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A *PLN* nonsense variant causes severe dilated cardiomyopathy in a novel autosomal recessive inheritance mode

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ABSTRACT

Background: Pathogenic variants in human phospholamban coding gene (*PLN*) are known to cause hereditary dilated cardiomyopathy with heart failure in an autosomal dominant mode.
Methods: We performed high-depth targeted next-generation sequencing using a cardiomyopathy-panel containing 80 disease-related genes in 650 unrelated patients with non-ischemic cardiomyopathy to identify potential pathogenic *PLN* variants. To comprehensively evaluate the genetic cause of the proband and his pedigree, whole-exome sequencing and Sanger sequencing were performed.
Results: A novel homozygous nonsense variant (p.Glu2Ter, c.4G>T) in *PLN* was identified in a 36-year-old male suffering from dilated cardiomyopathy with severe heart failure. No more cardiomyopathy-causing variant or likely pathogenic copy number variation was identified. This variant was not detected in 800 unrelated healthy controls. Furthermore, the variant is not in the Exome Aggregation Consortium or the Genome Aggregation databases. Western blots showed that this variant significantly reduced the expression of phospholamban. Furthermore, in pedigree analysis, we found that all five heterozygous *PLN*-p.Glu2Ter carriers (including four elder relatives) had normal heart size and cardiac function, which revealed a novel autosomal recessive inheritance mode.
Conclusions: Our study identified a novel pathogenic variant of *PLN*, and revealed a novel pathogenic inheritance mode of *PLN* causing dilated cardiomyopathy with heart failure.

1. Introduction

Hereditary dilated cardiomyopathy (DCM), with a prevalence of approximately 1 in 250–500 in the general population [1], is one of the most important causes of heart failure [2]. There are many pathogenic inheritance modes in DCM, however, the most common pattern is autosomal dominant pathogenic inheritance mode [1]. The manifestations of DCM include contractile function reduction, ventricular enlargement and pathological remodeling of the heart, but the potential molecular etiologies of this disease still largely remains unknown [3].

Phospholamban (*PLN*) is a 52-amino-acid transmembrane SR phosphoprotein. The key function of this protein is to reversibly regulate of the cardiac SR Ca^{2+}-adenosine triphosphoatase isoform 2a (SERCA2a) pump [4]. Pathogenic variants of *PLN* gene have been demonstrated as genetic causes of autosomal dominant dilated cardiomyopathy and hypertrophic cardiomyopathy (HCM) [5]. Furthermore, recent meta-analysis of DCM on >8000 individuals demonstrated a significantly higher prevalence of cardiac transplantation, sudden cardiac death and ventricular arrhythmias in *PLN*-pathogenic variants carriers compared to patients with pathogenic variants in classic sarcomeric gene (*MYH7*) [6,7].

To date, only three pathogenic coding variants in *PLN* gene have been reported to cause cardiomyopathy with heart failure in the ClinVar database [8]. Although incomplete penetrance of cardiomyopathy phenotype was observed in previous pedigrees, the inheritance mode of this gene in DCM is, for now, regarded as autosomal dominant [5,8–11]. In the previous reports, individuals carrying heterozygous *PLN* pathogenic variant usually manifested HCM or DCM, the homozygous carriers always suffered from severe DCM with heart failure during teenage years [9,12].

2. Methods

2.1. Ethics statement

Our study was approved by the ethics committee of Tongji hospital Tongji Medical College, Huazhong University of Science and Technology. Written informed consents were obtained from all participants for this study.

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2.2. Sequencing and bioinformatic analysis

To detect the genetic causes of 650 unrelated cardiomyopathy patients, we performed high-depth targeted next-generation sequencing by using a rapid custom-ordered "Cardiomyopathy panel" containing 80 cardiomyopathy related pathogenic genes (including *PLN*) on an Ion torrent Personal Genome Machine (PGM) (Life Technologies, USA). The library construction, sequencing and data analysis were performed as we previously described [13].

To comprehensively discover the genetic cause of the proband, we performed whole-exome sequencing (WES) with a 57.7 Mb target region Ion AmpliSeq™ Exome Panel (Hi-Q) on an Ion Proton™ System (Life Technologies, USA). In this way, we tried to analyze variants in the targeted sequencing uncovered genes and copy number variations (CNV) of the proband.

2.3. Pathogenic variant identification

We searched the Human Gene Mutation Database (HGMD) and the ClinVar database to find the reported known pathogenic variants. To filter non-pathogenic variants, we removed variants with minor allele frequency (MAF) >0.001 in the 1000-Genomes-Project, the Exome Sequencing Project (ESP), the Exome Aggregation Consortium (ExAC) and the Genome Aggregation Database (gnomAD) databases. We remove up/down stream, intron, and synonymous variants. We also removed the benign variants reported in the ClinVar database. Finally, we removed the identified non-pathogenic Chinese specific rare variants by using our local healthy control databases.

For a missense variant, we evaluated its evolutionary conservation across multiple species by phyloP score. We also predicted the functional consequence of the substitution by both SIFT (<0.05) and Polyphen-2 (>0.85) scores.

After bioinformatic analysis, the identified potential pathogenic variants were validated by bidirectional Sanger sequencing on an Applied Biosystems 3500xL Dx Genetic Analyzer (Applied Biosystems). Sanger sequencing were performed in the proband, all his relatives and extra 800 unrelated Chinese healthy controls. All identified potential pathogenic variants were performed co-segregate analysis with incidence of cardiomyopathy in the pedigree.

2.4. CNV analysis

The multiplex parallel ampliseq technique of Ion torrent platform allows us to analyze CNVs directly based on the sequencing data by using CNV workflow on Ion Reporter™ software 5.0. Among the 650 patients we performed CNV analysis of the 80 genes (including *PLN*) using the high-depth targeted next-generation sequencing data. We also analyzed whole exome ranged CNVs of the proband using exome sequencing data. All identified potential pathogenic CNVs were validated using quantitative real-time PCR.

2.5. PLN-mRNA expression analysis

Total RNAs were extracted from fresh leukocytes using TRIzol reagent (Invitrogen). The RNA was reverse-transcribed by using an M-MLV First-Strand cDNA Synthesis Kit (Invitrogen). The related expression level of *PLN* was measured by quantitative real-time PCR as we previously described [14]. The real-time PCR Primers were: PLN-rt-F 5′-CACCCG TAAGACTTCATACAACACAAT-3′ and PLN-rt-R 5′-GCTGAGCGA GTGAGGTATTGGAC-3′.

2.6. Phospholamban expression analysis

The wild type and mutant phospholamban was extracted from fresh leukocytes of seven family members. Western blots were performed as previously described [14]. Briefly, fresh blood cells were treated with erythrocyte lysis buffer (TIANGEN, China). The leucocytes were then extracted and homogenized in ice-cold lysis buffer. Lysates (30 μg protein/lane) were separated by 18% SDS-PAGE, and transferred to 0.22 μm PVDF membranes. The membranes were blocked with 3% BSA and 5% non-fat milk for 2 h. The protein blots were incubated with primary antibodies overnight (4 °C), and followed by peroxidase-conjugated secondary antibody for 2 h. Finally, the enhanced chemiluminescence was used to visualize proteins.

2.7. Statistical analysis

The statistical analysis was performed with SPSS20.0. Differences between groups were evaluated for significance using Person chi-Square test. A two-sided *P* value <0.05 was considered statistically significant.

3. Results

3.1. Clinical report

A 36-year-old male (Fig. 1A, II-4) was referred to our hospital with a 3 year history of angina, palpitations and dyspnea after exercise. The chest roentgenogram (Fig. 1B) and echocardiography revealed enlargement of heart: left ventricle (73 mm), left atrium (43 * 49 * 76 mm), right ventricle (55 mm), right atrium (45 * 65 mm) with a left ventricular ejection fraction of 25%. In laboratory tests, his NT-proBNP is 8802 (reference<62.9) pg/ml, hsTnI is 655.7 (reference<34.2) pg/ml. The 12-lead electrocardiogram (ECG) and 24-h Holter ECG monitoring revealed persistent atrial fibrillation, frequent ventricular premature beat (3753 per 24 h), and incomplete right bundle branch block (Fig. 1C). He had no family history of cardiomyopathy or sudden cardiac death.

The proband's parents were a consanguineous marriage. His father (I:1, 66 years-old) and mother (I:2, 64 years-old) are cousins. They are both heterozygous p.Glu2Ter carriers of *PLN* but had normal cardiac phenotype. No significant abnormalities were seen in their echocardiography and electrocardiograph tests. They did not have angina, palpitations or syncope histories. The proband's elder sister (II:2, 42 years-old), elder brother (II:3, 40 years-old) and the daughter (III:2, 12 years-old) were also heterozygous p.Glu2Ter carriers and all had normal echocardiography and electrocardiograph results as well (Fig. 1A and Table 1). All his relatives (including four elder relatives) did not show obvious anomalies during physical examination or echocardiography (Table 1).

3.2. Pathogenic variant identification

According to the guidelines [15,16], we identified a novel homozygous nonsense variant in the proband in *PLN* gene (p.Glu2Ter, c.4G>T). It has not been reported in any databases we previously mentioned (including ESP, ExAC and gnomAD databases).

We did not detect any other likely pathogenic variant in all reported cardiomyopathy-related genes of the proband. We did not identify any potential pathogenic CNV containing cardiomyopathy-related genes of the proband. We validated the novel *PLN* variant by direct Sager sequencing using the DNA of all family members of the proband as well as the 800 unrelated healthy controls. We found five of his healthy relatives (I:1, I:2, II:2, II:3, III:2) also carry this variant in heterozygous mode (Fig. 1D). From this point of view, it showed an autosomal recessive pathogenic inheritance mode. Furthermore, the following Sanger sequencing demonstrated that the identified pathogenic variant is not detected in the 800 unrelated healthy controls.

Notably, this nonsense variant introduced a stop codon at the second amino-acid-residue and is located in an evolutionary highly conservative area across multiple species (phyloP score = 2.7) (Fig. 1D).

3.3. Phospholamban expression evaluation

Western blots showed that the phospholamban of heterozygous p.Glu2Ter carriers decreased to about half of the wild type. The phospholamban of homozygous p.Glu2Ter carrier was almost vanished (Fig. 2A). The mRNA expression analysis of the pedigree revealed no significant differences of the proband comparing with other healthy relatives in this family (Fig. 2B).

4. Discussion

In this study, we identified a previously unreported *PLN* pathogenic variant, p.Glu2Ter (c.4G>T), which is associated with inherited severe DCM and heart failure in early age in a novel autosomal recessive pathogenic inheritance mode. All heterozygous carriers, including elder generations, presented normal phenotypes of cardiomyopathy.

By targeted next-generation sequencing of 80 cardiomyopathy-related genes and Sanger sequencing validation, we identified a novel likely pathogenic *PLN* homozygous variant in a 36-year-old male with severe DCM and heart failure out of 650 unrelated cardiomyopathy patients. Furthermore, to systematically investigate the potential genetic cause of the proband, WES was performed to evaluate both variants and CNVs in all cardiomyopathy pathogenic related genes. Finally, we found no more related positive founding except the *PLN*-p.Glu2Ter variants. By pedigree analysis, we demonstrated that this identified pathogenic variant causes DCM following a novel autosomal recessive hereditary mode, which is obviously different from previously reported autosomal dominant hereditary mode of *PLN* gene.

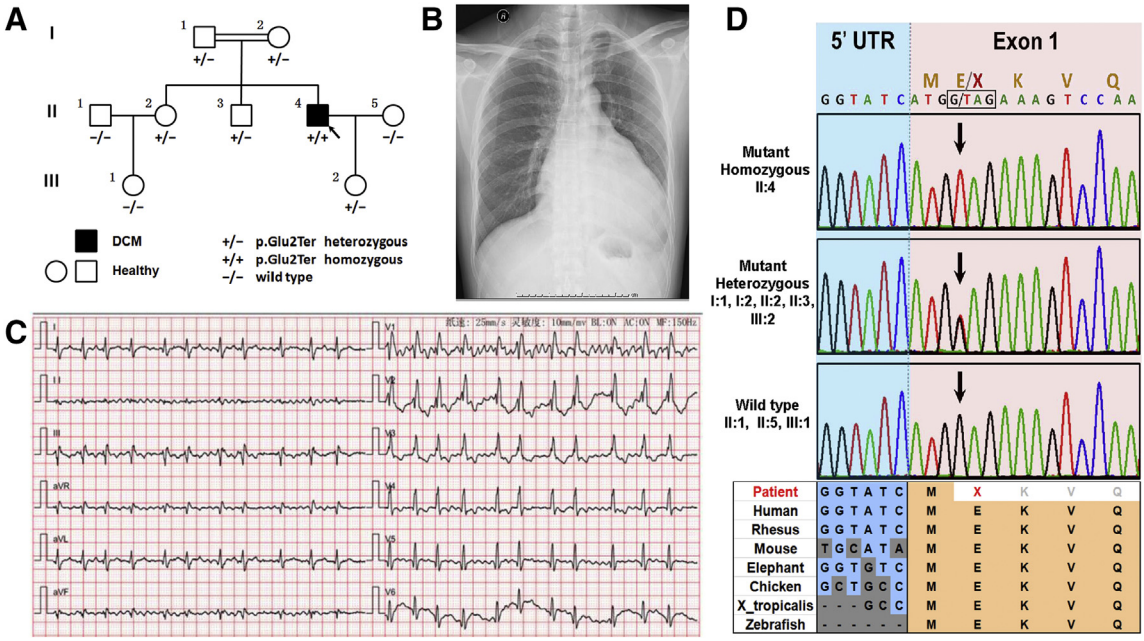

Fig. 1. Genotype of the pedigree and the chest roentgenogram of the proband. (A) The family tree of the HCM pedigree. Male and female are indicated by squares and circles, respectively. Filled symbols represent DCM affected individuals. Arrow shows the proband. +/+, represents homozygous *PLN* p.Glu2Ter variant. +/−, represents heterozygous *PLN* p.Glu2Ter variant. −/−, represents wild type. (B) The chest roentgenogram of the proband showed enlargement of the heart. (C) The 12-lead electrocardiogram of the proband revealed persistent atrial fibrillation and incomplete right bundle branch block. (D) Sanger sequencing was performed to validate the variant identified by targeted sequencing and whole-exome sequencing. The variant was located in an evolutionary highly conservative area across multiple species.

As is known, *PLN* is a causative gene responsible for autosomal dominant primary DCM type 1P (OMIM: 609909) and primary HCM type 18 (OMIM: 613874). So far, three pathogenic coding variants had been identified in this gene (p.Arg9Cys, p.Arg14del, p.Leu39Ter) [8] (Fig. 2C). As previously reported, *PLN* heterozygous pathogenic variant carrier exhibited cardiomyopathy (hypertrophy or dilated), and sometimes with incomplete penetrance of cardiomyopathy phenotype [5,9,12]. Strikingly, homozygous carriers developed DCM with heart failure in their early ages [9]. In our pedigree, the proband's parents (I:1, 66 years-old; I:2 64 years-old), his elder sister (II:2, 42 years-old) and elder brother (II:3, 40 years-old) as well as his daughter (III:2, 12 years-old) were all heterozygous p.Glu2Ter carriers and had normal echocardiography and electrocardiograph results (Fig. 1A and Table 1). Given the affected status of them, we considered autosomal recessive as the most likely inheritance model.

The phospholamban encoded by *PLN* gene is a major substrate for the cAMP-dependent protein kinase in cardiac muscle. All three previously reported dominant *PLN* pathogenic variants (p.Arg9Cys, p.Arg14del, p.Leu39Ter) were demonstrated as loss-of-function variants [3,4,9]. Importantly, the functional phospholamban is found as a pentamer [3]. Previous study suggested that the *PLN* dominant mutant exerts a "dominant negative effect" on wildtype phospholamban [4]. The detailed pathogenic mechanism includes that the mutant monomers result in a partial disruption of the stability of the phospholamban heteropentamers, leading to enhanced monomer concentration [4,17]. For example, the only reported pathogenic nonsense variant (p.Leu39Ter) follows an autosomal dominant inheritance mode [9]. The pathogenic variant stops the phospholamban at the 39/52 amino-acid-residual. The heterozygous p.Leu39Ter variants formed abnormal "poison" monomer protein product and disrupted the phospholamban heteropentamers. Besides forming loss-of-function heteropentamers in protein level, the p.Leu39Ter variant also reduced the *PLN* mRNA concentration [9].

In our case, it is quite different from the p.Leu39Ter variant, the p.Glu2Ter variant stops the phospholamban synthesis from the very beginning. Because the mutant translated product (p.Glu2Ter) is just a single methionine molecule. Therefore, the heterozygous p.Glu2Ter variant could not form "poison" loss-of-function phospholamban heteropentamers in protein level. For the p.Glu2Ter variant, PLN does not display haploinsufficiency in a heterozygous state, but rather requires a complete absence of protein product to be pathogenic (in the absence of a "poison monomer protein product "). Therefore, previously reported "dominant negative effect" of dominant *PLN* pathogenic variants could not be applied to our case. Furthermore, our study demonstrated that the p.Glu2Ter variant did not affect the phospholamban in mRNA level. In summary, we speculated that these discussed above may be the reasons why the heterozygous p.Glu2Ter-carriers present normal cardiac phenotype. The detailed pathogenic mechanism(s) need to be explored in the future.

In conclusion, we identified a novel nonsense variant (c.4G > T, p.Glu2Ter) in *PLN* gene causing severe DCM with heart failure in early

Table 1
Clinical characteristics of the subjects carrying the *PLN*-Glu2Ter variant in the pedigree.

Subject	Genotype*	Age, yrs	IVEDT, mm	LVEDD, mm	PWEDT, mm	LVEF, %	Phenotype
I:1	Heterozygous	66	9	47	9	61	Asymptomatic, normal ECG
I:2	Heterozygous	64	9	46	8	62	Asymptomatic, normal ECG
II:2	Heterozygous	42	9	41	9	66	Asymptomatic, normal ECG
II:3	Heterozygous	40	9	45	8	65	Asymptomatic, normal ECG
II:4	**Homozygous**	**36**	**9**	**73**	**9**	**25**	**Heart failure symptoms, arrhythmia**
III:2	Heterozygous	12	8	42	8	75	Asymptomatic, normal ECG

Bold indicate the clinical characteristics of the proband.
* indicate the genotype of the *PLN*-Glu2Ter. IVEDT indicates interventricular septum end diastolic thickness; PWEDT, posterior wall end diastolic thickness; LVEDD, left ventricular end diastolic dimension; LVEF, left ventricular ejection fraction; ECG, electrocardiogram.

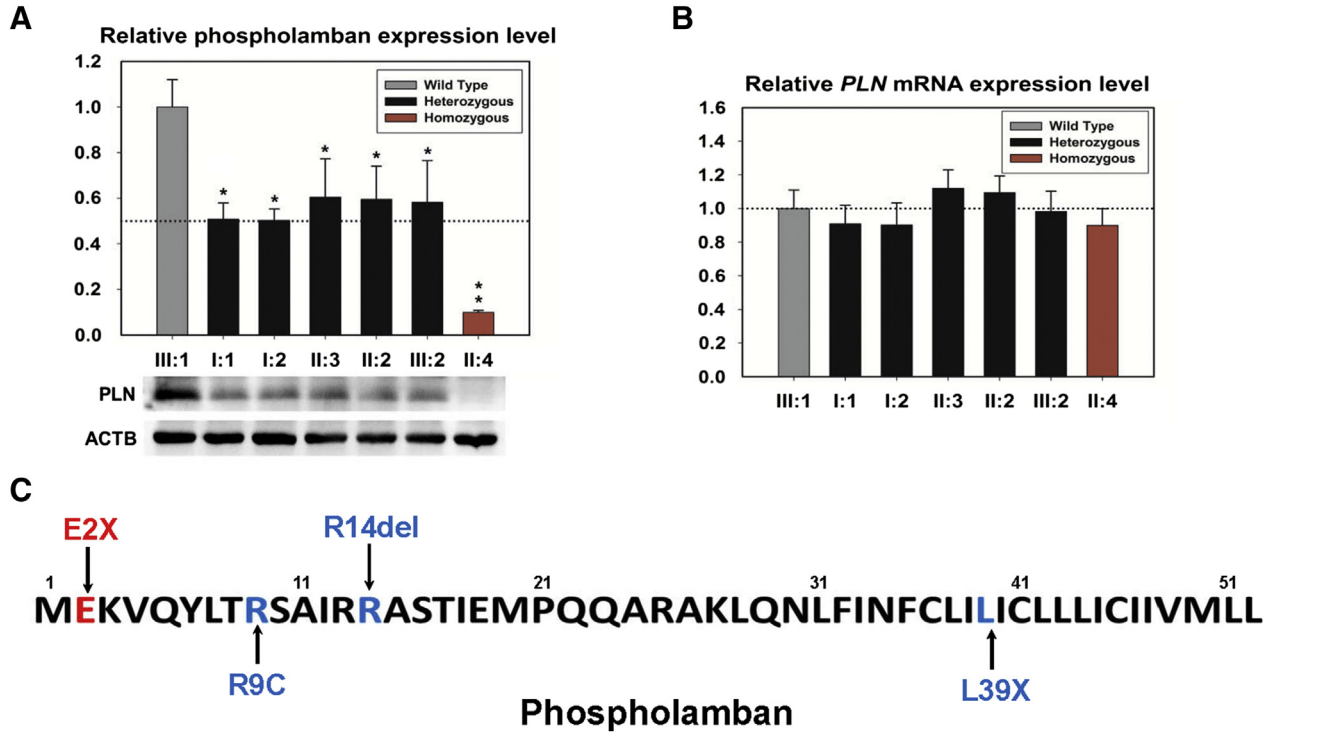

Fig. 2. Functional consequences of the *PLN* p.Glu2Ter variant. (A) The relative phospholamban expression level using western blot. Data listed as mean ± SD. * indicates $p < 0.05$ compared with wild type. ** indicates $p < 0.05$ compared with heterozygous mutant. (B) Quantitative reverse transcription PCR analysis presenting the expression of *PLN* mRNA in the proband and six healthy relatives. Comparative Ct method was used to calculate gene expression, and *GAPDH* was used as reference gene. Data listed as mean ± SD. (C) Reported pathogenic variants of *PLN*. Blue color indicate reported pathogenic coding variants. Red color indicates our variant.

age in a novel autosomal recessive inheritance mode. This study enriches the pathogenic spectrum of DCM and reveals a new pathogenic inheritance mode of *PLN* gene.

Conflict of interest

Nothing to declare.

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