Myocardial ultrastructure can augment genetic testing for sporadic dilated cardiomyopathy with initial heart failure

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Abstract

Aims The aim of the present study was to consider whether the ultrastructural features of cardiomyocytes in dilated cardiomyopathy can be used to guide genetic testing.

Methods and results Endomyocardial biopsy and whole-exome sequencing were performed in 32 consecutive sporadic dilated cardiomyopathy patients [51.0 (40.0–64.0) years, 75% men] in initial phases of decompensated heart failure. The predicted pathogenicity of ultrarare (minor allele frequency ≤0.0005), non-synonymous variants was determined using the American College of Medical Genetics guidelines. Focusing on 75 cardiomyopathy-susceptibility and 41 arrhythmia-susceptibility genes, we identified 404 gene variants, of which 15 were considered pathogenic or likely pathogenic in 14 patients (44% of 32). There were five sarcomeric gene variants (29% of 17 variants) found in five patients (16% of 32), involving a variant of MYBPC3 and four variants of TTN. A patient with an MYBPC3 variant showed disorganized sarcomeres, three patients with TTN variants located in the region encoding the A-band domain showed sparse sarcomeres, and a patient with a TTN variant in encoding the I-band domain showed disrupted sarcomeres. The distribution of diffuse myofilament lysis depended on the causal genes; three patients with the same TMEM43 variant had diffuse myofilament lysis near nuclei (P = 0.011), while two patients with different DSP variants had lysis in the peripheral areas of cardiomyocytes (P = 0.033).

Conclusions Derangement patterns of myofilament and subcellular distribution of myofilament lysis might implicate causal genes. Large-scale studies are required to confirm whether these ultrastructural findings are related to the causative genes.

Keywords Whole-exome analysis; Myofilament changes; Electron microscopy; Causative gene variants; Dilated cardiomyopathy; Initial decompensated heart failure

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Introduction

Dilated cardiomyopathy (DCM) is a severe heart disease characterized by enlarged ventricles and systolic dysfunction.1 DCM is a major cause of heart failure (HF) and heart transplantation (HTx). In some patients with DCM, optimal treatments for HF such as renin-angiotensin system inhibitors and beta-blockers can gradually improve left ventricular (LV) function and prognosis.2 Recently, the causative genes of several diseases have been detected by next-generation...
sequencing. TTN\(^3\) and LMNA\(^4\) have been reported to be involved in DCM, with associations between gene variants and clinical features such as prognosis\(^5\) and deterioration of cardiac function,\(^6\) have been evaluated.

Electron microscopy of endomyocardial biopsy (EMB) samples allows the detailed analysis of cardiomyocyte degeneration.\(^7\)–\(^9\) Myofilament changes in cardiomyocytes\(^7\) and abnormal nuclei\(^8\) could predict poor prognosis, and autophagic vacuoles\(^5\) were associated with better prognosis in DCM patients. Ultrastructural alterations were expected to have some relationships to variants in genes encoding myocardial constitutive proteins; however, direct association between individual gene variants and ultrastructural findings in patients with DCM has not been thoroughly studied.

A period-specific observational study\(^10\) showed that early diagnosis and intervention of DCM improved prognosis. Identification of the causative gene for DCM may lead to risk stratification of patients and enable individualized treatment; however, it is difficult to detect sporadic DCM at asymptomatic or early stages of HF. Indeed, beyond DCM, there is currently no evidence for risk reduction due to genomic medicine in routine general practice.\(^11\) In contrast, EMB to distinguish secondary cardiomyopathy is performed relatively early as part of insured medical care in Japan. Here, we investigated whether the ultrastructural features of cardiomyocytes might augment genetic testing, such as whole-exome sequencing.

**Methods**

**Study population**

The present study enrolled 32 Japanese patients with sporadic DCM. All patients underwent DNA collection from peripheral blood and EMB from the left ventricle during the period from October 2001 to December 2011, inclusive, at the Nippon Medical School Hospital (Figure 1). A DCM diagnosis was made from the combined results of trans-thoracic echocardiography, coronary angiography, left ventriculography, and EMB. Patients with secondary (metabolic, drug-induced, or inflammatory) cardiomyopathies, myocarditis (according to the Dallas criteria), neuromuscular disorders, congenital, ischaemic, or severe valvular heart disease were excluded. All patients enrolled in the study had systolic dysfunction (LV ejection fraction <50%) without significant coronary artery stenosis, as assessed by coronary angiography. Written informed consent was obtained from all patients prior to their inclusion in the study. The study protocol was approved by the committee overseeing clinical and genetics research at our institution and was performed in accordance with the Declaration of Helsinki.

**Clinical data collection, including endomyocardial biopsy**

On admission, all patients underwent routine laboratory analyses and trans-thoracic echocardiography. Two-dimensional, M-mode, and colour Doppler imaging was performed according to the standardized methods of the American Society of Echocardiography.\(^12\) Cardiac catheterization was performed together with EMB and performed under radiographic guidance with continuous electrocardiographic monitoring. Tissue samples were collected from the LV infero-posterior wall using a 7 Fr bioptome (Cordis; Johnson & Johnson Co, New Brunswick, NJ) by retrograde approach.

**Tissue preparation**

Preparation of biopsy specimens for light and electron microscopic analyses has been described previously.\(^7,9\) Ultrastructural variables such as myofilament changes were classified as positive (when identified in the cytoplasm of cardiomyocytes) or negative.\(^7\) Photomicrographs of 200 cardiomyocytes were evaluated per patient. Three of the authors evaluated all electron microscopy results for EMB samples (T. S., A. A., and Y. S.), with each sample examined three times in random order; these examiners were blinded to the clinical background and results of genetic testing of the patients. Any discrepancies in the ultrastructural evaluations were decided by consensus. The Z-line is a structure
with high electron density to which myofilament is bound. Abnormal Z-line was defined as a structure that has the same density as normal Z-line and can adhere to one or several myofilaments, even if myofilament lysis occurs. The M-line is in the centre of the sarcomere between the Z-lines. Obscured M-line was defined as obscured and discontinuous in nature.

**DNA isolation**

Genomic DNA was extracted from peripheral blood lymphocytes using Genomix Kit (Biologica Co., Nagoya, Japan) following the manufacturer’s protocol.

**Whole-exome sequencing**

Whole-exome sequencing was performed by Riken Genesis Co., Ltd. in Japan. Exon capture was performed using the SureSelectXT Human All Exon V6 Kit (Agilent Technologies, Santa Clara, CA), and sequencing was performed on the Illumina HiSeq 2500 platform (Illumina Inc, San Diego, CA). Sequence mapping was performed using the Burrows-Wheeler Aligner 0.7.10. Mapping results were corrected using Picard (Ver. 1.73) for removing duplicates and Genome Analysis Toolkit (GATK Ver. 1.6-13) for local alignment and quality score recalibration. Variant detection was performed with multi-sample calling with GATK.

**Variant filtering and pathogenicity assessment**

Variant annotation was performed using software developed in-house by Riken Genesis Co., Ltd. A series of filters were used to prioritize variants. Variants were given higher priority when (i) they had a high-quality score to coordinates with variant quality score recalibration passing and variant call quality score ≥30, (ii) they were non-synonymous variants (i.e. missense, nonsense, frameshift insertion/deletion, in-frame insertion/deletion, or splice error), and (iii) less common in reference databases [minor allele frequency (MAF) ≤0.0005 within genomAD in any ethnic group (n = 125 748, https://gnomad.broadinstitute.org/) or East Asian population group (n = 9197, https://gnomad.broadinstitute.org/)], 1000 Genome Project (n = 2504, https://www.internationalgenome.org/1000-genomes-browsers), the National Heart, Lung, and Blood Institute Grand Opportunity Exome Sequencing Project (n = 6503), and Human Genetic Variation Database (n = 3248, http://www.hgvd.genome.med.kyoto-u.ac.jp/) databases]. The MAF was calculated to be 0.0004 using an estimated maximum prevalence of 1:25013 and was set to ≤0.0005 cut-off. Variants meeting these criteria underwent a further gene-specific surveillance for all known 75 cardiomyopathy-susceptibility and 41 arrhythmia-susceptibility genes (N = 116; Table 1). The American College of Medical Genetics guidelines modified specifically for DCM14 for the interpretation of sequence variants were used to classify identified variants as pathogenic (P), likely pathogenic (LP), or variant of uncertain significance (VUS).

Those gene variants were confirmed using standard polymerase chain reaction and Sanger sequencing methods.

**Statistical analysis**

Continuous variables were expressed as median values reported with 25th and 75th percentiles. Variables were evaluated by one-way analysis of variance in the case of normally distributed data and by the Kruskal–Wallis test if data were not normally distributed, as determined by the Shapiro–Wilk test. The distribution of myofilament lysis in cardiomyocytes was evaluated by χ² or Fisher’s exact test. Statistical analyses were performed using the SPSS software package (SPSS Inc., Chicago, IL), and P < 0.05 was considered significant.

**Results**

**Patient characteristics**

The clinical, genetic, histopathological, and ultrastructural characteristics of the patients are summarized in Tables 2 and 3. During the follow-up period [7.6 (3.9–8.0) years], 12 patients (38%) were readmitted because of HF recurrence, one patient (P7, Table 4) received HTx, and four patients (13%) died. One death was from multiple organ failure due to decompensated HF with inability to control ventricular arrhythmia (P1, Table 4), and three were due to sudden cardiac death (P9 in Table 4 and P20 and P26 in Table 5).

**American College of Medical Genetics classifications of variants**

In 32 patients, a total of 404 variants were detected (Table 1). After filtering with MAF and the American College of Medical Genetics guidelines, 62 ultrarare variants remained, including 5 P and 10 LP variants in 14 patients (44% of 32, Table 4) and 35 VUSs in 24 patients (75% of 32, Table 5). VUS included two variants (6% of 35, Table 5) that could be upgraded to LP variants if there was additional evidence supporting pathogenicity, and six variants (17% of 35, Table 5) that could be downgraded to likely benign variants if there was additional evidence supporting benign impact.
| Gene symbol | NCBI RefSeq | Protein name | Location |
|-------------|-------------|--------------|----------|
| ACTC1       | NM_005159.5 | Actin, alpha, cardiac muscle 1 | 15q14 0 |
| ACTN2       | NM_001103.3 | Actinin alpha 2 | 1q43 2 |
| APLN        | NM_017413.5 | Apelin | Xq26.1 1 |
| BAG3        | NM_004281.3 | BAG coherperone 3 | 10q26.11 4 |
| CACNA2D1    | NM_000722.4 | Calcium voltage-gated channel auxiliary subunit alpha2delta 1 | 7q21.11 1 |
| CALR3       | NM_145046.5 | Calreticulin 3 | 19p13.11 2 |
| CAMK2D      | NM_001103.4 | Calcium/calmodulin-dependent protein kinase II delta | 4q26 1 |
| CAV3        | NM_033337.3 | Caveolin 3 | 3p25.3 2 |
| CCN2        | NM_001901.3 | Cellular communication network factor 2 | 6q2.3 0 |
| COX15       | NM_078470.6 | COX15, cytochrome c oxidase assembly homologue | 10q24.2 4 |
| CRYAB       | NM_001289807.1 | Crystallin alpha B | 1q23.1 2 |
| CSRP3       | NM_003476.5 | Cysteine-rich and glycine-rich protein 3 | 6q23.1 0 |
| CTF1        | NM_001330.3 | Cardiotrophin 1 | 1p11.2 0 |
| DES         | NM_001927.4 | Desmin | 2q35 1 |
| DMD         | NM_001221.4 | Disc large MAGUK scaffold protein 1 | 3q29 1 |
| DNM1L       | NM_012062.5 | Dynamin 1 like | Xp21.2-p21.1 19 |
| DSC2        | NM_004949.5 | Desmocollin 2 | 18q12.1 1 |
| DSG2        | NM_004415.4 | Desmoplakin | 6q24.3 12 |
| DTA2        | NM_001390.4 | Dystrobrevin alpha | 18q12.1 2 |
| EMD         | NM_000117.3 | Emerin | Xq28 0 |
| EYA4        | NM_004100.5 | EYA transcriptional coactivator and phosphatase 4 | 6q23.2 1 |
| FGFI2       | NM_021032.4 | Fibroblast growth factor 12 | 3q28-q29 0 |
| FHL2        | NM_001039492.3 | Four and a half LIM domains 2 | 2q12.2 3 |
| FKTN        | NM_006731.2 | Fukutin | 9q3.2 1 |
| GAA         | NM_000152.5 | Glucosidase alpha, acid | 17q25.3 8 |
| GATA5       | NM_004949.5 | GATA-binding protein 5 | 20q13.33 1 |
| GATA6       | NM_005257.5 | GATA-binding protein 6 | 18q11.2 1 |
| GATAD1      | NM_021167.5 | GATA zinc finger domain-containing 1 | 7q21.2 1 |
| GLA         | NM_000169.3 | Galactosidase alpha | Xq22.1 0 |
| HEY2        | NM_012259.3 | Hes related family bHLH transcription factor with YRPW motif 2 | 6q22.31 0 |
| JPH2        | NM_020433.5 | Junctophilin 2 | 20q13.12 1 |
| JUP         | NM_001352773.1 | Junction plakoglobin | 17q21.2 1 |
| LAMA4       | NM_01105206.3 | Laminin subunit alpha 4 | 6q21 11 |
| LAMP2       | NM_001122606.1 | Lysosomal-associated membrane protein 2 | Xq24 0 |
| LDB3        | NM_001080114.2 | LIM domain binding 3 | 10q23.2 5 |
| LMNA        | NM_170707.4 | Lamin A/C | 1q22 3 |
| MLIP        | NM_138569.2 | Muscular LMNA interacting protein | 6p12.1 12 |
| MYBPC3      | NM_000256.3 | Myosin-binding protein C, cardiac | 11p11.2 5 |
| MYH6        | NM_002471.3 | Myosin heavy chain 6 | 1q11.2 5 |
| MYH7        | NM_000257.4 | Myosin heavy chain 7 | 1q11.2 3 |
| MYL2        | NM_000432.4 | Myosin light chain 2 | 1q24.11 0 |
| MYL3        | NM_000258.3 | Myosin light chain 3 | 3p21.31 1 |
| MYL2K       | NM_033181.8 | Myosin light chain kinase 2 | 20q11.21 1 |
| MYL3K       | NM_182493.3 | Myosin light chain kinase 3 | 16q11.2 3 |
| MYOZ2       | NM_016599.5 | Myozenin 2 | 4q26 0 |
| MYPL1       | NM_032578.8 | Myophillin | 10q21.3 6 |
| NEBL        | NM_006393.2 | Nebulette | 10p12.31 9 |
| NEXN        | NM_144573.3 | Nexilin F-actin binding protein | 1p3.11 2 |
|PKP2        | NM_004572.3 | Plakophilin 2 | 12p11.21 1 |
|PLN         | NM_002667.5 | Phospholamban | 6q22.31 0 |
|PRKAG2      | NM_016203.4 | Protein kinase AMP-activated non-catalytic subunit gamma 2 | 7q36.1 4 |
|PSEN1       | NM_000214.1 | Presenilin 1 | 1q42.4 2 |
|PSEN2       | NM_000447.3 | Presenilin 2 | 1q42.13 2 |
|RBM20       | NM_001134363.3 | RNA-binding motif protein 20 | 10q25.2 8 |
|RYR2        | NM_001035.3 | Ryanodine receptor 2 | 1q43 4 |
|SCO2        | NM_005138.3 | SCO2, cytochrome c oxidase assembly protein | 2q13.33 1 |
|SOH4        | NM_004168.4 | Succinate dehydrogenase complex flavoprotein subunit A | 5p15.33 4 |
|SGCD        | NM_000337.5 | Sarcoglycan delta | 5q3.2-q3.3 0 |
|SLC25A4     | NM_001151.4 | Solute carrier family 25 member 4 | 4q35.1 1 |
|TBX20       | NM_020417.1 | T-box transcription factor 20 | 7p14.2 1 |

(Continues)
Table 1 (continued)

| Gene symbol | NCBI RefSeq   | Protein name                                | Location  | N   |
|-------------|---------------|---------------------------------------------|-----------|-----|
| TBX5        | NM_080717.3   | T-box transcription factor 5                | 12q24.21  | 2   |
| CAP         | NM_003673.4   | Titin-cap                                   | 17q12     | 0   |
| TGBF3       | NM_003239.4   | Transforming growth factor beta 3           | 14q24     | 0   |
| TEMEM43     | NM_024334.3   | Transmembrane protein 43                    | 3p25.1    | 7   |
| TMPO        | NM_003276.2   | Thymopoietin                                | 12q23.1   | 2   |
| TNNC1       | NM_003280.3   | Troponin C1, slow skeletal and cardiac type | 3p21.1    | 0   |
| TNNI3       | NM_003635.3   | Troponin I3, cardiac type                   | 19q13.4   | 2   |
| TNNT2       | NM_003636.4   | Troponin T2, cardiac type                   | 1q32.1    | 4   |
| TP63        | NM_003722.5   | Tumour protein p63                          | 3q28      | 0   |
| TPM1        | NM_001018004.2| Tropomyosin 1 (alpha)                      | 15q22.2   | 2   |
| TTN         | NM_133378.4   | Titin                                       | 2q31.2    | 142 |
| TTR         | NM_003773.1   | Transthyretin                               | 18q12.1   | 0   |
| TMEM43      | NM_024334.3   | Transmembrane protein 43                    | 3p25.1    | 7   |

Arrhythmia-susceptibility genes

| Gene symbol | NCBI RefSeq   | Protein name                                | Location  | N   |
|-------------|---------------|---------------------------------------------|-----------|-----|
| ABCC8       | NM_000352.6   | ATP-binding cassette subfamily C member 8   | 11p15.1   | 0   |
| ABCC9       | NM_005691.3   | ATP-binding cassette subfamily C member 9   | 12p12.1   | 0   |
| AKAP9       | NM_005751.4   | A-kinase anchor protein 9                    | 7q21.2    | 16  |
| ANK2        | NM_001148.6   | Ankyrin 2                                   | 4q25-q26  | 5   |
| ANKRD1      | NM_014391.2   | Ankyrin repeat domain 1                     | 10q23.1   | 0   |
| CACNA1C     | NM_00719.7    | Calcium voltage-gated channel subunit alpha | 12p13.33  | 4   |
| CACNB2      | NM_00724.4    | Calcium voltage-gated channel auxiliary subunit beta 2 | 10p12  | 0   |
| CALM1       | NM_001363669.1| Calmodulin 1                                | 14q32.11  | 0   |
| CASQ2       | NM_001232.3   | Casquestrin 2                               | 1p13.1    | 3   |
| DPP6        | NM_130797.4   | Dipeptidyl peptidase like 6                 | 7q36.2    | 1   |
| GJA1        | NM_000165.5   | Gap junction protein alpha 1                | 6q22.31   | 0   |
| GJA5        | NM_181703.4   | Gap junction protein alpha 5                | 1q21.2    | 0   |
| GJD4        | NM_153368.3   | Gap junction protein delta 4                | 10p11.21  | 2   |
| GPD1L       | NM_001514.1   | Glycerol-3-phosphate dehydrogenase 1        | 3p22.3    | 0   |
| HCN4        | NM_005477.3   | Hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 | 15q24.1  | 1   |
| KCNA5       | NM_002334.4   | Potassium voltage-gated channel subfamily A member 5 | 12p13.32  | 1   |
| KCND3       | NM_172198.2   | Potassium voltage-gated channel subfamily D member 3 | 1p13.2    | 0   |
| KCNE1       | NM_000219.6   | Potassium voltage-gated channel subfamily E regulatory subunit 1 | 21q22.12  | 1   |
| KCNE2       | NM_172201.1   | Potassium voltage-gated channel subfamily E regulatory subunit 2 | 21q22.11  | 0   |
| KCNE3       | NM_005472.4   | Potassium voltage-gated channel subfamily E regulatory subunit 3 | 11q13.4   | 0   |
| KCNE5       | NM_012282.4   | Potassium voltage-gated channel subfamily E regulatory subunit 4 | Xq23      | 0   |
| KCN2H       | NM_000238.4   | Potassium voltage-gated channel subfamily H member 2 | 7q36.1    | 2   |
| KCN2J       | NM_000891.3   | Potassium inwardly rectifying channel subfamily J member 2 | 17q24.3   | 0   |
| KCN5        | NM_000890.5   | Potassium inwardly rectifying channel subfamily J member 5 | 11q24.3   | 1   |
| KCN8        | NM_004982.4   | Potassium inwardly rectifying channel subfamily J member 8 | 12p12.1   | 0   |
| KCNQ1       | NM_000218.3   | Potassium voltage-gated channel subfamily Q member 1 | 11p15.5-p15.4 | 2 |
| NNX2-5      | NM_001166175.2| NK2 homeobox 5                              | 5q34      | 0   |
| NOS1AP      | NM_014697.3   | Nitric oxide synthase 1 adaptor protein     | 1q23.3    | 1   |
| RANGRF      | NM_016492.5   | RAN guanine nucleotide release factor        | 17p13     | 0   |
| SCN10A      | NM_006514.3   | Sodium voltage-gated channel alpha subunit 10 | 3p22.2   | 10  |
| SCN1B       | NM_001037.5   | Sodium voltage-gated channel beta subunit 1 | 1q13.11   | 4   |
| SCN2B       | NM_004588.5   | Sodium voltage-gated channel beta subunit 2 | 11q23.3   | 0   |
| SCN3B       | NM_018400.3   | Sodium voltage-gated channel beta subunit 3 | 11q24.1   | 0   |
| SCN4B       | NM_001142348.2| Sodium voltage-gated channel beta subunit 4 | 11q23.3   | 1   |
| SCN5A       | NM_198056.2   | Sodium voltage-gated channel alpha subunit 5 | 3p22.2   | 5   |
| SLC39A       | NM_007159.4   | Sarcotremata associated protein              | 3p14.3    | 0   |
| SNTA1       | NM_003098.3   | SNTA1                                       | 20q11.21  | 1   |
| TAZ         | NM_000116.5   | Tafazzin                                    | Xq28      | 0   |
| TRDN        | NM_001251987.2| Triadin                                     | 6q22.31   | 9   |
| TRPM4       | NM_017636.4   | Transient receptor potential cation channel subfamily M member 4 | 19q13.33  | 2   |
| TRPM7       | NM_017672.6   | Transient receptor potential cation channel subfamily M member 7 | 15q21.2   | 1   |
Ultrastructural features of cardiomyocytes and gene variants

Pathogenic or LP variants involved five sarcomeric gene variants in five patients (16% of 32): an MYBPC3 variant and four TTN variants. Electron microscopy revealed distinctive types of focal derangement of myofilaments (sarcomere damage) depending on the genes. Compared with normal cardiomyocytes (Figure 2A), a patient with a MYBPC3 variant (c.2833_2834delCG; P1, Table 4) showed disorganized
| Clinical characteristics | All patients (N = 18) | No myofilament changes (N = 3) | Focal derangement of myofilaments (N = 10) | Diffuse myofilament lysis (N = 5) | P-value |
|--------------------------|-----------------------|-------------------------------|--------------------------------|-------------------------------|---------|
| Age (years)              | 50.0 (40.0–64.8)      | 40.5 (40.3–40.8)              | 62.0 (39.5–67.0)              | 56.0 (40.0–62.0)              | 0.998   |
| Male                     | 15 (83%)              | 3 (100%)                      | 7 (70%)                      | 5 (100%)                      | 0.237   |
| Systolic blood pressure (mmHg) | 133.0 (117.5–155.3)  | 141.0 (136.5–145.5)           | 130.0 (119.0–140.0)           | 157.0 (102.0–162.0)           | 0.952   |
| Diastolic blood pressure (mmHg) | 78.0 (66.0–108.5) | 84.0 (81.0–87.0)              | 74.0 (66.0–94.0)              | 112.0 (62.0–117.0)            | 0.963   |
| Heart rate (b.p.m.)      | 86.5 (74.5–98.8)      | 79.0 (73.5–84.5)              | 88.0 (77.0–97.5)              | 85.0 (54.0–112.0)             | 0.775   |
| NYHA Scale III and IV    | 8 (44%)               | 1 (33%)                       | 5 (50%)                      | 2 (40%)                       | 0.854   |
| Co-morbidities           |                       |                               |                               |                               |         |
| Atrial fibrillation      | 5 (38%)               | 1 (33%)                       | 3 (30%)                      | 1 (20%)                       | 0.895   |
| Hypertension             | 11 (61%)              | 1 (33%)                       | 7 (70%)                      | 3 (60%)                       | 0.520   |
| Diabetes                 | 6 (33%)               | 1 (33%)                       | 4 (40%)                      | 1 (20%)                       | 0.741   |
| Renal dysfunction        | 4 (22%)               | 1 (33%)                       | 2 (20%)                      | 1 (20%)                       | 0.879   |
| Clinical chemistry       |                       |                               |                               |                               |         |
| B-type natriuretic peptide (pg/mL) | 435.1 (262.1–828.6) | 1114.9 (972.9–1256.8)         | 329.5 (260.0–539.0)           | 821.7 (434.5–1470.0)          | 0.092   |
| C-reactive protein (mg/dl) | 0.2 (0.1–0.7)        | 0.1 (0.1–0.2)                 | 0.1 (0.1–0.3)                 | 0.9 (0.9–0.9)                 | 0.058   |
| Haemoglobin (g/dL)       | 14.5 (13.4–16.3)      | 18.3 (18.1–18.6)              | 14.3 (12.8–15.4)              | 13.9 (13.7–15.7)              | 0.087   |
| Total bilirubin (mg/dl)  | 6.9 (0.7–1.1)         | 1.2 (1.0–1.5)                 | 0.9 (0.5–1.1)                 | 1.0 (0.9–1.3)                 | 0.339   |
| Echocardiographic data   |                       |                               |                               |                               |         |
| Left atrial dimension (mm) | 45.0 (42.0–50.8)     | 46.6 (44.3–48.8)              | 45.0 (43.5–50.0)              | 45.0 (42.0–51.0)              | 0.551   |
| Left ventricular ejection fraction (%) | 29.0 (21.5–41.0) | 31.0 (24.0–38.0)              | 33.0 (23.5–39.0)              | 25.0 (21.0–26.0)              | 0.834   |
| Left ventricular diastolic dimension (mm) | 63.5 (62.0–70.0) | 60.0 (58.0–62.0)              | 64.0 (60.0–71.0)              | 62.0 (62.0–67.0)              | 0.979   |
| Left ventricular systolic dimension (mm) | 54.5 (48.3–59.8) | 50.5 (46.8–54.3)              | 54.0 (48.5–61.0)              | 55.0 (54.0–60.0)              | 0.834   |
| Interventricular septum thickness (mm) | 10.0 (9.0–11.0) | 9.0 (8.0–10.0)                | 10.0 (9.5–11.0)               | 10.0 (9.0–10.0)               | 0.656   |
| Posterior wall thickness (mm) | 9.5 (7.3–10.0)     | 10.0 (8.5–11.5)               | 10.0 (7.5–10.0)               | 9.0 (8.0–13.0)                | 0.868   |
| Left ventricular reverse remodelling | 13 (72%)            | 3 (100%)                      | 6 (60%)                      | 4 (80%)                       | 0.487   |
| Outcome of morphometry   |                       |                               |                               |                               |         |
| Cellular diameter (μm)   | 18.4 (17.1, 19.5)     | 18.5 (18.3, 18.6)             | 17.4 (17.0–18.9)              | 19.8 (19.1–20.0)              | 0.277   |
| Nuclear diameter (μm)    | 8.3 (8.0, 9.0)        | 8.2 (8.1, 8.3)                | 8.3 (7.7–8.5)                 | 9.1 (8.1–9.3)                 | 0.509   |
| Proportion of fibrosis (%) | 10.3 (7.4, 18.3)   | 13.2 (10.3, 16.0)             | 10.0 (7.1–11.3)               | 25.0 (12.3–30.3)              | 0.147   |
| Genetic analysis         |                       |                               |                               |                               |         |
| Sarcomeric gene variants⁵ | 4 (22%)               | 0 (0%)                        | 2 (20%)                      | 2 (40%)                       | 0.007   |
| Non-sarcomeric gene variants | 10 (56%)             | 1 (33%)                       | 5 (50%)                      | 4 (80%)                       | 0.380   |
| Nuclear gene variant⁴     | 1 (6%)                | 0 (0%)                        | 0 (0%)                       | 1 (20%)                       | 0.252   |
| Gap junction gene variant ⁶ | 3 (17%)              | 1 (33%)                       | 1 (10%)                      | 1 (20%)                       | 0.619   |
| Channel gene variant⁶     | 4 (22%)               | 0 (0%)                        | 2 (20%)                      | 2 (40%)                       | 0.407   |
| Follow-up data           |                       |                               |                               |                               |         |
| Ajmalidine                | 1 (6%)                | 0 (0%)                        | 1 (10%)                      | 0 (0%)                        | 0.655   |
| ICVD or CRT-D implantation | 2 (11%)              | 0 (0%)                        | 1 (10%)                      | 1 (20%)                       | 0.675   |
| Ventricular tachyarrhythmia | 4 (22%)              | 0 (0%)                        | 2 (20%)                      | 2 (40%)                       | 0.407   |
| Heart failure recurrence  | 4 (22%)               | 0 (0%)                        | 2 (20%)                      | 2 (40%)                       | 0.407   |
| Heart transplantation     | 0 (0%)                | 0 (0%)                        | 0 (0%)                       | 0 (0%)                        | —       |
| Mortality                 | 2 (11%)               | 0 (0%)                        | 1 (10%)                      | 1 (20%)                       | 0.675   |
| Mean follow-up duration (years) | 8.0 (7.7–8.7)   | 8.1 (7.9–8.4)                 | 8.1 (7.9–8.9)                 | 7.9 (7.5–7.9)                 | 0.461   |

Abbreviations as in Table 2.
⁵Sarcomeric genes were NEXN, SNTA1, TTNA, DMD, MYLK3, and MUP.
⁶Nuclear gene was LMNA.
⁷Gap junction genes were DUSP, PKP2, and GJD4.
⁸Channel genes were SCN4B, SLC25A4, KCNA4, and KCNH2.
myofilaments with residual but abnormal Z-line structure (Figure 2B). Patients with TTN variants had ultrastructural alterations compatible with the location of the titin domain where their mutated nucleotide sequences were located. P2 had a nonsense variant, and P3 and P4 had frameshift variants. All variants were in exon 325 that encodes the A-band domain. Ultrastructural analysis showed obscured M-line and sparse myofilaments (Figure 2C and 2D). PS had a TTN frameshift variant (c.14488_14491delCAGT), which was in Exon 45, the I-band domain. Ultrastructural analysis showed sparse myofilaments with mitochondrial infiltrates and glycogen granules. The patient also had focal areas of disrupted sarcomere structure with lipid droplets (Figure 2E and 2F); this region had fewer glycogen granules and mitochondria than the surrounding areas, while the boundary was unclear. In five patients with sarcomeric gene variants, diffuse myofilament lysis was not found except in one case (P4), who also had a DSP variant.

Three patients had the same TMEM43 variant (c.271A>G). One had tiny nuclear changes with diffuse myofilament lysis expanded around the nuclei (P6, Table 4). The other two patients (P7 and P8), both with personal histories of potentially fatal ventricular tachyarrhythmia, had extensive diffuse myofilament lysis surrounding nuclei with lipofuscin deposition (Figure 2G). Diffuse myofilament lysis was observed in the perinuclear area of cardiomyocytes in all three cases (perinuclear vs. peripheral was 100% vs. 9%; P = 0.011).

Two patients with DSP variants (P4 and P9, Table 4) showed diffuse myofilament lysis spreading to the peripheral areas of cardiomyocytes (perinuclear vs. peripheral was 8% vs. 100%; P = 0.033), occurring at both sides of intercalated disc structures containing desmosome-derived elements with high electron density (Figure 2D).

There were three patients with same TRPM4 variant (c.1532T>A) and two patients with same TBX5 variant (c.52G>C); however, it was difficult to determine whether there were specific changes based on ultrastructural examination.

Case series with clinical implications

Some patients might have been treated earlier and more effectively if their causal variants in DCM genes were identified. A 36-year-old man (P1, Table 4) hosted a frameshift variant in MYBPC3 (c.2833_2834delCG), classified as LP. Despite optimal therapy, he developed HF due to sustained ventricular tachycardia, and insertion of an implantable cardioverter defibrillator was performed. After HF recurrence, implantable cardioverter defibrillator treatment was changed to cardiac resynchronization therapy defibrillator treatment. Seven years from the first hospitalization, his ventricular arrhythmia could not be controlled, and he died from multiple organ failure due to severe HF. While considering HTx, his condition could not be controlled, and he died from multiple organ failure due to severe HF. While considering HTx, his condition could not be controlled, and he died from multiple organ failure due to severe HF.
Table 5 Patients’ summary with variants of unknown significance

| Case | Age, sex | Ultrastructural findings | Genes | Mutation |
|------|---------|-------------------------|-------|----------|
| P1   | 36, male| Focal derangement        | MYH6  | c.5661G>A |
| P2   | 42, female| Focal derangement      | FHL2  | c.191A>G   |
| P3   | 51, female| Focal derangement      | SCN5A | c.2497G>A  |
| P5   | 48, female| Focal derangement      | RYR2  | c.3423+3G>A |
| P7   | 47, female| Focal derangement      | DMD   | c.4859A>G  |
| P8   | 58, male| Focal derangement        | LAMA4 | c.4494delT |
| P9   | 64, male| Focal derangement        | SCN10A| c.4205T>C  |
| P10  | 58, male| Focal derangement        | RBM20 | c.3067G>T  |
| P11  | 44, male| Focal derangement        | TPM1  | c.2T>C     |
| P12  | 72, male| Focal derangement        | MLIP  | c.1309C>T  |
| P14  | 50, male| Focal derangement        | TBX20 | c.374C>T   |
| P15  | 62, male| Focal derangement        | NEXN  | c.919C>A   |
| P16  | 39, male| Focal derangement        | SNTA1 | c.1432G>C  |
| P17  | 62, male| Focal derangement        | TTN   | c.37202-2G>T|
| P18  | 69, female| Focal derangement       | MYLK3 | c.844C>G   |
| P19  | 56, male| Focal derangement        | LMNA  | c.1123G>A  |
| P20  | 35, male| Focal derangement        | JUP   | c.1907G>A  |
| P21  | 41, male| Focal derangement        | PKP2  | c.592G>A   |
| P22  | 40, male| Focal derangement        | SCN4B | c.463+3A>T |
| P23  | 64, female| Focal derangement       | KCNAD | c.1103_1110delACTTCATC|
| P24  | 39, male| Focal derangement        | DTA  | c.2095C>T  |
|      |        |                         | KCHN2 | c.28C>T    |
|      |        |                         | TBX5  | c.1034C>T  |
| P25  | 71, male| Focal derangement        | RBM20 | c.1552C>T  |
| P26  | 44, male| Focal derangement        | OXTR  | c.1126C>T  |
| P27  | 70, male| Focal derangement        | CALR3 | c.28G>A    |

ACMG, American College of Medical Genetics.

*Can be upgraded to likely pathogenic variants if they have other evidence supporting pathogenicity.
*Can be upgraded to likely benign variants if they have other evidence supporting benign impact.

The present study compared the results of whole-exome sequencing and electron microscopy findings. We previously worsening and HTx was not implemented. Ultrastructural findings of EMB at his initial admission were not so severe, with only sarcomeric changes (Figure 2B). EMB was re-examined because of concern of acute myocarditis when his HF became uncontrollable immediately before his death. Acute myocarditis was negative histologically, but severe findings were observed by electron microscopy, especially diffuse myofilament lysis and lobulated nuclei with highly condensed chromatin (Figure 3A).

A 47-year-old woman (P7, Table 4) hosted a TMEM43 variant (c.271A>G) designated as LP. She had chest pain at admission due to HF, and the acetylcholine load test provoked coronary artery spasm. As atrial fibrillation was also observed, myocardial ischaemia and arrhythmia were thought to be the cause of HF. Beta-blockers were avoided to prevent exacerbating coronary spasms. After 13 months, severe decompensated HF recurred. After repeated HF attacks, she received an HTx 5 years after the onset of HF. Ultrastructural findings of EMB at her initial admission with HF showed diffuse myofilament lysis, areas where myofilaments were replaced with mitochondrial hyperplasia. Mitochondrial abnormality (Figure 3B) and mitophagy, as activated selective autophagy (Figure 3C), were also found.

**Discussion**

The present study compared the results of whole-exome sequencing and electron microscopy findings. We previously
Figure 2  Ultrastructural findings in cardiomyocytes. (A) Normal cardiomyocytes of a patient with dilated cardiomyopathy, without any genetic variants (40-year-old man). m, mitochondria; N, nucleus. (B) P1 with a MYBPC3 variant (c.2833_2834delCG) had disorganized sarcomeric thick filaments (yellow arrows). The Z-line (z) remained, but some aggregates appeared club shaped. m, mitochondria. (C) P2 hosted a TTN nonsense variant (c.71112T>A) in exon 325, encoding the A-band domain. The M-line was absent, and sparse but organized myofilaments without thin filament were found. The Z-line (Z) structure is also maintained, and Z-line interval is constant compared with (A). m, mitochondria. (D) P4 had a TTN frameshift variant (c.72233delT) in exon 325 and a DSP missense variant (c.4996C>T). The sparse myofilament pattern is similar to (C). Diffuse myofilament lysis (ML) spreads to both sides of cell adhesion with abnormal desmosomes (red arrows). Autophagic vacuoles (AV) appeared in areas of degeneration. Li, lipofuscin; m, mitochondria. (E) P5 had a TTN frameshift variant (c.14488_14491delACGT) in exon 45, encoding the I-band domain. The cardiomyocytes contained focal areas of disrupted sarcomeric structure (yellow asterisks) with lipid droplets (L). The nucleus (N) showed a normal form. m, mitochondria. (F) Higher magnification of (E) shows that thick myofilaments (yellow arrows) scatter to several directions. The boundary is unclear and includes fewer glycogen granules (g) and mitochondria (m) than surrounding areas of cardiomyocytes. Lipid droplets (L) are a finding suggestive of acute myocardial damage.16 Li, lipofuscin; m, mitochondria. (G) In cardiomyocytes of P8 with a TMEM43 variant (c.271A>G), diffuse myofilament lysis (ML) spreads near the abnormal-shaped nucleus (N). Scale bars = 2 μm (A, C), 1 μm (B), 5 μm (D, E, G), and 500 nm (F).
Figure 3 Ultrastructural findings in cardiomyocytes of patients with clinical manifestations of dilated cardiomyopathy. (A) At end-stage heart failure, cardiomyocytes of P1 with a MYBPC3 variant (c.2833_2834delCG) showed severe ultrastructural changes, such as abnormally shaped nuclei (N), diffuse myofilament lysis (ML) with autophagic vacuoles (AV) of various sizes and lipofuscin (Li). (B) P7 hosted a TMEM43 variant (c.271A>G). In the cardiomyocytes, mitochondrial hyperplasia (m) spreads to replace areas of myofilament disappearance, including degenerated mitochondrion (bold yellow arrows). AV, autophagic vacuole; L, lipid droplet. (C) In cardiomyocytes of P7, mitophagy is observed; an autophagic vacuole with a double membrane structure (surrounded by yellow arrowheads) envelops the abnormal mitochondrion (m) with swelling cristae. L, lipid droplet. Scale bar = 5 μm (A) and 2 μm (B, C).

showed that DCM patients with myofilament changes in LV cardiomyocytes had poor prognosis7 and difficulty recovering cardiac function.16 Myofilament changes were classified as either focal derangement of myofilaments (sarcomere damage) or diffuse myofilament lysis (disappearance of most sarcomeres in cardiomyocytes).7 In the present study, five patients with sarcomere-related gene variants were classified as P/LP; four of them (80%) showed focal myofilament derangement, and the ultrastructural findings were consistent for each gene variant. Our patient with a MYBPC3 variant (c.2833_2834delCG) had cardiomyocytes with disorganized myofilaments with Z-band and thin filaments remaining (Figure 2B). This is reminiscent of the electron microscopy findings of skeletal muscle sarcomeres in patients with myopathy associated with a MYBPC3 variant (c.2882C>T).17 Cardiac myosin-binding protein C binds to myosin filaments, consistent with the disorganization of thick filaments in cardiomyocytes, which appear to be myosin filaments. Titin is the largest human protein (33,000 amino acids), and a variety of ultrastructural forms have been reported because of TTN variants.18 Three of our patients had variants in exon 325 of TTN, which encodes the A-band domain of titin.15 In those patients, electron microscopy revealed that the area around the M-line was unclear, and thick filaments became sparse with a loss of thin filaments (Figure 2C). One patient had a variant in exon 45, encoding an I-band domain15 between the Z-line and A-band. In addition to sparse sarcomeres, this patient’s cardiomyocytes had small focal areas of disrupted sarcomere (Figure 2E) where scattered bundles of thick filaments were oriented in random directions (Figure 2F). These were similar to the ultrastructural findings in the skeletal muscle of patients with titin-related myopathy with mutations in the titin A-band and I-band domains, respectively.18

Diffuse myofilament lysis has previously been recognized in acute myocarditis due to Coxsackie virus infection19 and in doxorubicin-induced cardiomyopathy.20 It was considered to be a non-specific change due to various causes rather than as a result of the spread of focal myofilament derangement. We identified diffuse myofilament lysis in DCM associated with non-sarcomere-related gene variants, such as TMEM43 and DSP. Even in a patient with a MYBPC3 variant (P1), diffuse myofilament lysis was shown in cardiomyocytes obtained by EMB at the time of progressing to end-stage HF despite not being observed at the onset of HF (Figure 3A). Therefore, we consider diffuse myofilament lysis as an indication of a process leading to cardiomyocyte failure. In cardiomyocytes of patients with TMEM43 variants, diffuse myofilament lysis spreads around the nuclei (Figure 2H). TMEM43 encodes Luma, a nuclear membrane protein that transmits mechanical force from the cytoplasm to the nuclei, like Emerin and Lamin A/C.21 In contrast, patients with DSP variants had diffuse myofilament lysis in the periphery of cardiomyocytes, with abnormal cell adhesion on both sides (Figure 2D). DSP codes for desmoplakin, which is one of the proteins that make up the outer dense plaque of desmosomes. At the onset of HF in DCM patients, distribution patterns of diffuse myofilament lysis in cardiomyocytes correlate with variants in known causative genes.

When HF occurs and DCM is diagnosed, optimal treatment commences. Some DCM patients had improved cardiac function and elimination of HF symptoms by treatments to reduce mechanical overload.2 However, myocardial damage due to DCM may develop if there are underlying factors, like...
pathogenic gene variants, and there is subsequent exposure to triggering factors, such as mechanical stress. A randomized study indicated that HF symptoms and cardiac dysfunction relapse could be triggered by withdrawing optimal treatment after initial improvement of symptoms. This indicates that myocardial damage in DCM can progress subclinically, even after HF improves and cardiac function recovers. A genotype-phenotype correlation has begun to show that DCM caused by LMNA variants has a poorer prognosis than sarcomere-related gene variants. However, our patient with a MYBPC3 variant (P1) had intractable HF, and ultrastructural changes in cardiomyocytes reflected severe disease progression. The present study suggests that DCM involves several conditions caused by variants in known disease-causing genes. Clarifying the causative gene in each DCM patient might inform early decision on intervention methods, such as medication, mechanical therapy, or HTx.

The current medical approach for DCM is diagnosis based on the clinical phenotype and providing treatment for HF according to symptoms. This runs the risk of delaying care for DCM due to time spent excluding other causes of cardiac dysfunction, or giving priority to treatment of co-morbidities. For example, patient P7 also had vasospastic angina and paroxysmal atrial fibrillation. As such, priority was given to treating these co-morbidities and considering these as the cause of HF at her initial admission. Beta-blockers and diuretics were not introduced, resulting in a significant delay to the treatment of HF, which may have affected the subsequent outcome of progression to HTx. P7 had an LP variant in TMEM43 (c.271A>G). Electron microscopy revealed expanded areas of myofilament loss replaced by mitochondrial hyperplasia. There were also various abnormal mitochondrial lesions and mitophagy (Figure 3B and 3C). TMEM43 is one of the causative genes of arrhythmogenic cardiomyopathy, and there is a risk of sudden cardiac death, even with VUS. If the gene variant was known at the time of diagnosis, earlier consideration could have been given to treatment, including HTx. While electron microscopy findings are still developing as evidence to judge myocardial damage and might carry a risk of overestimation when considered alone, findings with established evidence, such as myofilament changes and mitochondrial abnormalities, which can be readily determined, become helpful in diagnosis. We propose using ultrastructural findings as supporting evidence to determine if gene variants are pathogenic.

Conclusions

While diffuse myofilament lysis in cardiomyocytes of DCM patients may be a non-specific finding, derangement pattern of myofilament and subcellular distribution of myofilament lysis might implicate particular causal genes. Future, large-scale studies are required to clarify the relationship between ultrastructural findings and the causative genes of DCM.

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Conflict of interest

None declared.

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