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The *trans KCNMA1-M744T* and *cis ANXA11-I457V* and *DYDC2-P123R* Variants are Associated with Familial Dilated Cardiomyopathy

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Abstract

Objectives: Cardiomyopathies are diseases of heart muscle caused by mutations in cytoskeletal genes. Disease severity and penetrance vary greatly among patients carrying the same mutation(s) and single-gene variants often do not reliably predict cardiomyopathy phenotypes.

Background: The chromosome 10q21-q23 locus was previously associated to familial dilated cardiomyopathy (DCM), arrhythmias, heart failure, Wolff-Parkinson-White (WPW) syndrome, mitral valve prolapse (MVP) and/or mitral regurgitation (MR). However, the exact variants responsible for heterogeneous DCM and arrhythmia phenotypes remained unknown.

Methods: A large family of 62 members was re-studied using whole exome and direct sequencing. Phenotype-genotype correlation and systems genetics analysis were performed.

Results: We identified missense *KCNMA1-M744T*, *ANXA11-I457V*, and *DYDC2-P123R* variants at 10q21-q23. The proband and ten family members carried *ANXA11-I457V* and *DYDC2*-P123R identified in *cis* (digenic heterozygosity). Seven digenic carriers were affected including DCM (n = 3), heart failure (n = 1), left ventricular dysfunction (n = 3), arrhythmia (n = 2), MVP (n=2) and MR (n = 3). A single *KCNMA1-M744T* variant was identified in 11 individuals, including 4 affected with MVP or MR (n = 4), ventricular arrhythmia (n = 2), and WPW (n = 1). All three variants were identified in three family members and all were affected. Rare variants in cardiomyopathy-related genes such as *CORIN-I52V*, *TTN-S21630P*, *TRPM7-Y537H*, *OTX1-H301del* and *FLNC- N47S* were identified in affected individuals.

Conclusions: This study facilitated the family-based predictive and personalized approach in identifying disease causative basis in patients with familial DCM and their relatives "at risk". Different variant combinations of *trans-KCNMA1-M744T* and *cis-ANXA11-I457V* and *DYDC2-P123R* identified at 10q21-q23 underlie the clinical heterogeneity of familial DCM. Rare variants identified beyond the 10q21-q23 were predicted to modify the function of *KCNMA1*, *ANXA11*, and *DYDC2* that may serve as the genetic modifiers in intra- and inter-familial phenotypic variability.

Keywords: Dilated cardiomyopathy; Mutation; Genotype-phenotype correlation; Complex traits; Systems genetics analysis

Introduction

Cardiomyopathies are genetic diseases of heart muscle with a high risk of heart failure (HF) and sudden cardiac death (SCD) in children and adults [1]. Types of cardiomyopathy are characterized by changes in cardiac chamber size, thickness and stiffness of the myocardial walls, cardiac function and rhythm disturbances. Dilated cardiomyopathy (DCM) is characterized by left ventricular (LV) dilation and systolic dysfunction and patients with DCM are prone to arrhythmias and SCD [2,3]. DCM cases with prominent ventricular arrhythmias are classified as arrthythmogenic DCM (aDCM), where the onset of ventricular arrhythmias may occur in the absence of signs and symptoms of overt HF and may not be related to the severity of LV dysfunction [4]. Criteria for aDCM include both DCM (LV systolic dysfunction with FS<25% and/or EF<50%, dilation with LVEDD>112% predicted corrected for age and body surface area) and arrhythmia criteria (history of sustained/non-sustained ventricular tachycardia [5 or more beats >150 bpm], >1000 PVCs/24 h, syncope of arrhythmic origin (suspected or documented), SCD (witnessed SCD with/without documented VF or death within 1 h of acute symptoms or nocturnal deaths with no prior history of worsening symptoms), appropriate ICD shock, cardiac arrest, CPR, or successful basic life support for a cardiac arrest, in any family member) [5,6]. The genetic bases of aDCM cases are complex with " double hits, " involving compound and digenic heterozygosity and, likely, polygenic etiology

is more common than recognized [7,8]. We previously identified the 3.9 Mbp region of chromosome 10q21-q23 as a candidate region for DCM and arrhythmias in a multigenerational family using genetic linkage studies, however, the primary causal mutations for aDCM remained undetected [9].

In this study, we re-studied our multigenerational family with familial DCM, HF, ventricular arrhythmias, SCD, and Wolff-Parkinson-White (WPW) syndrome using whole exome sequencing and identified three rare single nucleotide polymorphisms (SNPs) at the 10q21-q23 region, c.2231T>C (p.M744T) in *KCNMA1* (rs75573826), c.1372A>G (p.I457V) in *ANXA11* (rs1802932), and c. 371C>G (p.P123R) in *DYDC2* (rs36027713) that were associated with diverse clinical phenotypes in this family (Figure 1). We report that *KCNMA1-M744T* is inherited in *trans-*, while *ANXA11-I457V* and *DYDC2-P123R* heterozygosity is inherited in *cis*-heterozygous manner. The *KCNMA1-M744T* variant was associated with ventricular arrhythmias and *ANXA11* and *DYDC2*heterozygosity was associated with the DCM and HF, while compound heterozygosity of all three variants increased the penetrance of the aDCM phenotype. In addition, we identified multiple rare deleterious variants in cardiomyopathy- and arrhythmia-associated GWAS genes located on chromosomes other than 10q21-q23. To functionally associate all these variants, we further performed a systems genetics analysis by combining our WES findings with myocardial transcriptome data of the largest murine genetic reference population (GRP), consisting of 150 lines of BXD recombinant inbreed strains. Among the BXD strains, more than 5 million sequence variants have been identified being segregated similar to that of many human populations [10]. The cross-species systems genetics approach demonstrated that the genes beyond 10q21-q23 such as *CORIN*, *TTN*, *TRPM7* and *OTX1* are strongly correlated with *KCMNA1*, *ANXA11*, or *DYDC2*. We suggest that *CORIN-I52V*, *TTN-S21630P*, *TRPM7-Y537H*, *OTX1-H301del* and *FLNC-N47S* variants may serve as the potential modifiers affecting the activity of the genes at major 10q21-q23 locus and adding more diversity in intra-, inter- and sub-familial clinical heterogeneity in this multigenerational family.

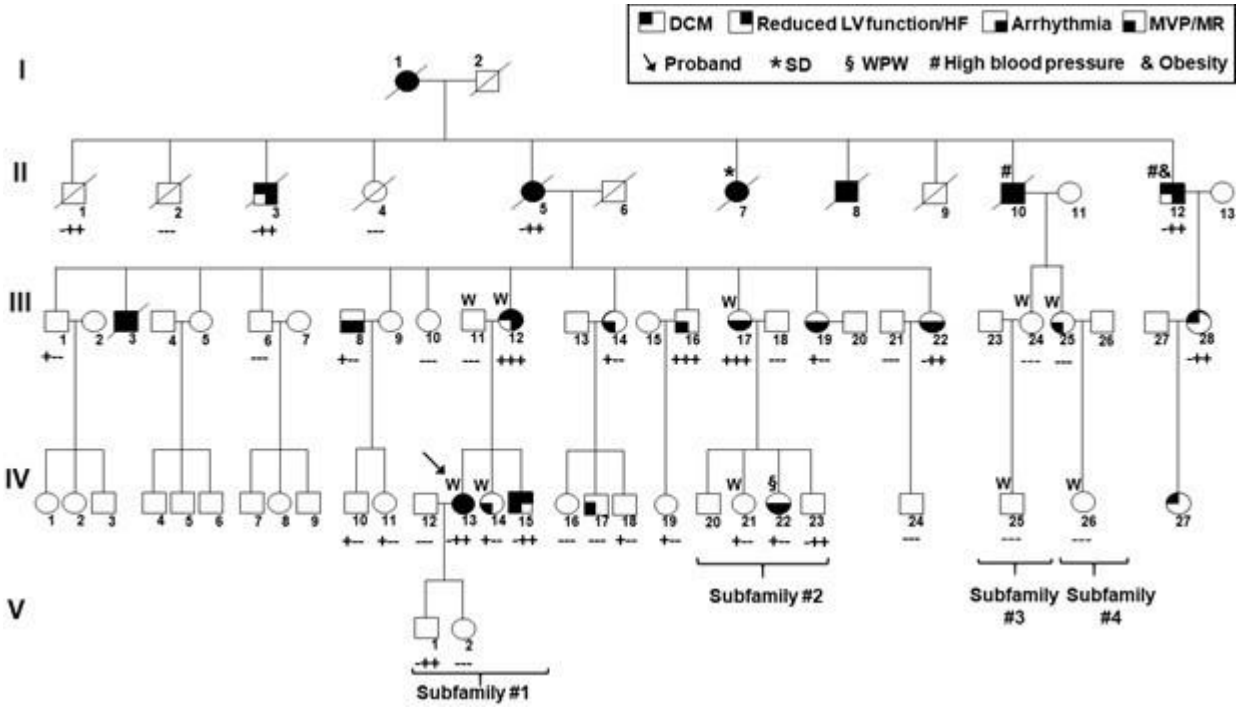

Figure 1: Family pedigree and identified variants.
Squares and circles indicate male and female individuals, respectively. Black filling in the squares and circles indicates subjects affected with DCM (upper left corner), reduced LV function/HF (upper right corner), MVP/MR (lower left corner) and arrhythmias (lower right corner). The arrow indicates the proband. *=sudden death, § = WPW syndrome, #=high blood pressure, &=obesity, W=whole exome sequencing, (+) = Sanger positive, (−)=Sanger negative. The -++ indicates negative for *KCNMA1*, positive for *ANXA11* and *DYDC2*; +-- indicates positive for *KCNMA1*, negative for *ANXA11* and *DYDC2*; --- indicates negative for *KCNMA1*, *ANXA11* and *DYDC2*; +++ indicates positive for *KCNMA1*, *ANXA11* and *DYDC2* variants.

Materials and Methods

Study family and ethics statement

A multigenerational family with a history of familial DCM and arrhythmias was re-evaluated [11,12]. The studies involving human subjects and experimental protocols were approved by the IRB committees at Cincinnati Children ' s Hospital Medical Center (CCHMC) and the University of Tennessee Health Science Center (UTHSC). Written informed consent was obtained from all participating individuals. Pedigree, health history, and medical records of consented individuals were used. Genomic DNA was extracted from de-identified samples (blood or immortalized lymphoblastoid cells lines) obtained from the Pediatric Cardiomyopathy Specimen

Repository (PCSR). WES was conducted in 10 members including the proband, parents and sibling (" trio " family), and affected and unaffected relatives from the extended family.

Molecular studies

DNA library preparation: Genomic DNA was extracted from blood or immortalized lymphoblastoid cells lines obtained from the proband and all consented family members using the Magna Pure Compact Instrument (Roche Molecular Systems, Inc). Coding regions of genomic DNA were enriched using NimbleGenSeqCap EZ Human Exome Library v2.0 (Roche NimbleGen, Madison, WI) according to the commercial protocols.

Whole exome sequencing: To expedite mutation discovery and identify potential modifiers, we conducted whole exome sequencing of the proband, 4 first degree family members and 6 individuals from extended families using Illumina HiSeq2500 paired-end sequencing (Illumina, San Diego, CA) performed at the CCHMC DNA Sequencing and Genotyping Core. A minimum of 20x depth was used to obtain a coverage for >95% of the bases targeted in the exomes.

Sanger sequencing validation, analysis and genotype-phenotype correlation: To identify and validate the primary mutation(s) and potential modifier(s), Sanger sequencing of *KCNMA1*, *DYDC2* and *ANXA11* genes and other genes of interest was conducted with primers designed using Primer3 software. Sequence alignment, variation and annotation was referenced using DNA Sequencher (Gene Codes, Ann Arbor, MI). To identify potential mutations, all variants were analyzed using available online databases. Co-segregation of all confirmed variants within the family was performed. Comparisons between sub-families, patients and other normal members within the extended family were also performed.

Bioinformatics analysis

Paired-end reads were mapped onto human reference genome (GRCh37) with BWA [13]. The reference Fasta file was indexed using Samtools 1.6, and a sequence directory created using Picard (http://broadinstitute.github.io/picard/) [14]. A vcf file was then produced using GATK 3.8 HaplotypeCaller, allowing for a maximum of 10 alternate alleles [15]. The variants were filtered out with Vcffilter implemented VCFlib toolkit (https://github.com/vcflib/vcflib) and annotated using wANNOVAR [16]. All nonsynonymous, stop-gain, or stop-loss variants with MAF < 0.01 in 1000G, ExAC, ESP6500, or gnomeAD_exome were prioritized by in silico algorithms. Seven prediction algorithms (SIF, Polyphen2, LRT, MutationTaster, FATHMM, PROVEAN, and MetaSVM) were used to predict the variant effect, Variant with a deleterious effect in less than five prediction programs were further filtered out.

Unidentifiable fastq data was deposited in NCBI (https://www.ncbi.nlm.nih.gov/sra).

Variant filtering

Filtering of variants of interest was performed by location (chromosome 10q21-q23) using Golden Helix software according to the instructions provided. Potential pathogenic variants and possible modifiers beyond chromosome 10q21-q23 were also filtered. For known variants, population allele frequencies from NCBI dbSNP (build 135) and NHLBI ESP Exome Variant Server (ESP5400) was used to identify rare variants [minor allele frequencies (MAF) < 1%] from low-frequency or common variants. In brief, all protein-coding genes were selected according to potential zygosity (heterozygous or homozygous) and predicted protein impact (change or no change). Variants that were predicted to alter the protein included non-synonymous amino acid changes (missense), truncation of the polypeptide (nonsense), or potentially altered RNA splicing (splice-site). We classified splice-site variants as those located in the splice consensus sequences (C/A)AGgt(a/g)agt and cagG for donor and acceptor sites, respectively. Variants that were predicted to result in no change to the encoded protein included those found in an intron, 5' or 3' untranslated regions (UTR), and upstream or downstream of the gene. Each variant was classified by allele frequencies. The filtered variants were compared with our cardiomyopathy-, arrhythmia-, or ion channel-related panel consisting of 740 genes listed in Supplemental Table 1. We defined "novel" variants as those absent from online databases. Finally, we used OMIM, Pubmed, Uniprot, Google etc. searches to find reported clinical associations for known variants.

Systems genetics analysis

To discover relationships between the genes of interest (i.e., *KCNMA1*, *ANXA11*, and *DYDC2*) and other modifier genes located beyond the 10q21-q23 locus, the transcript level of each of these genes was uploaded to the Gene network website and compared to that of across murine GRP consisting of 150 strains of recombinant inbred BXD mice derived from C57BL/6J and DBA/2J paternal strains. By comparing all 41,345 probes sets, the genetically correlated genes were identified and genetic correlative analysis was calculated using Pearson' s product correlations. All data were deposited into Gene Network and can be publically accessed online. Genes with significant ($P < 0.05$) correlation with *KCNMA1*, *ANXA11*, or *DYDC2* and other genes of interest were then selected for further analysis using web resources.

Web resources

The URLs for data presented herein are as follows:

Exome Aggregation Consortium Browser, http://exac.broadinstitute.org/

The Genome Aggregation Database (gnomAD), https://gnomad.broadinstitute.org/

Human Genetic Variation Database, http://www.genome.med.kyoto-u.ac.jp/SnpDB/

NHLBI Exome Sequencing Project, http://evs.gs.washington.edu/EVS/

dbSNP Short Genetic Variations, http://www.ncbi.nlm.nih.gov/SNP/

Novoalign, http://www.novocraft.com/products/novoalign/

The Genome Analysis Toolkit (GATK), https://www.broadinstitute.org/gatk/

Picard, http://broadinstitute.github.io/picard/

Annovar, http://annovar.openbioinformatics.org/en/latest/

Polyphen2, http://genetics.bwh.harvard.edu/pph2/

SIFT, http://sift.jcvi.org/

MutationTaster, http://www.mutationtaster.org/

RefSeq, http://www.ncbi.nlm.nih.gov/refseq/

UCSC Human Genome Browser, https://genome.ucsc.edu/

OMIM, http://www.omim.org/

ClinVar database, https://www.ncbi.nlm.nih.gov/clinvar

Genenetwork, http://www.genenetwork.org/

Results

Molecular studies identified *KCNMA1* in *trans*- and *ANXA11* and *DYDC2* in *cis*-state variants on chromosome 10q21-q23.

Based on the inheritance pattern in the pedigree shown in Figure 1, we hypothesized that the primary mutation was a heterozygous autosomal dominant variant that tracked with all affected family members. We then considered potential mutations that did not track completely with the disease as potential modifiers. Due to higher efficiency of WES compared to whole genome sequencing (WGS) in the identification of medically actionable variants, we performed WES in 10 family members to detect variants in coding regions [17]. By focusing on shared variants among affected family members and excluding variants present in unaffected individuals, three missense variants in three genes: *KCNMA1-M744T* (rs75573826, MAF = 0, GnomAD_exome), *ANXA11-I457V* (rs1802932, MAF = 0.00955, GnomAD_exome), and *DYDC2-P123R* (rs36027713, MAF = 0.01898, GnomAD_exome) were selected for validation out of ~3,545 variants identified in 10q21-q23 (Supplemental Table 2). None of these variants have been identified in previous linkage studies or reported in Clinvar database [11,18]. Sanger sequencing for *KCNMA1*, *ANXA11* and *DYDC2* variants in another 40 members from the extended family identified four different variant combinations (Figure 2A, Table 1). 1) three members carried all three variants *KCNMA1-M744T*, *ANXA11-I457V* and *DYDC2-P123R* (indicated as +++); 2) 10 individuals, including the proband, were negative for the *KCNMA1-M744T* variant and positive for the *ANXA11-I457V* and *DYDC2-P123R* variants (indicated as -++); 3) 11 individuals carried *KCNMA1-M744T*, but were negative for the *ANXA11-I457V* or *DYDC2-P123R* variants (indicated as +--); and 4) 16 individuals were negative for all three variants (indicated as ---). There were no subjects with a +-+ inheritance pattern, demonstrating that two variants, *ANXA11-I457V* and *DYDC2-P123R*, were in *cis* (i.e., located on the same allele at 10q21-q23) inherited from the index case (individual I.1, Figure 1).

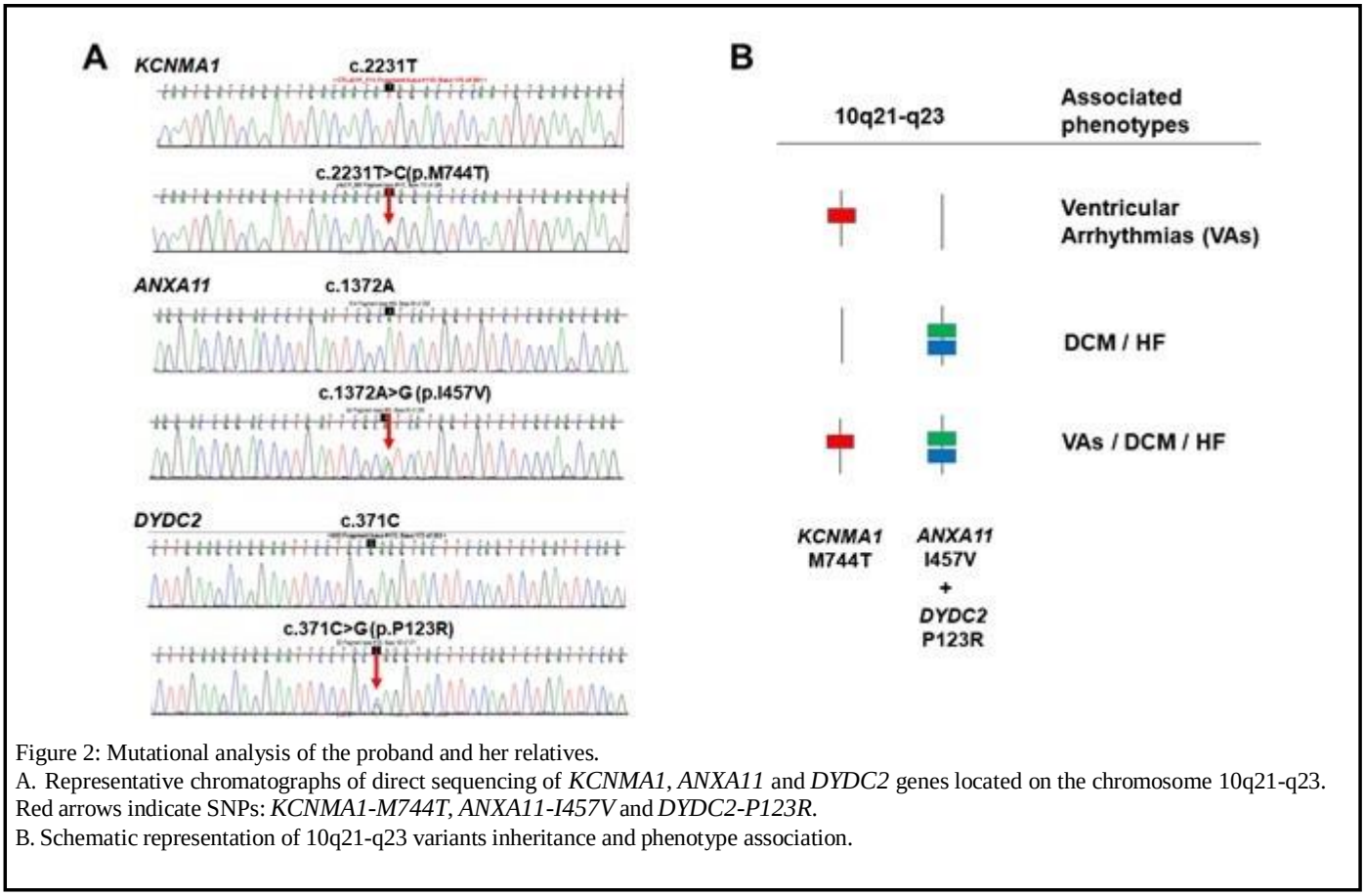

Figure 2: Mutational analysis of the proband and her relatives.
A. Representative chromatographs of direct sequencing of *KCNMA1*, *ANXA11* and *DYDC2* genes located on the chromosome 10q21-q23. Red arrows indicate SNPs: *KCNMA1-M744T*, *ANXA11-I457V* and *DYDC2-P123R*.
B. Schematic representation of 10q21-q23 variants inheritance and phenotype association.

Symptoms	(+ + +) N=3	(- + +) * N=10	(+ - -) N=11	(- - -) N=16	NO DNA (N=4)
Affected	3	7	4		4

History of heart problem		6			4
Died of heart problem		3			3
Died suddenly		1			1
Cardiomyopathy	1	3			4
Congestive heart failure		1			2
Decrease in left ventricular (LV) function	1	3			4
LV hypokinesis		1			3
Dilated LV		3			4
Dilated right ventricle (RV)		1			1
Biventricular hypertrophy	1				3
Mitral regurgitation (MR)	1	2	4	2	2
Mitral Valve prolapse (MVP)	1	2	3		2
Tricuspid regurgitation (TR)		1	1	5	2
Pericardial effusion					
Ventricular arrhythmias		2	2		
Tachycardia	1				2
Nonspecific ST-T wave changes	1				1
Normal sinus rhythm with pre-excitation			1		
Premature atrial contraction (PAC)			1		
Ventricular ectopic beats (VEB)			1		
WPW			1		
Heart palpitation	3	3	2	1	
Chest pain	1	1	2		3
Shortness of breath	1	2			1
Deep vein thrombosis					1
Dizziness		1			
Enlargement of the cardiac silhouette					1
Intermittent tingling of the left upper extremity			1		
Pulmonic insufficiency		1			
Pulmonary vascular prominence					1
Right bundle branch block (RBBB)					1
Sleep apnea					1
Systolic ejection murmur at the apex without a diastolic component or gallop					1
Comment: * indicates a proband, WPW = Wolff-Parkinson-White syndrome					

Table1: Correlation between inheritance pattern and cardiac symptoms collected from patients' report.

10q21-q23 genotype-phenotype association

A clinical summary available from 46 participating individuals is presented in Tables 1 and 2. To properly identify the primary mutation from potential modifier(s) on 10q21-q23, family co-segregation and genotype-phenotype association studies were performed by correlating inheritance patterns with cardiac-related signs (history of death due to heart disease, HF or SCD, DCM, decreased LV function, dilated LV, MVP, MR, ventricular arrhythmia, heart palpitations, chest pain, or shortness of breath). As shown in Figure 1 and Table 2, three siblings in generation III hosting the +++ genotype all had palpitations, MVP and MR, while DCM was diagnosed at 39 years of age in the mother of the proband. She had severe LV dysfunction and the cardiac biopsy revealed myocardial hypertrophy and fibrosis. Multifocal PVCs and related ventricular tachycardia with heart rates up to 189 bpm were noted on electrocardiographic tracings. The -++ genotype was associated with a history of cardiac-related disorders in 60% of carriers (6 out of 10), LV dilation and LV dysfunction (EF < 25%) occurred in 50% of subjects (5 out of 10 patients), including two young family members younger than 18 years, IV.13 (proband) and her brother IV. 15. Interestingly, these two -++ carriers did not display arrhythmias, while all older -++ relatives from generation III had palpitations or an identifiable rhythm disturbance documented by electrocardiographic tracings, suggesting that arrhythmias may have late-onset in patients with the -++ genotype. In contrast, no +-- carriers were diagnosed with DCM or LV dysfunction, while arrhythmias were documented in 3 patients out of 11 individuals with +-- genotype. Early-onset arrhythmias and WPW syndrome was diagnosed in one 9-year-old patient (IV.22, +--). Although the +-- genotype appeared to be closely associated with MVP/MR (5 out of 11 patients), two individuals with the -- genotype (III-25 and IV-17) also had MVP/MR. The sibling of the proband (IV.14, +--) had a normal heart on echocardiography and appeared healthy, having only trivial MR and TR. Taken together, we conclude that the -++ genotype (*ANXA11-I457V* and *DYDC2-P123R*) is primarily associated with DCM/HF phenotype in this family and compound trigenic heterozygosity of all 3 variants (*KCNMA1-M744T*, *ANXA11-I457V* and *DYDC2-P123R* or +++ genotype) increased the penetrance of the clinical phenotype.

In contrast, monogenic *KCNMA1-M744T* heterozygosity was associated with no DCM or HF, while milder phenotypes, such as non-life-threatening arrhythmias were common in +-- carriers. As MPV and MR were seen in many members of the family including individuals with --- genotype, we suggest there is no relationship between the 10q21-q23 genotypes and MPV or MR. We could not obtain genomic DNA from 6 affected members with DCM and HF between the ages of 22 to 89 years, due to death before the 1980s or lack of availability. Due to similar cardiac phenotypes seen in those affected individuals, we can hypothesize that these members could carry the +++ or -++ genotypes.

Genotype-phenotype association analysis in the extended family

Although we clearly associated the multigenic compound heterozygosity of 3 genes located at the chromosomal locus 10q21-q23 with DCM, HF or arrhythmia phenotypes in the extended family, we observed distinct phenotypic expressivity between generations and between subjects within small sub-families (Figure 1 and Table 2). For example, the average age of onset of clinical signs for affected individuals in generation IV (14.3±4.7 years old, n = 4) was significantly ($P < 0.001$) lower compared to that in generation III (34.1 ± 6.7 years old, n = 9) or in generation II (64.5 ± 3.5 years old, n = 4). Variability in clinical signs was also considerable among individuals and sub-families with the same 10q21-q23 genotypes. For example, the proband (IV.13) and her brother (IV.15; subfamily#1) had the same -++ genotype as their cousin (patient IV.23; subfamily#2), but the siblings from sub-family#1 had more severe symptoms and earlier-onset of DCM than their cousin (IV.23), who was healthy at the same age. Moreover, patient IV.22 had onset of ventricular tachycardia at nine years of age in contrast to her healthy sister (IV.21) and their cousins (IV.10, VI.11, VI.14, VI.18 and VI.19) who all have the same genotype (+--). Therefore, we hypothesize that the secondary variant(s) in modifier genes located on chromosomes other than the 10q21-q23 locus may modulate the effects of the primary variants at 10q21-q23 and change the phenotypic outcomes, age of onset, and severity of disease.

N	Family Position	Affected	Age of Diagnosis	Genotype	Sex	Symptoms
1	I.1	Y	82	No DNA	F	History of heart palpitations and chest pain, died of stroke
2	I.2	N		No DNA	M	
3	II.1	N/A		(- + +)	M	N/A
4	II.2	N		(- - -)	M	
5	II.3	Y	>60	(- + +)	M	Died of heart problem
6	II.4	N		(- - -)	F	
7	II.5	Y	66	(- + +)	F	Died of HF and DCM, chest pain, shortness of breath, EF=10-15, mitral regurgitation (MR), tricuspid regurgitation (TR), pulmonic insufficiency, RV and LV hypokinesis
8	II.7	Y	63	No DNA	F	Affected, died suddenly. Autopsy: dilated LV and RV, biventricular hypertrophy
9	II.10	Y	68	No DNA	M	Late-onset DCM, died of HF. EF=43%, SF=19%, hypokinetic ventricle, dilated LV with symmetrical hypertrophy. Right bundle branch block, nonspecific ST-T wave abnormality, ventricular arrhythmias.
10	II.12	Y	N/A	(- + +)	M	History of heart problems, high blood pressure, obesity, excessive smoking

11	III.1	N		(+ - -)	M	
12	III.3	Y	20	No DNA	M	Died at 22 years of age. Myocarditis, DCM and HF.
13	III.6	N		(- - -)	M	
14	III.8	Y	41	(+ - -)	M	Arrhythmia, mitral valve prolapse (MVP), MR
15	III.10	N		(- - -)	F	
16	III.11	N		(- - -)	M	
17	III.12	Y	39	(+ + +)	F	DCM, LV size enlarged, decrease in LV function, EF=45-50%. Ventricular tachycardia, nonspecific ST wave changes, no MR. Biopsy - myocardial hypertrophy and fibrosis
18	III.14	Y	35	(+ - -)	F	MVP, MR
19	III.16	Y	39	(+ + +)	M	Heart palpitations, arrhythmia
20	III.17	Y	33	(+ + +)	F	Heart palpitations, EF 60%, trace MR with normal LA size
21	III.18	N		(- - -)	M	
22	III.19	Y	36	(+ - -)	F	Arrhythmia, MVP, MR
23	III.21	N		(- - -)	M	
24	III.22	Y	30	(- + +)	F	Arrhythmia, MVP, MR
25	III.24	N		(- - -)	F	
26	III.25	Y	30	(- - -)	F	Heart palpitations, MR, TR
27	III.28	Y	N/A	(- + +)	F	N/A
28	IV.10	N		(+ - -)	M	
29	IV.11	N		(+ --)	F	
30	IV.12	N		(- - -)	M	
31	IV.13	Y	18	(- + +)	F	DCM, dilated LV, mildly depressed LV function, SF=21%, EF=33%, arrhythmia, MVP
32	IV.14	N		(+ - -)	F	No significant heart disease, MR, TR
33	IV.15	Y	16	(- + +)	M	Mild DCM, EF=30%, SF=36%, MR
34	IV.16	N		(- - -)	F	Normal heart function, FS=38%, trivial TR, trivial pulmonary regurgitation (PR)
35	IV.17	N		(- - -)	M	Normal heart function, FS=40%, MR, trivial TR, trivial PR
36	IV.18	N		(+ - -)	M	Normal heart function, SF=33%
37	IV.19	N		(+ - -)	F	
38	IV.21	N		(+ - -)	F	
39	IV.22	Y	9	(+ - -)	F	Heart palpitations, WPW, SF=31%, LV systolic dimension 4.1 cm, MR
40	IV.23	N		(- + +)	M	
41	IV.24	N		(- - -)	M	
42	IV.25	N		(- - -)	M	
43	IV.26	N		(- - -)	F	
44	IV.27	Y	~20	No DNA	F	DCM diagnosed
45	V.1	N		(- + +)	M	
46	V.2	N		(- - -)	F	

45	V.1	N		(- + +)	M	

Comments: N = not affected, Y = affected, N/A = not available, blank = no disease onset/symptoms, HF = heart failure, DCM = dilated cardiomyopathy, EF = ejection fraction, SF=shortening fraction, RV = right ventricle, LV = left ventricle, LA = left atrium, (+ + +) = positive for *KCNMA1-M744T*, *ANXA11-I457V* and *DYDC2-P123R* variants, (+ - -) = positive for *KCNMA1-M744T*, negative for *ANXA11-I457V* and *DYDC2-P123R* variants, (- + +) = negative for *KCNMA1-M744T*, positive for *ANXA11-I457V* and *DYDC2-P123R* variants, (- - -) = negative for all variants.

Table 2: Genotype-phenotype correlation of participating family members.

Rare variants identified beyond the 10q21-q23

To elucidate the phenotypic variability seen between generations and between subjects within small sub-families, we performed bioinformatics analysis in the members from extended family and identified ~130,000 unique variants outside of the 10q21-23 region. When the variants were filtered against our cardiomyopathy, arrhythmia, and ion channel gene panel, 97 unique rare deleterious variants were identified in sub-family #1, 124 rare deleterious variants were identified in sub-family #2, and 34 rare deleterious variants were identified in sub-families #3-4 (Supplemental Table 3). Further comparative analysis within sub-families identified *TTN*-S21630P and *CORIN*-I52V variants in the proband and her affected mother, while proband' s unaffected father and sister were negative (Table 3). In addition, the proband's mother had additional *TRPM7*-Y537H and *DGKB*-V239M variants. The proband's aunt (III.17, +++) from sub-family #2 with MVP and arrhythmias and no DCM, had the same *TTN*-S21630P and *TRPM7*-Y537H variants as well as her "private" variants in *FLNC*, *CXCL10*, and *NDUFA10*. Unaffected daughter of III.17 (IV.21, +--) had only the *OTX1*-H301del variant. This data suggests that *TTN*-S21630P (rs768267695) and *TRPM7*-Y537H (rs200922295) are not associated with DCM and HF phenotypes. Interestingly, we identified the *SPTB*-I1952T (rs138039383) variant in patient III.25 (---, sub-family #4) with MR, TR and heart palpitations with no documented arrhythmia.

Sub-family	Subject (Affected)	10q21-q23	Rare variants in cardiomyopathy/arrhythmia related genes	SNP	Significant association (P < 0.05)		
					KCNMA1	*ANXA11*	*DYDC2*
1	III-11	(- - -)	None				
	III-12 (Affected)	(+ + +)	*TTN* (NM_003319):c.64888T>C (p.S21630P)	rs768267695		Y	
			CORIN (NM_001278585): c.154A>G (p.I52V)		Y	Y	
			TRPM7 (NM_001301212): c.1609T>C (p.Y537H)	rs200922295	Y		
			DGKB (NM_001350711): c.715G>A (p.V239M)	rs751564836			
	IV-13	(- + +)	*TTN* (NM_003319):c.64888T>C (p.S21630P)	rs768267695		Y	
	Proband						
	(Affected)		*CORIN* (NM_001278585): c.154A>G (p.I52V)	rs587621330	Y	Y	
	IV-14	(+ - -)	None				
2	III-17 (Affected)	(+ + +)	*FLNC* (NM_001458): c.140A>G (p.N47S)	rs770861991			
			TTN(NM_003319):c.64888T>C (p.S21630P)	rs768267695		Y	
			TRPM7 (NM_001301212): c.1609T>C (p.Y537H)	rs200922295	Y		
			CXCL10 (NM_001565): c.85C>T (p.R29C)	rs11548618			
			NDUFA10 (NM_001322019): c. 674A>G (p.H225R)	rs202004542			
	IV-21	(+ - -)	*OTX1* (NM_001199770):c.873_875del (p.H301del)	rs773204989			Y
3	III-24	(- - -)	None				
	IV-25	(- - -)	None				
4	III-25 (Affected)	(- - -)	*SPTB* (NM_000347):c.5855T>C (p.I1952T)	rs138039383			
	IV-26 (N)	(- - -)	None				

Comment: Y indicates significant association between genes (P < 0.05).

Table 3: Variants in cardiomyopathy- and arrhythmia-associated genes identified by a whole exome sequencing.

Association between *KCNMA1*, *ANXA11* and *DYDC2* with modifier genes beyond the 10q21-q23

Systems genetics is a powerful approach to understand complex diseases by joining animal and human phenome-wide associations to test gene function, mechanisms and risk of disease controlled by environmental factors, epistatic interactions, and molecular networks [19]. To functionally predict whether the genes of interest (*KCNMA1*, *ANXA11* and *DYDC2*) interact with each other and whether each interact with any of genes beyond the 10q21-q23 locus where rare deleterious variants were identified, we combined our human WES findings with heart transcriptome data collected from 40 lines of BXD recombinant inbred strains and genetic correlative analysis and Pearson' s product correlations were calculated using Genenetwork (http://www.genenetwork.org/) (Figure 3). The systems genetics approach found no significant association between three genes located at the 10q21-q23 locus, while *CORIN* (chromosome 4) was significantly associated with two genes, *KCNMA1* and *ANXA11*; *TTN* (chromosome 2) was significantly associated with *ANXA11*; *TRPM7* (chromosome 15) was significantly related with *KCNMA1*; and *OTX1* (chromosome 2) was significantly related with *DYDC2*, suggesting that these genes may act as modifier genes for *KCNMA1*, *ANXA11* or *DYDC2* at the major 10q21-q23 locus (Figure 3 and Table 3). We further suggest that the variants, *CORIN-I52V*, *TTN-S21630P*, *TRPM7-Y537H*, and *OTX1-H301del*, may modulate the expressivity of *KCNMA1*, *ANXA11* and *DYDC2*, underlying the heterogeneity in disease onset, phenotypic outcomes, and severity between generations and subjects with the same genotype in this family.

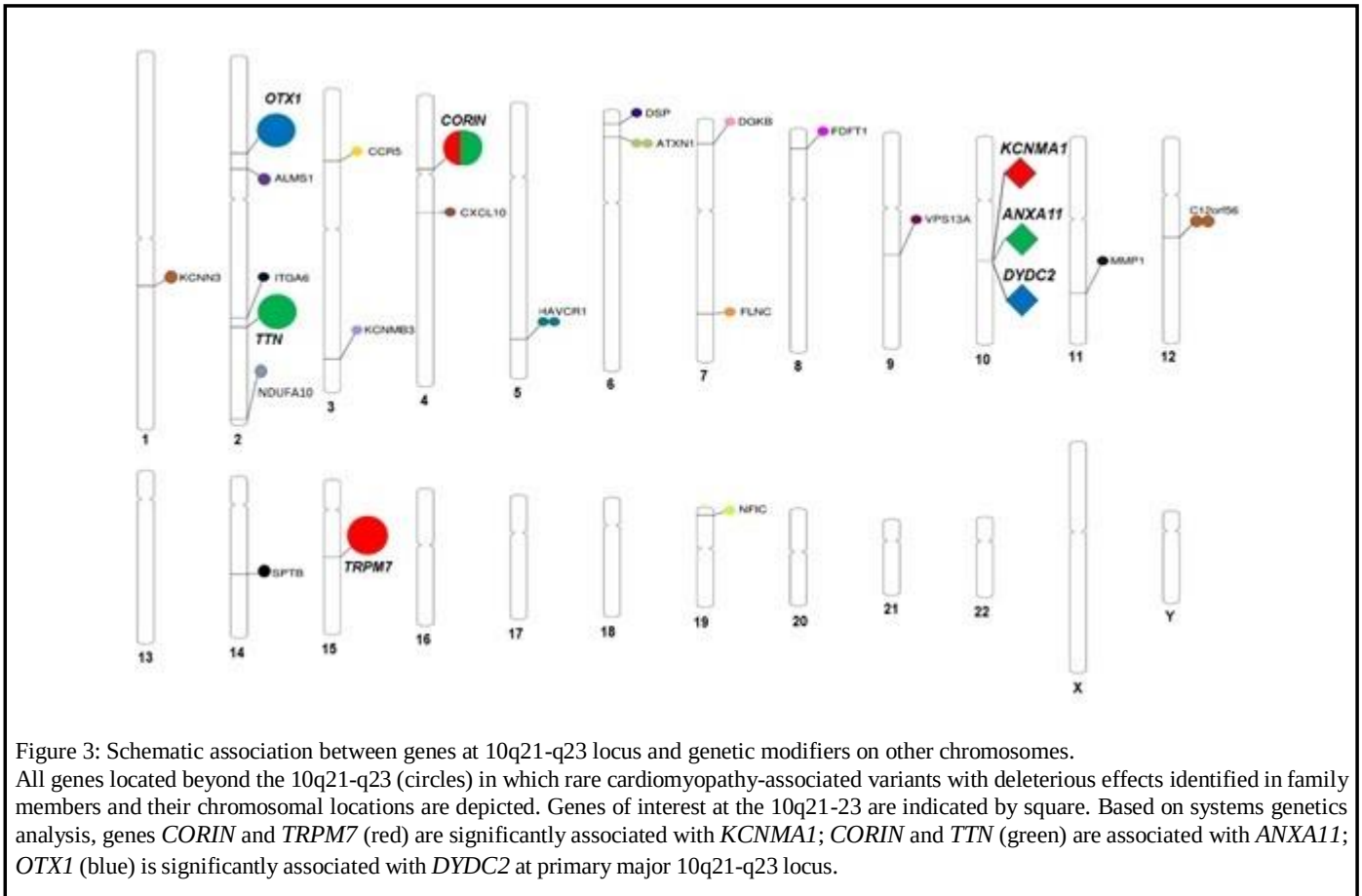

Figure 3: Schematic association between genes at 10q21-q23 locus and genetic modifiers on other chromosomes.
All genes located beyond the 10q21-q23 (circles) in which rare cardiomyopathy-associated variants with deleterious effects identified in family members and their chromosomal locations are depicted. Genes of interest at the 10q21-23 are indicated by square. Based on systems genetics analysis, genes *CORIN* and *TRPM7* (red) are significantly associated with *KCNMA1*; *CORIN* and *TTN* (green) are associated with *ANXA11*; *OTX1* (blue) is significantly associated with *DYDC2* at primary major 10q21-q23 locus.

Discussion

Substantial clinical variability is observed in many Mendelian diseases, including cardiomyopathies, while complex inheritance patterns including compound and polygenic heterozygosity, accompanied by incomplete penetrance and variable expressivity, add more complexity to the clinical manifestations [9]. Therefore, many challenges endure to reliably interpret and analytically evaluate the variants identified in clinically and genetically heterogeneous cardiomyopathy cases and the vast majority of cardiomyopathy causal genes, and in particular, modifier genes, remain elusive [20]. In this study, we re-studied a large multigenerational family with a history of autosomal dominant DCM, HF, ventricular arrhythmias, SCD, WPW syndrome, MVP, and MR, using WES followed by systems genetics

analysis. Although our previous genetic linkage studies identified the chromosome 10q21-q23 locus as the location of the disease-causing gene(s) in this family, no significant abnormalities were detected in numerous candidate genes mapped in this region, suggesting "unusual mutations" may be a cause for diverse clinical phenotypes [11,18]. Interestingly, the same 10q21-q23 region has also been associated with myofibrillar myopathy and arrhythmogenic ventricular cardiomyopathy 7 (ARVC7) [21].

Given to higher coverage of targeted coding regions and efficacy compared to WGS, we employed WES in the proband and members of the extended family to detect causative variants [17]. Current study identified a unique composition of *trans*-monogenic variant in KCNMA1 combined with an in *cis*-digenic heterozygosity in *ANXA11* and *DYDC2*, all with predicted deleterious effects and located at 10q21-q23. We report that aDCM and HF phenotypes co-segregated with the *cis*-digenic *ANXA11-I457V* and *DYDC2-P123R* variants, while milder phenotypes, such as non-life-threatening ventricular arrhythmias were associated with the *KCNMA1-M744T* variant. Polygenic inheritance with compound trigenicheterozygosity of all 3 variants (*KCNMA1-M744T*, *ANXA11-I457V* and *DYDC2-P123R*) increased the penetrance of the clinical aDCM/HF phenotype in this family. Interestingly, *KCNMA1* (potassium channel, calcium-activated, large conductance, subfamily m, alpha1) gene have been shown to be associated with essential hypertension, myocardial infarction and high mortality among adults with HF [22]. Pathological phenotypes collectively referred to as "*KCNMA1*-linked channelopathy" have been primarily linked to neurological conditions, including seizures, movement disorders, developmental delay, and intellectual disability as well [23]. The fact that *KCNMA1-M744T* has MAF = 0 suggests that this variant is a novel arrhythmia-associated mutation and further molecular and functional studies are required.

In contrast, the frequency of *ANXA11-I457V* (MAF = 0.009) and, especially, *DYDC2-P123R* (MAF = 0.019) is too high to consider as the candidate causal variants of DCM if they are identified in *trans*. Likewise, no GWAS data available on the frequency of the healthy people who carry both of these variants and our system genetics analysis found no relation between these two genes at major 10q21-q323 locus. It is largely accepted that highly penetrant rare variants in known disease-associated genes are responsible for 1–10% of familial disease incidence, while a mixture of common variants with small effects (determined by polygenic risk scores) in combination with rare variants of moderate effect have been considered as elevated risk for familial diseases [24]. Recently, common *SCN5A*, *SCN10A* and *HEY2* variants have been associated with susceptibility to Brugada syndrome [25]. Due to clear genotype-phenotype association in this family, we presume that a unique in *cis*-state digenic combination of *ANXA11-I457V* and *DYDC2-P123R* may be the cause of DCM and HF phenotypes. Supporting this, *ANXA11* encoding annexin11, a member of the large family of calcium-dependent membrane-binding proteins, is one of the DCM and ARVC7 susceptibility genes [26]. While limited data exists for association of *DYDC2* gene with cardiac phenotypes, both *ANXA11* and *DYDC2* have been associated with sarcoidosis [27]. Interestingly, our previous study identified *ANXA11-R230C* (rs1049550) variant in members of this family, which was not associated with DCM phenotype [18].

Among the factors that may explain differences in complex disease expression are modifier genes in which genetic variation modifies the effects of the pathogenic variant(s) at a major locus [9]. The power to detect the genetic modifiers, epistatic interactions and polygenic risk effects in cardiomyopathies by GWAS is limited due to a small number of patient cohorts and extremely diverse clinical expression in humans. Systems genetics analysis using large animal GRP is a powerful technique to detect the genetic and epistatic interactions [19]. Thus, we applied systems genetics approach to elucidate any associations between the three genes at the major 10q21-q23 locus and the genes outside of the 10q21-q23 variants which were identified in the proband and her relatives by comparing them with cardiac transcriptome data of BXD mouse strains used as GRP. Although no associations are found between 3 genes of interest at the 10q21-q23, we identified that *CORIN* (chromosome 4) is significantly associated with both, *KCNMA1* and *ANXA11*. *CORIN* encodes a protease enzyme that converts the atrial natriuretic peptide (ANP) precursor (pro-ANP) to mature ANP and regulates salt-water balance and blood pressure [28]. Several mutations in *CORIN* were previously identified in patients with hypertension and cardiac hypertrophy [29]. In mice, corin deficiency prevents pro-ANP processing and elevated levels of plasma corin are inversely correlated with cardiac function, reflecting the severity of myocardial damage [30]. Therefore, we speculate that the novel *CORIN-I52V* variant identified in the proband (-++) and in her affected mother (+++) may play a crucial role in modulation of the aDCM phenotype through *KCNMA1* and *ANXA11*. Another candidate modifier variant may be *TTN-S21630P* and *TRPM7-T537H* identified in the proband and her mother. Interestingly, both *TTN-S21630P* and *TRPM7-T537H* variants were also identified in subject III.17 (+++) from subfamily #2 with arrhythmias and MR but no DCM, who also carried *FLNC*-N47S. We suggest that *FLNC* is a strong candidate modifier gene, as it has recently been implicated in the development of various types of cardiomyopathies including truncation mutations identified in aDCM cases [31]. Regarding sub-families #3-4, all members carried the --- genotype and were phenotypically negative, except the cousin of proband's mother (III. 25), who had MR, TR and palpitations. As subject III.25 carried the p.I1952T variant in *SPTB*, the gene that encodes spectrin B, a member of the spectrin protein family involved in cell membrane organization and stability, we consider further cellular and molecular studies for its functional effects [32]. Regarding MVP and MR, these phenotypes were present in many family members with different 10q21-q23 genotypes, including the individuals with --- genotype, suggesting no genotype-phenotype relationship between MVP/MR and 10q21-q23.

Conclusion

In conclusion, we report that heterogeneity of aDCM phenotypes in this family (disease-onset, signs, and severity between generations and subjects with the same genotype) was partially dependent on different combinations of *KCNMA1-M744T* with *cis-ANXA11-I457V* and *DYDC2-P123R* variants at chromosome 10q21-q23. We also conclude that *CORIN-I52V*, *FLNC-N47S*, *TTN-S21630P*, *TRPM7-T537H* and *SPTB-I1952T* variants discovered outside the 10q21-q23 locus may serve as potential modifiers of inter- and intra-familial phenotypic variabilities. Our detailed investigation of genotype-phenotype association combined with systems genetics approach represents an example of how the genetics of familial cardiomyopathy is complex and "unusual". This study could be useful for determining polygenic risks of rare and common variants in complex phenotypes and facilitating the family-based predictive and personalized management in patients and individuals "at risk".

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Contributorship Statement

EP acquired conception and design of the study, supervised all experimentation, drafted and edited the manuscript; WZ supervised a whole exome sequencing and edited the manuscript; Uz.M collected clinical data and performed genotype-phenotype association studies; NG and RM acquired Sanger sequencing, variant analysis and assisted in collecting clinical data, BS assisted in sequencing and genotype-phenotype studies; Un.M performed whole exome sequencing and prepared figures; FX and LL acquired mouse gene expression data and performed systems genetics analysis; JAT recruited all study participants and edited the manuscript.

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WES Data Citation

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