Patient-specific induced-pluripotent stem cell-derived cardiomyocytes recapitulate the pathogenic phenotypes of dilated cardiomyopathy due to a novel \( \text{DES} \) mutation identified by whole exome sequencing

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In this paper, we report a novel heterozygous mutation of A285V codon conversion on exon 4 of the desmin (\( \text{DES} \)), using whole exome sequencing (WES) in an isolated proband with documented dilated cardiomyopathy (DCM). This mutation is predicted to cause three-dimensional structure changes of \( \text{DES} \). Immunohistological and electron microscopy studies demonstrated diffuse abnormal \( \text{DES} \) aggregations in DCM-induced-pluripotent stem cell (iPSC)-derived cardiomyocytes, and control-iPSC-derived cardiomyocytes transduced with A285V-DES. DCM-iPSC-derived cardiomyocytes also exhibited functional abnormalities \textit{in vitro}. This is the first demonstration that patient-specific iPSC-derived cardiomyocytes can be used to provide histological and functional confirmation of a suspected genetic basis for DCM identified by WES.

INTRODUCTION

Mutations in >30 genes related to different components of the cytoskeleton, the sarcomere or the nuclear lamina have been implicated in 30–35% of patients with dilated cardiomyopathy (DCM) (1). Desmin (\( \text{DES} \)), encoded by \( \text{DES} \), is a muscle-specific intermediate filament protein that maintains the overall structure and cytoskeletal organization within cardiomyocytes and striated muscle cells (2). Mutations in the \( \text{DES} \) can lead to dilated, hypertrophic and restrictive cardiomyopathies (1,3), and even arrhythmogenic right ventricular cardiomyopathy (4). \( \text{DES} \)-related cardiomyopathies are

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characterized by accumulation of DES-positive protein aggregates that disrupt the filamentous network of cardiomyocytes with consequent myocardial fibrosis and dysfunction (5). Detection of pathogenic mutations in patients with DCM nonetheless remains a challenge as known causal variants, including those in DES, account for <5% of cases. Whole exome sequencing (WES) is a potentially powerful and cost-effective strategy for identifying rare and novel pathogenic mutations in various genetic disorders, including DCM (6,7). Its use in sporadic cases of DCM, nonetheless, remains challenging, with the inability to confirm co-segregation between mutation and disease (8). Here, we report the use of patient-specific cardiomyocytes derived from human induced-pluripotent stem cells (iPSCs) to provide histological confirmation of a novel DES mutation identified by WES in an isolated proband with documented DCM.

RESULTS

Patient

A 43-year-old Caucasian man presented with palpitations and pre-syncope attack. His father and brother had died suddenly at age 54 and 38 years, respectively, presumably due to previously unidentified cardiac disease (Fig. 1A). Holter monitoring in our patient revealed frequent runs of non-sustained ventricular tachycardias. Transthoracic echocardiogram revealed dilated left ventricle with an impaired left ventricular (LV) ejection fraction of 32%. Coronary angiogram showed normal coronary arteries. Cardiac magnetic resonance imaging demonstrated diffuse gadolinium enhancement over the LV lateral wall and impaired LV ejection fraction of 30% (Supplementary Material, Video S1). He did not report any muscle weakness and no evidence of skeletal myopathy was noted on physical examination or biochemical test on creatine kinase. He underwent implantation of a cardioverter defibrillator and received multiple appropriate device therapies over the LV lateral wall and impaired LV ejection fraction (0.001 vs. wild-type and vector control). 

Whole exome sequencing analysis

The WES was carried out by DeCODE, using a blood sample from the patient. The sequencing procedure captured the paired-end reads in two FASTQ files which contained a total of 92788582 76-bp paired-end sequence read. The raw sequence read passed all of the quality checks in FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). After read alignment in the primary pipeline, the coverage statistics showed that about 62% of the reads were mapped to the targets (as listed in the BED file provided by DeCODE) or within the flanking 150 bp regions. Of the whole targeted exome, 91% were covered by at least 1 read, 75.7% by at least 5 reads and 63.04% by at least 10 reads.

By combining the result sets from the primary pipeline and the secondary pipeline set, a total of 26481 variants were called. Among the called variants, there were 43 stop-gain or stop-loss variants (Table 1). 5 of them were novel mutations but with unknown functional relevance. We investigated the remaining stop-gain and stop-loss mutations for their functional significance and diseases linkage. Among them, there was only one candidate, the Q45X mutation in the AMP deaminase 1 (AMPD1; rs17602729) gene, appeared to be relevant to the present study. This mutation was previously related to metabolic myopathy (9), however, upon further investigation, we found out that this mutation is presented in ~3.3% of asymptomatic Caucasians, which is not rare enough to be considered as a highly penetrate-causative candidate variant.

Other than the stop-gain/stop-loss mutations, there were 7832 unique nonsynonymous mutations. According to our prioritization criteria for causal variant candidacy (see Materials and Methods), high ranking was assigned to 5308 variants that were called by all the pipelines. Among these variants, 208 were novel and only 26 of them were predicted as damaging by SIFT and were found to be residing within a protein domain (Supplementary Material, Table S1). Through extensive literature search and functional analysis using online resources, one single mutation stood out as our prime causal variant candidate. We identified a heterozygous C→T mutation in the DES gene, physically located at chr 2:219993579. DES was previously related to DCM (1,3,10), the phenotype that closely matched to our case. This placed the DES mutation to the top of our priority list. We proceeded to do more subsequent analyses to characterize this particular novel DES mutation.

Functional effects of A285V mutation

To validate the presence of C→T mutation at base e940, Sanger sequencing was performed (Fig. 1B). This mutation causes an A285V codon conversion on exon 4 of the DES gene, and is predicted to cause changes in the three-dimensional protein structure of DES (Fig. 1C). The function of DES may be relevant to the phenotype of our patient, as DES mutations have been reported in DCM (1,3,10). More importantly, the novel A285V mutation site has been found to reside on the coiled 2A region of the encoded DES which is highly conserved throughout evolution in mammals (Fig. 1D).

The functional effects of A285V mutation on DES expression were characterized by cloning of wild-type and mutant A285V-DES and expression in mammalian HEK293 cells (DES−ve, Vimentin+ve) using lentivirus expression vector. Western blot analysis revealed that A285V-DES is expressed as an intact protein with the same molecular size as wild-type-DES (Fig. 1E). As shown in Figure 1F, wild-type-DES interacted with vimentin, another intermediate filament protein, to form organized filament in mammalian cells as reported previously (10). On the other hand, over-expression of A285V-DES in HEK293 cells resulted in diffuse abnormal DES aggregation in up to 70% of cells without clear organized filament network (Fig. 1F, P < 0.001 vs. wild-type and vector control).

Human iPSC-derived cardiomyocytes

We successfully generated iPSCs from primary fibroblasts derived from our DCM patient with an A285V-DES mutation (DCM-iPSC) using retroviral transduction of the reprogramming factors SOX2, OCT4, c-MYC and KLF4. Human
embryonic stem cell-like colonies were typically observed 14–21 days after retroviral transduction and were manually dissected and clonally expanded on murine embryonic fibroblasts. Several clones of DCM-iPSCs were generated, two of which were continuously propagated and used for cardiomyocyte differentiation and characterization. Human control iPSCs were created from fibroblasts from a healthy individual as reported previously (11). The authenticity of these human iPSCs were confirmed with the expression of a panel of pluripotent markers, transgene silencing, OCT4 promoter demethylation and teratoma formation after inoculation into severe combined immunodeficiency mice (Supplementary Material, Fig. S2). The genomic DES locus was sequenced in all the iPSC clones, confirming the integrity of the locus and the absence of retroviral DNA. The expected A285V-DES C→T mutation was detected in all iPSC clones derived from the DCM patient but not in those cells derived from the control subject.

Undifferentiated DCM- and control-iPSCs were co-cultured with endoderm-like cells to induce cardiac differentiation. Spontaneously beating clusters (Supplementary Material, Videos S2 and S3) started to appear at 12–14 days of differentiation, and were isolated and then plated for subsequent use. Immunofluorescence staining was performed to confirm cardiac phenotype and expression of DES protein. As shown in Figure 2A, there was co-localization of immunostaining for DES and sarcomeric protein cardiac Troponin-T with full network of DES filaments in control-iPSC-derived cardiomyocytes. Similarly, DES was co-localized with the cytoskeleton-binding protein α-Actinin (Supplementary Material, Fig. S3A) and F-actin (Phallolin staining, Supplementary Material, Fig. S3B) in control-iPSC-derived cardiomyocytes as revealed by immunostaining. Only poor co-localization of DES with cardiac Troponin-T (Fig. 2A), α-Actinin (Supplementary Material, Fig. S3A) or F-actin (Supplementary Material,
Fig. S3B) and incapable of assembling into DES network was observed in DCM-iPSC-derived cardiomyocytes, whilst diffuse isolated aggregations of DES-positive protein were observed in up to 70% of these cells (Fig. 2A, \(P, 0.01\) vs. control-iPSC-derived cardiomyocytes). Disruption of the F-actin filament was also noted in DCM-iPSC-derived cardiomyocytes, probably due to malformation of the Z-disc with its protein interacting partners (Supplementary Material, Fig. S3B).

To further confirm the causal relationship between A285V-DES mutation and the phenotypes observed in DCM-iPSC-derived cardiomyocytes, wild-type-DES or A285V-DES was transduced into control-iPSC-derived cardiomyocytes using lentivirus-mediated transduction. As shown in Figure 2B, co-localization of DES and cardiac Troponin-T with full network of DES filaments was observed in control-iPSC-derived cardiomyocytes transduced with wild-type-DES or vector alone. In contrast, control-iPSC-derived cardiomyocytes transduced with A285V-DES simulated the phenotypes of DCM-iPSC-derived cardiomyocytes in which poor co-localization of DES with cardiac Troponin-T and incapable of assembling into DES network were seen, and diffuse isolated aggregations of DES were observed (Fig. 2B).

We next used transmitted electron microscopy to examine the ultra-structures of the iPSC-derived cardiomyocytes. Control-iPSC-derived cardiomyocytes had normal architectures in their sarcomeres, whilst DCM-iPSC-derived cardiomyocytes showed Z-disk streaming with accumulation of granulofilamentous materials or pleomorphic dense structures in adjacent to the Z-disk or between the myofibrils (Fig. 2C).

There was no significant difference in the differentiation efficiency of cardiomyocytes among the various DCM- and control-iPSC clones (Supplementary Material, Fig. S3C–E). Moreover, similar abnormal immunostaining (data not shown) as well as ultrastructure changes on electron microscopy (Fig. 2C) were observed from DCM-iPSC-derived cardiomyocytes obtained from two different clones of iPSC generated from this patient. Nevertheless, DCM-iPSC-derived cardiomyocytes in which poor co-localization of DES with cardiac Troponin-T and incapable of assembling into DES network were seen, and diffuse isolated aggregations of DES were observed (Fig. 2B).

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Table 1. A list of 43 stop-gain or stop-loss variants identified in the DCM patient

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cardiomyocytes exhibited significant functional abnormalities compared with the control-iPSC-derived cardiomyocytes as demonstrated by the diminished maximum rate of calcium ion re-uptake (Fig. 3A), slower spontaneous beating rate (Fig. 3B, \( P < 0.05 \)) and failure to have sustained respond to the inotropic stress induced by administration of isoproterenol (Fig. 3C; Supplementary Material, Videos S2 and S3).

**DISCUSSION**

This is the first demonstration that patient-specific iPSC platform can be used to generate cardiomyocytes for validation and recapitulating the pathogenic phenotypes of DCM caused by a novel mutation identified by WES. In this case, we opted to apply multiple next-generation sequencing analysis pipelines to ensure high sensitivity detection of the variants due to limited familial information of our patient. The search for a causal variation in our patient led to the identification of a novel mutation in the DES gene. It encodes a main muscle-specific inter-filament protein-DES, known to interact with other protein molecules to support myofibrils at the Z-disc level (2). However, the pathogenicity of this novel DES mutation could not be validated by demonstrating co-segregation of phenotypes over one or more generations within a family (6). Alternatively, detailed histological examination can be used to demonstrate the pathological features as predicted by the functional and morphological changes that result from the mutations. However, this approach is limited by the need for invasive
tissue biopsy or on postmortem samples (12). Latest iPSC technology (13) provides a unique opportunity to generate different patient-specific tissue samples, including cardiomyocytes via more accessible somatic cell sources (14). We used a human iPSC platform to create patient-specific iPSC-derived cardiomyocytes with this novel mutation to provide histological proof for possible DES-related DCM in this patient.

To date, there are about 49 mutations reported on DES. Many of them are reported as pathogenic (15) and some have been linked to familial cardiomyopathies (3,4,16,17). The A285V mutation described here is novel and located in the coil 2A domain in which only two other mutations have been reported at a different codon location (L274P, L274R) (18). The molecular mechanisms underlying the DES-related cardiomyopathy are intriguing, since they can be manifested as DCM, hypertrophic and restrictive cardiomyopathies (3,16,17) and arrhythmogenic right ventricular dysplasia (4); similar phenotypes are observed in mutations related to the accessory proteins associated with DES, such as αB-crystallin, myotilin, Z band alternatively spiced PDZ-motif protein, Bcl-2-associated athanogene 3 and Filamin C (2,5).

Pathologically, DES-related cardiomyopathy is characterized by sarcoplasmic protein aggregates formed by the misfolded mutant proteins that lead to weakening and malfunctioning of cardiomyocytes. Our observations in patient-specific iPSC-derived cardiomyocytes as well as in mammalian cells and control-iPSC-derived cardiomyocytes with focused expression of A285V-DES are consistent with the idea that abnormal DES-positive protein aggregates due to DES mutation can cause structural and functional abnormalities in cardiomyocytes. Protein aggregation was observed even when A285V-DES was expressed in HEK293 cells (without any baseline DES expression) and the phenotype was recapitulated in the wild-type control-iPSC-cardiomyocytes (with normal baseline DES expression). Similarly, protein aggregation had also been reported in mammalian cells after over-expression of other DES mutation located in the coil 2A domain (18). These observations support the notion that the novel DES mutation reported in this study also exhibited a dominant-negative manner as described previously (19,20). Furthermore, electron microscopy of patient-specific iPSC-derived cardiomyocytes demonstrated features consistent with those early changes observed in human desminopathy (21).

Figure 3. Physiological characterization of induced-pluripotent stem cells derived cardiomyocytes. (A) Calcium (Ca\(^{2+}\)) handling property of control-iPSC-derived cardiomyocytes (n = 15); and DCM-iPSC-derived cardiomyocytes (n = 15). Left panel shows the representative tracing of rhythmic spontaneous Ca\(^{2+}\) transients of control-iPSC-derived cardiomyocytes and DCM-iPSC-derived cardiomyocytes. Right panel shows the relative amount of Ca\(^{2+}\) released (upper), the maximum Ca\(^{2+}\) released (middle) and the maximum Ca\(^{2+}\) reuptake rate (bottom). F/F0 represents changes in the fluorescence intensities and F/F0/S denotes the F/F0 rate per second. (B) Beating rates (beats per minute) of the control (n = 15) and DCM-iPSC-derived cardiomyocytes clusters (n = 4) in the absence or presence of isoproterenol (ISP). At baseline, the beating rate of DCM-iPSC-derived cardiomyocytes was significantly slower than the control-iPSC-derived cardiomyocytes but their beating rates accelerated after administration of ISP. (C) Representative tracings of the rate of spontaneous Ca\(^{2+}\) transient in the beating control (upper) and DCM-iPSC-derived cardiomyocytes (lower) at baseline and after administration of 10 μM of ISP (red arrow). ∗P < 0.05 vs. control-iPSC-derived cardiomyocytes; #P < 0.05 vs. DCM-iPSC-derived cardiomyocytes.
MATERIALS AND METHODS

DNA sample, exome capture and sequencing

Genomic DNA sample was extracted from EDTA peripheral blood using the QIAamp DNA blood kit (QIAGEN GmbH, Hamburg, Germany). The WES was carried out by DeCODE. Exome capture by hybridizations and coding sequence enrichment was performed using the Agilent SureSelect Human All Exon Kit (Agilent Technologies Inc, Santa Clara, CA, USA). The enrichment was designed to cover 1.22% of the human genome (~38 Mb) corresponding to the exons defined in the Consensus Coding Sequences database. The targeted regions cover 165637 coding exons that constitute 17134 genes. Massive parallel sequencing was performed using Illumina GAIIx. The exome sequences were captured as 76-bp paired-end reads in standard FASTQ format.

We used FASTQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) to screen the quality of the raw read data. After quality check, the reads were submitted to a pipeline developed primarily for WES analysis. Its goal was to align the reads to the reference genome, filter out reads with poor quality, and then call variants from the exome profile for further downstream analysis. One limitation of this study was not to obtain other case samples from the family for validation by means of segregation study. To ensure the robustness of the analysis process and to minimize the chance of false positive in detecting the potential causative variants, we also analyzed the exome sequencing data through a secondary WES pipeline set.

Based on the family history of sudden death as well as no apparent cause for the phenotype of the patient, we considered the underlying disease to be Mendelian and likely caused by a rare variant of high penetrance, and prioritize the variants based on this hypothesis. As validating the WES findings by family segregation study was not feasible in this patient, we had to be more cautious that any candidate variants called were not incidental findings and any credible potential candidates would be filtered out by one single WES analysis pipeline. In this line, we set up a secondary WES analysis pipeline set with additional aligners and variant calling programs to increase the sensitivity of variant calling (Supplementary Material, Fig. S1A). It was supposed that variants called consistently by multiple pipelines were less likely to be false positives. Furthermore, this could increase the sensitivity of the WES analysis as we cannot rule out whether one aligner may perform better than the others in a particular sequence region. We gathered the called variants and then assessed them against a set of stringent prioritization criteria for causative variation candidacy. The top candidate variants were subjected to further scrutinization for functional significance. They were examined individually for fitness in protein coding and functional relevance to the cardiac phenotype.

The primary whole exome sequencing analysis pipeline

The Novoalign program (www.novocraft.com/main/page.php s=doc_novoalign_refmanual) was used to strip the adapters and align the sequenced paired-end reads against the human reference genome Build 36 downloaded from UCSC genome browser. Novoalign scored the alignment locations. Reads with excess low-quality base positions were automatically excluded from alignment. Reads that mapped to multiple locations were also excluded from downstream analysis. We enabled quality recalibration in the Novoalign command using the ‘-k’ option.

After alignment, polymerase chain reaction duplications were marked by Picard tool and excluded from downstream analysis. The sequence depth and breadth coverage were calculated using the BED file from DeCODE and the BEDTools software (22).

SAMtools pileup was used to compute consensus sequence, calling single-nucleotide polymorphisms (SNPs) and short indels. Variant quality filtering was performed using SAMTools varFilter and awk programming script. The minimum mapping quality scores for indel and SNP calls were set to 50 and 20, respectively. Quality variants were annotated using VariantClassifier. Variant novelty check was performed by comparison with dbSNP131, 1000 Genome pilot SNP calls and variants identified from an internal exome project database. For nonsynonymous variants that cause amino acid substitution, prediction of functional deleteriousness for the called variants was obtained using the SIFT program (http://sift.jcvi.org/). Downstream follow-up multiple sequence alignment was performed for putative variants using ClustalX (23).

The secondary whole exome sequencing analysis pipeline set

The challenge for analyzing WES data for a Mendelian disorder is to identify a single causative variant. When one tries to identify a rare variant with only one sample, it is extremely important to formulate a pipeline with high sensitivity so that the causative variant will not be missed due to alignment bias or call bias. To increase the sensitivity of our exome sequencing analysis, we deployed three additional aligners and two different variant callers in our secondary WES pipeline set (Supplementary Material, Fig. S1B). The three aligners aligned the sequenced reads to the same reference human genome hg18 downloaded from UCSC genome browser.

The alignment files output from the three aligners were subsequently processed using the GATK toolkit (24). Duplicated reads were marked and excluded from downstream processing as in the primary pipeline. To aid variant detection, we re-aligned the reads using GATK IndelRealigner and recalibrated the base quality scores. SAMtools mpileup and GATK UnifiedGenotyper were applied independently for variant calling. For any variant to be called in either tool, we set the requirement for minimum read depth to 8 and a minimum mapping quality score to 30, and requiring the called variant to be situated within the captured regions. When calling with mpileup, we also enabled the extended BAQ calculation that helped to increase the sensitivity of variant detection. Gene-based annotation of the called variants was done using ANNOVAR (http://www.openbioinformatics.org/annovar/) (25).
Prioritizing candidate variants

The challenge of the present case was analyzing WES data for a Mendelian disorder without familial data. The causative genetic variation has to be rare and functionally deleterious to a great extent. Our secondary WES processing pipeline set was employed to supplement findings from the primary pipeline. The variants called by all pipelines were gathered and analyzed collectively.

We applied the following selection criteria to rank the called variants: (i) variant calling consistency: variants called concordantly by multiple variant caller programs were preferred, (ii) type of protein coding effect: called variants were prioritized according to the type of mutation. For example, stop-gain or stop-loss mutations were ranked higher than nonsynonymous mutations, (iii) variant novelty: variants that were not present in the 1000 Genome Project (CEU population) and the dbSNP132 databases were ranked higher, (iv) variant locality: variants located within a conserved protein domain were of higher interest, hence ranked higher. Domain mapping and search was performed using KGGSeq (26) that accomplishes the task by searching through the Uniprot (27) database, and (v) functional deleteriousness: nonsynonymous variants that were predicted as damaging by SIFT and KGGSeq were assigned a higher rank.

After ranking the called variants, we performed a literature search on the genes where the candidate variants reside using PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and OMIM (http://www.ncbi.nlm.nih.gov/omim). For genes that have been previously reported as functionally significant to DCM or similar phenotypes, we conducted further study to learn more about the feature of the candidate variants.

Validation of whole exome sequencing finding

Direct sequencing using the Sanger sequencing was performed with the specific primers as listed in Supplementary Material, Table S2, e.g. Primer sets J553 and J554 were used for DES-Exon 4 and J614-J615 were used for sequencing the cDNA from clone 1 and clone 2 of iPSC-derived cardiomyocytes from the patient with DCM, respectively.

Family co-segregation testing of the causative variation in this patient was not possible: his father and brother had died and his son refused to undergo screening. The validation process was therefore carried out on an iPSC platform. This involved generation of patient-specific iPSC, differentiation of cardiomyocytes from iPSCs, and functional and histological characterization of patient-specific iPSC-derived cardiomyocytes. The functional effects of the mutation were verified by cloning and expression of the identified candidate gene (i.e. DES) in mammalian HEK293 cells and iPSC-derived cardiomyocytes generated from a healthy control subject (Supplementary Material, Methods).

The study protocol was approved by the local Institutional Review Board (HKCTR-725, http://www.hkclinicaltrials.com) and written informed consent was obtained from the patient.

Detailed protocols on confocal calcium imaging, electrophysiological characterization, transgenic study and electron microscopy were described in Supplementary Material, Methods.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed with the unpaired Student t-test or paired sample t-test as appropriate. Calculations were performed using SPSS software (version 16.0). A P-value of <0.05 was considered statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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