Exon 3 deletion of RYR2 encoding cardiac ryanodine receptor is associated with left ventricular non-compaction

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Aims
Ryanodine receptor gene (RYR2) mutations are well known to cause catecholaminergic polymorphic ventricular tachycardia (CPVT). Recently, RYR2 exon 3 deletion has been identified in patients with dilated cardiomyopathy (DCM) and/or CPVT. This study aimed to screen for the RYR2 exon 3 deletion in CPVT probands, characterize its clinical pathology, and confirm the genomic rearrangement.

Methods and results
Our cohort consisted of 24 CPVT probands. Polymerase chain reaction (PCR)-based conventional genetic analysis did not identify any mutations in coding exons of RYR2 in these probands. They were screened using multiplex ligation-dependent probe amplification (MLPA). In probands identified with RYR2 exon 3 deletion, the precise location of the deletion was identified by quantitative PCR and direct sequencing methods. We identified two CPVT probands from unrelated families who harboured a large deletion including exon 3. The probands were 9- and 17-year-old girls. Both probands had a history of syncope related to emotional stress or exercise, exhibited bradycardia, and were diagnosed with left ventricular non-compaction (LVNC). We examined 10 family members and identified six more RYR2 exon 3 deletion carriers. In total, there were eight carriers, of which seven were diagnosed with LVNC (87.5%). Two carriers under the age of 4 years remained asymptomatic, although they were diagnosed with LVNC. Using quantitative PCR and direct sequencing, we confirmed that the deletions were 1.1 and 37.7 kb in length.

Conclusion
RYR2 exon 3 deletion is frequently associated with LVNC. Therefore, detection of the deletion offers a new modality for predicting the prognosis of patients with LVNC with ventricular/atrial arrhythmias, particularly in children.

Keywords
RYR2 exon 3 deletion • Catecholaminergic polymorphic ventricular tachycardia • Left ventricular non-compaction • Bradycardia • Genetic screening • Multiplex ligation-dependent probe amplification

Introduction
Cardiac ryanodine receptor (RyR2) is an essential Ca²⁺ release channel of the sarcoplasmic reticulum (SR) and plays a central role in excitation–contraction coupling in cardiomyocytes. Abnormal Ca²⁺ leak from SR due to RyR2 dysfunction generates delayed after-depolarization (DAD) and causes catecholaminergic polymorphic ventricular tachycardia (CPVT). Catecholaminergic polymorphic ventricular tachycardia is characterized by exercise-induced ventricular tachyarrhythmias and sudden cardiac death in structurally normal hearts.

In 2007, Bhuiyan et al.² reported two families who carried a 1.1 kb deletion of ryanodine receptor gene (RYR2) including exon 3. The carriers displayed sinoatrial and atrioventricular node dysfunction, atrial fibrillation, and atrial standstill, in addition to exercise-related ventricular arrhythmias. Some affected members also displayed left ventricular dysfunction and were diagnosed with dilated cardiomyopathy (DCM). Other CPVT families were subsequently identified.
RYR2 exon 3 deletion in LVNC

What’s new?
- Ryanodine receptor gene (RYR2) exon 3 deletion was suspected as a genetic cause of left ventricular non-compaction.
- Left ventricular non-compaction associated with RYR2 exon 3 deletion was related with catecholaminergic polymorphic ventricular tachycardia and/or bradycardia.
- The deletion range varied among each family. To confirm the deletion and to characterize the precise location of the genomic deletion, we performed quantitative polymerase chain reaction (PCR), long-range PCR, and direct sequencing.

Methods

Study population
The study cohort consisted of 24 CPVT probands (13 females), who were referred to two institutes in Japan, Shiga University of Medical Science and Kyoto University Graduate School of Medicine, for genetic evaluation. Their mean age at registration was 15.0 ± 2.2 years. In addition to CPVT probands, we included five probands with LVNC identified by echocardiography—four of them were diagnosed with DCM (one female) and one female was diagnosed with sick sinus syndrome (SSS). The mean age of the five probands with DCM and SSS was 47.4 ± 10.5 years. All subjects submitted written informed consent in accordance with the guidelines approved by each institutional review board. They were evaluated according to medical history, physical examination, standard 12-lead electrocardiography (ECG), exercise stress test, ambulatory 24 h ECG (Holter) monitoring, and echocardiography. Left ventricular non-compaction was diagnosed according to previously proposed echocardiographic criteria: (i) the absence of coexisting cardiac abnormalities, (ii) the presence of a two-layer structure, including a compacted thin epicardial band and a much thicker non-compacted endocardial layer of trabecular meshwork with deep endomyocardial spaces, a maximal end systolic ratio of non-compacted to compacted layers of >2, and (iii) colour Doppler evidence of deep perfused intertrabecular recesses.

Genetic analysis
Catecholaminergic polymorphic ventricular tachycardia probands were found to be negative for mutations in all coding exons of RYR2, CASQ2, KCNJ2, KCNQ1, and SCN5A, as verified by polymerase chain reaction (PCR)-based direct sequencing. cDNA sequence of RYR2 was identified on the basis of GenBank reference sequence NM 001035.2. To identify RYR2 exon 3 deletion, we performed multiplex ligation-dependent probe amplification (MLPA) analysis. The probes for MLPA analysis of RYR2 exons 3 and 97 (SALSA MLPA Kit P168) were purchased from MRC Holland. Because the MLPA Kit P168 consists of ARVC/D-related genes, only RYR2 exons 3 and 97 were included. Multiplex ligation-dependent probe amplification was performed according to the manufacturer’s instructions. To confirm the precise location of the deletion, long-range PCR was performed using Tks G flex polymerase (Takara Bio) for reactions using previously reported primer pairs or KOD FX neo (TOYOBO) for newly designed primer pairs (Table 1). Conventional direct sequencing was performed using ABI PRISM-3130 sequencer (Applied Biosystems).

Quantitative polymerase chain reaction analysis for detecting deletion range
Quantitative PCR (qPCR) primers (Table 1) for Universal Probe Library system (Roche Diagnostic GmbH) were designed with the Universal

<table>
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<th>Primer set</th>
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<th>Primer Reverse</th>
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Table 1 Primer design for long-range PCR and qPCR
Probe Library Assay Design Center (https://www.roche-applied-science.com/). We performed relative quantitation using the Light Cycler probe master mix (Roche). With the exception of the exon 3 primer setting, we introduced the human G6PD gene assay (Roche) labelled with LC yellow 555 as the reference and performed dual-colour analysis. For quantification of exon 3, we used the reference probe–primer set designed for KCNE1 located on chromosome 21 and performed monocolour analysis. For exon 4, we used the SYBR green method because we could not design the probe set for the exon (Table 1). All qPCRs were performed using LightCycler 480 instrument (Roche Diagnostic GmbH), and the data were analysed using \( \Delta \Delta C_T \) method.

### Results

**Multiplex ligation-dependent probe amplification analysis**

No mutations were detected in any exons of RYR2 of 24 CPVT probands using conventional PCR methods. We next performed MLPA analysis to identify the RYR2 exon 3 deletion and identified two probands with the deletion. Figure 1A (indicated as arrows) shows the results of MLPA analysis in the two probands. The ratio of copy
number for RYR2 exon 3 was half compared with that of controls, suggesting that these probands carried a deletion in exon 3. We further performed MLPA analysis in 10 family members from the two families and identified 6 family members carrying the exon 3 deletion (Figure 1B and C). Although we screened for RYR2 exon 3 deletion in five other patients with LVNC, no one carried the deletion.

Clinical characteristics
Table 2 summarizes the clinical characteristics of the eight carriers with RYR2 exon 3 deletion.

Family 1
The proband (III-4) was a 17-year-old girl diagnosed with CPVT, with a history of repeated syncope related to exercise (Figure 1B). Her ECG at rest showed sinus bradycardia (54 b.p.m.) and intermittent atrioventricular dissociation (Figure 2A). In addition, bidirectional ventricular tachycardia was detected by Holter recording (data not shown). Frequent premature ventricular contractions (PVCs) were observed with increasing heart rate during the treadmill exercise test (TMT) (Figure 2B), and echocardiography revealed the presence of LVNC (Figure 2C). β-Blocker therapy failed to suppress the syncope and worsened the bradycardia. Therefore, she was administered flecainide after implantable cardioverter-defibrillator (ICD) implantation. Her sister (III-2) had also experienced syncope while swimming in her teens and was diagnosed with LVNC on echocardiography (Figure 2D). Her heart rate at rest was 44 b.p.m., and PVCs were also observed in TMT with increasing heart rate. She was also diagnosed with CPVT and was administered flecainide after ICD implantation. Her 3-year-old daughter (IV-1) and 1-year-old son (IV-2) were asymptomatic, although their ECGs showed mild bradycardia at age 83 and 78 b.p.m., respectively. Furthermore, they were diagnosed with LVNC on echocardiography. The proband’s mother (II-2) was asymptomatic but had persistent atrial fibrillation, and verapamil was prescribed to control her heart rate since the age of 47. Her echocardiography did not show LVNC. The proband’s maternal grandmother (I-4) underwent pacemaker implantation due to severe bradycardia. Moreover, she was diagnosed with LVNC on echocardiography. Other family members in the family tree did not complain of any cardiac symptoms and showed no apparent ECG abnormality.

Family 2
The proband was a 9-year-old girl (II-1) who lost consciousness while playing a game to beat a drum (Figure 1C). When she was newborn, premature atrial contractions were observed and was diagnosed with peripheral pulmonary stenosis. She was followed up until the age of 2 years, during which she did not exhibit any morphological or neurological abnormalities. She experienced syncope at the age of 1 and 4 but had recovered immediately both times. She suffered a third episode of syncope at the age of 9 and was admitted to the emergency hospital by an ambulance. Her ECG showed bradycardia (54 b.p.m.), Mobitz type 1 atrioventricular block, and T-wave inversions in lead V1–3 (Figure 3A). In an exercise stress test, PVCs appeared immediately and changed to polymorphic ventricular tachycardia (VT) with increasing heart rate (Figure 3B), and she was diagnosed with CPVT. Left ventricular non-compaction diagnosis was confirmed on echocardiography (Figure 3C) and cardiac magnetic resonance imaging (MRI). In both examinations, her left ventricular compaction layer was thin with a prominent trabeculation. In contrast MRI image, non-compaction area was clearly visualized. For the evaluation of bradycardia, she was performed electrophysiological study and was diagnosed with complete atrioventricular block. A pacemaker was implanted, and she was treated with carvedilol (0.1 mg/kg), which successfully suppressed her syncope thereafter. Her mother (I-2) also experienced syncope while exercising at the age of 12 and 35. Her mother’s ECG (Figure 3D) was similar to the proband’s, showing bradycardia (52 b.p.m.), T-wave inversions from V1 through V5 leads, and atrioventricular dissociation was suspected. Polymorphic VT was recorded in the exercise stress test.
Clinical characteristics of Family 2. Twelve-lead electrocardiography (ECG) at rest of the proband (II-1) (A) and her mother (I-2) (B) showed Morbitz type 1 AV block (A) and sinus bradycardia (B). Polymorphic ventricular tachycardia was recorded in an exercise stress test of the proband (C) and RYR2 exon-3 deletion in LVNC her mother (D). (E) Apical four chamber view of the proband, which showed a noncompaction layer and numerous trabeculations. Scale bars indicate 1 mV and 400 ms.
Figure 3E and Holter recordings. Therefore, her mother was diagnosed with CPVT. In addition to the diagnosis of CPVT, her echocardiography revealed LVNC as well. We could not obtain further clinical information of maternal side because they did not give the consent to our study.

Of the eight carriers of RYR2 exon 3 deletion, seven had LVNC (87.5%), whereas four were diagnosed with CPVT (50%). These results are summarized in Table 2. No carriers showed left ventricular dysfunction (Table 3).

In remaining 22 CPVT probands without RYR2 exon 3 deletion, we did not identify any other cases of LVNC.

### Analysis of detailed genomic rearrangement

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were the first to report a large genomic rearrangement in RYR2. Here, we performed long-range PCR using primer pairs (long PCR1) located in introns 2 and 3, as previously designed. We confirmed the presence of PCR products in the two probands (Figure 4A). In one proband (F2-II-1), we detected two clear bands similar to the previous report. The sizes of the PCR products were 3.9 and 2.8 kb. In contrast, only one band was detected in the other proband (F1-III-4), the intensity of which was faint compared with the control. To confirm the deletion range for proband F2-II-1, we performed direct sequencing and confirmed the same deletion (Figure 4B and C) reported in 2007. The deletion was c.169-198_c.273+823 del1126, and the deletion caused an in-frame deletion of 35 amino acids p.Asn57_Gly91. To identify the specific range of the deletion in F1-III-4, we designed primer pairs (Table 1) and performed multiple qPCR spanning from intron 1 to exon 4. Figure 4D summarizes the results of qPCR; the deletion started 35 kb after exon 2 and ended before exon 4. To confirm the exact deletion range, we designed new primer pairs (Table 1, long PCR2), the location of forward and reverse primers were shown in arrows in Figure 4D, and performed a long-range PCR reaction. As a result, we could confirm a PCR product in the proband, which was longer than 10 kb (Figure 4E, arrow), the product was not visible in the control sample. In the sequence analysis, we confirmed that the deletion was c.169-22924_c.273+14653 del37682 insCAT, corresponding to a 35 amino acid deletion (p.Asn57_Gly91-del35), which was consistent with another proband (Figure 4F and G).

### Discussion

#### Clinical phenotype of RYR2 exon 3 deletion carriers

Since the first report from Bhuiyan et al. in 2007, RYR2 exon 3 deletion has been identified in six families with an atypical CPVT phenotype. Table 3 summarizes the clinical manifestations of 29 deletion carriers from eight different families, including the two families examined in this study. Of the 29 carriers, 23 (79.3%) patients suffered from ventricular arrhythmias, and they were highly complicated with atrial arrhythmias (n = 16, 55.2%) or sinoatrial dysfunction (n = 16, 55.2%). The frequency of supraventricular arrhythmias was higher than that reported for CPVT probands harbouring RYR2 mutations. Although the first report emphasized left ventricular dysfunction, cardiac function was reduced in only five patients...
Figure 4  Detailed analysis of genomic rearrangement. (A) Long-range PCR amplification with the primers designed in introns 2 and 3. Polymerase chain reaction products of 3.9 and 2.8 kb (arrows) were obtained with DNA from F2-II-1. The PCR product with DNA from F1-III-4 was faint compared with that of the control. (B) DNA sequencing electropherogram of F2-II-1 showed the starting point of the large deletion. (C) The scheme simplified the genomic rearrangement in the proband F2-II-1. (D) The upper bar graphs display the relative ratio of the qPCR from DNA from family1-III-4. The ratios of intron 2-2 to exon 3 decreased. The lower scheme denotes the primer locations (arrows) in introns 2 and 4. (E) Long-range PCR amplification with the primers designed in introns 2 and 4. Polymerase chain reaction product longer than 10 kb (arrow) was obtained with DNA from F1-III-4 but was not obtained from control. (F) Upper sequence shows the 3’ end of the break point of the deletion and lower sequence denotes the 5’ end. Electropherogram displays three nucleotides (CAT) insertion between the break point. (G) Schema of the deletion in the proband of Family 1.
CONCLUSIONS

Here, we reported for the first time that RYR2 exon 3 deletion co-segregated with LVNC in two families, with both families exhibiting phenotypes that included sinus bradycardia, atrioventricular conduction disturbances, or atrial arrhythmias in addition to CPVT. Although we could not identify any RYR2 exon 3 deletion in probands without CPVT phenotype, further studies are required to elucidate the association between LVNC and RYR2 exon 3 deletion.

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