

# Impact of cardiac myosin light chain kinase gene mutation on development of dilated cardiomyopathy

Akihiko Hodatsu<sup>1</sup>, Noboru Fujino<sup>1</sup>, Yuki Uyama<sup>2</sup>, Osamu Tsukamoto<sup>2</sup>, Atsuko Imai-Okazaki<sup>3</sup>, Satoru Yamazaki<sup>4</sup>, Osamu Seguchi<sup>5</sup>, Tetsuo Konno<sup>1</sup>, Kenshi Hayashi<sup>1</sup>, Masa-aki Kawashiri, Yoshihiro Asano<sup>3</sup>, Masafumi Kitakaze<sup>6</sup>, Seiji Takashima<sup>2</sup> and Masakazu Yamagishi<sup>1,7\*</sup>

<sup>1</sup>Department of Cardiovascular and Internal Medicine, Kanazawa University Graduate School of Medicine, Kanazawa, Japan; <sup>2</sup>Department of Medical Biochemistry, Osaka University Graduate School of Frontier Biosciences, Suita, Japan; <sup>3</sup>Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan; <sup>4</sup>Department of Cell Biology, National Cerebral and Cardiovascular Center, Suita, Japan; <sup>5</sup>Department of Transplantation, National Cerebral and Cardiovascular Center, Suita, Japan; <sup>6</sup>Department of Clinical Research and Development, National Cerebral and Cardiovascular Center, Suita, Japan; <sup>7</sup>Osaka University of Human Sciences, Settsu, Japan

## Abstract

**Aims** Cardiac myosin light chain kinase (cMLCK) phosphorylates ventricular myosin regulatory light chain 2 (MLC2v) and regulates sarcomere and cardiomyocyte organization. However, few data exist regarding the relationship between cMLCK mutations and MLC2v phosphorylation, particularly in terms of developing familial dilated cardiomyopathy (DCM) in whom cMLCK gene mutations were identified. The purpose of the present study was to investigate functional consequences of cMLCK mutations in DCM patients.

**Methods and results** The diagnosis of DCM was based on the patients' history and on echocardiography. We screened cMLCK gene mutations in DCM probands with high resolution melting analysis. Known DCM-causing genes mutations were excluded by exome sequencing of family members. MLC2v phosphorylation was analysed by Phos-tag sodium dodecyl sulfate–polyacrylamide gel electrophoresis assays. We also performed ADP-Glo assays for determining the total amount of adenosine triphosphate used in the kinase reaction. Unrelated DCM probands (109 males and 40 females) were enrolled in this study, of which 16 were familial and 133 sporadic. By mutation screening, a truncation variant of c1915-1 g>t (p.Pro639Valfs\*15) was identified, which was not detected in 400 chromosomes of 200 healthy volunteers; it is listed in the Human Genetic Variation Database with an allele frequency < 0.001. In the proband, the presence of mutations in known DCM-causing genes was excluded with exome analysis. Familial analysis identified a 19-year-old male carrier who manifested slight left ventricular dilation with preserved systolic function. Phosphorylation assays analysed by Phos-tag SDS-PAGE revealed that the identified p.Pro639Valfs\*15 mutation results in a complete lack of kinase activity, although it did not affect wild-type cMLCK activity. ADP-Glo assays confirmed that the mutant cMLCK had no kinase activity, whereas wild-type cMLCK had a  $K_m$  value of  $5.93 \pm 1.47 \mu\text{M}$  and a  $V_{max}$  of  $1.28 \pm 0.03 \text{ mol/min/mol kinase}$ .

**Conclusions** These results demonstrate that a truncation mutation in the cMLCK gene p.Pro639Valfs\*15 can be associated with significant impairment of MLC2v phosphorylation and possibly with development of DCM, although a larger study of DCM patients is required to determine the prevalence of this mutation and further strengthen its association with disease development.

**Keywords** Dilated cardiomyopathy; Genetics; MYLK3

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\*Correspondence to: Masakazu Yamagishi, Department of Human Sciences, Osaka University of Human Sciences, 1-12-13 Shoya, Settsu, 566-8501 Osaka, Japan.

Tel: +81 6 6105 7106; Fax: +81 6 6381 3502. Email: m-yamagishi@kun.ohs.ac.jp

## Introduction

Dilated cardiomyopathy (DCM) includes a large group of heterogeneous myocardial disorders characterized by ventricular dilation and depressed myocardial contractility.<sup>1,2</sup> Although DCM is a leading cause of refractory heart failure, no fundamental therapy exists due to extremely heterogeneous genetic aetiologies. Indeed, over 40 genes have been reported as a cause of DCM, resulting in the identification of disease-causing mutations in 20% of DCM probands.<sup>3</sup> Nonetheless, continued efforts to identify novel disease-causing genes are important because they provide valuable information on novel disease pathways and functional relevance that may contribute to novel therapeutic strategies.<sup>4</sup>

Recently, cardiac myosin light chain kinase (cMLCK), which phosphorylates ventricular myosin regulatory light chain 2 (MLC2v) and regulates sarcomere and cardiomyocyte organization,<sup>5,6</sup> was cloned from the myocardia of patients with heart failure.<sup>5</sup> Interestingly, in animal models, knockout mice show reduced MLC2v phosphorylation and DCM-like phenotypes such as reduced left ventricular (LV) systolic function and LV dilation.<sup>7–10</sup> In humans, reduced MLC2v phosphorylation was observed in LV biopsy samples from end-stage heart failure including DCM.<sup>11–13</sup> However, few data exist regarding the information for the relationship between cMLCK mutations and MLC2v phosphorylation, despite the fact that cMLCK gene (*MYLK3*) mutations have been identified in DCM pedigrees.<sup>14</sup> Therefore, we screened for mutations in the cMLCK gene and investigated the functional consequences of cMLCK mutations in DCM patients.

## Methods

### Study populations and clinical evaluations

This investigation conforms with the principles outlined in the Declaration of Helsinki.<sup>15</sup> The study was approved by the Bioethical Committee on Medical Research, Kanazawa University. Informed consent was obtained from all subjects or their guardians.

Standard M-mode and two-dimensional echocardiographic studies were performed to identify and quantify morphological features of the LV. The LV ejection fraction was calculated using Teicholtz's method.<sup>16</sup> The diagnosis of DCM was based on the patients' history and on echocardiographic demonstration of a dilated and thinned LV with decreased systolic dysfunction in the absence of coronary artery disease.<sup>1</sup>

### Mutation screening and confirmation

Genomic DNA was isolated from peripheral white blood cells of all subjects using the DNA extractor 341 Nucleic Acid

Purification System (GENEPURE, PE Biosystems) or a Wizard Genomic DNA Purification Kit (Promega). Exon coding sequences and exon-intron boundaries for *MYLK3* were amplified by PCR. The primers are shown in *Table 1*. High resolution melting analysis (LightScanner System, Idaho Technology) was used for screening mutations. We performed direct sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) when irregular profiles were detected. The presence or absence of mutations was confirmed by digestion of PCR products with restriction enzyme *Bcl*I. This method was also used to determine the genotype of DNA from family members of the probands and 400 chromosomes of 200 healthy individuals without cardiovascular disease.

To identify an aberrant transcript that resulted from the splice acceptor site mutation, total RNA was isolated from lymphocytes using a BiOstic® Blood Total RNA Isolation Kit (MO BIO Laboratories). RNA was subjected to reverse transcription, and the DNA products were amplified by PCR. The primers were forward: 5'-ggaccggaggacgtgaagaac-3'; reverse: 5'-aagtccttgccctcctccgagag-3'.

### Exome sequencing

Whole exome sequencing of the proband and a clinically unaffected sibling were performed. Genomic DNA was fragmented using a DNA shearing system (Covaris Inc.). Genomic DNA was captured using the SureSelectXT Human All Exon V4 (51 Mb) kit (Agilent Technologies) and sequenced on a HiSeq 2000 (Illumina) with 100 bp paired-end reads. The reads were mapped to the hg19 human reference using BWA-0.6.1.<sup>17</sup> Optical or PCR duplicates were removed using Picard.<sup>18</sup> Single nucleotide variants (SNVs) and insertions and/or deletions were called using the Genome Analysis Toolkit (GATK) v. 1.6–13.<sup>19,20</sup> Variants were annotated using ANNOVAR.<sup>21</sup>

**Table 1** Primers used for amplifying *MYLK3* exons

Exon		Sequence (5'-3')	Annealing temperature (°C)
2	F	AGCTGGGCGCTCCTCTTT	64–67 (touchdown)
	R	CCTGGCATCAGACTGCACC	
5	F	GTGCCGGGAGACTGGGTTTGA	64–67 (touchdown)
	R	CCTGCCCGTGACTCCTGCTCTAA	
6	F	TTCGGGATAGCCAGCAGGGTTTTG	62
	R	AGGCCCCACAGGCAGCTTACAGA	
7	F	GTGGGGCCTGGGGCAAGAAGGT	64
	R	GCCGCCCGCCTCTGATACTCA	
8	F	CTACCCGGGCTGGCCTTTTTG	67
	R	GTGGCCTGTCCCAGACTCCAGAAC	
9	F	CCAGCCCCTTCATGATTTCCATT	67
	R	AACAAGGCCTGTCTGCAAGTCAAA	
10	F	AGCCCCAGCACCATCTCC	70
	R	CCTTCCCTGGCTCCTTCCCTTC	
11	F	CCGCTTCTCCCTTTAATGAACA	69
	R	TGCCAGCTCCCAGACCTG	

Non-synonymous SNVs, splice site mutations, and insertions and/or deletions were retained and filtered for a minor allele frequency < 0.001 in the dbSNP135, 1000 Genomes database, the NHLBI Exome Sequencing Project (ESP6500), and the Human Genetic Variation Database<sup>22</sup> for discovery of rare *de novo* mutations. Variants potentially affecting protein function, including non-synonymous variants, frameshifts in the coding sequence, or variants potentially affecting splicing were analysed. Variants were filtered against the quality of exome sequencing, rarity, functional significance predicted high impact by SnpEff<sup>23</sup>, and segregation. Further filtering using Ingenuity Pathway Analysis software (Ingenuity Systems) was performed to examine the association with cardiac function and/or structure.

### Purification of recombinant cardiac myosin light chain kinase proteins from HEK293T cells

Human *MYLK3* cDNA was cloned using pENTR/D-TOPO Cloning Kits (Invitrogen). Mutant constructs of p.Pro639Valfs\*15 were subsequently introduced by primer-derived mutagenesis. The primers for mutant cMLCK were forward: 5'-gtacaagctc gagagaagctgaaggtgaac-3'; reverse: 5'-cttgaggtccaggtcaggatg tagtgctggt-3'. Wild-type and mutant plasmids were recombined into N terminal FLAG destination vectors (pEF-DEST51/nFLAG plasmid) using GATEWAY LR recombinase (Invitrogen). HEK293T cells transfected with wild-type cMLCK or mutant cMLCK vectors were lysed in lysis buffer (10 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 1% NP40, 1 mM EDTA, and protease inhibitor cocktail) and immunoprecipitated with anti-FLAG M2 agarose (Sigma-Aldrich) at 4°C for 30 min. The beads were washed three times with washing buffer (10 mM Tris-HCl, pH 7.2, 0.3 M NaCl, 1% NP40, and 1 mM EDTA) and eluted with elution buffer (10 mM Tris-HCl, pH 7.2, 0.15 M NaCl, 1% NP40, 1 mM EDTA, and 0.05 mg/mL FLAG peptide) at 4°C for 30 min. After centrifugation, the supernatants were used as recombinant FLAG-tagged proteins.

### Purification of recombinant regulatory light chain proteins from *Escherichia coli*

To prepare substrate proteins, cDNA fragments encoding human MLC2v (gene accession no. NM\_000432) was PCR amplified using cDNA from heart. The primers for MLC2v were as follows: the forward, 5'-caccatggcacctaagaagcaaagaagagagcc-3'; the reverse, 5'-ctagctctctctctcctggtgatgat-3'. Amplified cDNA was subcloned into pENTR/D-TOPO vector and inserted into the pDEST17 vector by Gateway Technology System (Invitrogen) according to the manufacturer's protocol. The pDEST17 constructs possessing each regulatory light chain sequence was transformed into the BL21 chemically competent *Escherichia coli*. A 5 mL culture using lysogeny broth containing

100 µg/mL of ampicillin was grown overnight shaking at 37°C and used to inoculate 200 mL of lysogeny broth containing ampicillin (in a 1 L flask) until the culture optical density (600 nm) reaches at 0.5. Arabinose was added at final concentration of 0.2% and subsequent culturing for 3 h at 37°C. Cells were harvested by centrifugation at 5000 *g* at 4°C for 10 min, and resulting cell pellet was resuspended in 10 mL of BugBuster Master Mix (Merck Millipore) containing EDTA-free protease inhibitor cocktail and rotated at room temperature for 20 min. The inclusion body that contains N-terminus His-tagged MLC2v protein was isolated from the supernatant by centrifugation at 16 000 *g* at 4°C for 10 min.

The resulting inclusion body was washed with two-fold diluted BugBuster Master Mix one time and 10-fold diluted BugBuster Master Mix twice. The inclusion body pellet was solubilized in immobilized metal ion affinity chromatography binding buffer [10 mM HEPES (pH 7.4), 0.5 M NaCl, 1 mM MgCl<sub>2</sub>, and 6 M urea] by repeatedly passing the inclusion body through an 18 g syringe and incubated at 4°C for 1 h. After centrifugation at 20 000 *g* at 4°C for 20 min, any insoluble material was centrifuged out. The resulting resuspended protein solution was loaded onto a column of TALON Metal Affinity Resin (Clontech) equilibrated with immobilized metal ion affinity chromatography binding buffer at 4°C. The bound His-tagged MLC2v protein was eluted with elution buffer [50 mM sodium phosphate (pH 8.0), 0.3 M NaCl, 0.1% CHAPS, and 0.15 M imidazole], refolded, and concentrated by centrifugation at 5000 *g* at 4°C using centrifugal filter (Amicon Ultra-15, Millipore) and stored at -80°C until use.

### Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The stacking gel was composed of 12% (wt/vol) acrylamide, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 125 mM Tris-HCl (pH 6.8), 0.1% (wt/vol) ammonium persulfate, and 0.5% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine. The resolving gel was composed of 12% (wt/vol) acrylamide, 0.1% (wt/vol) SDS, 375 mM Tris-HCl (pH 8.8), 0.05% (wt/vol) ammonium persulfate, and 0.25% (wt/vol) *N,N,N',N'*-tetramethylethylenediamine. Electrophoresis was performed in 0.1% (wt/vol) SDS, 25 mM tris, and 192 mM glycine at 180 V per gel for 50 min. Proteins were transferred to poly(vinylidene difluoride) membranes (0.2 µm; Bio-Rad) at 15 V for 30 min in transfer buffer. Membranes were incubated with 3% (wt/vol) non-fat dry milk in Tris-buffered saline containing Tween [TTBS; 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, and 0.1% (vol/vol) Tween 20] for 1–2 h and then in anti-FLAG M2-HRP antibody (1:4,000; Sigma-Aldrich, A8592) in TTBS for 2 h at room temperature. After washing with TTBS (three times for 5 min each), membranes were incubated with the ECL Western Blotting Detection Reagent (GE Healthcare). The emitted light was detected and quantified

with a chemiluminescence imaging analyzer (LAS4000, Fujifilm), and images were analysed with Adobe Photoshop Elements 13.

### In vitro kinase activity

Kinase activities were assayed in 20 mM HEPES (pH 7.5), 1 mM CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 150 μM ATP, 0.01% Tween 20, and 150 nM calmodulin with 2.5 or 5 nM purified FLAG-tagged MLCKs and indicated concentration of purified nHis-MLC2v in 40 μL total volume. Reaction mixtures were pre-incubated for 5 min, and the kinase reactions were started by the addition of ATP and incubated for 1 or 3 h at 25°C. Reactions were terminated, and kinase activities were measured by Phos-tag sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) or ADP-Glo assay. For measurement of MLC2v Km values of wild-type and mutant cMLCK, 5 nM kinase were assayed with 0.625–20 μM nHis-MLC2v. Km values were calculated by non-linear fit to the Michaelis–Menten equation by using Igor Pro software.

### Phos-tag sodium dodecyl sulfate–polyacrylamide gel electrophoresis

For analysis of MLC2v phosphorylation, samples of kinase reactions were subjected to phosphate affinity SDS-PAGE using an acrylamide-pendant phosphate-binding tag (Phos-tag SDS-PAGE<sup>24</sup>). The stacking gel was composed of 12% (wt/vol) acrylamide, 0.1% (wt/vol) SDS, 125 mM Tris–HCl (pH 6.8), 0.1% (wt/vol) ammonium persulfate, and 0.5% (vol/vol) *N,N,N,N*-tetramethylethylenediamine. The resolving gel was composed of 12% (wt/vol) acrylamide, 30 μM Phos-tag acrylamide (Wako), 60 μM MnCl<sub>2</sub>, 0.1% (wt/vol) SDS, 375 mM Tris–HCl (pH 8.8), 0.05% (wt/vol) ammonium persulfate, and 0.25% (wt/vol) *N,N,N,N*-tetramethylethylenediamine. Electrophoresis was performed in 0.1% (wt/vol) SDS, 25 mM Tris, and 192 mM glycine at 150 V per gel for 80 min. After electrophoresis, the gel was soaked in EDTA (+) transfer buffer

[10 mM EDTA, 50 mM Tris, 380 mM glycine, 0.000375% (wt/vol) SDS, and 20% (wt/vol) EtOH] for 10 min and then in transfer buffer for 10 min. Proteins were transferred to poly(vinylidene difluoride) membranes (0.2 μm; Bio-Rad) at 15 V for 30 min in transfer buffer. Membranes were then blocked with non-fat dry milk, incubated with primary antibody (anti-MLC2v 1:4000, Abcam ab92721) followed by secondary antibody (HRP-coupled goat anti-rabbit 1:8000; Cappel, #55696) and detection as with SDS-PAGE. Phosphorylated and non-phosphorylated MLC2v were quantified by densitometry using ImageJ software.<sup>25</sup>

### ADP-Glo kinase assay

Kinase activity was measured by quantifying the amount of adenosine diphosphate (ADP) produced during a kinase reaction using the ADP-Glo Kinase Assay (Promega) according to the manufacturer's protocol. In brief, the kinase reactions were stopped by adding 40 μL of ADP-Glo reagent. After incubation at room temperature for 40 min, 80 μL of kinase detection reagent was added per well and incubated for 30 min. Luminescence was measured using the chemiluminescence imaging analyzer TriStar2 LB942 (Berthold) with an integration time of 0.3 s/well. The luminescence intensity was converted to ADP amount using calibration curve. The amount of phosphates used for MLC2v phosphorylation was calculated as follows: total ADP produced during kinase reaction was measured as earlier; background ADP including cMLCK autophosphorylation was measured by the reaction without MLC2v; and the amount of ADP used for MLC2v phosphorylation was assessed by subtracting background ADP from total ADP.

### Data analysis

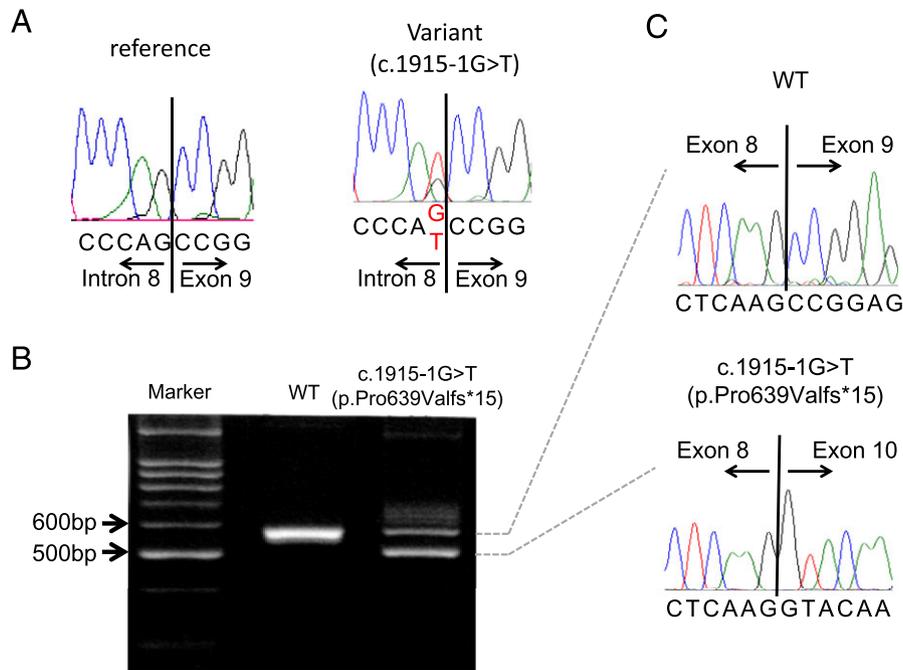
Statistical analyses were performed using SPSS statistics software version 22 (IBM). Data were analysed using one-way analysis of variance and Bonferroni post hoc testing. A *P* value below 0.01 was considered statistically significant.

**Table 2** Clinical characteristics of pedigrees of DCM-155

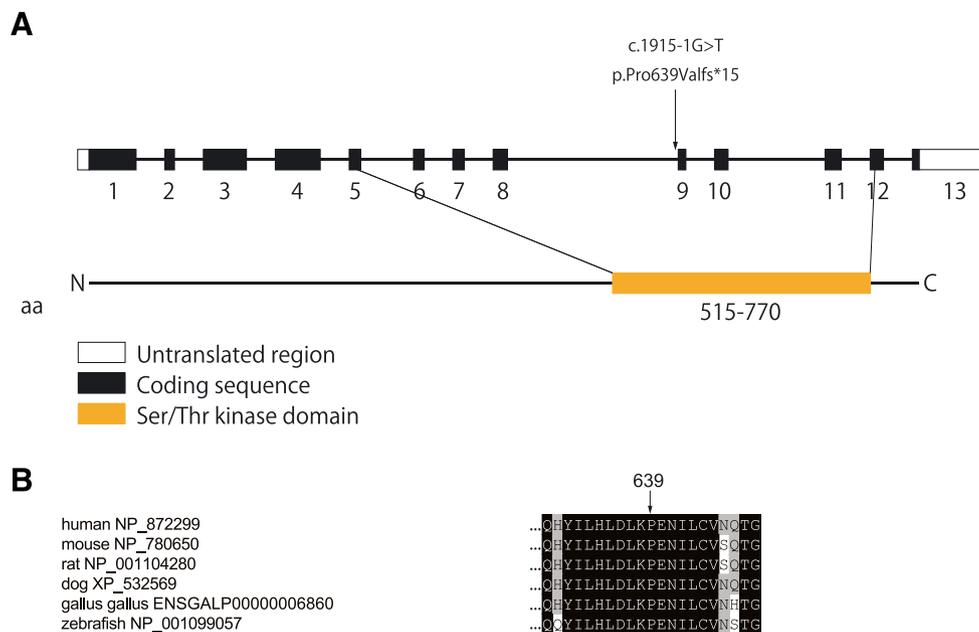
Member	Genotype	Age	Gender	Echocardiographic data				ECG findings
				LVDd/Ds (mm)	LVEF (%)	IVS/PW (mm)	LAD (mm)	
II-1	Hetero	49	M	70/55	42	8/9	42	IVCD, mild ST-T change
II-3	WT	47	M	50/33	63	10/10	38	Early repolarization
III-1	WT	24	M	51/35	59	10/10	31	WNL
III-2	WT	22	M	51/36	56	10/10	31	WNL
III-3	WT	21	F	50/34	60	9/9	33	PAC
III-4	Hetero	19	M	55/36	63	8/8	31	CR, IVCD, mild ST-T change

CR, clockwise rotation; DCM, dilated cardiomyopathy; Ds, end-systolic dimension; ECG, electrocardiogram; IVCD, intraventricular conduction delay; IVS, intraventricular septum; PW, posterior wall; LAD, left atrial diameter; LVDd, left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction; PAC, premature atrial contraction; WNL, within normal limits; WT, wild type.

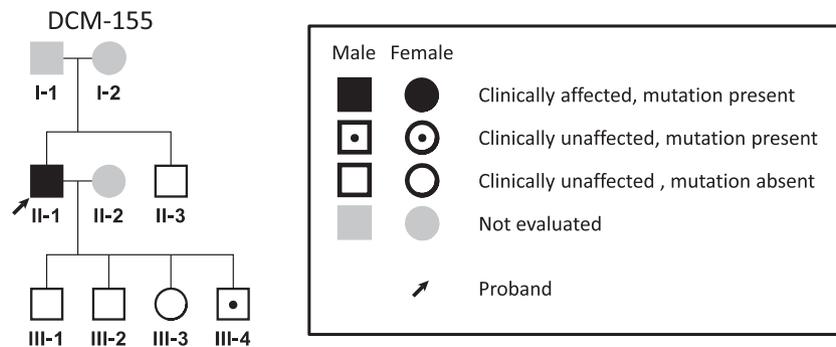
**Figure 1** *MYLK3* variant identified in a patient with dilated cardiomyopathy (DCM). (A) Sequence trace of the *MYLK3* variant p.Pro639Valfs\*15 (DCM-155). (B and C) Reverse transcription PCR and sequence analysis of the reverse transcription PCR product derived from mRNA of the DCM-155 proband and a control showing out-frame skipping of exon 9 (71 bp) caused by the c.1915-1g>t mutation. WT, wild type.



**Figure 2** *MYLK3* variant and scheme of cardiac myosin light chain kinase structure. (A) Schematic of the genomic structure of human *MYLK3* and functional domains of myosin light chain kinase. p.Pro639Valfs\*15 mutations locate in the serine/threonine kinase domain. (B) Protein sequence alignment of cardiac myosin light chain kinase in vertebrates. Residue 639 is highly conserved across species. DCM, dilated cardiomyopathy.



**Figure 3** Pedigree of the proband identified with *MYLK3* mutations. Familial analysis was performed in the pedigree of DCM-155. A 19-year-old male carrier (III-4) showed dilation of the left ventricle on echocardiography and ST-T abnormalities on electrocardiography, although these findings did not meet clinical criteria for DCM. All non-carriers had the normal ventricular size on echocardiographic and also had normal electrocardiographic findings. DCM, dilated cardiomyopathy.



## Results

### Genetics

One hundred and forty-nine unrelated DCM probands (109 male and 40 female) were enrolled in this study. There were 16 familial and 133 sporadic DCM cases. Among these patients, we identified one SNV at the splice acceptor site of exon 9 (c.1915-1G>A; p.Pro639Valfs\*15 in DCM-155) (Table 2 and Figure 1). This variant was not detected in 400 chromosomes of 200 healthy volunteers and is listed in the Human Genetic Variation Database<sup>15</sup> with an allele frequency < 0.001. Sequence analysis of the reverse transcription PCR product derived from mRNA demonstrated that p.Pro639Valfs\*15 caused exon skipping and created a premature terminal codon (Figure 1B and C) that is predicted to result in protein truncation. Pro639 locates to the kinase active site of the serine/threonine kinase domain (Figure 2).

The family analysis of patient DCM-155 revealed a 19-year-old male carrier (DCM-155; III-4) with borderline dilation and preserved ejection fraction of the LV on echocardiography and ST-T abnormalities on electrocardiography (Figure 3 and Table 2), whereas all non-carriers showed normal echocardiographic and electrocardiographic findings. The mother of the proband (DCM-155; I-2) had died from gastric cancer at the age of 35.

To exclude the possibility of the presence of mutations in known DCM-causing genes in the DCM-155 pedigree, we performed whole exome sequencing in the proband (II-1) and his clinically unaffected sibling (II-3) (Table 3). This revealed 80 variants from 74 genes that were not shared between these two subjects. However, none of the 74 genes has been reported as a DCM disease-causing gene or implicated in other forms of cardiomyopathies. Ingenuity Pathway Analysis software (Ingenuity Systems) showed that only 2 of the 74 genes (STAB1 and AKAP9) were associated with cardiac function and/or structure. STAB1 encodes stabilin 1, a multifunctional

**Table 3** Whole exome sequencing analyses in the pedigree of DCM-155

	Proband (DCM-155; II-1)	Sibling (DCM-155; II-3)
All variants	85 682	85 982
Quality passed	52 681	53 126
Non-synonymous mutation, not in dbSNV151, and <0.5% in 1000 genome	1091	1107
Deleterious variants (predicted high impact by SnpEff)	169	178
Segregation	80 variants (in 74 genes) ABCA2 <sup>a</sup> , ACAD9, AKAP9, AKD1 <sup>a</sup> , ATAD3B, ATG4B, C1R, CAMKK2, CAPN8, CCDC17, CD151, CDH23, CHL1 <sup>a</sup> , CLTC, CLTCL1, CNKSR1, D2HGDH <sup>a</sup> , DCHS2 <sup>a</sup> , DNAH17, DNM1L, DTL, EPSTI1, EWSR1, EYS, FAM81B, FAP, HLA-DQA1, HLA-DQB1, HLA-DRB1, HPS3, HYOU1, ITPR1, KALRN, KIAA0430, KIR3DX1, LAPTM4B, LCMT2, LDLRAP1, LILRB5, LRP5L, LTF, MICAL1, MON2, MYLK3, MYO7B, NADK, NEDD4, NFIA, NHLRC3, NLRP8, NOTCH2, NPHP1, OLFM1, OXCT1, PDC, PDE4DIP, PNPLA7, PTGES, RGS11, RNF212, SAT1, SCARF2, SELPLG, SERINC4, SLC25A25, SNF8, SPEF2, STAB1 <sup>a</sup> , TUBGCP6, UNC50, VLDLR, WDR90, ZDHHC11, ZNF527	
On IPA gene list	Three variants in two genes (STAB1 <sup>a</sup> and AKAP9)	

DCM, dilated cardiomyopathy; IPA, Ingenuity Pathway Analysis.

<sup>a</sup>Two variants in one gene.

scavenger receptor selectively expressed in tissue macrophages and sinusoidal endothelial cells, and is reported to be a candidate gene for bipolar disorder.<sup>26</sup> AKAP9 mutations were reported as a cause of long QT syndrome.<sup>27</sup> Thus, the presence of mutations in known DCM-causing genes was excluded in the pedigree of DCM-155, suggesting that *MYLK3* p.Pro639Valfs\*15 could be a disease-causing mutation.

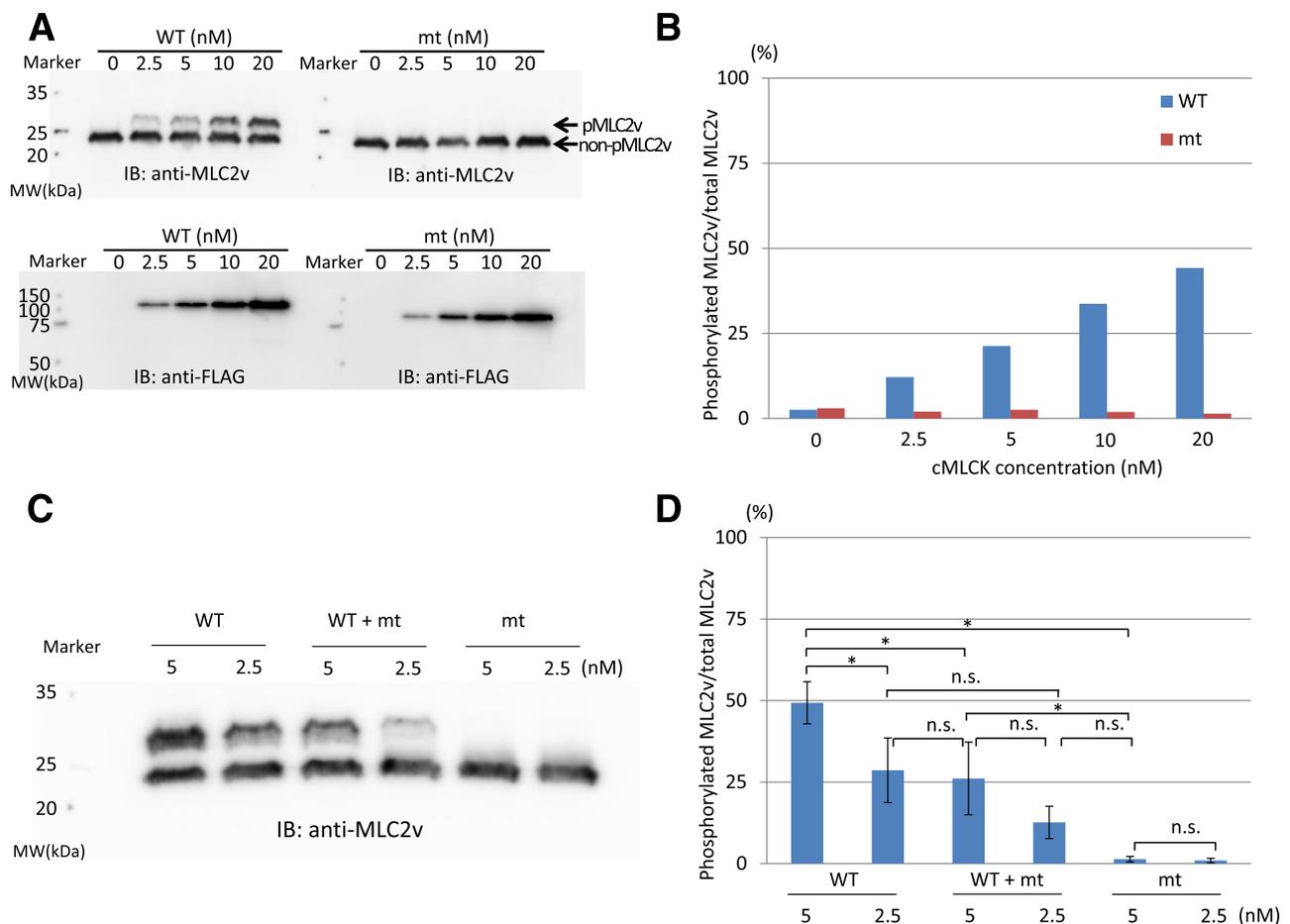
## Functional analysis

To assess the functional consequences of possessing this variant, we examined kinase activity *in vitro*. Purified nHis-tagged MLC2v proteins from *E. coli* were phosphorylated by purified

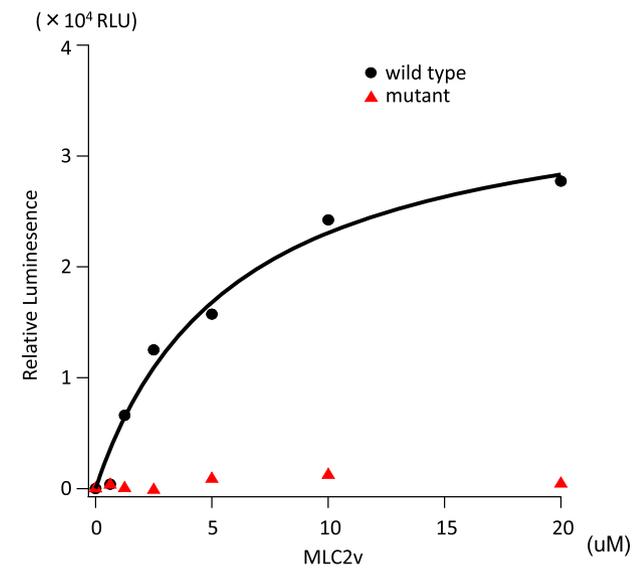
nFlag-tagged wild-type or p.Pro639Valfs\*15 cMLCK *in vitro*. We used Phos-tag SDS-PAGE<sup>24</sup> to separate phosphorylated and non-phosphorylated MLC2v. Importantly, the kinase activity of the mutant p.Pro639Valfs\*15-cMLCK was completely abrogated, although wild-type MLC2v showed kinase activity in a dose-dependent manner (Figure 4A and B). Kinase assays with a mixture of wild-type and mutant cMLCK showed that mutant cMLCK did not inhibit the former (Figure 4C and D).

Calculating MLC2v  $K_m$  and  $V_{max}$  values by ADP-Glo assay indicated lack of kinase activity of mutant cMLCK, although wild-type cMLCK had a  $K_m$  value of  $5.93 \pm 1.47$  and a maximum luminescence of  $(3.67 \pm 0.37) \times 10^4$  relative light unit that corresponded to a  $V_{max}$  of  $1.28 \pm 0.03$  mol/min/mol kinase (Figure 5).

**Figure 4** Phosphorylation assays analysed by Phos-tag sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). (A) Purified nHis-tagged ventricular myosin regulatory light chain 2 (MLC2v) proteins were phosphorylated by purified nFlag-tagged wild-type (WT) or p.Pro639Valfs\*15 cardiac myosin light chain kinase (cMLCK) *in vitro* at 25°C for 1 h. Phosphorylated MLC2v (pMLC2v) and non-phosphorylated MLC2v (non-pMLC2v) were then separated by Phos-tag SDS-PAGE followed by immunoblotting for MLC2v. Note that MLC2v is phosphorylated in a dose-dependent manner only by WT cMLCK. Mutant (mt) cMLCK has completely lost kinase activity. (B) Quantitative analysis also showed that MLC2v is phosphorylated in a dose-dependent manner only by WT cMLCK. (C) Phos-tag SDS-PAGE of WT cMLCK, mixture of WT and mt cMLCK, and mt cMLCK. *In vitro* kinase assay by cMLCK was performed at 25°C for 3 h; mt cMLCK did not affect WT cMLCK activity. (D) Densitometries of Phos-tag SDS-PAGE. WT + mt, the same amount of wild-type and mutant cMLCKs. \* $P < 0.01$ ;  $n = 4$  for each point; n.s., not significant.



**Figure 5** Michaelis–Menten saturation curve measured with the ADP-Glo assay. MLC2v dose-dependence of the nFlag-tagged wild-type or p.Pro639Valfs\*15 cMLCK activities measured by ADP-Glo assay. The reaction that contained no MLC2v was set as a background. The luminescence readings of the reactions were corrected with background subtraction and finally fit with the Michaelis–Menten equation using Igor Pro software to obtain  $K_m$  (MLC2v). Wild-type cMLCK had a  $K_m$  value of  $5.93 \pm 1.47 \mu\text{M}$  and a maximum luminescence of  $(3.67 \pm 0.37) \times 10^4$  relative light units that corresponded to a  $V_{\text{max}}$  of  $1.28 \pm 0.03 \text{ mol/min/mol}$  kinase. Mutant cMLCK had no kinase activity.  $n = 2$  for each point. RLU, relative light unit.



## Discussion

In the present study, we identified a p.Pro639Valfs\*15 mutation in cMLCK in a DCM family. Assessing cMLCK kinase activity by Phos-tag SDS-PAGE and ADP-Glo assays revealed a complete lack of phosphorylation even at high concentrations of mutant cMLCK. In contrast, kinase activity of wild-type cMLCK was well preserved.  $K_m$  value of wild-type cMLCK was  $5.93 \pm 1.47 \mu\text{M}$ , which was almost the same as the previously reported value of  $4.3 \pm 1.5 \mu\text{M}$ .<sup>6</sup> Additionally, this mutation was observed only in a DCM patient. These results demonstrate that this mutation is closely related to the occurrence of myocardial dysfunction associated with DCM.

Ventricular myosin regulatory light chain 2 functions are regulated by phosphorylation by its specific kinase and some phosphatases.<sup>28</sup> Mutations in this gene are reported as a cause of DCM.<sup>29</sup> Thus, MLC2v dephosphorylation was associated with heart failure including DCM in humans.<sup>11–13</sup> Mice overexpressing myosin phosphatase exhibited MLC2v dephosphorylation and DCM phenotypes.<sup>30</sup> In fact, a conditional *MYLK3* knockout mouse showed reduced MLC2v phosphorylation and DCM phenotype like LV dilation, wall thinning, and decreased fractional shortening.<sup>8–10</sup> Ding *et al.*

demonstrated that heterozygous cMLCK knockout mice exhibited decreased systolic function of the LV,<sup>7</sup> which also supports our hypothesis that the truncation mutation in *MYLK3*, p.Pro639Valfs\*15, causes DCM in humans. This is reasonable considering that Pro639 locates to the kinase active site of serine/threonine kinase domain<sup>6</sup> (Figure 2).

Of note, one young carrier in the proband's family also exhibited borderline LV dilation on echocardiography, indicating that the *MYLK3* truncation mutation, p.Pro639Valfs\*15, may cause pathological LV remodelling even in adolescents. Tobita *et al.*<sup>14</sup> reported five *MYLK3* mutation carriers from two pedigrees. Three carriers had read-through mutation c.2459A>C (p.\*820Sext\*19); the level of expression of the protein and phosphorylated MLC2v level was greatly decreased. The other two carriers had a truncation variant c.1879\_1885del (p.L623 fs\*41) with diminished kinase activity. Interestingly, three of the five carriers were diagnosed with DCM in their 40s and 50s. The exact relationship between the onset of cardiac dysfunction and *MYLK3* mutation requires further investigation.

Cardiac myosin light chain kinase is a not structural but is a functional protein. Therefore, cMLCK could present a therapeutic modality in patients with DCM caused by *MYLK3* mutations. From this point of view, further studies are warranted to determine whether harbouring p.Pro639Valfs\*15 is associated with increased risk of heart failure in the general Japanese population, because this variant identified in a DCM family was listed in the reference database of genetic variations in the Japanese population at an allele frequency  $< 0.001$ .

There are several limitations to the present study. First, we did not assess the phosphorylation level of MLC2v because of lack of heart tissue samples from biopsy. In addition, determination of the MLC2v phosphorylation level in experimental models is difficult, because it depends on heart load conditions.<sup>11–13</sup> However, under these conditions, it is possible that MLC2v phosphorylation levels could be decreased in the proband, because MLC2v phosphorylation was shown to decrease in a heterozygous cMLCK knockout mouse model.<sup>7</sup> Mutant cMLCK did not interfere with the wild-type activity *in vitro*, and truncation variants such as this mutation are generally degraded by nonsense-mediated mRNA decay, suggesting that this mutation can cause DCM with haploinsufficiency. Determination of MLC2v phosphorylation levels in induced pluripotent stem cells derived from mutation carriers may verify these functional data. Second, the prevalence of this cMLCK mutation in clinical DCM is still unclear, although we identified the possible pathogenic mutation of cMLCK in a patient with clinical DCM. Therefore, large-scale, multicentre studies are needed to clarify the prevalence and spectrum of *MYLK3* mutations.

In conclusion, a truncation mutation in the cMLCK gene (p.Pro639Valfs\*15) can be associated with clinical DCM. We suggest that cMLCK could be a therapeutic target for DCM, although a larger study of DCM patients to identify the

prevalence of this mutation and further strengthen its association with disease through family analysis is necessary.

## Conflict of interest

None declared.

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