Next-Generation Sequencing Identified Novel Desmoplakin Frame-shift Variant in Patients with Arrhythmogenic Right Ventricular Cardiomyopathy

Xiaoping Lin
the Second affiliated hospital, Zhejiang university school of medicine
Yuankun Ma
the second affiliated hospital, Zhejiang university school of medicine
Zhejun Cai
the second affiliated hospital, Zhejiang univeristy school of medicine
Qiyuan Wang
the second affiliated hospital, Zhejiang university school of medicine
Lihua Wang
the second affiliated hospital, Zhejiang univeristy school of medicine
Zhaoxia Huo
Zhejiang University
Dan Hu
Wuhan University Renmin Hospital
Jian'an Wang
Zhejiang University
Meixiang Xiang (xiangmx@zju.edu.cn)
second affiliated hospital, school of medicine, zhejiang university https://orcid.org/0000-0003-0255-6056

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Abstract

Background Arrhythmogenic right ventricular cardiomyopathy (ARVC) is one of the leading causes for sudden cardiac death (SCD). Recent studies have identified mutations in cardiac desmosomes as key players in the pathogenesis of ARVC. However, the specific etiology in individual families remains largely unknown. Methods A 4-generation family presenting with syncope, lethal ventricular arrhythmia and SCD was recruited. Targeted next generation sequencing (NGS) was performed and validated by Sanger sequencing. Plasmid containing the mutation and wild type (WT) was constructed, western-blot and immunofluorescence were performed to detect the functional change due to the mutation. Results The proband, a 56-year-old female, presented with recurrent palpitation and syncope. An ICD was implanted due to her family history of SCD/ aborted SCD. NGS revealed a novel heterozygous frame-shift variant (c.832delG) in Desmoplakin (DSP) among 5 family members. The variant led to a frame-shift and a premature termination codon, producing a truncated protein. Cardiac magnetic resonance (CMR) of the family members carrying the same variant shown myocardium thinning and fatty infiltration in the right ventricular, positive bi-ventricular late gadolinium enhancement and severe RV dysfunction, fulfilling the diagnostic criteria of ARVC. HEK293T cells transfected with mutant expressed truncated DSP protein, upregulation of nuclear junction plakoglobin (PG) and downregulation of β-catenin, when compared with WT. Conclusion We infer that the novel c.832delG variant in DSP was associated with ARVC in this family, likely through Wnt/β-catenin signaling pathway.

Background

Arrhythmogenic right ventricular cardiomyopathy (ARVC), characterized by gradual myocardium loss and fibrofatty replacement predominately in the right ventricle[1], is one of the primary causes for life-threatening ventricular arrhythmia and sudden cardiac death (SCD), particularly in young and athletes[2]. The clinical presentation varies, including palpitation, syncope, symptomatic ventricular tachycardia, right heart failure and SCD. However, it's not rare that SCD presents as the initial and final manifestation, posting tremendous challenges to the diagnosis post mortem[2, 3]. Diagnosis of ARVC, according to the guideline proposed by the international task force[4], is mainly based on findings of electrophysiological, structural and histological features, family history and genetic testing, hence, for those SCD patients, their family screening is of utmost importance. The current treatments for ARVC are mostly supportive and palliative[5], aiming at alleviation of arrhythmic and heart failure symptom and prevention of SCD, and heart transplantation are the final solution for end-stage patients. However, reversal or a complete cure of the disease requires further in-depth understanding of its etiology and pathogenesis.

Known as genetically determined cardiomyopathy, ARVC is mainly inherited in an autosomal dominant pattern with genetic and phenotypic heterogeneity[6]. Genetic studies have identified mutations in 5 components of cardiac desmosomes as main etiology of ARVC[6], namely Plakophilin 2 (PKP2), Desmoplakin (DSP), Desmoglein 2 (DSG2), Desmocollin 2 (DSC2), and Junction plakoglobin (JUP/PG). Genetic defects of above genes can be found in 40-60% of ARVC patients[4]. However, the specific etiology in individual cases remains largely unknown. First identified in a recessive disorder of keratoderma, woolly hair, and ARVC with left ventricle predominance (Carvajal syndrome)[7], DSP mutations are responsible for nearly 2-12% of ARVC patients[8, 9]. Recent study interestingly found that the left ventricle predominance or bi-ventricle involved phenotype was associated with DSP non-missense mutation[10], but the genotype-phenotype correlations remain uncertain due to small sample size and need to be further characterized in individual families as well as large sample cohorts. Recent studies also suggested mutations that impaired ion channel activities may be causal or modifier to ARVC[11, 12], however, their prevalence is unsure.

In the current study, the underlying genetic defects in a 4-generation family presenting syncope, life-threatening ventricular arrhythmia and SCD were explored using next generation high-throughput sequencing platform, and a novel frame-shift variant *c.832delG* in *DSP* were identified. Cardiac magnetic resonance (CMR) further reveled the diagnosis of ARVC on two asymptomatic family members carrying the identical *DSP* variant. Through co-segregation and genotype-phenotype association analysis, and functional study on HEK293t cells, we infer that the novel frame-shift variant *DSP c.832delG* was associated with ARVC in this family.

Methods

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by Institutional Review Board (IRB) at the Second Affiliated Hospital, Zhejiang University School of Medicine (2016-087). Written informed consent was obtained from all participants. 10 out of total 31 family members in a 4-generation SCD family were recruited in the current study. A complete clinical information including family history, medical history, physical examination, lab test, 12-lead echocardiogram (ECG), 24-hour Holter monitoring, transthoracic echocardiography and CMR were collected.

DNA extraction, target region capture and next-generation sequencing

The proband was selected for next generation sequencing using a commercial capture array (Roche NimbleGen, United States) covering the exons and 50 base pairs of adjacent introns of 1876 cardiovascular diseases associated genes, including inherited cardiomyopathy, arrhythmogenic diseases, congenital heart diseases, mitochondrial diseases, etc.

Genomic DNA was extracted from peripheral blood lymphocytes by standard procedures using Axygen® AxyPrep™-96 Blood Genomic DNA Kit (Axygen，United States). The DNA libraries were constructed and sequenced using the Illumina 2000 platform (Illumina, United States), providing an average sequencing depth of > 100-fold of targeted exons.

Data filtering and bioinformatics analysis

Minor allele frequencies (MAF) of the identified variants in the matched population were compared with 3 major SNP databases: ExAc (http://exac.broadinstitute.org/), 1000 genomes (http://www.1000genomes.org/) and ESP6500 (http://evs.gs.washington.edu/EVS/), as well as in-house

database. Known or published genetic determinants for the human inherited disease were reviewed with HGMD (http://www.hgmd.cf.ac.uk/ac/), OMIM (http://www.omim.org/) and ClinVar databases (https://www.ncbi.nlm.nih.gov/clinvar/). The in silico prediction analysis of non-synonymous variants were performed using 3 known prediction tools, namely PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/) and MutationTaster (http://www.mutationtaster.org/). Sanger sequencing was performed bidirectionally for the verification of *AKAP9 c.10714C>G*, *FLNC c.7778C>G* and *DSP c.832delG* in all participants.

Plasmids construction and site-directed mutagenesis

AICSDP-9:DSP-mEGFP was a gift from The Allen Institute for Cell Science (Addgene plasmid # 87424 ; http://n2t.net/addgene:87424 ; RRID:Addgene_87424) [13]. In order to facilitate the observation following transfection of mutant plasmid, GFP were cleaved and inserted in between the promoter and *DSP* gene. The frame-shift mutation was introduced into a wild-type DSP clone using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The clones were sequenced to confirm the desired mutation and to exclude any other sequence variations.

Western Blots

HEK293T cells were transfected with either wild type or mutant plasmids using lipofectamine 3000 (Invitrogen, USA). Total cell extracts were lysed by RIPA lysis buffer. Nuclear and cytoplasmic extracts were separated using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, China). Protein samples were separated by SDS-PAGE, transferred to PVDF membranes and immunoblotted using the corresponding antibodies.

Confocal Microscopy

Cells seeded on cover slips were fixed with 4% paraformaldehyde (PFA)/PBS, permeabilized in 0.5%(v/v) Triton X-100 (Sigma-Aldrich, USA) and blocked with 5% (w/v) BSA. Then the cells were immunoblotted using the corresponding antibodies, and the cover slips were mounted on microscope slides using mounting medium containing DAPI (Burlingame, CA, USA). Optical sections of cells were obtained using Leica TCS SP8 (Leica Microsystems Inc., Buffalo Grove, IL, USA) with a 63× objective lens.

Statistical analysis

Data were presented as the means ± SEM of at least three independent experiments. Student T test was performed to evaluate differences of continuous variables between two groups. P values of less than 0.05 were considered statistically significant. Statistical calculations were carried out using GraphPad Prism 8.0.1.

Results

Demographic and clinical features of family members

The pedigree of the family was shown in Figure 1B. The proband (Ⅱ-1), a 56-year-old female, was admitted to our hospital due to ICD battery depletion. She presented with a history of recurrent palpitation and syncope for 10 years. An ICD was implanted when she was 49 years old due to a positive family history of SCD/aborted SCD. Since no discharge was detected upon ICD implantation and she remained asymptomatic, she was not on medication therapy. Her paternal grandmother (Ⅰ-2), uncle (Ⅰ-4), and cousin (Ⅱ-16) died suddenly. Her youngest sister (Ⅱ-7) experienced 2 episodes of syncope in her 38 and 40 years old, and an ICD was implanted in her 40 years old following resuscitation from a VT/VF event. Six appropriate discharges were detected in the following 6 years, and a second ICD was replaced when she was 46 years old. She was generally asymptomatic, treating with β-blocker. 10 out of 31 family members were available and recruited for subsequent clinical and genetic evaluation (Figure 1B).

The complete clinical features of all available family members were summarized in Table 1. No obvious depolarization and repolarization or structural abnormalities were detected by either ECG or transthoracic echocardiography tests for all participants. Though Ⅱ-3, Ⅱ-5 and Ⅲ-3 were asymptomatic, CMR were performed due to their potential positive genotype. Myocardium thinning and fatty infiltration was detected in the right apical area in Ⅲ-3 when cardiac function was preserved. However, other than myocardium thinning and fatty infiltration in the right ventricle, positive bi-ventricular late gadolinium enhancement (LGE) and sever right ventricular dysfunction were detected in Ⅱ-5 and Ⅲ-3. In addition, left ventricular function was moderately affected in Ⅲ-3 (Table 1 and Figure 2). Thus, CMR manifestation of Ⅱ-5 and Ⅲ-3 fulfilled the international Task Force criteria for the diagnosis of ARVC[4].

Identification of pathogenic variant

Next generation sequencing was performed on the proband. The average sequencing depths of sample on the targeted regions were 18,992 fold. More than 93.60% targeted regions were covered. We identified a total of 11,583 variants in the proband, including 1,232 non-synonymous variants, 1,494 synonymous variants, 8,857 intronic variants and variants in un-translated regions (UTRs). After filtering common ones, 82 non-synonymous variants distributed in 42 genes were left. Through screening of SCD associated genes, 4 novel heterozygous non-synonymous variants, including 2 missense variants, 1 non-sense variant and 1 frame-shift variant were selected for further in silico analysis (Table 2). Prediction tools yielded controversy results on A-kinase anchoring protein 9 (*AKAP9*) *c.10714C>G* and filamin C (*FLNC*) *c.7778C>G*, favoring them as harmless polymorphisms, thus their clinical significance was uncertain. Though spectrin repeat containing nuclear envelope protein 1 (*SYNE1) c.25954C>T* non-sense variant was predicted to be disease-causing by MutationTaster, none of the family member presented with neuromuscular disorder as previously reported[14]. The *DSP c.832delG* (Figure 1A) was predicted to be disease-causing by MutationTaster, PolyPhen-2 and SIFT. Sanger sequencing further revealed that the proband's father (Ⅰ-2), her two sisters (Ⅱ-3 and Ⅱ-7) and her daughter (Ⅲ-1) carried *AKAP9 c.10714C>G.* The proband's mother (Ⅰ-3), her youngest sister (Ⅱ-7) and her niece (Ⅲ-4) carried *FLNC c.7778C>G.* The proband's father (Ⅰ-2), her

youngest sister (Ⅱ-7), her younger brother (Ⅱ-5) and her nephew (Ⅲ-3) carried *DSP c.832delG* (Table 1). Hence only *DSP c.832delG* was co-segregated with positive phenotype in those characterized members of this family (Table 1; Figure 1B), supporting the possible pathogenic role of this novel variant.

DSP c.832delG led to truncated protein, increased *PG* and decreased β-catenin expression in the nuclear

The DSP c.832delG led to a frame shift and a premature termination codon (p.A278Pfs*39) (Figure 1C), producing a truncated protein of 315 amino acid, compared with full-length of 2,871 amino acids. Western-bolt did not shown a difference of protein expression between wild type and DSP c.832delG when using a DSP primary antibody, hence, protein truncation was examined using GFP antibody. Over-expression of plasmid carrying DSP c.832delG presented with significantly shortened protein, when compared with wild type (Figure 3), suggesting a truncating effect caused by the mutation. We then tested the down-stream protein change separately in cytoplasm and nuclear. DSP c.832delG over-expression led to upregulation of Junction plakoglobin (PG) and downregulation of β-catenin in the nuclear, without affecting their expression in the cytoplasm (Figure 4), when compared with wild type plasmid. Immunofluorescence through confocal microscopy confirmed the up-regulation of nuclear PG upon transfection of mutant type plasmid (Figure 5), indicating accumulation of nuclear PG and activation of Wnt/β-catenin signaling pathway may play a key role in the pathogenesis of ARVC due to DSP c.832delG.

Discussion

In the current study, through targeted next generation sequencing platform covering a board rang of inherited cardiovascular disease genes, a novel frame-shift variant *DSP c.832delG* was identified in a large SCD family. CMR unveiled the typical manifestations of myocardium thinning, fatty replacement and severely impaired heart function, particular in the right heart of the variant carriers, fulfilling the international Task Force criteria for the diagnosis of ARVC[4]. Functional study on HEK293t cells revealed truncation of *DSP* protein, down-regulation of *PG* and up-regulation ofβ-catenin expression in nuclear but not cytoplasm upon transfection of plasmid with *DSP c.832delG*.

Desmoplakin, a member of the plakin family, anchors other desmosome components to intermediate filaments as to maintain the integrity of desmosome structure[15]. SCD was reported to be more prevalent in *DSP* defect patients, especially truncations[16], when compared with other desmosome defects[9]. In our ARVC family, 4 family members presented with SCD/aborted SCD as first clinical manifestation, and the VT/VF survivor carried *DSP c.832delG* truncation, consistent with previous findings. It has been proposed that *DSP* missense mutation exert a negative dominant effect whereas non-missense mutation exert haploinsufficiency[17], leading to phenotypic discrepancy. *DSP* missense mutation presented with more severe phenotype than non-missense mutation[18], such as earlier disease onset and more prevalence of lethal arrhythmia. However, this correlation was inconsistently reported in clinical studies. Up to date, the largest ARVC cohort with *DSP* mutation recruiting 27 patients suggested that non-missense mutations was only associated with left-dominant forms[10]. In the current study, despite normal TTE, CMR exam sensitively detected that 2 of our *DSP c.832delG* carriers presented mild to moderate left ventricle involvement, nevertheless, right ventricular impairment was dominant, suggesting phenotype is possibly mutation-dependent. Apparently, larger sample of ARVC cohort with various types of *DSP* mutation will be needed to further explore the genotype-phenotype correlation.

The canonical Wnt/β-catenin signaling was considered to play a central role in the pathogenesis of ARVC with *DSP* defects[19]. Non-specific heterozygous *DSP*-deficient mice demonstrated substantial adiposity and fibrosis in the ventricular myocardium, recapturing the human ARVC phenotype[20]. Nuclear translocation of the desmosomal protein plakoglobin (PG) and suppression of Wnt/β-catenin signaling pathway activity were found to be the underlying mechanism[20]. However, cardiac-restricted *DSP*-deficient mice developed a biventricular form of ARVC and no significant changes in PG or β-catenin expression were detected[21], indicating that mechanisms other than Wnt pathway were responsible. In addition, silencing in HL-1 cells resulted in decreased expression and redistribution of the $Na_v1.5$ protein and reduced sodium current[22], indicating an orchestra of canonical and non-canonical pathways synergically modulated the disease pathogenesis. Hence, immortal lymphoblastoid cell lines from the *DSP c.832delG* carriers and non-carriers in this family were established as to investigate the molecular pathogenesis. However, in our study no obvious *DSP* expression was detected by either western-blot or flow cytometry (data not shown), hindering the utilization of this cell line in downstream study. Therefore, plasmid carrying *DSP c.832delG* was constructed and transfected into HEK293T cells. Upregulation of *PG* and downregulation of β-catenin in the nuclear suggested canonical Wnt/β-catenin signaling pathway is likely to play a central role in the development of ARVC phenotype as previously reported [20]. However, HEK293t cells was unable to simulate the character of cardiomyocyte, hindering further studies on non-canonical pathways and cardiac phenotype.

Various cell models have been established to explore the potential effect of mutations[23]. Buccal mucosa cells from ARVC patients exhibited redistribution of desmosomes and gap junction protein, similar to those observed in heart[24]. However, in-depth phenotypic and mechanistic studies are not possible due to its distinct cellular features from cardiomyocytes. Patients-specific induced pluripotent stem cells (iPSc) derived cardiomyocytes contain the unique mutations and complete genetic background[25], thus providing us an ideal model to investigate the precise etiology and molecular mechanism. Moreover, the combination of iPSc and latest genome editing technology, such as CRISPR/Cas9, has been succeeded in correcting LQT causal mutations and reversing phenotype[26, 27], promoting it as a promising approach towards precision medicine, and thereby should be introduced in our future study.

Conclusions

We found the novel *DSP c.832delG* variant was likely causal in our ARVC family. CMR was a powerful alternative approach for the diagnosis of ARVC with high spatial and temporal resolution, especially in asymptomatic and echo negative patients. Future studies using patient-specific stem cell or animal models on the impact of the novel mutations, will be warranted to elucidate its pathogenesis of ARVC.

Abbreviations

ARVC: Arrhythmogenic right ventricular cardiomyopathy;

SCD: sudden cardiac death;

NGS: next generation sequencing;

DSP: Desmoplakin;

CMR: Cardiac magnetic resonance;

PG: Junction plakoglobin;

ECG: echocardiogram;

AKAP9: A-kinase anchoring protein 9;

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of Second Affiliated Hospital, Zhejiang University School of Medicine (2016-087). Written informed consent was properly obtained from all participants.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MX and XL designed the study; XL also enrolled patients, collected data, and was a major contributor in writing the manuscript; YM performed the cell experiments; QW, LW and ZH analyzed the data and prepared the manuscript; ZC, DH and JW acted as supervisor for data analysis and/or experiments; All authors have read and approved the final version of this manuscript.

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Not applicable

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Tables

Table 1 Clinical featuresand genotypes of family members

No.	Gender	Age rangs (y)	Medical history	ECG	Holter	ECHO	CMR	ARVC Criteria	Genotype
II2	M	80-85	CHD, Dual-chamber pacemaker	DDD pacing rhythm, LBBB, old MI	DDD pacing rhythm	LA enlargement	-	-	AKAP9+/-FLNC-/-DSP+/-
III3	F	50-55	Palpitation	PVCs	-	-	RV apical myocardium thinning and fatty infiltration	Unfulfilled	AKAP9+/-FLNC-/-DSP-/-
IV1	F	30-35	Asymptomatic	Normal	-	Normal	-	-	AKAP9+/-FLNC-/-DSP-/-
IV4	M	15-20	Asymptomatic	Normal	-	-	-	-	AKAP9-/-FLNC+/-DSP-/-

II3	F	80-85	HTN	PVC
III5	M	50-55	Asymptomatic	Nor
IV2	F	25-30	Asymptomatic	PVC

ECG, electrocardiogram; ECHO, echocar
CHD, coronary artery disease; HTN, hype
infarction; LA, left atrium; LV, left ventric
contractions; PVCs, premature ventricula
ventricular tachycardia; VF, ventricular fi

Table 2. In silico predictions of 4 novel non-synonymous variants

Gene	cDNA alteration	AA alteration	Effect	Mutation Taster	PolyPhen-2	SIFT
AKAP9	*c.10714C>G*	*p.P3572A*	Missense	Polymorphism0.99	Benign 0.003	Tolerate0.86
FLNC	*c.7778C>G*	*p.T2593S*	Missense	Disease causing0.99	Benign0.055	Tolerate0.25
SYNE1	*c.25954C>T*	*p.R8652X*	Nonsense	Disease causing0.99	Disease causing	Disease causing
DSP	*c.832delG*	*p.A278Pfs*39*	Frame-shift	Disease causing0.99	Disease causing	Disease causing

DSP, Desmoplakin; *AKAP9,* A-kinase anchoring protein 9; *FLNC,* filamin C; *SYNE1,* spectrin repeat containing nuclear envelope protein 1; AA, amino acid

Figures

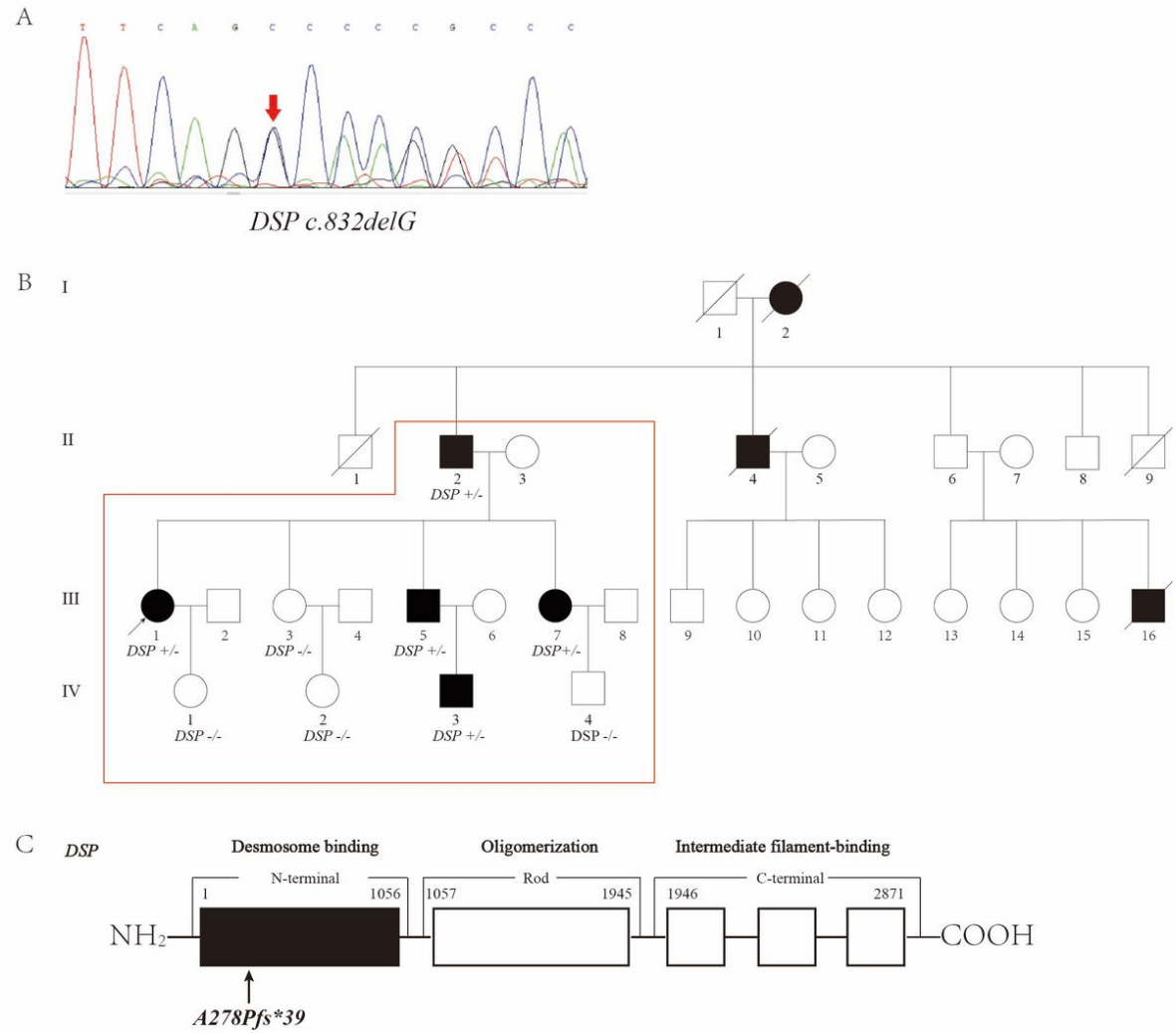

Figure 1

enetic analysis and in silico prediction. A. A heterozygous frame-shift variant DSP c.832delG was identified through targeted next generation sequencing; B. Pedigree and genotype. Family members in the red frame were genotyped. Arrow indicates the proband; squares indicate male family members; circles indicate female members; black filled indicate family members diagnosed with ARVC or experienced sudden cardiac death; diagonal lines indicate deceased family member; C. Schematic diagram of the location of DSP p.A278Pfs*39.

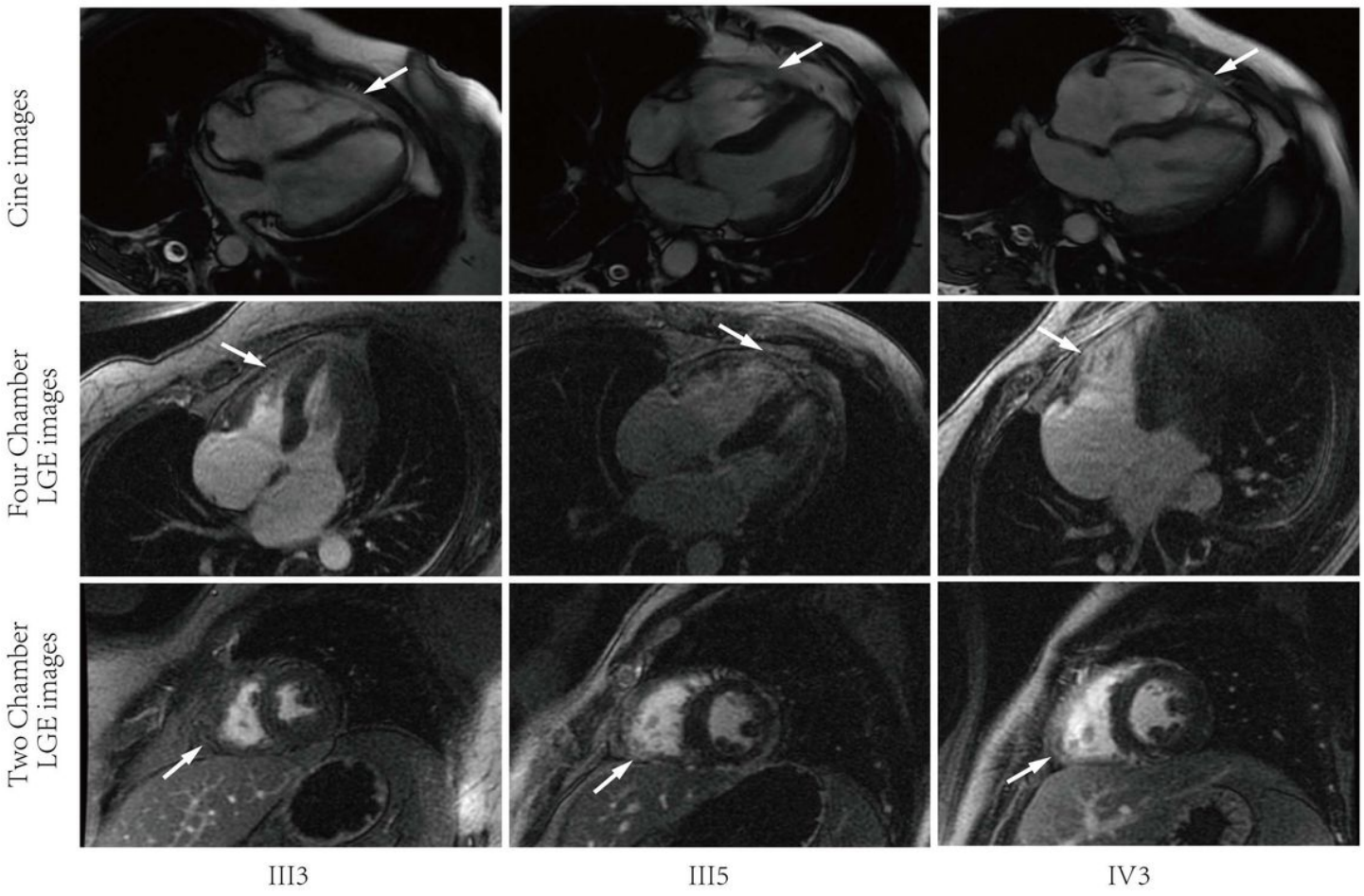

Figure 2

Representative cardiac magnetic resonance images. Myocardium thinning and fatty infiltration (arrow) in the right ventricular and positive bi-ventricular late gadolinium enhancement were detected in Ⅲ5 and Ⅲ3. Myocardium thinning and fatty infiltration (arrow) were detected in the right apical region in Ⅳ-3. LGE, late gadolinium enhancement.

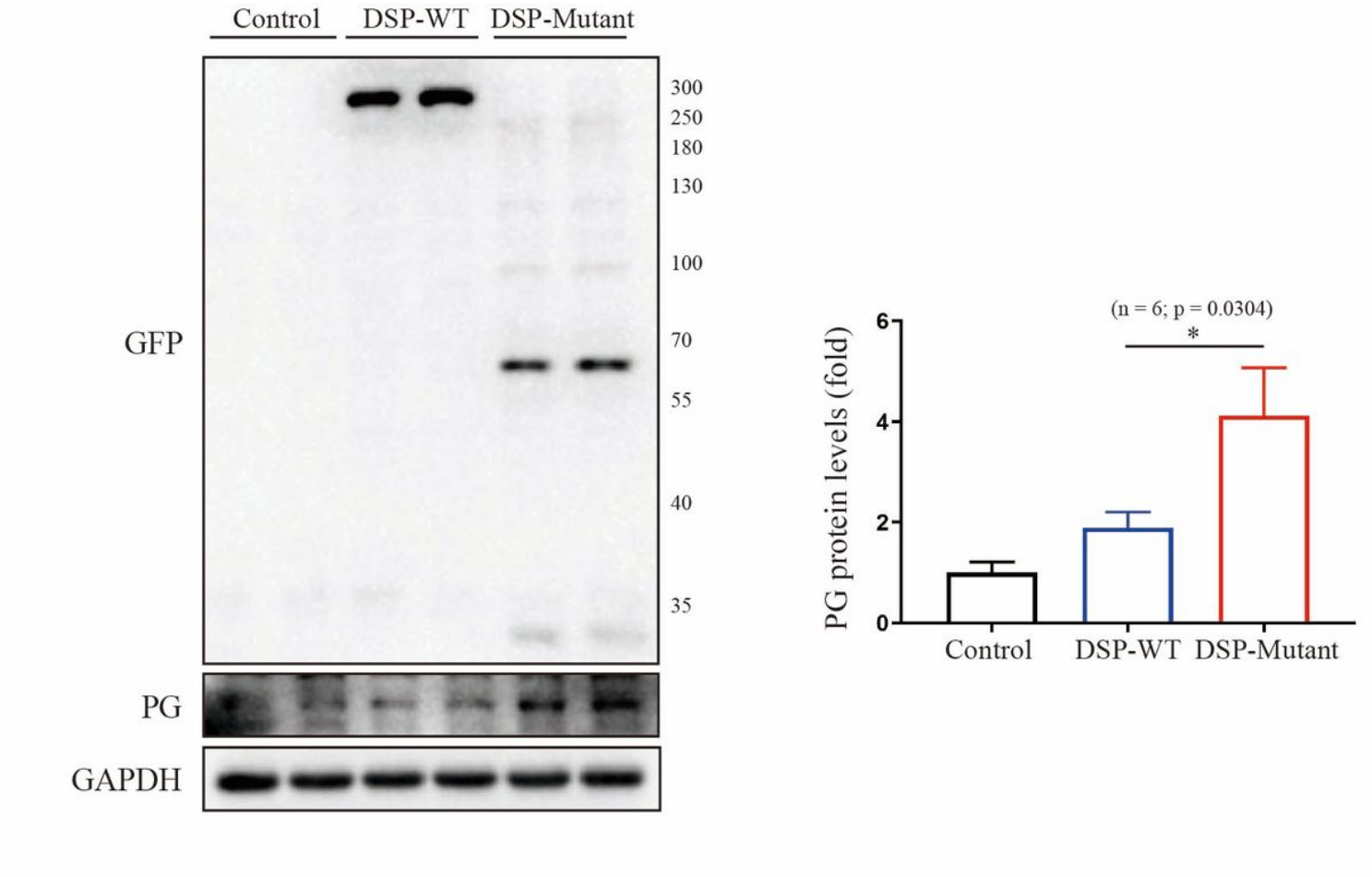

Figure 3

Total DSP and PG protein expression. GFP antibody was used to exam the length of protein expressed by either wild type or mutant DSP. Mutant DSP protein was much shorter than wild type, suggesting truncation effect of the mutation. PG expression was significantly increased in the mutant group. GAPDH served as an internal control. Blank plasmid without DSP gene served as control group. DSP, Desmoplakin; PG, Junction plakoglobin; WT, wild type.

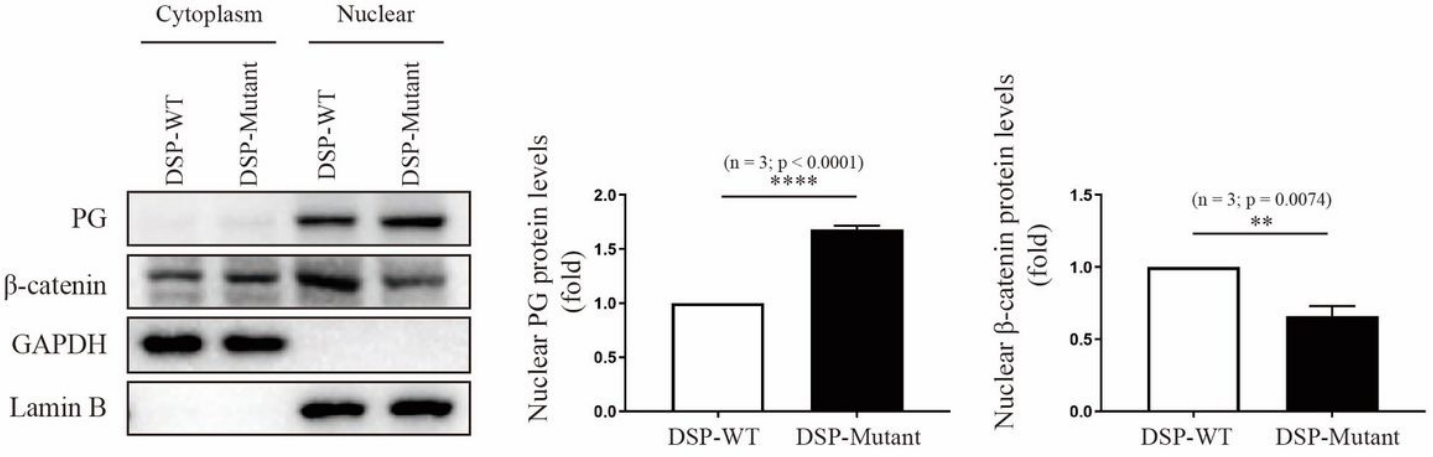

Figure 4

PG and β-catenin expression in cytoplasm and nuclear, separately. PG was found to be upregulated andβ-catenin was significantly downregulated expressed by mutant type in the nuclear, but not cytoplasm, when compared with wild type DSP. β-catenin was GAPDH served as an internal control in the cytoplasm and Lamin B served as an internal control in the nuclear. Blank plasmid without DSP gene served as control group. DSP, Desmoplakin; PG, Junction plakoglobin; WT, wild type.

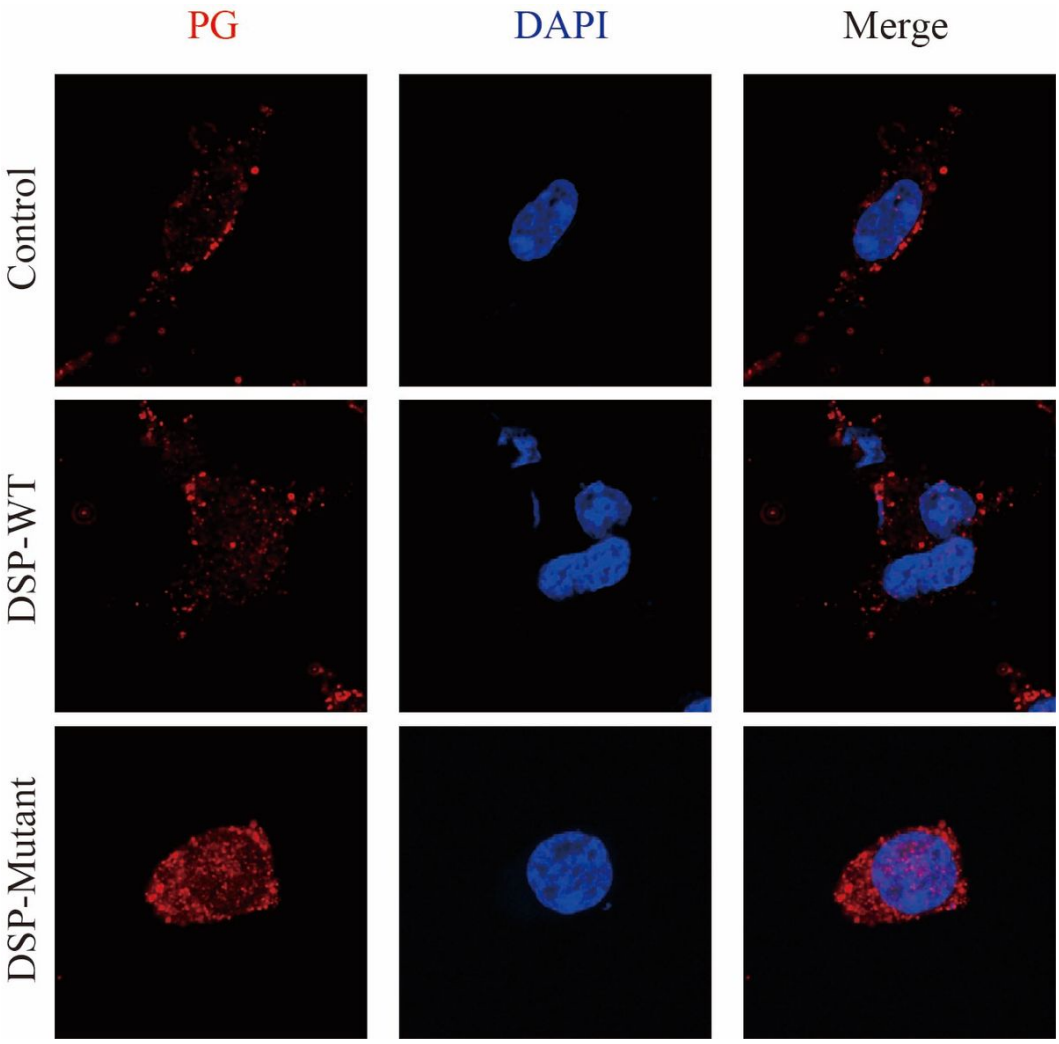

Figure 5

Confocal microscopy detected PG expression level with either wild type or mutant DSP. Blank plasmid without DSP gene served as control group. Blue indicate nuclear and red indicate PG. DSP, Desmoplakin; PG, Junction plakoglobin; WT, wild type. (n=4)