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AN INVESTIGATION OF MOLECULAR LESIONS IN TWO JAPANESE FAMILIES WITH FAMILIAL PAROXYSMAL KINESIGENIC DYSKINESIA

Hiroki Kubota, Atsuko Noguchi, Tamami Yano, Daiki Kondo and Tsutomu Takahashi

Department of Pediatrics, Akita University Graduate School of Medicine

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Abstract

Familial paroxysmal kinesigenic dyskinesia (PKD) is an episodic involuntary movement disorder characterized by recurrent and brief attacks induced by sudden voluntary movement. Proline-rich transmembrane protein 2 (*PRRT2*) has been identified as a gene responsible for PKD and its related disorders. Recently, the protein encoded by *PRRT2* was identified as a synaptic protein with a regulatory role in neurotransmitter release, which indicated that PKD may be a synaptopathy. At present, more than 50 *PRRT2* mutations have been identified, but the molecular mechanisms underlying the heterozygous mutations that cause the disorder remain unclear. A novel *PRRT2* mutation, c.649delC (p.R217Efs*12), was identified as a heterozygous allele in one of two Japanese families with PKD. The mutation encodes a truncated PRRT2 protein, which consists of 216 amino acid residues compared to the full length protein of 429 amino acid residues. To examine the subcellular localization of the wild and mutant PRRT2 proteins, we induced the transient expression of the PRRT2 protein fused with fluorescent proteins, pAcGFP1-C1 and pDsRed-monomer-C1, in COS7 cells. Although the transient intracellular expression of wild PRRT2 protein fused with pAcGFP1-C1 confirmed its subcellular localization at the cell membrane, the mutant p.R217Efs*12 PRRT2 protein fused with pDsRed-monomer-C1 was detected in the cytosol and nucleus of COS7 cells. In the co-transfection experiment, the mutant truncated PRRT2 protein did not inhibit the subcellular localization of the wild-type PRRT2 protein. The results suggested that a heterozygous *PRRT2* mutation might cause the disorder through a reduction in the amount of the prot ein encoded by the *PRRT2* gene.

Key words : paroxysmal kinesigenic dyskinesia, PRRT2 gene, subcellular localization

Background

Familial paroxysmal kinesigenic dyskinesia (PKD) is an episodic involuntary movement disorder characterized by recurrent and brief attacks induced by sudden voluntary movement, such as standing up too quickly or being startled. The attacks of involuntary movements, which usually last for between seconds and one minute, may involve many forms, such as dystonia, chorea, athetosis, or ballism, and usually affect one side of the body or one limb[1]. The form of involuntary movement depends on the affected individual and may even vary between members of the same family. Patients with familial PKD usually begin to show signs and symptoms of the disorder during childhood or adolescence ; the number of attacks generally increases during puberty and then decreases in the second and third decades of the patient's life. Many patients with familial PKD demonstrate a pattern of

Correspondence : Tsutomu Takahashi, M.D., PhD.
Department of Pediatrics, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan
TEL : 81-18-884-6159
FAX : 81-18-836-2620
E-mail : tomy@med.akita-u.a.jp

symptoms, often described as a crawling or tingling sensation in the affected sites, which precede the attacks as an aura. The patients appear fully healthy between attacks and do not lose consciousness during an episode. Some patients with familial PKD are known to have the complication of recurring afebrile seizures in infancy, known as benign familial infantile convulsions (BFIC)[2], which disappear before the age of 3 years old. The association of infantile convulsions with PKD is known as infantile convulsions and choreoathetosis (ICCA)[3-5], although PKD and BFIC can occur independently.

Proline-rich transmembrane protein 2 (*PRRT2*) has been identified as a gene responsible for PKD[6], BFIC[7], and ICCA. Recently, the protein encoded by *PRRT2* was identified as a synaptic protein with a regulatory role in neurotransmitter release, which indicated that PKD may be a synaptopathy. To date, more than 50 different mutations have been reported[8,9]. Among them, nonsense mutations that introduce a premature stop codon into a part of the gene are the most common type, with c.649dupC (p.R217Pfs*8) being a notable hot-spot[10-12].

In this study, we have reported a novel *PRRT2* mutation, c.649delC (p.R217Efs*12), which was identified in one of two unrelated Japanese families with familial PKD. We investigated the molecular background of the c.649delC (p.R217Efs*12) mutation that caused PKD in this family.

Materials and Methods

Case reports

Patient 1 (Fig. 1, family 1, III-1) was a boy born to nonconsanguineous Japanese parents ; the patient weighed 4,025 g at birth after a 41-week gestation period. His growth was normal during infancy and the preschool ages. He was diagnosed with a tic disorder at the age of 8 years, which was treated by Chinese herbal medicine without apparent effectiveness but eventually disappeared within 2 years. He started showing episodic involuntary movement induced by voluntary movements at approximately 10 years old. For example, while running, the voluntary movements induced involuntary movements, which led to clumsiness in his physical movements. He was referred to our hospital because of the above complaints at 11 years of age. The neurological and laboratory examinations did not reveal any abnormalities, but the mother of the patient asked the physician if PKD was a possible diagnosis. The mother had been made aware of a PKD website and suggested the idea of PKD to her child's physician. Carbamazepine, which is well known to be effective for PKD, was administered to the patient and episodes of the attack dramatically disappeared. When his mother (family 1, II-3) was approaching puberty, she had complained of clumsiness of physical movements induced by voluntary movements, during running or playing basketball.

Patient 2 (Fig. 1, family 2, III-2) was a boy born after an uneventful delivery to nonconsanguineous Japanese parents ; the patient weighed 3,540 g at birth after a 39-week gestation period. He grew normally during infancy and preschool age and had no episodes of epilepsy, including febrile convulsion. He was referred to us at the age of 10 years and 7 months after complaining of episodic involuntary movements, which were characterized by recurrent and brief attacks induced by sudden voluntary movements. The attacks usually lasted from seconds to

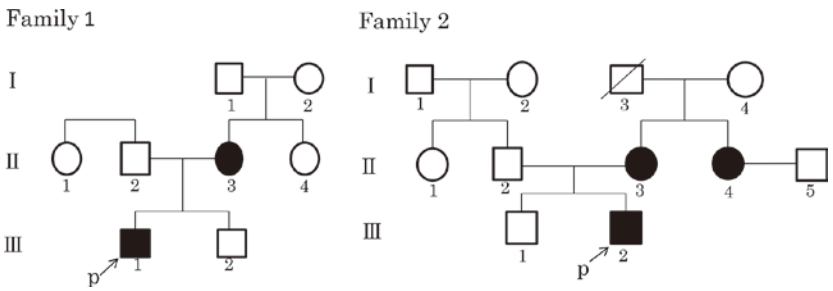

Fig. 1. Pedigrees of the two Japanese families with paroxysmal kinesigenic dyskinesia.
Filled-in symbols indicate the individuals with paroxysmal kinesigenic dyskinesia.

one minute without a loss of consciousness. In his family, a similar pattern of episodes was observed in his mother (family 2, II-3) and mother's elder sister (family 2, II-4) during puberty and then gradually decreased, but still occurred occasionally.

Mutational analysis of *PRRT2*

The gene, *PRRT2*, of the patients and their parents was sequenced by the Sanger DNA sequencing method. Genomic DNA was purified from peripheral blood. The forward and reverse PCR primers were designed by using Primer3Plus software (http://primer3plus.com/) and the PCR products were purified by using Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA). PCR amplification and sequencing was performed by using a Veriti® Thermal Cycler (Applied Biosystems®, Japan). The sequencing products were purified by using Performa® DTR Gel Filtration Cartridges (EdgeBio, San Jose, CA, USA). The PCR products were directly sequenced on an ABI PRISM3100 Genetic Analyzer and the sequence analysis was computed by Sequencing Analysis v5.3.1. This study was approved by the Institutional Review Board and Ethical Committee of Akita University Graduate School of Medicine. Written informed consent was obtained from the parents of the patients.

To analyze the mRNA sequence of the *PRRT2* gene from patient 2, Epstein-Barr (EB) virus-transformed lymphoblast cells were established from a normal individual and patient 2 using standard techniques The cells were cultured in modified RPMI1640 medium supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO2. To block the functional nonsense-mediated mRNA decay (NMD) system of mammalian cells, two inhibitors of NMD, emetine dihydrochloride hydrate and cycloheximide, were added to the medium for 6 h and 5 h, respectively, to immobilize the lymphoblast cells from the subjects[10]. Total RNA was extracted from EB virus-transformed lymphoblast cells by using the RNAgents SV Total RNA Isolation System (Promega, Madison, WI, USA). The purified RNA samples were reverse transcribed and PCR-amplified using the SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen, Carlsbad, CA, USA) and a set of sense and antisense primers designed to amplify the region encompassing the target. Subsequently, the RT-PCR products were purified on a 1.0% agarose gel and directly sequenced in the forward direction.

Construction of expression vectors and confocal fluorescence microscopy

A full-length cDNA encoding the wild-type *PRRT2* was custom-synthesized by FASMAC Co., Ltd (Atugi, Japan), digested with the appropriate restriction enzymes, and cloned into the pAcGFP1-C1 vector by using a T4 DNA ligase (TaKaRa, Otsu, Japan). Another full-length cDNA encoding a mutant *PRRT2* was similarly custom-synthesized, digested with the appropriate restriction enzymes, and cloned into the pDsRed-Monomer-C1 vector. The initial clones were identified through the transformation of JM109 (Toyobo, Osaka, Japan) and sequencing to confirm the presence of the plasmids. The vector plasmids were transfected into COS7 cells by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. After transfection, the cells were observed by confocal fluorescence microscopy. COS7 cells were obtained from RIKEN Cell Bank (RCB0539) and cultured in Dulbecco's modified Eagle's medium (SIGMA, St. Louis, MO, USA) or RPMI1640 medium (pH 7.4) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere with 5% CO_2.

The cells were observed using a Zeiss-LSM 780 confocal microscope equipped with a UV laser.

Results

Mutational analysis of *PRRT2*

The analysis of the sequence of *PRRT2* identified a heterozygous single base deletion of C at the cDNA position of 649, designated as c.649delC, in the patients of family 1 (Fig. 2A and 2B). The c.649delC mutation was predicted to cause a frame shift at codon 217, which resulted in an arginine (CGA) to glutamic acid (GAG) substitution ; this frameshift mutation caused a termination at codon 228, which was predicted to encode a prematurely truncated PRRT2 protein, designated as

p.R217Efs*12 (Fig. 3).

The c.649delC mutation was also identified as a heterozygous mutation in patient 2. However, low amplification of the mutant sequence was observed in the sequencing chart (Fig. 2C) and in the normal subject (Fig. 2D), which suggested that it might be an artifact sequence. We analyzed mRNA isolated from the immortalized lymphoblasts of the patient 2 by RT-PCR sequencing and found the c.649delC mutation, but it was also present at low amplification in the sequencing chart. In mammalian cells, a specific transcriptional regulatory mechanism, designated as the nonsense-mediated mRNA decay (NMD) pathway, has been known to degrade mRNA with premature termination codons, such as c.649delC, to diminish the production of potentially deleterious truncated proteins. This NMD system is known to be inhibited by emetine dihydrochloride hydrate or cycloheximide ; therefore, we analyzed mRNA isolated from the immortalized lymphoblasts incubated with the NMD inhibitors. As the results showed that the low amplification of the sequence was not changed by inhibitors of NMD, it was confirmed as an artifact sequence (Fig. 4A, 4B, and 4C).

Confocal fluorescence microscopy

To investigate the subcellular localization of wild-type

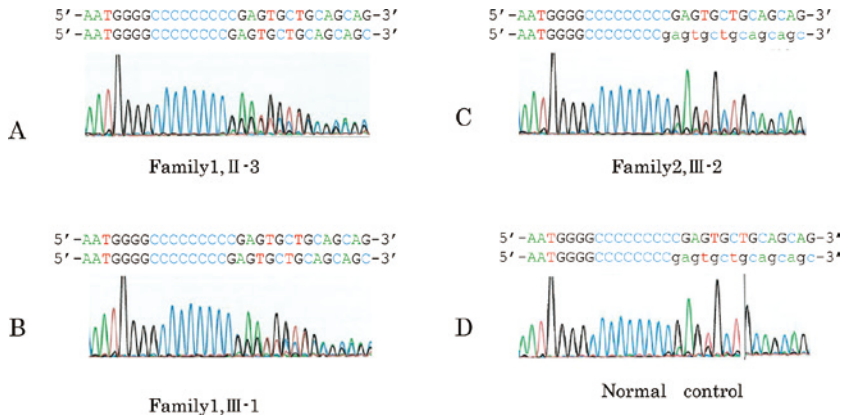

Fig. 2. Sequence analyses of PRRT2 in the two Japanese families with paroxysmal kinesigenic dyskinesia.
Two sequences of the patients from family 1 had a heterozygous single base deletion of C at the cDNA position of 649 (A : II-3 and B : III-1). Two sequences of a patient from family 2 and normal control showed low amplification of the heterozygous single base deletion of C at the cDNA position of 649 (C : III-2 and D : normal control).

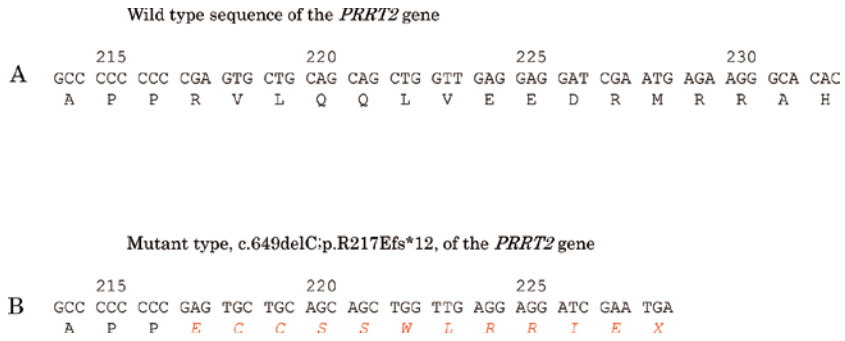

Fig. 3. Predicted amino acid residues of the truncated mutant PRRT2 protein.
The wild-type sequence of PRRT2 and the amino acid residues are shown in A. The mutant type c.649delC of PRRT2 caused a frame-shift at codon 217, which resulted in an arginine (CGA) to glutamic acid (GAG) substitution and this frameshift mutation caused a termination at codon 228 (B).

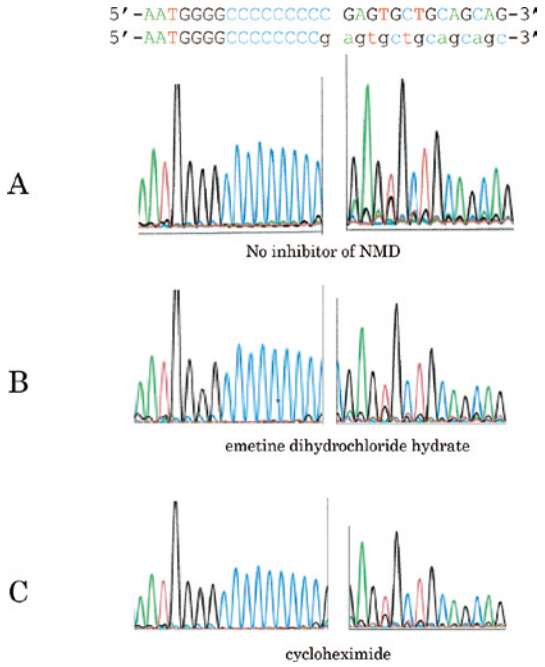

Fig. 4. The sequence of RT-PCR products isolated from lymphoblastic cells in family 2.
The RT-PCR sequence indicated low amplification of the heterozygous c.649delC mutation (A). Two analyses of the mRNAs from the lymphoblast treated with emetine dihydrochloride hydrate (B) or cycloheximide (C) are shown.

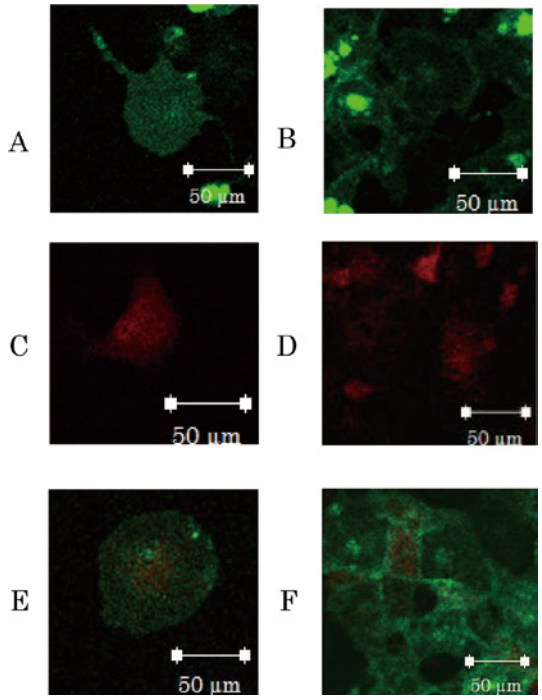

Fig. 5. Subcellular localization of wild-type and mutant PRRT2 proteins.
A single transfection of wild-type PRRT2 cDNA subcloned into the pAcGFP1-C1 vector resulted in green fluorescence in COS7 cells (A and B). A single transfection of wild-type PRRT2 cDNA subcloned into the pDsRed-Monomer-C1 vector resulted in red fluorescence in COS7 cells (C and D). The co-transfection of wild-type and mutant DNA resulted in green and red fluorescence, respectively, in a single cell ; the two colors were not observed as merged yellow within a single cell (E and F).

and truncated PRRT2 proteins, COS7 cells were transfected with either wild-type plasmid cloned into the pAcGFP1-C1 vector or mutant plasmid cloned into the pDsRed-Monomer-C1 vector. Although wild-type PRRT2 protein was localized mainly in the cell membrane, the mutant PRRT2 protein was localized to the cytoplasm and nucleus (Fig. 5A, 5B, 5C, and 5D). Co-transfection of the wild-type and mutant plasmids showed the same localization of the individual proteins as each protein transfected individually (Fig. 5E and 5F). It was observed that the mutant truncated protein did not influence either the expression or the subcellular localization of the wild-type protein in COS7 cells (Fig. 5E and 5F).

Discussion

A novel heterozygous *PRRT2* mutation, c.649delC, was identified as a molecular lesion in one of two Japanese families with PKD. This mutation caused a frameshift at codon 217, such that the truncated PRRT2 protein, designated as p.R217Efs*12, differed from the wild-type PRRT2 protein in 11 amino acid residues at the C-terminus. Codon 217 is a hot-spot for mutations in *PRRT2*. To date, three mutations, R217Pfs, R217Efs, and R217X, have been reported to be responsible for PKD[8,13]. Among them, R217Pfs (alternatively designated as c.649dupC), accounted for nearly 80% of the

PRRT2 mutant families[8], which indicated the hypermutability of the region encompassing codon 217[14]. Compared with the c.649dupC mutation, which is the duplication of the last base in nine serial cytidine bases, the mutation identified in our patients, c.649delC, has a deletion of the last base in the same nine serial bases, which suggest the diversity of molecular lesions, even in the same region of hypermutability.

In this report, we examined the subcellular localization of wild-type and p.R217Efs*12 mutant PRRT2 proteins. Although the transient intracellular expression of wild-type PRRT2 protein fused with pAcGFP1-C1 showed the subcellular localization to the cell membrane as expected, the transient intracellular expression of the mutant p.R217Efs*12 PRRT2 protein fused with the pDsRed-monomer-C1 showed subcellular localization in the cytosol and nucleus of COS7 cells. From the membrane topology, PRRT2 contains a long N-terminal domain and the short M1-M2 loop towards the cytoplasm and maintains a C-terminal anchor with three transmembrane domains[8]. The p.R217Efs*12 mutation was expected to produce the truncated PRRT2 protein without the C-terminal domains, which was compatible with the results of our study that showed the truncated protein was located in the cytosol and nucleus.

In the co-transfection experiment, the mutant truncated PRRT2 protein did not inhibit the subcellular localization or the intracellular expression of the wild-type PRRT2 protein. The results suggested that a heterozygous *PRRT2* mutation might cause the disorder through a reduction in the amount of the protein encoded by *PRRT2*. This result might support the association of PKD caused due to mutations in *PRRT2* with haploinsufficiency.

We did not find any mutations in the *PRRT2* gene in one of two families with PKD (family 2). However, there are limitations to conventional Sanger sequencing for the detection of different types of mutations in genomic DNA[15]; for example, whole exon deletions and duplications are difficult to detect. Thus, the next step in the analysis of the molecular lesion in family 2 would be to utilize a multiplex ligation-dependent probe amplification (MLPA) analysis. If MLPA analysis does not yield any results, whole exome sequencing should be applied in our study, because the PRRT2 protein is now known to play a role in presynaptic vesicular secretion and is a component of the multiprotein machinery; another component of the machinery might be responsible for synaptopathies such as PKD.

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