

Prevalence and spectrum of LRRC10 mutations associated with idiopathic dilated cardiomyopathy

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Received August 8, 2014; Accepted May 1, 2015

DOI: 10.3892/mmr.2015.3843

Abstract. Dilated cardiomyopathy (DCM) is the most common form of primary myocardial disease. It is the most common cause of chronic congestive heart failure and the most frequent reason for heart transplantation in young adults. There is increasing evidence demonstrating that genetic defects are involved in the pathogenesis of idiopathic DCM. Recent studies have shown that genetically defective LRRC10 predisposes animals to DCM. However, the association of LRRC10 with DCM in humans has not been reported. In the current study, the whole coding region and flanking splice junction sites of the *LRRC10* gene were sequenced in 220 unrelated patients with idiopathic DCM. The available relatives of the index patients harboring identified mutations and 200 unrelated ethnically matched healthy individuals used as controls were also genotyped for *LRRC10*. The functional effect of the *LRRC10* mutations was analyzed *in silico*. As a result, two novel heterozygous LRRC10 mutations, p.L41V and p.L163I, were identified in two families with DCM, respectively, with a mutational prevalence of ~0.91%. Genetic analyses of the pedigrees showed that in each family, the mutation co-segregated with DCM was transmitted as an autosomal dominant trait with complete penetrance. The missense mutations were absent in 400 control chromosomes and the altered amino acids were completely conserved evolutionarily across various species. Functional analysis *in silico* indicated that the *LRRC10* mutations were causative. This study firstly reports the association of LRRC10 mutations with enhanced susceptibility to DCM in humans, which provides novel insight into the molecular mechanism underpinning DCM,

and contributes to the development of novel prophylactic and therapeutic strategies for DCM.

Introduction

Dilated cardiomyopathy (DCM) is characterized by ventricular chamber enlargement and contractile dysfunction with normal ventricular wall thickness in the absence of associated conditions, such as coronary heart disease, hypertension and cardiac valve disease. It is the most common form of primary myocardial disease, affecting ~1:2,500 (1). It is the most prevalent cause of chronic congestive heart failure and sudden cardiac death in individuals between the ages of 20 and 60 years-old, and is the most frequent reason for heart transplantation in humans (1,2). A previous study identified that mortality or cardiac transplantation occurred in 26% of patients with childhood DCM within 1 year of diagnosis and ~1% per year thereafter (3). Although certain acquired risk factors have been implicated in DCM, including viral myocarditis, myocardial infarction, arrhythmias and autoimmune disorders (4), in the majority of patients with DCM no secondary etiologies can be identified, this is defined as idiopathic DCM, among which 25-50% of DCM occur in at least two close relatives, hence termed familial DCM, and familial transmission of DCM has been observed to occur in an autosomal dominant, autosomal recessive or X-linked manner with variable expressivity and penetrance (1). Increasing evidence demonstrates that genetic defects are involved in the pathogenesis of idiopathic DCM, and mutations in >50 genes have been causally linked to idiopathic DCM (1,5-21). Nevertheless, these established DCM-associated genes are observed in less than a third of all cases and the genetic determinants underpinning DCM in an overwhelming majority of patients remain unclear (1,5-22).

Leucine rich repeat containing proteins (LRRCs), which contain multiple LRR motifs forming solenoid-shaped structures ideal for protein-protein interactions, have been involved in diverse cellular functions, including cell adhesion, signal transduction, ion channel expression, mechanical-stretch sensing, DNA repair and development (23-25). As a family member of LRRCs, LRRC10 is a cardiac-specific factor exclusively expressed in embryonic cardiomyocytes and in the hearts of adult zebrafish, mice and humans, exhibiting pivotal roles in cardiac development and function (26-28). In zebrafish,

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Key words: dilated cardiomyopathy, genetics, LRRC10

knockdown of *LRRC10* using morpholinos resulted in severe cardiac morphogenic defects, including a cardiac looping failure accompanied by pericardial edema, and embryonic lethality between day 6 and 7 post fertilization. The *LRRC10* morphants exhibited cardiac impairments, with decreased ejection fraction and cardiac output as well as reduced numbers of cardiomyocytes (29). In mice, targeted deletion of *LRRC10* led to antenatal cardiac dysfunction and the development of DCM in early postnatal life (30). Furthermore, *LRRC10* has been substantiated to interact physically with α -actin and α -actinin in the heart, forming a cytoskeletal complex, and mutations in α -actin and α -actinin have been causally linked to human DCM (30-32). These previous studies warrant the screening of *LRRC10* as a novel DCM candidate gene in humans.

Materials and methods

Study subjects. To evaluate the prevalence and spectrum of *LRRC10* mutations in patients with idiopathic DCM, a cohort of 220 genetically unrelated patients with idiopathic DCM was enrolled from the Han Chinese population, who were referred to Shanghai Chest Hospital between January 1st 2011 and December 31st 2013. The available relatives of the index patients harboring the identified *LRRC10* mutations were also included in this study. A total of 200 ethnically-matched unrelated healthy individuals were recruited as controls. All participants underwent a detailed medical history, physical examination, electrocardiogram, chest radiography, echocardiography and exercise tolerance test. Cardiac catheterization, coronary angiography and cardiac magnetic resonance imaging were applied only if there was a strong clinical indication. Medical records were also reviewed for deceased or unavailable relatives. Diagnosis of idiopathic DCM was made according to the criteria established by the World Health Organization/International Society and Federation of Cardiology Task Force on the Classification of Cardiomyopathy: A left ventricular end-diastolic diameter >27 mm/m² and an ejection fraction $<40\%$ or fractional shortening $<25\%$ in the absence of abnormal loading conditions, coronary artery disease, congenital heart disease and other systemic diseases (11,16,18,33). Subjects were excluded if they had poor echocardiographic image quality, or coexistent entities that may lead to cardiac systolic dysfunction, such as essential hypertension, coronary artery disease, and valvular heart disease. Familial DCM was defined when DCM occurred in two or more first-degree relatives. Peripheral venous blood samples from all the participants were collected. The clinical investigations were conducted with researchers blinded to the genotyping data. The study was conducted in accordance with the principles outlined in the Declaration of Helsinki of 1975 as revised in 2008. The study protocol was reviewed and approved by the local institutional ethics committee of Shanghai Chest Hospital, Shanghai Jiao Tong University (Shanghai, China). Prior to commencement of the study, all participants provided written informed consent for the use of their blood specimens for genetic analysis.

Genetic analysis of *LRRC10*. Genomic DNA was isolated from blood lymphocytes of each participant with Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). According to the referential genomic DNA sequence

of the *LRRC10* gene (GenBank accession no. NC_000012), the primers to amplify the entire coding region and flanking splice junction sites of *LRRC10* by polymerase chain reaction (PCR) were designed as shown in Table I. The coding exon and exon-intron boundaries of *LRRC10* were PCR-sequenced in 220 unrelated patients with idiopathic DCM. When *LRRC10* mutations were detected in index patients, the available relatives of the mutation carriers and 200 unrelated healthy controls were subsequently genotyped for *LRRC10*. PCR was performed using HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany) on a Verti Thermal Cycler (Applied Biosystems, Foster, CA, USA) with standard conditions and concentrations of reagents. Both strands of each PCR product were sequenced with a BigDye[®] Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) under an ABI PRISM 3130 XL DNA analyzer (Applied Biosystems).

DNA sequences were analyzed with the DNA Sequencing Analysis Software v5.1 (Applied Biosystems). A sequence variation was verified by re-sequencing of an independent PCR-generated amplicon from the same subject. Additionally, for an identified variation, the public databases for human sequence variations, including single nucleotide polymorphism (SNP; <http://www.ncbi.nlm.nih.gov/SNP>) and human gene mutation (HGM; <http://www.hgmd.org>) databases, were analyzed to confirm its novelty.

Multiple alignments of *LRRC10* amino acid sequences among species. Using the online MUSCLE program (version 3.6; http://www.ncbi.nlm.nih.gov/homologene?cmd=Retrieve&dopt=MultipleAlignment&list_uids=17154), the human *LRRC10* amino acid sequence was aligned with those of chimpanzee, monkey, dog, cattle, mouse, rat, fowl, zebrafish and frog.

Functional analysis of the *LRRC10* sequence variations in silico. The disease-causing potential of *LRRC10* sequence variations were evaluated by MutationTaster (an online program at <http://www.mutationtaster.org>), which automatically gave a probability for the variation to be either a causative mutation or a benign polymorphism. Notably, the P-value used here is the probability of the correct prediction rather than the probability of error as used in t-test statistics (i.e., a value close to 1 indicates a high accuracy of the prediction).

Statistical analysis. Quantitative values are expressed as the mean \pm standard deviation. Continuous data were tested for normality of distribution and Student's unpaired t-test was used for the comparison of numeric variables between two groups. Comparison of the categorical variables between two groups was made using Pearson's χ^2 test or Fisher's exact test when appropriate. A 2-tailed $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Baseline clinical characteristics of the study participants. A detailed evaluation of clinical data from 220 unrelated patients with idiopathic DCM and 200 control individuals was made. None of them had acquired risk factors for DCM. All the patients manifested with a progressive DCM phenotype without congenital cardiovascular defects. The control

Table I. Primers to amplify the whole coding region and splice junction sites of the *LRRC10* gene.

Exon	Forward primer (5' to 3')	Reverse primer (5' to 3')	Amplicon (bp)
1-a	tag, gat, agg, ccc, cag, agc, ag	gga, gac, tca, gct, cac, aga, cc	520
1-b	gca, cct, tga, aac, agc, tct, gc	aga, aca, tgc, tgc, agt, tgg, gt	600

bp, base pairs.

Table II. Baseline clinical characteristics of the patients with DCM and control individuals.

Variable	No. of patients (n=220)	No. of controls (n=200)	P-value
Age (years)	44.7±13.5	45.1±12.9	0.7569
Male (%)	104 (47.3)	95 (47.5)	1.0000
Family history of DCM (%)	62 (28.2)	0 (0)	<0.0001
SBP (mmHg)	115.2±14.8	125.4±10.6	<0.0001
DBP (mmHg)	77.0±8.5	84.9±6.2	<0.0001
HR (beat per minute)	86.7±10.0	75.1±9.4	<0.0001
LVEDD (mm)	69.3±7.5	46.8±6.3	<0.0001
LVESD (mm)	58.8±7.2	34.5±5.6	<0.0001
LVEF (%)	39.3±8.2	62.8±6.4	<0.0001
NYHA function class (%)			
I	35 (15.9)	NA	NA
II	47 (21.4)	NA	NA
III	92 (41.8)	NA	NA
IV	46 (20.9)	NA	NA

DCM, dilated cardiomyopathy; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association; NA, not applicable or not available.

individuals had no evidence of structural cardiac diseases, and their echocardiographic results were normal. The baseline clinical characteristics of the study participants are summarized in Table II.

Identification of *LRRC10* mutations. Using PCR-sequencing of the *LRRC10* gene, two mutations were identified in 2 out of 220 unrelated patients with idiopathic DCM, respectively, with a mutational prevalence of ~0.91%. Specifically, a substitution of guanine for thymine in the first nucleotide of codon 41 (c.121T>G), predicting the transition of leucine (L) into valine (V) at amino acid position 41 (p.L41V), was identified in the index patient from family 1. A change of cytosine into adenine at nucleotide 487 (c.487C>A), equivalent to a displacement of leucine at amino acid 163 by isoleucine (p.L163I), was detected in the proband from family 2. The sequence chromatograms showing the identified heterozygous *LRRC10* mutations compared with their control sequences are displayed in Fig. 1. The missense mutations were neither identified in the 400 control chromosomes nor reported in the SNP or HGM databases. Genetic screening of the families revealed that in each family the mutation was present in all

affected living family members, but absent in unaffected family members examined. Analysis of the pedigrees demonstrated that in each family the mutation co-segregated with DCM was transmitted in an autosomal dominant manner with complete penetrance (Fig. 2). Notably, during the most recent follow up, atrial fibrillation was recorded by standard 12-lead electrocardiogram in 2 patients with DCM (I-1 and II-1 from family 2), implying that DCM may be a key cause of atrial fibrillation. The phenotypic characteristics and status of *LRRC10* mutations of the affected living family members are listed in Table III.

Alignment of multiple *LRRC10* protein sequences across various species. As shown in Fig. 3, a cross-species alignment of *LRRC10* protein sequences exhibited that the altered amino acids of p.L41 and p.L163 were evolutionarily conserved, indicating that these amino acids are functionally important.

Causative potential of the *LRRC10* variations. The *LRRC10* sequence variations of c.121T>G and c.487C>A were predicted by MutationTaster to be disease-causing mutations with P-values of 0.982216 for c.121T>G and 0.999719 for

Table III. Phenotypic characteristics and status of *LRRC10* mutations of the affected living pedigree members.

Subject	Gender	Age (years)	Cardiac phenotype	LVEDD (mm)	LVESD (mm)	LVEF (%)	<i>LRRC10</i> mutation
Family 1							
II-2	F	57	DCM	78	67	22	L41V +/-
III-1	M	32	DCM	64	55	38	L41V +/-
Family 2							
I-1	M	61	DCM, AF	72	58	30	L163I +/-
II-1	M	36	DCM, AF	80	69	25	L163I +/-
II-7	F	29	DCM	62	50	36	L163I +/-

F, female; M, male; DCM, dilated cardiomyopathy; AF, atrial fibrillation; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; LVEF, left ventricular ejection fraction; +/-, heterozygous mutation.

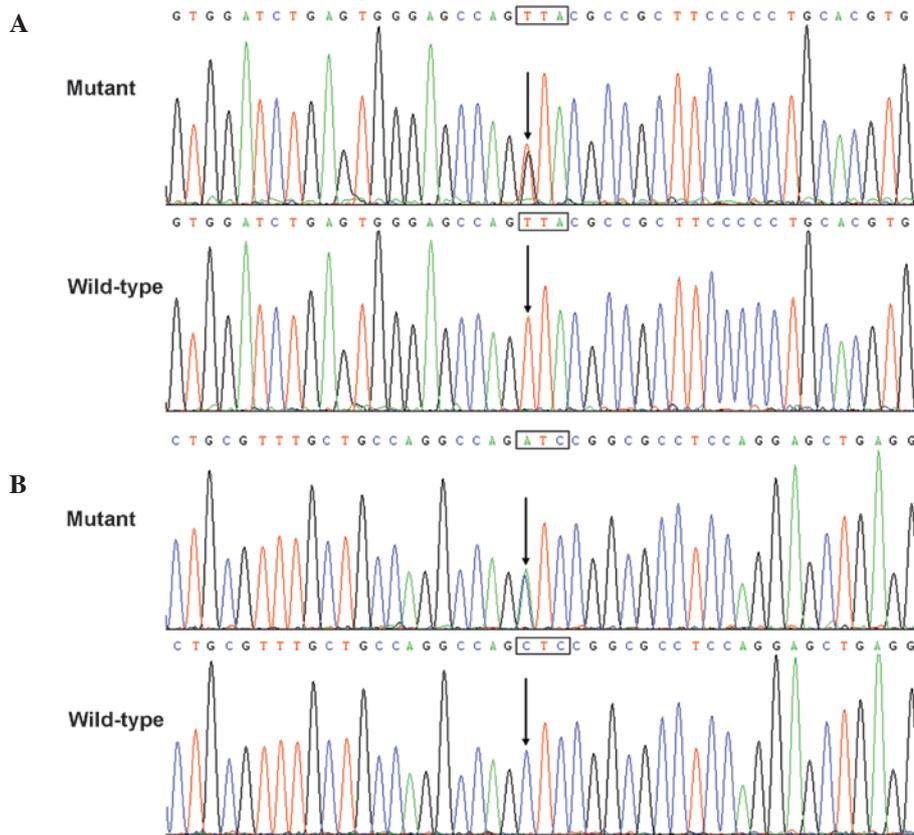


Figure 1. Sequence electropherograms showing the heterozygous *LRRC10* mutations compared with their controls. The arrow points to the heterozygous nucleotides of T/G (A) or C/A (B) in the patient with idiopathic dilated cardiomyopathy (mutant) or the homozygous nucleotides of T/T (A) or C/C (B) in the corresponding control individual (wild-type). The rectangle signifies the nucleotides comprising a codon of *LRRC10*.

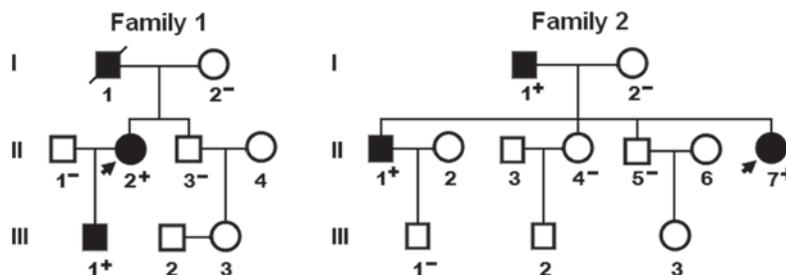


Figure 2. Pedigree structures of the families with idiopathic dilated cardiomyopathy. Families are designated as family 1 and family 2, respectively. Family members are recognized by generations and numbers. Square, male; circle, female; symbol with a slash, deceased; closed symbol, affected; open symbol, unaffected; arrow, proband; '+', carrier of the heterozygous mutation; '-', non-carrier.

		L41V	
		22	60
NP_963844.2	(Human)	--- VRDLREMP LDKMVDLSGSQ	L RRFPLHVCSFRELVKLYLS ---
XP_522465.1	(Chimpanzee)	--- VRDLREMP LDKMVDLSGSQ	L RRFPLHVCSFRELVKLYLS ---
XP_001117377.1	(Monkey)	--- VRDLHEMP LDKMVDLSGSQ	L RRFPLHVCSFKELVKLYLS ---
XP_005625671.1	(Dog)	--- ARDPRDMP PDKTVDLSSGRQ	L RRLPAHVCSFRELAKLYLS ---
NP_001069225.1	(Cattle)	--- ARDLREMP LDRMVDLSGSQ	L RRFVHVCSFQELVKLYLS ---
NP_666354.1	(Mouse)	--- VGD LREMP LDRMVDLSGSQ	L RRFPLHVCSFTELVKLYLS ---
NP_001101566.1	(Rat)	--- VGD LREMP LDRMVDLSGSQ	L RRFPLHVCSFTELVKLYLS ---
XP_004937622.1	(Fowl)	--- LEDLEEMP VDKMVDLSGRQ	L RRLPLHICSFRELVKLYLS ---
NP_001009559.1	(Zebrafish)	--- VGD LKEMP LDR TL DLS SRQ	L RRFVVRASAFDELVKLYLS ---
XP_002938552.2	(Frog)	--- LGELEEMP VDKMVDLSKSH	L RRFPLRVCAFTELVKLYLS ---
L41V			
		144	182
NP_963844.2	(Human)	--- SLLKTLHAGSNALRLLPGQ	L RRLQELRTIWLSGNRLTDF ---
XP_522465.1	(Chimpanzee)	--- SLLKTLHAGSNALRLLPGQ	L RRLQELRTIWLSGNRLTDF ---
XP_001117377.1	(Monkey)	--- SLLKTLHAGSNALRLLPGQ	L RHLQELRTIWLSGNRLTDF ---
XP_005625671.1	(Dog)	--- AVLRTLHAGSNGLRGLPGR	L QRLRELRTIWLSGNLLTDF ---
NP_001069225.1	(Cattle)	--- SLLKTLHAGSNALRLLPGQ	L QRLRELRTIWLSGNLLTDF ---
NP_666354.1	(Mouse)	--- SLLKTLHAGSNALRLLPGQ	L RRLRELRTIWLSGNQLADF ---
NP_001101566.1	(Rat)	--- SLLKTLHAGSNALRLLPGQ	L RRLRELRTIWLSGNQLADF ---
XP_004937622.1	(Fowl)	--- SLLKTLHAGSNALRTLPTTR	L RGLCELRSIWLSGNLLAEF ---
NP_001009559.1	(Zebrafish)	--- PNIKTLHLGYNQLRSLPAE	L GRLEELRSIWLAGNLLNDF ---
XP_002938552.2	(Frog)	--- TLLKTLHAGSNPIRQLPNE	L KNLKELRSIWNMSGTLLTEF ---

Figure 3. Multiple alignments of LRRC10 protein sequences across species. The altered amino acids of p.L41 and p.L163 are completely conserved evolutionarily among species.

c.487C>A. No SNPs in the altered regions were found in the MutationTaster database.

Discussion

In this study, two novel heterozygous LRRC10 mutations, p.L41V and p.L163I, were identified in two families with idiopathic DCM, respectively. In each family the missense mutation co-segregated with DCM was inherited as an autosomal dominant trait with complete penetrance. The two mutations, which were absent in the 400 reference chromosomes from an ethnically-matched control population, altered the amino acids that were evolutionarily conserved. Functional analysis *in silico* indicated that the mutations were pathogenic. Hence, it is likely that LRRC10 mutations predispose these mutation carriers to DCM.

In humans, *LRRC10* maps to chromosome 12q15, coding for a protein of 227 amino acids. LRRC10 is exclusively expressed in the precardiac region in early embryos and in the adult heart, and exhibits dynamic intracellular expression patterns in cardiomyocytes (26). Cardiomyocytes from embryos and newborns show diffuse cytoplasmic and nuclear distribution of LRRC10. By contrast, a striated expression pattern of LRRC10 is observed in adult cardiomyocytes, which colocalizes with the markers for the Z-disc, sarcoplasmic reticulum and transverse tubule. A further study by electron micrograph demonstrate that LRRC10 localizes predominantly to the diad region where the sarcoplasmic reticulum interacts with

the transverse tubule, adjacent to the Z-disc (26). The Z-disc appears as a fine dense line forming sarcomere boundaries in striated muscles, where actin myofilaments are crosslinked primarily by α -actinin (34). Therefore, the Z-disc is not only associated with lateral force transmission between sarcomeres, but also provides a mechanical link from the Z-disc myofilament to proteins in the peripheral subsarcolemmal costamere and eventually sarcolemma and extracellular matrix (35,36). Moreover, the Z-disc is crucial in sensing and transducing signals in response to biomechanical stress in the cardiomyocyte (35,37). Genetic deletion of several Z-disc and costameric proteins has been reported to cause DCM in mice, including Cypher (38), muscle LIM protein (39), enigma homologue protein (40), integrin-linked kinase (41) and vinculin (42). Furthermore, mutations in Cypher (43), muscle LIM protein (44), nexilin (45), myopalladin (46), integrin-linked kinase (47) and desmin (48) have been implicated in DCM in humans, indicating a key role for the dysfunction of Z-disc and costamere proteins in the pathogenesis of DCM. Given the fact that LRRC10 physically interacts with α -actin and α -actinin in the heart and directly interacts with all actin isoforms *in vitro* (30), mutant LRRC10 may confer increased vulnerability to DCM by disturbing the mechanical link between the Z-disc and the sarcolemma or cytoskeletal proteins.

The finding that genetically defective LRRC10 contributes to DCM may be partially ascribed to the abnormal development of the heart. As a cardiac-specific protein, LRRC10 is exclusively expressed in embryonic cardiomyocytes and the

adult heart, exhibiting an essential role in cardiac development and function, possibly by interacting with other factors that are required for cardiac development and function (26-28). In zebra fish, the *LRRC10*-knockdown morphants manifested with a reduced ejection fraction and cardiac output, as well as a decreased quantity of cardiomyocytes. There was also deregulation of two cardiac genes, including an increase in atrial natriuretic factor, a hallmark for heart failure, and a decrease in cardiac myosin light chain 2, an essential protein for cardiac contractility (29). In *LRRC10*-null mice, diminished cardiac systolic performance occurred *in utero*, prior to ventricular dilation observed in young adults. Gene expression profiling of the *LRRC10*-deletious embryonic hearts revealed dysregulation of the actin cytoskeleton as an early defective molecular signal in the absence of *LRRC10* (30). By contrast, microarray analyses of adult *LRRC10*-knockout hearts identified upregulation of oxidative phosphorylation and cardiac muscle contraction pathways during the progression of DCM (30). These experimental data demonstrate that *LRRC10* is necessary for proper cardiac development and cardiac function. However, the exact mechanism by which the identified *LRRC10* mutations result in or confer susceptibility to DCM remains to be elucidated by experiments *in vivo* and *in vitro*.

Notably, atrial fibrillation was documented in two DCM patients harboring an *LRRC10* mutation. Furthermore, *LRRC10* has been recognized as a transcriptional target of *Nkx2.5* and *GATA4* (49), and *Nkx2.5* and *GATA4* have been causally linked to DCM and atrial fibrillation (11,16,18,50-59). These observational results imply that atrial fibrillation and DCM may share a common genetic origin.

In conclusion, the current study firstly provides genetic evidence supporting that functionally compromised *LRRC10* contributes to the pathogenesis of DCM, and suggests potential implications of prenatal prophylaxis and gene-specific treatment of DCM.

Acknowledgements

The authors are thankful to the participants for their dedication to the study. This study was supported in part by grants from the National Natural Science Fund of China (grant no. 81270161), the Natural Science Fund of Shanghai, China (grant nos. 13ZR1438400 and 14ZR1438000) and the Key Program for Basic Research of Shanghai, China (grant no. 14JC1405500).

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