Whole exome sequencing identifies novel candidate mutations in a Chinese family with left ventricular noncompaction

YE ZHOU[1,2*], ZHIYONG QIAN[1*], JING YANG[3*], MENG ZHU[4], XIAOFENG HOU[1], YAO WANG[1], HONGPING WU[1] and JIANGANG ZOU[1]

[1]Department of Cardiology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu 210029; [2]Department of Cardiology, The Affiliated Hospital of Jiangsu University, Zhenjiang, Jiangsu 212000; [3]Department of Cardiology, Huai'an First People's Hospital, Huai'an, Jiangsu 223300; [4]Department of Epidemiology and Biostatistics, Ministry of Education, Key Laboratory for Modern Toxicology, School of Public Health, Nanjing Medical University, Nanjing, Jiangsu 211166, P.R. China

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Abstract. Left ventricular noncompaction (LVNC) is an inherited cardiomyopathy involving numerous genes. To identify novel candidate causal mutations, a whole exome sequencing study was performed on a Chinese LVNC family. Exons of the most prevalent pathogenic genes of LVNC (myosin heavy chain 7 and actin, α-cardiac muscle 1) were sequenced, although no mutations were identified. Following this, Burrows-Wheeler Aligner, PICARD and Genome Analysis Toolkit (v.2.8) were used to analyze the exome sequencing data. Non-silent single nucleotide variants (SNVs) that were identified in patients with LVNC, although not in the healthy individual, were investigated further using SNV prioritization via the integration of genomic data (SPRING) based on P-values. Co-expressed gene enrichment analysis was performed using Genotype Tissue Expression (GTEx) data in order to investigate the potential roles of the genes containing SNVs in the myocardium. In the Chinese LVNC family, seven novel SNVs were identified that were only present in patients with LVNC and annotated by SPRING with P<0.05. Among these SNVs, hemicentin 1 [c. thymine (T) 9776 cytosine (C)], tolloid like 2 [c. cytosine (C) 2615 thymine (T)], fms related tyrosine kinase 3 [c. guanine (G) 976 adenine (A)] and nucleotide binding protein like [c. guanine (G) 91 thymine (T)] were located in conserved regions and annotated as deleterious by PolyPhen2, LRT and MutationTaster database analyses. Based on GTEx data, it was revealed that *NUBPL* was co-expressed with almost all previously established LVNC pathogenic genes. Furthermore, the results of the present study demonstrated that genes co-expressed with *NUBPL* were additionally enriched in the Notch signaling pathway. In addition, the results revealed numerous novel mutations that may be causal SNVs for the development of LVNC in the family involved in the present study.

Correspondence to: Dr Jiangang Zou, Department of Cardiology, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing, Jiangsu 210029, P.R. China
E-mail: jgzou@njmu.edu.cn

*Contributed equally

Abbreviations: LVNC, left ventricular noncompaction; WES, whole exome sequencing; GATK, Genome Analysis Toolkit; SNVs, single nucleotide variants; *MYH7*, myosin heavy chain 7; *TAZ*, tafazzin; *MIB1*, mindbomb E3 ubiquitin protein ligase 1; *SCN5A*, sodium voltage-gated channel α-subunit 5; *MYBPC3*, myosin binding protein C, cardiac; *TPM1*, tropomyosin 1; *YWHAE*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein-ε; *ACTC1*, actin, α-cardiac muscle 1; *TNNT2*, troponin T2, cardiac type; SPRING, single nucleotide variant prioritization via the integration of genomic data; PCR, polymerase chain reaction; IGV, integrative genomics viewer; GTEx, genotype-tissue expression; KEGG, Kyoto Encyclopedia of Genes and Genomes; BMP-1/TLD, bone morphogenetic protein-1/tolloid; TGF-β, transforming growth factor-β

Key words: WES, LVNC, mutations, cardiomyopathy, genetic testing

Introduction

Left ventricular noncompaction (LVNC) is an inherited cardiomyopathy that is characterized by a thick spongy endocardial layer and a thin compacted epicardial layer of the left ventricular myocardium, numerous prominent trabeculations and deep intertrabecular recesses (1). With the introduction of various diagnostic methods, LVNC has become a common cardiomyopathy in population, but the exact prevalence of LVNC has yet to be determined ranging from 0.01 to 0.3% (2-6). Symptoms exhibited by patients with LVNC may vary from no symptoms to severe heart failure, malignant arrhythmias, circulatory embolism and sudden mortality (1,7). The mortality rate of adult patients with LVNC is 5-12% per year (4,8,9). LVNC is widely considered to be a genetic cardiomyopathy in which myocardial dysplasia is observed during embryogenesis. Despite the diagnosis of LVNC having been improved following the introduction of echocardiography, cardiac magnetic resonance imaging and computed

tomography (10), the etiology and mechanism underlying LVNC remain undetermined. LVNC is genetically heterogeneous in humans, and numerous genes have been revealed to be associated with LVNC, including: Myosin heavy chain 7 (*MYH7*); tafazzin (*TAZ*); mindbomb E3 ubiquitin protein ligase 1 (*MIB1*); sodium voltage-gated channel-α subunit 5 (*SCN5A*); myosin binding protein C, cardiac (*MYBPC3*); tropomyosin 1 (*TPM1*); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein-ε (*YWHAE*); actin, α-cardiac muscle 1 (*ACTC1*); and troponin T2, cardiac type (*TNNT2*) (11-13). Previous studies have revealed part of the pathogenesis of LVNC. For example, Luxán, *et al* (14) found that *MIB1* mutations could prevent trabecular maturation and compaction and induce LVNC by NOTCH signaling pathway. Finsterer (15) found that *MYH7* was the most important pathogenic genes belonging to sarcomere proteins gene accounting for 22% of all LVNC mutations. The more causal genes and pathogenic mechanisms are found, the more possible it is to find effective treatments. Recently, a novel mutation in *MYH7* [c. cytosine (C) 1492 guanine (G)] was identified via whole exome sequencing (WES) as a potential causal mutation in a Chinese family suffering from LVNC (11). However, the already established mutations associated with LVNC are only exhibited in subset of patients suffering from LVNC, and thus the genetic basis of LVNC has not been fully determined. The aim of the present study was to further investigate the potential causal mutations of LVNC by performing WES on a Chinese family with LVNC.

Materials and methods

Patients and clinical characteristics. In the present study, the enrolled individuals (2 male and 2 female, age ranging between 19 and 50 years) were a nuclear family from Jiangsu Province, China. A 19-year-old male (patient II2) was the proband and exhibited no symptoms of chest pain or dizziness; however, a heart murmur was detected during a regular check-up in 2015. Following further physical examination, the proband sought medical treatment at Huai'an First People's Hospital and The First Affiliated Hospital of Nanjing Medical University (Nanjing, China). During treatment, the patient underwent echocardiography and 12-lead electrocardiography, and two observers carefully and independently examined the results. The patient met the following echocardiographic diagnostic criteria for LVNC: (i) A noncompacted/compacted ratio for a two-layered endocardium of >2; (ii) left ventricular deep endomyocardial trabeculations; and (iii) deep recesses filled with blood visualized on color Doppler imaging (16,17). Subsequently, the parents of the proband, in addition to other relatives, underwent a general medical history review, physical examination and echocardiography. A brief questionnaire was additionally administered in order to collect information on family history. Finally, it was determined that the father (I1) of the proband additionally matched the criteria of a LVNC diagnosis (Fig. 1), whereas the mother of the proband (I2) and the sister (II1) were revealed to be healthy.

Ethics statement. The present study was supported and approved by the Human Ethics Committee of The First Affiliated Hospital of Nanjing Medical University, and written informed consent was provided by the subjects prior to medical examination.

WES and mutation analysis

Samples collection and DNA extraction. Blood samples from the proband and his parents were collected during August 2015 in The First Affiliated Hospital of Nanjing Medical University and analyzed. Genomic DNA was extracted using a Genomic DNA Purification kit (Qiagen, Inc., Valencia, CA, USA).

Genome sequencing. Exons of *MYH7* and *ACTC1* were sequenced by the Department of Epidemiology and Biostatistics, School of Public Health, Nanjing Medical University via Sanger sequencing to identify whether the family trio harbored mutations in the already established LVNC-associated causal genes; however, no mutations were revealed in said genes. Thus, the sequencing results suggested that unknown mutations may be responsible for the development of LVNC. Therefore, WES was subsequently performed using the TruSeq® DNA HS Sample Preparation Kit on the Illumina TruSeq Exome Enrichment platform kit libraries (Illumina, Inc., San Diego, CA, USA), which includes 20,794 target genes, with the aim of targeting 62 Mb genomic regions using 95-base DNA probes. In addition to covering the RefSeq and Ensembl coding sequences, the enriched sequences also include 28 Mb of RefSeq untranslated regions (UTR). Following this, genomic libraries were prepared, according to the manufacturer's protocol. First, 100-500-bp DNA fragments were prepared using genomic DNA (5 mg) in elution buffer (80 ml) via sonication at 200 W and for 30 sec (10 cycles) at 4˚C using a Bioruptor (Diagenode Inc., Denville, NJ, USA). Following this, DNA fragments ranging between 150 and 250 bp were isolated via 2% gel electrophoresis. End repair was performed using T4 DNA poly and Klenow poly cleave 3' (supplied with the TruSeq® DNA HS Sample Preparation kit) followed by a size selection procedure (as set out in the TruSeq Enrichment Guide and instructions in the MinElute Gel Extraction kit). An additional adenosine base was then added to the 3' end by Klenow 3' to 5' exo minus (3'→5' exo-), and following this, DNA fragments containing the Illumina multiPE-adaptor (Illumina, Inc.) were isolated (PE Adapters sequence: 5'P-GATCGGAAGAGCGGT TCAGCAGGAATGCCGAG, 5'ACACTCTTTCCCTAC ACGACGCTCTTCCGATCT; TruSeq® DNA HS Sample Preparation kit). Following this, DNA products were amplified via 12 cycles of polymerase chain reaction (PCR) using Illumina multiPE primer #1 (PE-1.0: 5'-AATGATACGGCG ACCACCGAGATCTACACTCTTTCCCTACACGACGCT CTTCCGATC* T-3'; 1 ml; 25 mM; Illumina, Inc.), Illumina multiPE primer #2 (PE-2.0: 5'-CAAGCAGAAGACGGC ATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCT CTTCCGATC* T-3'; 1 ml; 0.5 mM; Illumina, Inc.) and Illumina index primer (1 ml; 25 mM; Illumina, Inc.) to generate DNA libraries. The thermocycling conditions were: 98˚C for 30 sec followed by 10 cycles of 98˚C (10 sec), 60˚C (30 sec), 72˚C (30 sec), 72˚C (5 min) and holding at 10˚C.

Following this, the Illumina HiSeq1500 (Ilumina, Inc.) was used to sequence the enriched DNA libraries according to the

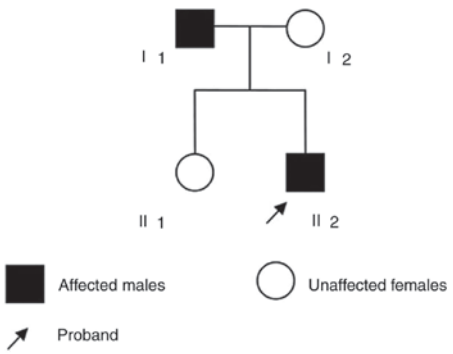

Figure 1. Pedigree of the LVNC Chinese family. Circles represent females; squares represent males. Filled shapes represent the patients with LVNC. Arrow indicates the proband. LVNC, left ventricular noncompaction; I1, father of proband; I2, mother of proband; II1, sister of proband; II2, proband.

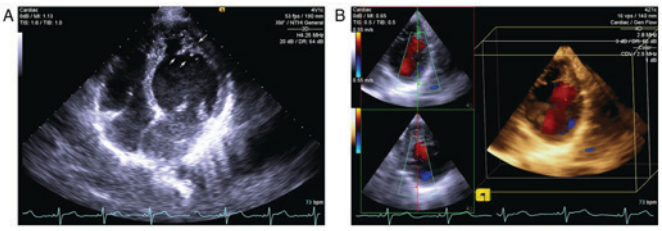

Figure 2. Echocardiography of the proband. (A) Apical four-chamber view of a two-dimensional echocardiogram of the proband revealing a thickened endocardial layer (short arrows) with prominent trabeculations and deep recesses, in addition to a thin epicardial layer (long arrow). (B) Apical 2-chamber view of a four-dimensional echocardiogram of the proband, demonstrating blood flow from the ventricular cavity into the deep recesses.

manufacturer's protocol. Burrows-Wheeler Aligner (18) was subsequently utilized to align the sequences to the human reference genome (http://hgdownload.cse.ucsc.edu/goldenPath/hg19/chromosomes/). Picard software (version 2.14.1, https://broadinstitute.github.io/picard/) was used to exclude PCR duplicates, and following this, new regional realignment and recalibration of quality scoring was performed using the Genome Analysis Toolkit (GATK; v.2.8) (19). Variations were identified by GATK using recommended parameters (https://gatkforums.broadinstitute.org/gatk/profile/GATK_Team). Integrative Genomics Viewer (IGV; https://igv.org/), a highly efficient and specialized tool for examining integrated genomic datasets, was used to determine genetic variations based on genomic position (20). Following this, known variants listed by the HAPMAP (http://www.internationalgenome.org/category/hapmap/), 1000 Genomes Project (http://www.internationalgenome.org/) or Project of dbSNP137 (http://varianttools.sourceforge.net/Annotation/DbSNP) were excluded. In the following analysis, the remaining single nucleotide variants (SNVs) were investigated further to identify whether they were present only in the patients suffering from LVNC and not in the healthy subjects in the family (the sister and mother of the proband). Following this, the remaining variants of nonsynonymous single nucleotides identified by SNV prioritization via the integration of genomic data (SPRING) (21) were prioritized for further analysis.

Statistical analysis. P-values were calculated by SPRING, combining six deleterious scores (LRT, SIFT, PolyPhen2, PhyloP, GERP and Mutation Taster) and five associated scores from multiple sources of genomic data (including protein-protein interactions, gene ontology, protein sequences, gene pathway annotations and protein domain annotations). Co-expression analysis was performed based on data obtained from Genotype-Tissue Expression (GTEx; gtexportal.org) on the ventricular myocardium. The R package 'clusterProfiler' (22) was used to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis. $P<0.05$ was considered to indicate a statistically significant result.

Results

Clinical characteristics. The proband (II2) exhibited no symptoms, and the electrocardiogram results were normal. The echocardiogram of the proband demonstrated prominent trabeculations associated with deep recesses in the apex and bicuspid aortic valve. A thickened endocardial layer and a thin epicardial layer were additionally observed (Fig. 2). The father (I1) of the proband was diagnosed with LVNC by echocardiography, which revealed a noncompacted area in the lateral wall of the left ventricle. The father of the proband additionally did not exhibit any clinical symptoms, and the electrocardiogram results were normal. The mother and sister of the proband were validated as healthy by electrocardiographic and echocardiographic testing.

Mutation screening. A total of 8,354 MB high-quality reads were obtained for patient II2, 2,689 M reads for patient I1 and 7,408 M reads for healthy control I2. A total of 21,337 variants or indels were detected in the proband, 18,230 were detected in the father of the proband additionally suffering from LVNC (I1) and 17,865 were detected in the healthy mother of the proband (I2). Following exclusion of variants revealed in multiple databases (HAPMAP, dbSNP137 and 1000 Genomes Project), 1,633, 1,750 and 1,524 variants or indels were retained, respectively. For subsequent analysis, synonymous variants and non-frameshift indels were excluded. Finally, a total of 43 non-silent coding variants (*LIN7C, PDCD6, FLT3, NALCN, HMCN1, TLL2, NUBPL, LIM2, OSBPL8, SH3PXD2A, ATP13A5, SPTB, NUP188, C16orf71, FKBP9, TP53BP2, MVP, LRRC3B, ARID1B, TXNIP, NR5A2, MUC5B, ANKAR, MGAT4C, USP24, DOK1, HMGN2, ANKRD20A4, SSC5D, ATP9A, DLG5, TAX1BP1, ZNF814, SMC1B, ELAVL3, PLEKHH2, REPIN1, TCEANC2, PLEKHA4, DOK3, PCDHA5, ZNF707* and *MUC12*) were revealed to be present in the two patients suffering from LVNC and not in the healthy control. SPRING was then used to prioritize the disease-causing nonsynonymous SNVs, and seven SNVs with $P<0.05$ were subsequently identified: hemicentin 1 (*HMCN1*) [c. thymine (T)9776C], programmed cell death 6 (*PDCD6*) [c. adenine (A)418T], tolloid like 2 (*TLL2*) (c.C2615T), lin-7 homolog C, crumbs cell polarity complex component (*LIN7C*) (c.C91T), fms related tyrosine kinase 3 (*FLT3*) (c.G976A), sodium leak channel, non-selective (*NALCN*) (c.G4975A) and nucleotide binding protein like (*NUBPL*) (c.G91T; Table I). In addition, the results demonstrated that *TLL2* (c.C2615T) and *FLT3* (c.G976A) are located in conserved regions and are annotated as deleterious by the SIFT, PolyPhe2, LRT and MutationTaster databases. Furthermore, *NUBPL* (c.G91T) and

Table I. Single nucleotide variants with P<0.05 and their categorization, as determined by single nucleotide variants prioritization via the integration of genomic data analysis.

Location of mutation	Gene	Amino acid change	P-value	PhyloP	SIFT	PolyPhen2	LRT	Mutation taster
Chr1: 186059938 T>C	HMCN1	p.L3259P	$2.05x10^{-2}$	Conserved	Tolerate	Deleterious	Deleterious	Deleterious
Chr5: 311458 A>T	PDCD6	p.R140W	$7.57x10^{-3}$	Neutral	Deleterious	Deleterious	Neutral	Deleterious
Chr10: 98133400 G>A	TLL2	p.S872L	$4.44x10^{-2}$	Conserved	Deleterious	Deleterious	Deleterious	Deleterious
Chr11: 27523414 G>A	LIN7C	p.P31S	$5.40x10^{-3}$	Conserved	Tolerate	Deleterious	Neutral	Deleterious
Chr13: 28623581 C>T	FLT3	p.G326R	$1.13x10^{-2}$	Conserved	Deleterious	Deleterious	Deleterious	Deleterious
Chr13: 101710339 C>T	NALCN	p.D1659N	$1.73x10^{-2}$	Conserved	Tolerate	Possibly damaging	Deleterious	Deleterious
Chr14: 32295867 G>T	NUBPL	p.A31S	$4.86x10^{-2}$	Conserved	Tolerate	Deleterious	Deleterious	Deleterious

Chr, chromosome; HMCN1, hemicentin 1; PDCD6, programmed cell death 6; TLL2, tolloid like 2; LIN7C, lin-7 homolog C, crumbs cell polarity complex component; FLT3, fms related tyrosine kinase 3; NALCN, sodium leak channel, non-selective; NUBPL, nucleotide binding protein like; SPRING, single nucleotide variant prioritization via the integration of genomic data.

HMCN1 (c.T9776C) were revealed to be located in conserved regions and annotated as deleterious by the PolyPhe2, LRT and MutationTaster databases. However, these variants (Table I) have not been previously reported in the Human Gene Mutation Database (www.hgmd.org). Following this, the reliability of the seven identified mutations was further validated by investigating the mapping quality around these mutations using IGV (data not shown; are available from the corresponding author on reasonable request).

Co-expressed gene enrichment analysis. Co-expression analysis on the seven identified genes and previously revealed LVNC-associated genes was performed using on data from the ventricular myocardium in GTEx (https://www.gtex-portal.org/home/). The results demonstrated that *NUBPL* was co-expressed with almost all of the established LVNC-associated genes, including *SCN5A, MYH7, ACTC1, YWHAE, MYBPC3, TAZ, MIB1* and *TPM1*. A further gene, *PDCD6,* was revealed to be co-expressed with the established LVNC-associated genes *SCN5A, MYH7, ACTC1, YWHAE, MYBPC3, TAZ* and *MIB1,* although not *TPM1*. KEGG pathway analysis revealed that genes co-expressed with *NUBPL* and *PDCD6* were enriched in the Notch signaling pathway (data not shown; are available from the corresponding author on reasonable request).

Discussion

WES is an important tool for genetic research; however, investigations regarding LVNC using WES have not been extensively performed. In the present study, WES was performed on a Chinese family with members suffering from LVNC and seven novel mutations were revealed that, to the best of our knowledge, have not previously been reported to be associated with LVNC. Among them, *TLL2* (c.C2615T) and *FLT3* (c.G976A) are located in conserved regions and were revealed to be deleterious by SIFT, PolyPhe2, LRT and MutationTaster database analyses. Thus, these mutations may represent potential causal mutations of LVNC.

TLL2 encodes an astacin-like zinc-dependent metalloprotease that is a subfamily member of the bone morphogenetic protein-1 (BMP-1)/tolloid (TLD) protein family. The BMP-1/TLD protein family is conserved from *Drosophila* to *Homo sapiens*. and includes BMP-1, TLD, TLL-1 and TLL-2 proteins, which are encoded for by the *BMP1*, *TLL1* and *TLL2* genes, respectively (23). TLL-1 and TLL-2 are important for normal morphogenesis and extracellular matrix deposition (24). Previous studies have revealed that TLL-1 is highly expressed in the endocardium and developing septa of the mouse heart and is involved in the regulation of heart morphogenesis via activation of transforming growth factor-β (TGF-β)-like molecules (25,26). In addition, mutations in *TLL1* have been demonstrated to be associated with atrial septal defects in humans (27). Despite the structure of TLL-2 being similar to that of TLL-1, TLL-2 is predominantly expressed in the developing skeletal muscle, where it activates latent myostatin and regulates muscle growth (23,25,28). Therefore, it may be suggested that the novel SNV (c.C2615T) in *TLL2* revealed in the present study may be associated with heart development in

humans; however, further research is required to investigate this result.

FLT3 encodes for a membrane-bound receptor tyrosine kinase that is associated with hematopoietic cell proliferation and differentiation (29,30). In addition, a further study revealed that FLT3 expression is upregulated following myocardial infarction in mouse hearts and that the activation of FLT3 by FLT3 ligand protects cardiomyocytes from oxidative stress-induced apoptosis via a protein kinase B-dependent mechanism (31). However, whether mutations in *FLT3* lead to abnormal myocardial development remains undetermined.

Despite other novel mutations in conserved regions of genes not being annotated as deleterious by SIFT, PolyPhe2, LRT and the MutationTaster database analyses, it was revealed that a number of said mutations are associated with cardiac function. In the present study, a mutation in *HMCN1* (c.T9776C), which encodes for an extracellular protein belonging to the immunoglobulin superfamily, was annotated as deleterious by PolyPhe2, LRT and the MutationTaster databases (32). Furthermore, HMCN1 has previously been revealed to be predominantly expressed in the vascular endothelial cells of coronary arteries and sparsely expressed in the endocardial endothelium in the mouse heart, which may have an important role in myocardial remodeling following myocardial infarction by affecting cardiac fibroblast migration via TGF-β1 signaling (33). Furthermore, a previous study using animal models and induced pluripotent stem cell-derived cardiomyocytes demonstrated that abnormal regulation of TGF-β may represent a potential mechanism underlying LVNC (34). Thus, it may be suggested that there may be an association between *HMCN1* and LVNC.

The mutation in *NUBPL* (c.G91T) was revealed to be deleterious by PolyPhe2, LRT and MutationTaster database analyses. The results of the present study revealed that *NUBPL* is co-expressed with genes that have been previously demonstrated to be associated with LVNC, including *SCN5A*, *MYH7*, *ACTC1*, *YWHAE*, *MYBPC3*, *TAZ*, *MIB1* and *TPM1,* although not *TNNT2,* based on GTEx. In addition, enrichment analysis revealed that *NUBPL* is enriched in the Notch pathway. Notably, Luxán *et al* (14) demonstrated that Notch pathway dysfunction may lead to LVNC. Furthermore, *NUBPL* encodes for a protein that is required for nicotinamide adenine dinucleotide dehydrogenase (human complex I) assembly in the respiratory chain (35). Loeffen *et al* (36) and Benit *et al* (37) demonstrated that human complex I deficiency is associated with hypertrophic cardiomyopathy. Therefore, it may be suggested that mutations in *NUBPL* may induce human complex I deficiency and abnormal myocardial development. Based on the previous evidence and the results of the present study, it may be hypothesized that *NUBPL* represents a potential causal gene of LVNC.

Furthermore, the present study identified *PDCD6* as an additional gene that is enriched in the Notch signaling pathway. *PDCD6* is a calcium-binding modulator associated with cell proliferation and death (38). Numerous studies have revealed that *PDCD6* is associated with tumors; however, the underlying mechanism remains unclear (39-42). Furthermore, one study demonstrated that overexpression of *PDCD6* suppressed angiogenesis *in vitro* via the phosphatidylinositol 3-kinase/protein kinase mTOR/ribosomal protein S6 kinase B1 pathway (43). However, as *PDCD6* was annotated by the LRT database analysis as neutral, it is less likely to be a causal gene of LVNC.

In conclusion, the results of the present study revealed numerous novel mutations [*TLL2* (c.C2615T), *FLT3* (c.G976A), *NUBPL* (c.G91T) and *PDCD6* (c.A418T)] that may be associated with LVNC in a Chinese family. Among them, mutations in *NUBPL* are more likely to represent causal SNVs for LVNC. Considering that this was an exploratory study using a small sample population, additional investigations, including expanding the sample size and performing reverse transcription-PCR, may be required to verify the preliminary results of the present study and to determine the mechanisms underlying the pathogenesis of LVNC.

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Availability of data and materials

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

Authors' contributions

YZ, ZQ, JY and JZ performed the study and were major contributors to writing the manuscript. MZ analyzed and interpreted the patient data regarding LVNC. XH, YW and HW collected clinical data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was supported and approved by the Human Ethics Committee of The First Affiliated Hospital of Nanjing Medical University, and written informed consent was provided by the subjects prior to medical examination.

Consent for publication

Written informed consent was provided by the subjects prior to medical examination.

Competing interests

All authors declare that there are no competing interests.

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