeting to the plasma membrane by *PRRT2* variants may be a key mechanism in causing PKD and related neurological disorders.

KEYWORDS
epilepsy, paroxysmal kinesigenic dyskinesia, protein localization, PRRT2, variants

1 | INTRODUCTION

Genetic variations in human *PRRT2* have been found to cause paroxysmal kinesigenic dyskinesia (PKD), infantile convulsions with choreoathetosis, benign familial infantile epilepsies, and hemiplegic migraine.[1–5] Most of the reported variants are nonsense or frameshift variants that result in truncated PRRT2 proteins. Among them, the p.Arg217Profs*8 variant has been detected in 77%-93% of all *PRRT2* variants and thus has been identified as a "hot spot mutation."[6–10]

PRRT2 is a membrane protein containing an N-terminal proline-rich domain and two transmembrane (TM) domains with an unconventional topology. Both the N- and C-termini were initially predicted to hang extracellularly; however, recent studies demonstrated that PRRT2 is a type II membrane protein with the N-terminus residing intracellularly.[11] The first TM domain (TM1) contains an unconventional helix-loop-helix kink that results in both ends of the TM1 domain protruding from the same intracellular face of the plasma membrane.[12]

Most truncating variants of *PRRT2* lead to a shortened protein that lacks both C-terminal TM domains. Previous functional studies have confirmed that truncated PRRT2 failed to localize to the plasma membrane and distributed in the cytoplasm and sometimes in the nucleus.[1,13,14] Thus, mislocalization of *PRRT2* mutations may lead to the loss of its normal functions at the synapses.[15–18] Currently, known missense variants of *PRRT2* have clustered around the C-terminal TM domains,[6] but the functional consequences of these variations have not been systematically investigated before. Determining the pathogenicity of missense variants has been cumbersome for clinicians, especially when supportive functional data are lacking.

Here, we report two novel variants of *PRRT2* in patients with PKD and infantile seizures, including one missense and one frameshift variant. We further studied the functional impact of these variants as well as selected known missense variants. We found that likely pathogenic missense variants at the C-terminus often lead to failure of protein targeting to the plasma membrane. Therefore, protein mislocalization may represent an important mechanism for *PRRT2* variants to cause PKD and related neurological disorders.

Key Points

- Two novel *PRRT2* variants, p.His232Glnfs*10 and p.Leu298Pro, impair localization of PRRT2 proteins
- *PRRT2* missense variants cluster near the C-terminus, whereas truncating variants spread throughout the gene
- *PRRT2* missense variants near C-terminus often lead to defects in its localization to the plasma membrane
- PRRT2 mislocalization in both truncating and missense variants is likely an important mechanism in causing PRRT2 dysfunctions

2 | MATERIALS AND METHODS

2.1 | Clinical and molecular genetic study

Patients diagnosed with PKD and having a history of benign infantile epilepsies (BIE) were recruited from the outpatient clinics at the Department of Neurology, Kaohsiung Chang Gung Memorial Hospital. Clinical data and a detailed family history were obtained during clinical visits and review of medical records, including electroencephalography and neuroimaging investigations. This study was approved by the human research ethics committees of Kaohsiung Chang Gung Memorial Hospital, Taiwan, and written consent was obtained from all subjects.

Whole venous blood DNA was extracted from peripheral leukocytes using a Qiagen DNA extraction kit according to the manufacturer's protocol. Family A was diagnosed using bidirectional Sanger sequencing. Coding exons 2 and 3 of the *PRRT2* gene including at least 10 base pair flanking regions were amplified and bidirectionally sequenced on the

ABI 3730XL automatic sequencer (Applied Biosystems) using the Big Dye kit (PerkinElmer). Family B was diagnosed through a microfluidic multiplex polymerase chain reaction–based multigene panel (Fluidigm Access 48.48 Array). Amplified products were barcoded and sequenced using an Illumina MiSeq system followed by standardized bioinformatic analysis and Sanger sequencing.[19,20] The functional impact of variant was predicted using Polymorphism Phenotyping 2 (PolyPhen-2; http://genetics.bwh.harvard.edu/pph2/),[21] Protein Variation Effect Analyzer (PROVEAN; http://provean.jcvi.org/index.php),[22] and MutationTaster (http://www.mutationtaster.org).[23] Segregation analysis was performed in the family members where DNA samples were available.

We searched the literature for previously published *PRRT2* variants with particular emphasis on missense variants. To avoid variants unlikely to be pathogenic, we limited our analysis to missense variants with a population frequency

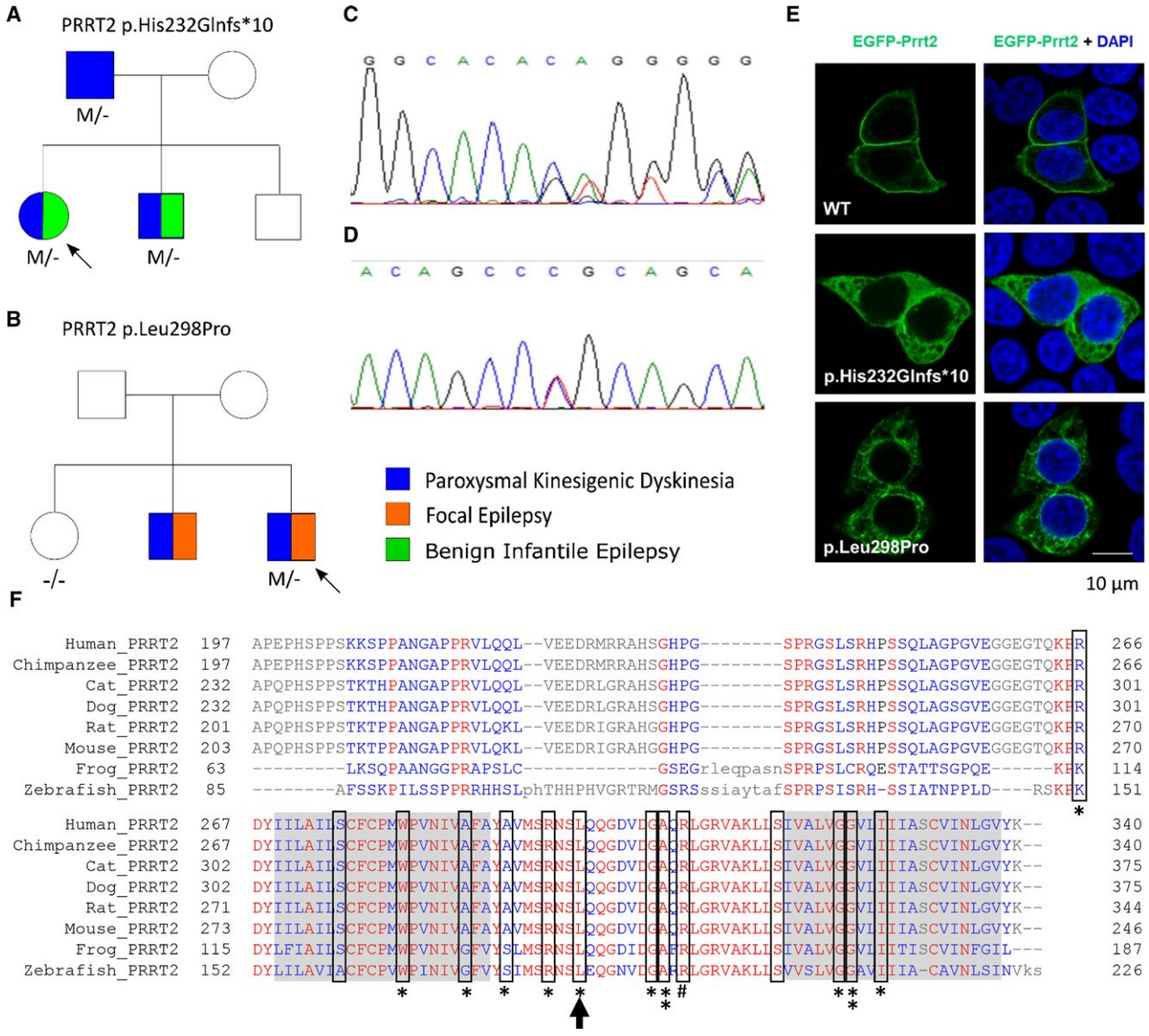

FIGURE 1 Clinical and functional studies of the two novel *PRRT2* variants. A, B, Pedigrees of two variant-positive families of paroxysmal kinesigenic dyskinesia and epilepsies. M, male. C, D, The chromatography of two novel variants confirmed by Sanger sequencing. E, Whereas wild-type (WT) enhanced green fluorescent protein–tagged Prrt2 (EGFP-Prrt2) exhibits localization to the plasma membrane, the prematurely truncating Prrt2 mutant p.His232Glnfs*10 is retained in the cytoplasm. The missense p.Leu298Pro mutant protein is localized to the endomembrane structures. DAPI, 4,6-diamidino-2-phenylindole. Scale bar = 10 µm. F, The primary amino acid sequences of PRRT2 protein from multiple species, including human, chimpanzee, dog, cat, rat, mouse, and zebrafish, were compared and aligned using the National Center for Biotechnology Information protein BLAST algorithm. Identical amino acids among all species are labeled in red. Highly conserved amino acids are in blue. Others are in gray. Reported missense variants are boxed. *Localization assayed in current study. **Two variants found in one position. #Localization reported in Liu et al.[13] The arrowhead indicates the novel missense mutation in the current study

< 1/100 000 in any subpopulation in the Thousand Genome Project (TGP), the Exome Variant Server (EVS), the Exome Aggregation Database (ExAc), or the Genome Aggregation Database (gnomAD), as the prevalence of PKD has been estimated as 1/150 000.[24,25] We selected 13 missense variants for subsequent functional analysis.

We also selected five benign *PRRT2* missense variants as controls. Two variants (p.Pro138Ala and p.Pro216Leu) were selected for the N-terminal domain based on the interpretation of the ClinVar database and the highest allele frequency in the gnomAD database. As for the C-terminal domain, no benign/likely benign variants existed in the ClinVar database. Therefore, three variants (p.Ala289Thr, p.Arg311Trp, and p.Ala333Ile) were selected from the TM1, ICD, and TM2 domains, respectively, based on the highest allele frequency in the gnomAD database.

2.2 | Multiple sequence alignment analysis

The multiple sequence alignment was performed using the National Center for Biotechnology Information protein BLAST algorithm (US National Institutes of Health). The primary amino acid sequences of PRRT2 from multiple species were compared.

2.3 | Plasmids

The mouse *Prrt2* cDNA construct was purchased from Origene (MG214094; GenBank accession # NM_01102563). The enhanced green fluorescent protein–tagged Prrt2 (EGFP-Prrt2) constructs of the wild-type and mutant *Prrt2* were generated and cloned into the pEGFP-C1 vector (Clontech). All mutant constructs were fully sequenced to verify the mutated nucleotides.

2.4 | Transfection and image acquisition

The procedure was performed according to a previous report.[13] Briefly, HEK293T human embryonic kidney cells were cultured in Dulbecco modified Eagle medium (Life Technologies) with 10% fetal bovine serum. Transfection was performed using Lipofectamine 2000 and 3000 lipofection reagents (Life Technologies), and cells were fixed and costained with 4,6-diamidino-2-phenylindole 24-48 hours after transfection. Cells

TABLE 1 Reported pathogenic *PRRT2* missense variants

Number	cDNA change	Codon change	Phenotype	PolyPhen-2	PROVEAN	MutationTaster
Chr16: 29825171	c.796C>T	p.Arg266Trp	PKD	D	D	N
Chr16: 29825199	c.824C>T	p.Ser275Phe	ICCA	D	D	D
Chr16: 29825216	c.841T>C	p.Trp281Arg	PKD	D	D	D
Chr16: 29825234	c.859G>A	p.Ala287Thr	PKD	P	N	N
Chr16: 29825247	c.872C>T	p.Ala291Val	PKD	D	N	D
Chr16: 29825658	c.884G>A	p.Arg295Gln	PKD, migraine	D	N	D
Chr16:29825667	c.893T>C	p.Leu298Pro	PKD	D	D	D
Chr16: 29825687	c.913G>T	p.Gly305Trp	PKD	D	D	D
Chr16: 29825687	c.913G>A/C	p.Gly305Arg	PKD	D	D	D
Chr16: 29825690	c.916G>A	p.Ala306Thr	ICCA, migraine	D	D	D
Chr16: 29825691	c.917C>A	p.Ala306Asp	ICCA	D	D	D
Chr16: 29825696	c.922C>T	p.Arg308Cys	PKD	D	D	D
Chr16: 29825724	c.950G>A	p.Ser317Asn	ICCA	D	D	D
Chr16: 29825742	c.968G>A	p.Gly323Glu	BFIE	D	N	D
Chr16: 29825744	c.970G>A	p.Gly324Arg	ICCA, migraine	D	D	D
Chr16: 29825745	c.971G>A	p.Gly324Glu	ICCA, migraine	D	D	D
Chr16: 29825755	c.981C>G	p.Ile327Met	BFIE	D	N	D

Note. Reference sequence: NM_145239.2. Selection criteria for probably pathogenic missense variants: reported in the literature, not present in the control database for >1/100 000. The population frequency was based on gnomAD with the highest allele frequency in any subpopulation.

Abbreviations: ACMG/AMP, American College of Medical Genetics and Genomics/Association for Molecular Pathology; BFIE, benign familial infantile epilepsy; D, damaging, deleterious, or disease causing; ICCA, infantile convulsion with choreoathetosis; N, neutral; N/A, not available; P, probably damaging; PKD, paroxysmal kinesigenic dyskinesia; VUS, variant of uncertain significance.

[a]ClinVar database: https://www.ncbi.nlm.nih.gov/clinvar/ (assessed October 2018).

[b]The variants are classified as likely pathogenic due to being cosegregated with disease in a large family with multiple affected members plus being reported in ClinVar as pathogenic, or a different missense change in the same amino acid has been reported before despite no supportive functional study.

expressing EGFP-Prrt2 proteins were then imaged by a confocal microscope (Zeiss LSM700) with a 100× Plan-Apochromat numerical aperture = 1.4 oil objective. Final composite images were processed by ImageJ (US National Institutes of Health).

2.5 | Cell surface biotinylation assay

Cell surface biotinylation assay was performed as described previously.[26] EGFP-Prrt2 constructs carrying Prrt2 variants were transfected into HEK293T cells. Twenty-four hours after transfection, cells were treated with 1 mg/mL of Sulfo-NHS-SS-biotin (Pierce) for 30 minutes at 4°C. Unbound biotin was quenched by biotin-blocking buffer (25 mmol·L^{-1} of Tris-HCl, pH 8.0; 133 mmol·L^{-1} of NaCl; 10 mmol·L^{-1} of KCl). Cells were then lysed with 1% Triton/0.1× SDS/PB (20 mmol·L^{-1} of sodium phosphate buffer, pH 7.5; 150 mmol·L^{-1} of NaCl). The biotinylated proteins were pulled down with NeutrAvidin beads (Pierce) overnight at 4°C. Sodium-potassium adenosine triphosphatase (ATPase) subunit alpha 1 (Cell Signaling Technology) was used as the positive control of plasma membrane biotinylation. The relative amount of biotinylated versus total Prrt2 protein was then analyzed by Western blotting. Sodium-potassium ATPase subunit alpha 1 (Cell Signaling Technology) was used as the internal control of biotinylated plasma membrane fraction. Beta-actin was used as the internal control of total protein extracts and the quality control of the surface biotinylation procedure. Green fluorescent protein (GFP; Abcam) and our in-house Prrt2 antibody[13] were used to detect the amount of EGFP-Prrt2 protein both in the total protein extracts and in the biotinylated membrane fraction. The relative amount of biotinylated Prrt2 protein in total Prrt2 protein was calculated by the following formula: (intensity of GFP in the biotinylated membrane fraction/intensity of Na^+/K^+ ATPase)/(intensity of GFP in the total protein extracts/beta-actin).

3 | RESULTS

3.1 | Genetics and clinical characteristics of two novel *PRRT2* variants in familial PKD and BIE

In Family A (Figure 1A), the proband was a 24-year-old female who had infantile seizures at the age of 4 months. The

Segregation	Population	ClinVar significance[a]	Mislocalization	ACMG/AMP guidelines	Reference
Mother-daughter pair	1/245 892	Pathogenic (literature)	No	VUS	29
Yes, in single family with 5 affected	1/245 366	Not provided	N/A	VUS	30
No, sporadic	Not present	Uncertain significance	Yes	Likely pathogenic	31
Yes	Not present	Not present	Yes	Likely pathogenic	31
Yes	Not present	Not present	Yes	Likely pathogenic	32
No, sporadic	Not present	Uncertain significance	Yes	Likely pathogenic	33
No	Not present	Not present	Yes	Likely pathogenic	This study
No	Not present	Not present	N/A	VUS	5
Yes, single family with reduced penetrance	2/241 012	Uncertain significance	No	VUS	34
Yes	Not present	Uncertain significance	No	Likely pathogenic[b]	3
Yes	Not present	Not present	Yes	Likely pathogenic	35
No	Not present	Conflicting interpretation of pathogenicity	No[13]	VUS	36
Yes, single large family	Not present	Pathogenic (literature)	N/A	Likely pathogenic[b]	4
N/A	Not present	Not present	No	VUS	7
Yes	Not present	Not present	Yes	Likely pathogenic	3
Yes	Not present	Not present	Yes	Likely pathogenic	3
No	Not present	Not present	No	VUS	37

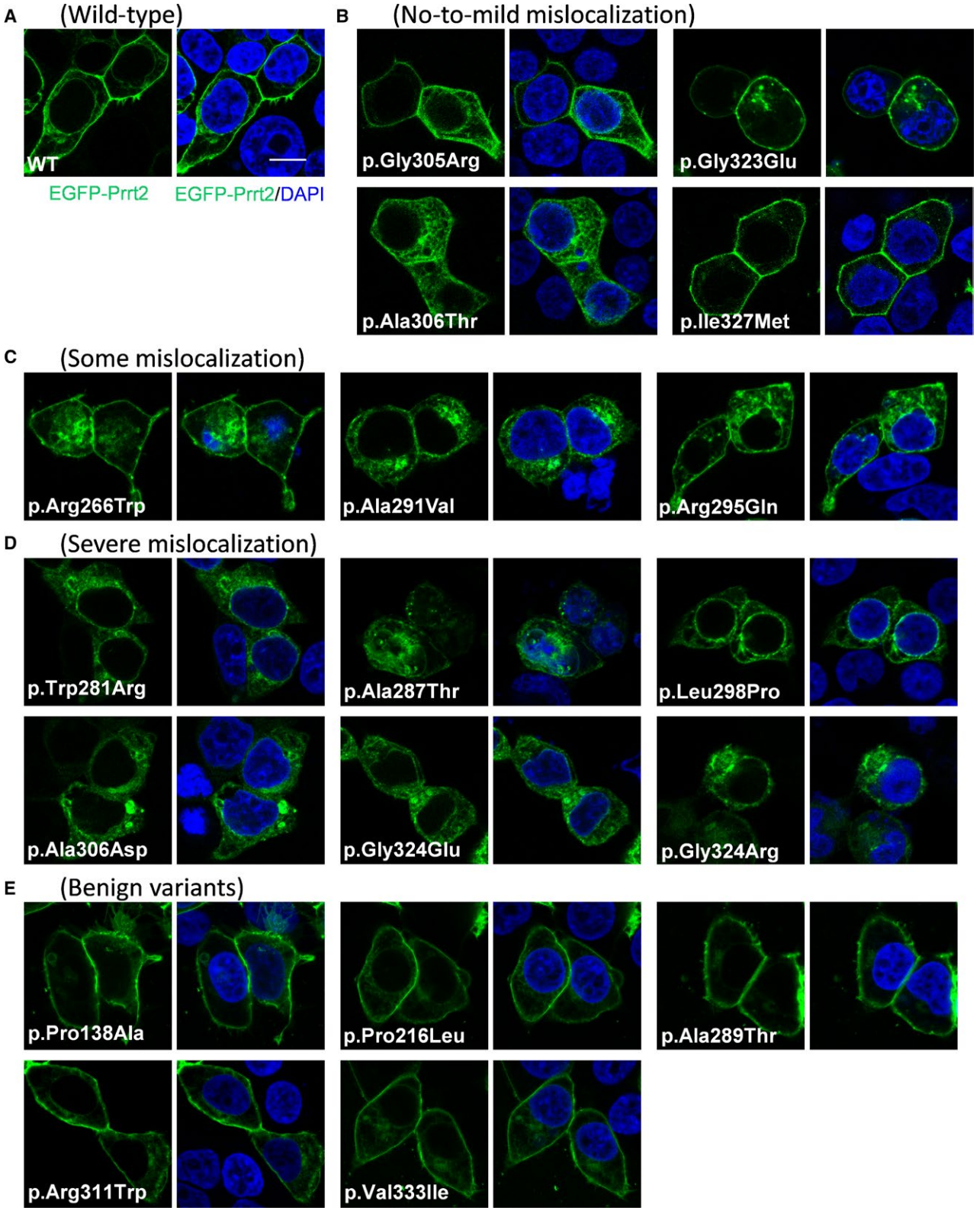

FIGURE 2 Subcellular localization of Prrt2 missense mutants at the C-terminus in cultured HEK293T cells. A, enhanced green fluorescent protein (EGFP)-tagged wild-type (WT) Prrt2 (green) was predominantly localized to the plasma membrane in cells. B, Prrt2 missense variants that exhibited localization predominately at the plasma membrane, with no or mild mislocalization. C, Prrt2 missense variants that have both intracellular and membrane localization. D, Prrt2 variants that are localized to endomembrane structures within cells and sometimes form large aggregates. E, Selected benign Prrt2 variants exhibit predominant membrane localization. All cells are costained with 4,6-diamidino-2-phenylindole (DAPI; blue) to indicate nuclei. Scale bar = 10 μm

seizures spontaneously subsided by the age of 1 year. She had intermittent involuntary right leg kinesigenic dyskinesia, especially at the start of movements, since the age of 22 years. The duration of the events lasted from several seconds to 5 minutes. Electroencephalogram (EEG) was normal, and her condition was easily controlled with low-dose oxcarbazepine. Her younger brother also had afebrile infantile seizures five times at the age of 4 months and then two times at 5 months. EEG was unremarkable. The brother and their father also suffered from the same involuntary movements. The early history of the father was not available. None of the three affected family members had a history of migraine or episodic ataxia. Sanger sequencing of *PRRT2* gene revealed a two-nucleotide deletion c.696-697delCA, which resulted in a frameshift variant with premature stop codon 10 amino acids downstream, p.His232Glnfs*10 (Figure 1C). This resulted in a truncated protein that lacked the two C-terminal TM domains. The variant was segregated in all affected individuals in the family.

In Family B (Figure 1B), the proband was a 25-year-old male who had had PKD since adolescence. During the attacks, he felt a strange sensation in the legs, sometimes accompanied by mild dyskinesia. At the age of 22 and 23 years, he also had two witness episodes of bilateral convulsive seizures with impaired consciousness preceded by ictal screaming. EEG showed focal sharp waves over the left temporal-occipital area. Brain magnetic resonance imaging was normal. Both PKD and seizures responded to carbamazepine. His elder brother was followed at another hospital for epilepsy and PKD. Both affected siblings did not have a history of migraine or episodic ataxia. Multigene panel and Sanger sequencing revealed a missense variant c.T893C, p.Leu298Pro, which was not present in the unaffected sister (Figure 1D). The variant was found in the intracellular loop between the two C-terminal TM domains. In silico programs (PROVEAN, PolyPhen-2, and MutationTaster) predicted a damaging effect.

Neither variant (c.696-697delCA and c.T893C) was presented in ClinVar or in other public databases, including TGP, EVS, ExAc, and gnomAD. In addition, they were also absent in our in-house Taiwanese controls (n = 265) using whole genome sequencing.

3.2 | Subcellular localization of novel Prrt2 mutant proteins

To investigate the potential pathogenic effects of these two variants, we first examined the subcellular localization of these mutant proteins. EGFP-Prrt2 constructs carrying these variants were transfected into HEK293T cells in culture. Subcellular localization of these PRRT2 proteins in HEK293T cells was visualized by confocal microscopy (Figure 1E). We found that whereas wild-type Prrt2 was localized to the plasma membrane, p.His232Glnfs*10 was localized predominantly in the cytoplasm, presumably due to the loss of its TM domains. The p.Leu298Pro mutant protein, whose variant is in the intracellular loop, could localize to the plasma membrane. However, the protein was also found in the endomembrane structures in transfected cells. Therefore, it is possible that p.Leu298Pro was trapped in the endoplasmic reticulum (ER)-Golgi membrane system. The mislocalization of these two mutant proteins may be critical to the dysfunction of PRRT2 in these patients.

3.3 | Missense variants in the conserved C-terminal amino acid residues

Using amino acid sequence alignment among different species, including human, chimpanzee, dog, cat, rat, mouse, and zebrafish, we confirmed that the amino acid sequence within the C-terminus of PRRT2, including the TM domains and intracellular loop, was highly conserved among the vertebrates (Figure 1F).[6] Coincidently, most currently known missense variants lie at the amino acid residues within this region (Figure 1F, Table 1), indicating the importance of these conserved residues. Supporting this notion, most missense variants occurred at the conserved amino acid residues (Figure 1F). However, the impact of these amino acid alternations on the function of PRRT2 remained elusive.

3.4 | Subcellular localization of other known missense Prrt2 proteins

Previously, we have shown that although wild-type Prrt2 is a membrane protein, all six truncated forms of Prrt2 we investigated failed to localize to the plasma membrane, with predominant cytoplasmic and nuclear distribution.[13] However, the subcellular localizations of most missense Prrt2 mutant proteins have not been investigated before. To test whether Prrt2 missense variants may also cause protein mislocalization, we expressed most of the reported missense mutants and looked at their localization within the cell (Figure 2). Whereas the wild-type Prrt2 predominantly localized to the plasma membrane with some found in the endomembrane (Figure 2A), nine of the 13 missense variants we tested led to different degrees of protein mislocalization, with six localizing predominantly in the endomembrane, including the nuclear envelope (p.Trp281Arg, p.Ala287Thr, p.Leu298Pro, p.Ala306Asp, p.Gly324Glu, and p.Gly324Arg; Figure 2D), and three exhibiting membrane as well as prominent intracellular localization (p.Arg266Phe, p.Arg295Gln, and p.Ala291Val; Figure 2C). The other four variants had prominent membrane as well as some intracellular localization (p.Gly305Arg, p.Ala306Thr, p.Gly323Glu, and p.Ile327Met; Figure 2B). As a comparison, we examined the localization of benign *PRRT2* variants p.Pro138Ala, p.Pro216Leu,

p.Ala289Thr, p.Arg311Trp, and p.Val333Ile selected across all domains. We found that, similar to the wild-type Prrt2, all of these variants displayed predominant membrane localization (Figure 2E). These findings suggested that potential pathogenic Prrt2 missense variants may affect the targeting to the plasma membrane at different degrees.

To quantitatively measure the amount of Prrt2 protein on the plasma membrane, we employed cell surface biotinylation assay in Prrt2-transfected cells. The protein expression levels of all but 1 (p.Gly324Arg) missense variants were comparable to the wild-type Prrt2. Proteins present on the plasma membrane were labeled with biotin for 30 minutes and then pulled down by NeutrAvidin beads. The relative amount of biotinylated versus total Prrt2 protein was then analyzed by Western blots. The membrane protein Na^+/K^+ ATPase was blotted as the positive control for the surface biotinylation assay. We found that eight of the 13 missense variants tested (p.Trp281Arg, p.Ala287Thr, p.Ala291Val, p.Arg295Gln, p.Leu298Pro, p.Ala306Asp, p.Gly324Glu, and p.Gly324Arg) exhibited lower levels of biotinylation in comparison to the wild type (Figure 3). By contrast, none of the benign variants tested had significant changes in the level of biotinylation. These results further supported defects of these mutant proteins in targeting to the plasma membrane.

4 | DISCUSSION

In the current study, we report two Taiwanese families with PKD and BIE and identify two novel *PRRT2*

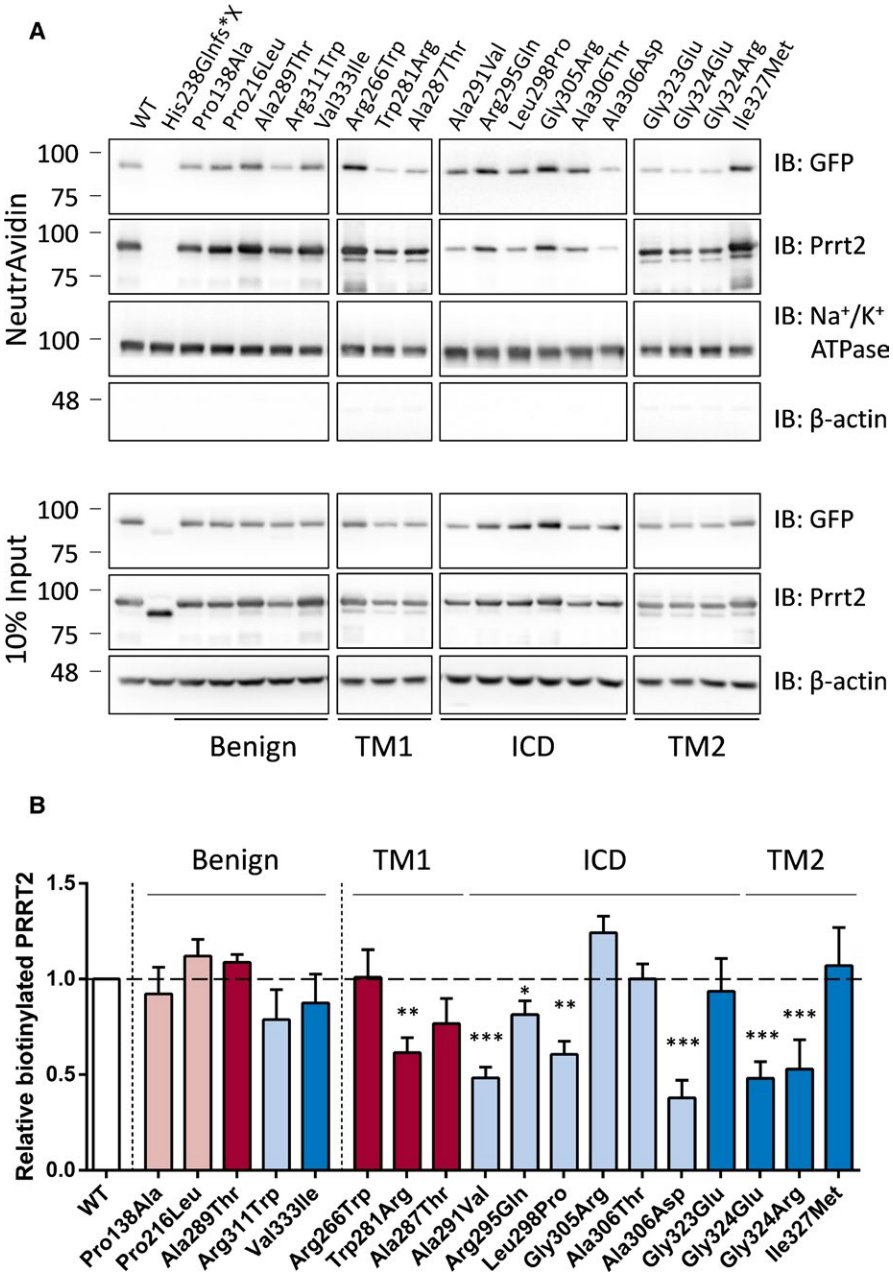

FIGURE 3 Protein targeting of mutant Prrt2 to cell plasma membrane. A, Western blots of biotinylated membrane fraction and 10% input of wild-type (WT), reported benign variants, and mutant Prrt2 proteins from transfected cells treated with biotin for 30 minutes. Na^+/K^+ adenosine triphosphatase (ATPase) and β-actin were also blotted as the positive and negative controls, respectively. The truncated mutation p.His238Glnfs*X, which lost both transmembrane (TM) domains, serves as the negative control. GFP, green fluorescent protein; IB, immunoblotting. B, Bar graph shows biotinylation levels of Prrt2 variants normalized to the WT, indicating their relative degree of membrane localization. Corresponding domains of each variant are color coded (pink, N-terminus; red, TM1; light blue, intracellular domain [ICD]; dark blue, TM2). Data are presented as mean ± standard error of the mean from at least three independent experiments. Statistical analysis was performed by one-way analysis of variance, followed by Fisher's least significant difference test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ when compared with WT group

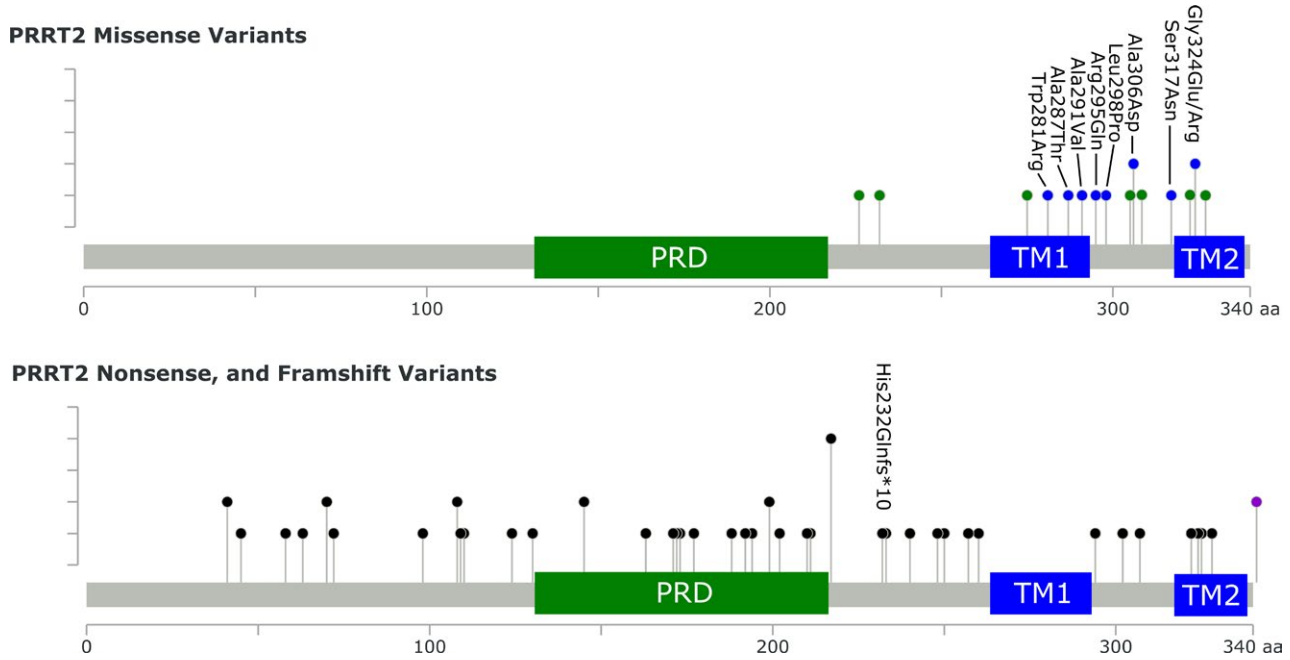

FIGURE 4 Hot zone for likely pathogenic *PRRT2* missense variants. The upper panel illustrates the distribution of published pathogenic missense variants (with allele frequency < 1/100 000 in control databases) of PRRT2 protein (1-340 amino acids [aa]), which cluster around the C-terminus transmembrane domains (TM1, 269-289 aa; TM2, 318-338 aa). In contrast, the distribution of truncating variants, including nonsense and frameshift variants (as shown in lower panel), appeared to spread throughout the entire PRRT2 protein. TM1, first transmembrane domain; TM2, second transmembrane domain. Blue dots indicate likely pathogenic missense variants based on American College of Medical Genetics and Genomics/Association for Molecular Pathology guidelines. Green dots indicate published pathogenic missense variants without supportive functional data. Black dots indicate nonsense or frameshift variants with premature stop codon. Purple dot indicates stop loss variants. The lollipop plots were generated by MutationMapper v1.0.1 (http://www.cbioportal.org)[28] and annotated using Inscape v092.2. PRD, proline-rich domain

variants: a frameshift variant (p.His232Glnfs*10) and a missense variant (p.Leu298Pro). The frameshift variant (p.His232Glnfs*10) leads to a truncating protein that exhibits predominant cytoplasmic distribution and is unable to localize to the plasma membrane, and the PRRT2 protein with a missense variant (p.Leu298Pro) has decreased membrane targeting on the plasma membrane. Hence, both mutant proteins are likely to lose their normal functions as a transmembrane protein. We further investigated the subcellular localization of an additional 12 published missense variants using fluorescent microscopy and cell surface biotinylation assay. We found that missense variants at the C-terminus often exhibit defects in targeting to the plasma membrane (eight of 13 variants tested). Therefore, protein mislocalization may hinder normal PRRT2 functions on the plasma membrane and contribute to the pathogenesis of PKD and related disorders.

Our results support the concept of a "hot zone" for missense variants near the C-terminus of PRRT2.[6] These pathogenic *PRRT2* missense variants mainly occur at conserved amino acid residues, suggesting the importance of these residues in normal PRRT2 functions. In contrast, the known truncating variants distribute more evenly throughout all domains of the gene, resulting in the absence of the two C-terminal TM domains (Figure 4).

Our study also suggests that the incorporation of PRRT2 into the plasma membrane, perhaps at the synapse, is crucial for its function. Previously, we have demonstrated that truncated Prrt2 proteins resulted from nonsense or frameshift variants distributed to the cytoplasm, and sometimes to the nucleus.[13] Here, we find that missense variants at the PRRT2 C-terminus often cause decreased localization to the plasma membrane, suggesting defects in plasma membrane targeting. It is possible that changes of hydrophobicity of these transmembrane amino acids alter the distribution of PRRT2. Alternatively, protein misfolding due to these amino acid changes may also trigger quality control response, which will retain the mutant protein in the ER. How amino acid residue changes affect protein folding and potentially trigger ER retention requires further investigation.

In addition, we also found that the previously reported missense variants p.Arg266Trp, p.Gly305Arg, p.Ala306Thr, p.Gly323Glu, and Ile327Met are predominantly localized to the plasma membrane, similar to the wild-type Prrt2, as well as the p.Arg308Cys variant.[13] It is unclear how these residue substitutions change the properties of Prrt2. Although we did not find obvious changes in the subcellular localization of these six missense variants, it is still possible that the variants affect the protein function through other mechanisms, such as altered protein stability, structure, or interaction with other proteins. The

interpretation of missense variants of uncertain significance is subjective to further changes to benign or pathogenic, with accumulating evidence provided by more functional studies.[13]

The determination of pathogenicity for missense variants is not a straightforward task and frequently fraught with lack of information and uncertainties.[27] Many computational predictive programs have been developed to aid in the interpretation, but they are far from perfect. Combining multiple in silico programs has been recommended, but they at times disagree with each other. Population and segregation data may sometimes be useful to exclude polymorphisms or to add support to the strength of pathogenicity but also have limitations such as nonpenetrance.[25,27] Functional studies are powerful tools to decide the impact of genetic variants on protein function. Here, we demonstrated that pathogenic *PRRT2* missense variants tend to occur around the C-terminal TM domains. These disease-causing missense *PRRT2* variants, like truncating variants, are likely to affect its subcellular distribution. Hence, the correct incorporation of PRRT2 protein to plasma membrane is crucial to its normal function.

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DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

AUTHOR CONTRIBUTIONS

All authors contributed to the acquisition, analysis, and interpretation of the data and revising the manuscript for intellectual content. M.-H. Tsai, F.-S. Nian, and J.-W. Tsai contributed to the design and conceptualization of the study, and drafting, revising, and final approval of the manuscript for intellectual content.

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