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ARTICLE

Genetic background of Japanese patients with pediatric hypertrophic and restrictive cardiomyopathy

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Abstract

Hypertrophic cardiomyopathy (HCM) and restrictive cardiomyopathy (RCM) present a high risk for sudden cardiac death in pediatric patients. The aim of this study was to identify disease-associated genetic variants in Japanese patients with pediatric HCM and RCM. We analyzed 67 cardiomyopathy-associated genes in 46 HCM and 7 RCM patients diagnosed before 16 years of age using a next-generation sequencing system. We found that 78% of HCM and 71% of RCM patients carried disease-associated genetic variants. Disease-associated genetic variants were identified in 80% of HCM patients with a family history and in 77% of HCM patients with no apparent family history (NFH). *MYH7* and/or *MYBPC3* variants comprised 76% of HCM-associated variants, whereas troponin complex-encoding genes comprised 75% of the RCM-associated variants. In addition, 91% of HCM patients with implantable cardioverter-defibrillators and infant cases had NFH, and the 88% of HCM patients carrying disease-associated genetic variants were males who carried *MYH7* or *MYBPC3* variants. Moreover, two disease-associated *LAMP2*, one *DES* and one *FHOD3* variants, were identified in HCM patients. In this study, pediatric HCM and RCM patients were found to carry disease-associated genetic variants at a high rate. Most of the variants were in *MYH7* or *MYPBC3* for HCM and *TNNT2* or *TNNI3* for RCM.

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Introduction

Hypertrophic cardiomyopathy (HCM) and restrictive cardiomyopathy (RCM) are primary cardiac muscle disorders that manifested with diastolic dysfunction of the left ventricle. HCM is characterized by unexplained left ventricular hypertrophy (LVH) and myofibrillar disarrays in the ventricle. HCM is one of the main causes of sudden cardiac death in children [1]. HCM affects 0.2% of the general population, the highest frequency among genetic cardiovascular disorders; in addition, 50–70% of HCM patients show a family history consistent with autosomal dominant traits [2]. Patients diagnosed at children and young adolescents, and especially infants, show worse prognosis than those diagnosed at adults [3]. However, most HCM patients are asymptomatic. Earlier diagnosis and management allow for the better prognosis [4]. RCM is characterized by severe diastolic dysfunction similar to HCM; however, it is not accompanied by apparent LVH. Pediatric RCM is a rare cardiac disease with very poor prognosis, with a 2-year survival rate of ~50% [5].

Over the past quarter century, numerous disease-associated genetic variants in the sarcomere-encoding

genes have been reported for HCM [6, 7]. However, disease-associated genetic variants in eight sarcomere-encoding genes (cardiac β myosin heavy chain, *MYH7*; cardiac troponin T, *TNNT2*; cardiac myosin-binding protein c, *MYBPC3*; cardiac troponin I, *TNNI3*; α tropomyosin, *TPM1*; myosin essential light chain, *MYL3*; myosin regulatory light chain, *MYL2*; and cardiac α actin, *ACTC1*) were identified in only 50–70% of familial cases and 10–30% of non-familial cases [7, 8]. Over the past few years, several genetic analyses of HCM using next-generation sequencing (NGS) have been reported; however, in these studies, the patients were predominantly adults [9, 10]. For pediatric HCM, there have only been a few reports of genetic variants, and these studies used Sanger sequencing [11–13]. Moreover, these analyses were mostly concerned with sarcomere-encoding genes, and were performed in European and American populations. There have been no comprehensive genetic analyses of cardiomyopathy-associated genes in Japanese patients with pediatric HCM. Similarly, most currently recognized RCM disease-associated genes are sarcomere-encoding [14]. However, there have been a few genetic analyses of pediatric RCM [13]. Disease-associated genes for HCM and RCM were found to partially overlap with those of dilated cardiomyopathy (DCM), left ventricular non-compaction cardiomyopathy (LVNC), and arrhythmogenic right ventricular cardiomyopathy (ARVC), and these genes encoded non-sarcomere proteins [7, 15].

Therefore, in this study, we analyzed a total of 67 cardiomyopathy-associated genes, comprising not only sarcomere- but also non-sarcomere-encoding genes, in Japanese pediatric patients with HCM and RCM, to determine the prevalence of disease-associated genetic variants.

Materials and methods

Sample collection

All patients were proband, who were diagnosed with HCM or RCM before the age of 16, and were living in different areas of Japan [16–18]. Complicating conditions such as neuromuscular disease, metabolic disorders, and malformation syndromes were excluded. Positive family history was defined as having one or more first-degree relatives with cardiomyopathies (HCM, RCM, DCM, LVNC, and ARVC) or sudden death, defined as sudden cardiac death or sudden death with unknown causes in this study. Some cases were associated with a family history (FH) of cardiomyopathy, abbreviated as HCM/FH, whereas the other cases were associated with no apparent family history (NFH), abbreviated HCM/NFH. Of these, eight and one HCM and RCM proband patients, respectively, had been analyzed by direct sequencing of eight sarcomere-encoding genes (*MYH7, MYBPC3, TNNT2, TNNI3, MYL3, MYL2, TPM1*, and *ACTC1*) and *MYP*, encoding myopalladin, before this genetic study using NGS [16–18]. Written informed consent was obtained for genetic analysis from parents of the patients, as well as family members 16 years of age or older. This study was approved by the ethics committee of the Medical Research Institute, Tokyo Medical and Dental University, Japan (No. 2013–16).

Variant analysis and the evaluations

Genomic DNA was purified from the blood samples using a Wizard DNA purification kit (Promega Corporation, WI, USA) according to the manufacturer's instruction. Genomic DNA was analyzed using the Ion Torrent PGM™ system (Thermo Fisher Scientific, CA, USA) for 67 disease-associated genes previously reported as inherited and related to secondary cardiomyopathy (Supplementary Table 1). Analysis of *TTN*, encoding Titin, missense variants, as well as *OBSCN*, encoding Obscurin, *MTO1*, encoding Mitochondrial TRNA Translation Optimization 1, and *SDHA*, encoding Succinate Dehydrogenase Complex Flavoprotein Subunit A, variants, will be reported in future papers.

Primer pairs for each gene were designed using the AmpliSeq Designer (Thermo Fisher Scientific, CA, USA). In total, 2250 primer pairs were designed for all coding regions of the target genes. DNA libraries were prepared using the Ion Ampliseq Library Kit 2.0 (Thermo Fisher Scientific, CA, USA). In total, 10 ng of genomic DNA was quantified using the Qubit 2.0 Fluorometer and Qubit dsDNA assay kit (Thermo Fisher Scientific, CA, USA), and was amplified by multiplex PCR using the designed primers. The amplicons were ligated with the barcode adaptors using the Ion Xpress™ Barcode adaptor kit (Thermo Fisher Scientific, CA, USA) and purified with Agencourt AMPure XP (Beckman Coulter, CA, USA). The DNA libraries were quantified using Qubit 2.0 Fluorometer and Qubit dsDNA assay kit (Thermo Fisher Scientific, CA, USA), and adjusted to 100 pM in low TE buffer. The libraries were subjected to emulsion PCR by the Ion One Touch 2 system using the Ion PGM™ template OT2 400 kit (Thermo Fisher Scientific, CA, USA). Enrichment of Ion Sphere Particles (ISPs) was performed using the Ion One Touch ES (Thermo Fisher Scientific, CA, USA). The prepared ISPs were loaded into the Ion 318 sequence chip, and sequenced using the Ion PGM 400 Sequence Kit (Thermo Fisher Scientific, CA, USA).

The sequencing data were analyzed using Torrent Suite 4.4 (Thermo Fisher Scientific, CA, USA). The filtered reads were mapped to hg19 as the reference genome, and the variants were extracted. Further, using the CLC genomic workbench (Qiagen, Venlo, Netherland), we then filtered out variants, for which the minor allele frequency (MAF)

Table 1 Patients' backgrounds

	Total HCM	FH	NFH	Total RCM	FH	NFH
Probands	46	20	26	7	3	4
Male	27 (59%)	11 (55%)	16 (62%)	6 (86%)	3 (100%)	3 (75%)
Age at diagnosis	9.4 ± 4.8	10.5 ± 4.4	8.6 ± 5.0	6.1 ± 3.3	6.3 ± 5.0	6.0 ± 2.2

HCM hypertrophic cardiomyopathy, *RCM* restrictive cardiomyopathy, *FH* patients associated with a family history, *NFH* patients with no apparent family history

was < 0.002 (0.2%) in the following databases: 1000 genomes project (TG), exome aggregation consortium (ExAC), and HGVD (human genetic variation database), which was used as the Japanese control variant database [19–22]. Nonsynonymous substitutions were selected from the variants. PCR primers were designed to confirm all the selected variants; PCR products were purified using ExoSAP-IT (Affymetrix, CA, USA) and sequenced using the BigDye terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, CA, USA) on an ABI 3130 (Thermo Fisher Scientific, CA, USA). Primer sequences and PCR conditions are available upon request. If the family samples were available, the segregation analyses were performed.

In silico analysis of each missense variant was performed using Polyphen-2, SIFT, Mutation Taster, PROVEAN, and fathmm [23–27]. The results were classified "Damaging" as follows; if they were determined to be "probably damaging" or "possibly damaging" in Polyphen-2, "damaging" in SIFT, "deleterious" in PROVEAN, "damaging" in fathmm, "disease causing" in Mutation Taster. Moreover, variants were evaluated by ClinVar [28]. These variants were classified by the American College of Medical Genetics and Genomics (ACMG) standards and guidelines [29]. When variants were classified as "pathogenic (P)" or "likely pathogenic (LP)" according to the guideline, they were defined as disease-associated genetic variants in this study.

Statistical analyses were performed using the Student's *t*-test and Fisher's exact test using JMP version 13.0 (SAS Institute Inc. NC, USA). A p value < 0.05 was considered to be statistically significant.

Results

Patients' backgrounds

In total, 46 genetically unrelated proband patients with HCM were registered; of these, 20 had FH, whereas 26 patients who had NFH. The average age at diagnosis was 9.4 ± 4.8 years for all patients. There was no significant difference between the HCM/FH and HCM/NFH patients regarding the age at diagnosis and sex (Table 1). Six patients were diagnosed with HCM before 1 year of age. In these cases, 83% (5/6) had NFH, and 67% (4/6) were males. The five cases of HCM with ICD, all had NFH, and 80% (4/5) were males.

Seven genetically unrelated RCM proband patients were also registered. The average age at diagnosis was 6.1 ± 3.3 years of age (Table 1).

Disease-associated genetic variants in HCM and RCM patients

We analyzed 67 cardiomyopathy-associated genes in 53 (46 HCM and 7 RCM) cases using NGS. The sequence quality was sufficiently reliable for analysis, with a total average of coverage depth was 187, and an average proportion of regions covered by > 20 reads was 96.3%.

In total, 78% (36/46) of HCM patients carried disease-associated genetic variants (Table 2, Supplementary Table 2). HCM patients carried disease-associated genetic variants in 12 out of 67 genes. The highest frequency of disease-associated genetic variants was observed in *MYH7* (33%), followed by *MYBPC3* (28%) (Table 2). There were no identified *TTN* truncation/frameshift variants. Eight patients (17%) carried double disease-associated genetic variants.

For HCM/FH, disease-associated genetic variants were identified in 80% (16/20) of cases. Surprisingly, for HCM/NFH, disease-associated genetic variants were identified in 77% (20/26) cases, although such variants were reported to be found in only 10–30% of adult HCM/NFH [7, 8]. For HCM/NFH, 19% (5/26) of patients carried double or homozygous variants, and 15% (4/26) harbored de novo variants (Table 2). Of the disease-associated genetic variants in HCM/FH cases, 11 variants were able to be investigated in affected family members. Of these, 91% (10/11) and 9% (1/11) of the variants were confirmed to exhibit cosegregation and penetration, respectively (Supplementary Table 2). Of the disease-associated genetic variants in HCM/NFH cases, nine variants were analyzed in the parents, and 44% (4/9) and 56% (5/9) of variants were identified to be de novo and penetrance, respectively (Supplementary Table 2).

In total, 37 different disease-associated genetic variants were identified in HCM (Supplementary Table 1). All had MAF value < 0.02% in ExAc and 94.6% (35/37) had MAF values < 0.002% in ExAc. In addition, 32% (12/37) of the

Table 2 Genetic profiling of disease-associated variants in HCM and RCM

Variant positive probands	HCM (*N*=46)			RCM (*N*=7)		
	Total (*N*=46)	FH (*N*=20)	NFH (*N*=26)	Total (*N*=7)	FH (*N*=3)	NFH (*N*=4)
	36 (78%)	16 (80%)	20 (77%)	5 (71%)	2 (66%)	3(75%)
Sarcomere						
MYH7	15 (33%)	5 (25%)	10 (38%)			
MYBPC3	13(28%)	7 (35%)	6 (23%)			
TNNT2	1(2.2%)	1 (5%)		1(14%)		1(25%)
TNNI3	2 (4.3%)		2 (7.7%)	3(43%)	1(33%)	2(50%)
TPM1	2 (4.3%)	1(5%)	1 (3.8%)			
ACTC1	1 (2.2%)	1(5%)				
MYH6	2 (4.3%)		2 (7.7%)	1(14%)	1(33%)	
Non-sarcomere						
LAMP2	2 (4.3%)	1(5%)	1 (3.8%)			
DES	1(2.2%)		1 (3.8%)			
FHOD3	1(2.2%)	1(5%)				
BAG3	1(2.2%)	1(5%)				
SCN5A	1(2.2%)		1(3.8%)			
MYPN				1(14%)	1(33%)	
Double variants (or homozygous)	8 (17%)	3 (15%)	5 (19%)	1(14%)	1(33%)	0
de novo variants	4 (8.7%)		4 (15%)	1(14%)		1(25%)

HCM hypertrophic cardiomyopathy, *RCM* restrictive cardiomyopathy, *FH* patients associated with a family history, *NFH* patients with no apparent family history

variants were novel, and 32 different missense variants were identified. Of these disease-associated genetic variants in pediatric HCM cases, 76% (28/37) were in two major genes, *MYH7* and *MYBPC3* (Table 2).

Regarding RCM, total 71% (5/7) of patients carried disease-associated genetic variants in four genes. Six different variants were identified (Table 2, Supplementary Table 3). All the variants had MAF value < 0.02% in ExAc, and 83.3% (5/6) of the variants had MAF value < 0.002% in ExAc. Of these, 67% (4/6) of the variants were found in the troponin complex-encoding genes, *TNNT2* and *TNNI3*.

According to the Genome Aggregation Databases (gnomAD), all of the disease-associated genetic variants in HCM and RCM exhibited MAF values similar to those in ExAc [21]. Moreover, according to Combined Annotation Dependent Depletion analysis, the score of the variants were higher than 20, indicating that the variants were rare and predicted to be deleterious [30].

Genetic evaluation of ICD and infant case

In total, 60% (3/5) of HCM with ICD patients carried *MYH7* variants, specifically *MYH7* R453C, M539I, and Q815P, combined with *MYH6* K1983N (Supplementary Figure 1). These three patients were all HCM/NFH cases and males.

Patients with infant onset was reported to be at a high risk for cardiac death [3]. Of the six HCM patients diagnosed before 1 year of age, 83% (5/6) carried disease-associated genetic variants: three infants carried *MYH7* variants at codon 870 of (R870C and R870L), one carried *MYBPC3* R820Q and one carried double variants (*MYBPC3* R495Q and *MYH6* R1691C) (Supplementary Figure 1). All the five infants harboring variants were HCM/NFH cases, and the 80% (4/5) were males.

Non-sarcomere-encoding gene variants

In non-sarcomere-encoding genes, six disease-associated genetic variants were identified in five different genes in HCM patients (Table 2). Of these, four variants in *LAMP2*, encoding lysosome associated membrane protein; *DES*, encoding desmin, and *FHOD3*, encoding formin homology 2 domain containing 3, did not exist in ExAc (Supplementary Table 2). The other two variants, in *BAG3*, encoding BCL2-associated athanogene 3, combined with *MYH7*, and in *SCN5A*, encoding cardiac Na channel, combined with *MYBPC3*, were double disease-associated genetic variants. The *BAG3* I339V and *SCN5A* V2016M had MAF values < 0.002% in ExAc (Supplementary Table 2). Two disease-associated genetic variants in *LAMP2*, the disease-causing gene for Danon disease, were identified in two HCM

patients (Supplementary Table 2). *LAMP2* Q353X was identified in a male patient, and disease onset for this individual was 9 years of age. His mother harbored the same variant, and was diagnosed with HCM. *LAMP2* K280fs was identified in a female HCM patient found to have an abnormal electrocardiogram upon school screening at 12 years of age. She did not manifest any other symptom as Danon disease besides heart. She was an HCM/NFH patient.

A disease-associated genetic variant in the *DES* gene, encoding desmin, an intermediate filament, was identified in an HCM/NFH patient. This male carried a homozygous variant of *DES* T219P (Supplementary Table 2). The parents of this patient carried the *DES* variants in a heterozygous state, and were cousins not diagnosed with HCM. The genetic trait was thus recessive. Detailed information of this family has been recently reported [31].

FHOD3 is involved in the regulation of actin assembly and sarcomere organization during myofibrillogenesis [32]. One male patient with HCM/FH was found to harbor *FHOD3* S963L (Supplementary Table 2).

Discussion

In this study, we found that 78% of HCM and 71% of RCM patients carried disease-associated genetic variants. In HCM patients, 76% (28/37) of the variants were in *MYH7* or *MYBPC3*, whereas 8.1% (3/37) were in troponin complex-encoding genes (*TNNT2* and *TNNI3*). For HCM/FH, 80% (16/20) of patients carried disease-associated genetic variants; surprisingly, 77% (20/26) of pediatric HCM/NFH patients carried disease-associated genetic variants, despite the fact that this proportion was reported to be only 10–30 % in the adult HCM/NFH patients [7, 8].

We previously analyzed disease-associated genetic variants in adult HCM patients, and found that 28% of the variants associated with HCM/FH were in *MYH7* and *MYBPC3*, whereas 13% of variants were in troponin complex-encoding genes (*TNNT2* and *TNNI3*) [7, 33]. Compared to adult patients, pediatric HCM patients tended to carry disease-associated genetic variants of *MYH7* and *MYBPC3* at a higher frequency.

In total, 60% (3/5) of HCM with ICD patients carried disease-associated genetic variants. These three patients were all males, in the HCM/NFH group, and carried *MYH7* variants (*MYH7* R453C, M539I, Q815P with *MYH6* K1083D) (Supplementary Figure 1). Of these, *MYH7* R453 is located on the S-1 domain (subfragment-1 of myosin motor domain), in between the nucleotide-binding pocket and actin-binding site. *MYH7* R453 is located at a site that interacts with the β6-sheet, β7-sheet, O-helix and HO-linker [34]. Thus, *MYH7* R453C could alter these structural interactions, decreasing the binding to ATP and consequently altering ATP hydrolysis [35]. The patient carried the same variant that was previously reported to be associated with poor prognosis [36, 37]. In the current study, another male patient who carried *MYH7* M539I was the only one with dilated phase HCM with ICD, he was diagnosed at 14 years of age (Supplementary Figure 1).

Patients with infant onset are reported to be at high risk for cardiac death [3]. In this study, 60% (3/5) of HCM/NFH infants carried disease-associated genetic variants in codon 870 of *MYH7*. *MYH7* R870 is located in the proximal domain of subfragment 2, which binds to cardiac myosin-binding protein c, and *MYH7* R870H reduces the binding for this interaction [38]. Therefore, *MYH7* R870C and R870L, identified at the same codon, may also affect the binding to cardiac myosin-binding protein c. We also identified *MYH7* R870C in an adult onset case of RCM/FH in a Japanese population (unpublished data). Although we were unable to investigate the variants for both parents, *MYH7* R870C and R870L observed in this study might be de novo variants. Recent whole-exome studies reveal that de novo variants are a major cause of severe early-onset genetic disorder; therefore, if the disease-associated genetic variants in patients with NFH were confirmed de novo, the variants might be significant for their disease onset [39].

The other two infants with HCM carried *MYBPC3* R820Q and double variants (*MYBPC3* R495Q and *MYH6* R1691C). *MYBPC3* R820 is located in the C6 domain of cardiac myosin-binding protein c. 'Ragdoll cats' that sometimes manifested with HCM, carried heterozygous or homozygous *MYBPC3* R820W variants [40]. *MYBPC3* R820W has also been reported to be associated with HCM in human [41]. One HCM patient presented with LVNC, which represents a severe phenotype for cardiomyopathy [41]. Across these species, variants in the same codon were associated with cardiomyopathy. *MYBPC3* R820Q is classified as "conflicting" in the ClinVar database; however, the variant was found to be located at a hot spot, and returned four "damaging" results from five different software programs based on in silico analysis; moreover, it is classified as "likely pathogenic" according to ACMG standards and guidelines. Therefore, this variant might be pathogenic in Japanese.

Six HCM/NFH patients carried *MYBPC3* variants, including some carrying double variants, and all of these were males (Supplementary Figure 1). In adults, HCM patients carrying *MYBPC3* variants tended to have later disease onset, compared with those carrying *MYH7* variants [42]. However, in pediatric HCM patients, the age at diagnosis in those carrying *MYBPC3* variants was similar to that in patients with *MYH7* variants (Supplementary Figure 1). A previous report identified 11 patients with childhood onset cardiac hypertrophy in the NFH group, who carried disease-associated genetic variants in the sarcomere-

encoding genes [11]. Of these, 73% (8/11) of patients were males, and 64% (7/11) carried *MYBPC3* variants. In addition, 86% (6/7) of the patients carrying *MYBPC3* variants were males. In a case of twins, a male carried *MYBPC3* D605N and presented with hypertrophy; however, his sister carried the same variant but did not exhibit the phenotype [11]. Thus, in pediatric onset HCM, androgens may contribute to disease severity [43]. After the age of 12 years, males exhibit higher testosterone levels than females. At the same age, the annual mortality rate in males has been found to exceed that observed in females [43]. In addition, infants who died suddenly were found to have significantly higher testosterone levels than unaffected individuals [44]. Thus, the male gender might be a risk factors for pediatric HCM.

Regarding troponin complex-encoding genes, *TNNI3* K178H and R192H, which were associated with RCM, were previously shown to encode proteins with higher calcium sensitivity than *TNNI3* K206E, which was identified in HCM [45, 46]. Thus, *TNNI3* and *TNNT2* variants, associated with pediatric RCM, might encode proteins with the higher calcium sensitivity than variants associated with adult onset HCM variants. Therefore, this may suggest that proteins with disease-associated genetic variants conveying higher calcium sensitivity results in increased severity of diastolic dysfunction.

LAMP2 variants were found in 4.3% (2/46) of HCM patients. In addition, HCM patients carrying heterozygous *LAMP2* variants did not show the other symptoms of Danon disease. Therefore, regarding *LAMP2*, it is necessary to determine the genetic background of the HCM patient, in addition to identifying variants in sarcomere-encoding genes.

For *FHOD3*, which is a DCM-associated gene, we identified a novel heterozygous missense variant, S963L, in a patient with pediatric HCM [47, 48]. The FHOD3 protein, located in the A-band region of cardiomyocytes, is involved in the sarcomere organization during myofibrillogenesis. The S963L variant of *FHOD3* is located in the FH2 domain, and is functionally important for actin assembly [32]. Further investigations are required to determine the effects on sarcomere organization that result from these variants.

In this study, we evaluated each variant using a family study, the control variant database, the phenotype–genotype relationship database in the USA, and in silico analysis. To more accurately characterize each variant, in vitro or in vivo functional analysis is required for each variant [29, 49]. Moreover, a phenotype–genotype relationship database regarding cardiomyopathy in the Japanese population and a validated functional evaluation tool for cardiomyopathy-associated gene variants, such as *GLA* (encoding α-galactosidase) variants in Fabry disease, are required [50].

In Conclusions, pediatric HCM and RCM patients were found to carry cardiomyopathy-associated genetic variants at a high rate, even in the HCM/NFH cohort. Most of disease-associated genetic variants were in *MYH7* or *MYPBC3* for HCM and *TNNT2* or *TNNI3* for RCM. HCM with ICD and infant cases mostly consisted of males and individuals in the NFH group. In addition, these patients carried HCM-associated *MYH7* or *MYBPC3* variants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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