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*Original Article*

# Leucine-rich repeat neuronal protein 4 (LRRN4) potentially functions in dilated cardiomyopathy

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**Abstract:** Leucine-rich repeat neuronal protein-4 (LRRN4 or NLRR4) has been identified as a new member of LRRN family, which is a group of proteins that contain leucine-rich repeat domains and functioned as regulators in a variety of pathologic processes including cardiac remodeling. However, the exact pattern of expression and function of LRRN4 in the human hearts is still unclear. In our study, the western blot test and real-time PCR were performed to detect the LRRN4 level in hearts of patients with dilated cardiomyopathy (DCM), ischemia heart disease (IHD) hearts respectively. Interestingly, the LRRN4 was highly expressed in donor hearts, but significantly reduced in hearts with DCM. While a comparable level of expression was detected in the IHD hearts when compared with donor hearts. Immunohistochemistry assay showed that LRRN4 was particularly expressed in cardiomyocytes and responsible for its decreased expression in the DCM hearts. Furthermore, we found LRRN4 was expressed in the ventricular cardiomyocytes of mice and apparently reduced after pressure overload treatment in the wild type mice. Therefore, our hitherto unrecognized findings provided the first evidence that the highly expressed LRRN4 is critical for maintaining morphology and function of heart. In addition to that, since its expression level decreased in DCM hearts but not IHD hearts, which indicated LRRN4 might be a therapeutic target clinically for DCM disease.

**Keywords:** LRRN4, DCM, IHD, cardiac remodeling

## Introduction

Dilated cardiomyopathy (DCM) is defined as left ventricular or biventricular dilation with systolic dysfunction in the absence of ischemia heart disease or abnormal loading conditions proportionate to the degree of LV impairment. It is one of the leading cause of heart failure and the most frequent indication for cardiac transplantation [1, 2]. Cardiac remodeling, characterized by interstitial fibrosis and cardiomyocyte hypertrophy, is the primary pathologic change of DCM [3, 4]. Yearly, a considerable number of patients with DCM progressed into end-stage heart failure every year, with the exception of heart transplantation, there is no curative therapy available because of the irreversible cardiac remodeling. Thus, it is important to ascertain the mechanisms of DCM with cardiac remodeling to offer options that are more therapeutic.

Leucine-rich repeat (LRR) is a common domain of proteins, consisting of 20-29 amino acid segment, characterized by abounding with hydrophobic leucine [5]. The LRR protein family existed extensively in both eukaryotes and prokaryotes and is expressed in various cells and organs including heart. The specific localization and complicated interaction contributed to the LRR protein functional diversity. It has been proven that LRR proteins functioned as tyrosine kinase receptor, cellular adhesion molecule and extracellular matrix in a variety of biological processes including hormone-receptor interaction, cytoadhesion and intracellular transport [6]. More importantly, it was reported that leucine-rich repeat proteins played a vital role in regulation of cardiac disease. Recently an increasing number of studies have focused on the crucial role of cardiac leucine-rich repeat proteins during the process of dilated cardiomyopathy [7-10]. Leucine-rich repeat neuronal pro-

tein (LRRN4), identified as a new member of leucine-rich repeat neuronal protein family (NLRR), was first identified in 2005 by *Tkayoshi Bando et al.* as a type I transmembrane protein with 640 amino acids [11]. Previous studies demonstrated that LRRN4 expressed in various tissues including lung, ovary, hippocampus, and cortex, and involved in hippocampus-dependent memory retention [11]. However, the expression pattern of LRRN4 in human normal hearts and those with cardiomyopathy have yet to be identified.

Therefore, in the present study, our hitherto unrecognized findings demonstrated that LRRN4 was expressed in the human and mouse heart, and its mRNA and protein level were significantly decreased in DCM hearts and mouse hypertrophic hearts, but not ischemia heart disease (IHD) hearts. These data suggested that LRRN4 might be a novel remedy for the treatment of human DCM.

## Materials and methods

### *Human heart samples*

Samples of failing human hearts were collected from the left ventricles of DCM patients and IHD patients undergoing heart transplantation. Samples collection was as described before [12, 13]. Control samples were obtained from left ventricles of normal heart donors whose heart were not suitable for transplantation for noncardiac reasons. All the procedures involving human samples were conformed to the principles outlined in the Declaration of Helsinki and approved by the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology Review Board in Wuhan, China.

### *Transverse aortic constriction in mice*

Transverse aortic constriction (TAC) was conducted in mice in order to produce pressure overload induced-cardiac remodeling and heart failure as described before [12, 14]. In brief, adult male mice were anesthetized by sodium pentobarbital (50 mg/kg, intraperitoneal, Sigma), and then placed in a supine position on a heating pad. After endotracheal intubation was completed, thoracotomy was performed. We used a chest retractor to retract sternum and then separated the thymus and fat tissues from the aortic arch. A 6.0 silk suture and a 27-gauge blunt needle were placed between the right innominate and left carotid artery. The first knot was tied against the needle fast, followed by the second and the needle promptly removed which induced 60-80% aortic constriction on the mice as described before [15]. For sham control mice, a sham surgery without occlusion was performed on age matched and weight matched mice.

### *Western blotting*

Total proteins were extracted from ventricles by RIPA lysis buffer (900 μl RIPA, 20 μl PMSF, 10 μl protease and phosphatease inhibitor cocktail (Thermo fisher scientific, 78440), 10 μl EDTA solution (Thermo fisher scientific, 78440), 50 μl NaF, 10 μl $Na_3VO_4$). The protein concentration was determined using the Pierce™ BCA Protein Assay Kit (ThermoFisher scientific, 23225). Twenty micrograms of protein were used for SDS-PAGE, and the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, IPVH00010). The membrane was incubated primary antibodies (GAPDH, Cat No. 5174, Cell signaling technology; LRRN4, Cat No. AP13663b, Abgent) overnight at 4°C. After incubated with Peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, 111-035-003, at 1:25000 dilution) for 1 hour at room temperature, signals were visualized with an ChemiDoc™ XRS+ imaging system (Bio-Rad) and then analyzed by Image lab software (version 5.2.1, Bio-Rad). The specific protein expression levels were normalized to GAPDH for the total lysates.

### *Real-time PCR*

Total mRNA was extracted from ventricles by using TRI Reagent®Solution (AM9738, ThermoFisher Scientific). The precipitated mRNA was dissolved by nuclease-free water and the RNA concentration was determined by Nanodrop2000 (ThermoFisher Scientific). Then, cDNA was synthesized using oligo (dT) primers with the Transcriptor First Strand cDNA Synthesis Kit (4896866001, Roche). Selected gene mRNA levels differences were detected by CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using iQ™SYBR® Green Supermix (1708884, Bio-Rad). The results were normalized against GAPDH gene expression. The coding sequence of human LRRN4 was

**Table 1.** The information of human donor hearts, DCM hearts and IHD hearts

| Parameter | Donor Hearts | DCM Hearts | IHD Hearts |
|---|---|---|---|
| No. | 6 | 14 | 14 |
| Age, y | 31.8±10.2 | 43.9±13.2 | 51.2±8.5 |
| Sex, male/total (%) | 2/6 (33.3) | 2/14 (14.3) | 0/14 (0) |
| BMI (kg/m$^2$) | N/A | 22.8±1.8 | 23.3±2.2 |
| LVEDD (mm) | N/A | 78.5±8.7 | 72.1±6.6 |
| LVEF (%) | 62±3.0 | 24.2±7.1 | 25.6±8.3 |
| Heart rate, /min | N/A | 92±11 | 85±10 |
| Blood pressure, mmHg | | | |
| Systolic | N/A | 105.7±14.8 | 111.3±9.9 |
| Diastolic | N/A | 59.1±6.2 | 68.2±10.0 |

BMI: body mass index; LVEDD: left ventricular end-diastolic dimension; LVEF: left ventricular ejection fraction; N/A: not available.

reverse-transcribed using the primers 5'-TCTGTCGCCACACACATCTT-3' (forward) and 5'-CGTGAGGAGCCAAGACAAGT-3' (reverse). The primers of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were as described before [15].

*Histological analysis*

Hearts were immediately fixed in 10% formalin after the hearts retrieval. Then the hearts were hydrated and embedded in paraffin by using standard histological procedures. Subsequently, these hearts were sectioned transversely at 5 μm. Sections were stained with hematoxylin-eosin (HE) for histopathology and myocytes cross sectional area analysis or picrosirius red (PSR) for collagen deposition evaluated as performed before.

*Immunohistochemical analysis*

The immunohistochemistry analysis for LRRN4 was followed the standard protocol. The paraffin sections were put to boil in 10 Mm sodium citrate buffer (Ph 6.0) and maintain at a boiling temperature for 10 min after being deparaffinized and hydrated. After that the slides were blocked with blocking buffer (5% normal goat serum (16210-064, ThermoFisher Scientific) in TBST) for 1 hour at room temperature. Then the slides were treated with 3% hydrogen peroxide dilution for 10 min. Subsequently, the LRRN4 primary antibody (1:25 dilution) was added to the slides for incubating overnight at 4°C after removing blocking solution. The peroxides-conjugated secondary antibody (Jackson immunoResearch Laboratories, 111-035-003, at 1:2500 dilution). The DAB kit was used to develop color, and then counter stain sections with hematoxylin. The slices were mounted with mounting solution after dehydration.

*Statistics analysis*

Data were represented as the mean ± standard error (SEM) and the statistical differences are shown in the figure legends. Student's two-tailed t-test was used to compare the means of two groups. $P<0.05$ is considered as statistical significance. The SPSS software (version 13.0) was used to perform all the statistical analysis of present study.

## Results

*LRRN4 was expressed in human left ventricle and its expression level was remarkably decreased in DCM hearts*

Previous research showed that LRRN4 was observed in various regions of the brain, peripheral nervous system, lung, and ovary of C57BL/6J mice [11, 16]. Nevertheless, its expression pattern in human normal hearts and in cardiomyopathy hearts was still largely unknown. To study this, we collected normal donor hearts and DCM human hearts. As shown in **Table 1**, the DCM patients had severe cardiac dysfunction defined by poor ejected fraction and enlargement of left ventricular compared to normal donor hearts. HE and PSR staining demonstrated striking cardiomyocytes hypertrophy and cardiac fibrosis (**Figure 1A** and **1B**). Meanwhile, DCM hearts showed higher mRNA level of the hypertrophic marker genes ANP and BNP (**Figure 1C**). Subsequently, we tested the mRNA level of LRRN4 in human left ventricular. Our results demonstrated that LRRN4 expressed in donor left ventricular and was remarkably decreased in DCM hearts (**Figure 2A**). Furthermore, we detected the protein level of LRRN4 which is also reduced comparably in DCM hearts (**Figure 2B** and **2C**).

*Cellular localization of LRRN4 in human left ventricular*

It is well known that human hearts were composed of several kinds of cells, such as cardiomyocytes, fibroblasts, endothelial cells and immune cells [17]. Therefore, it is valuable to

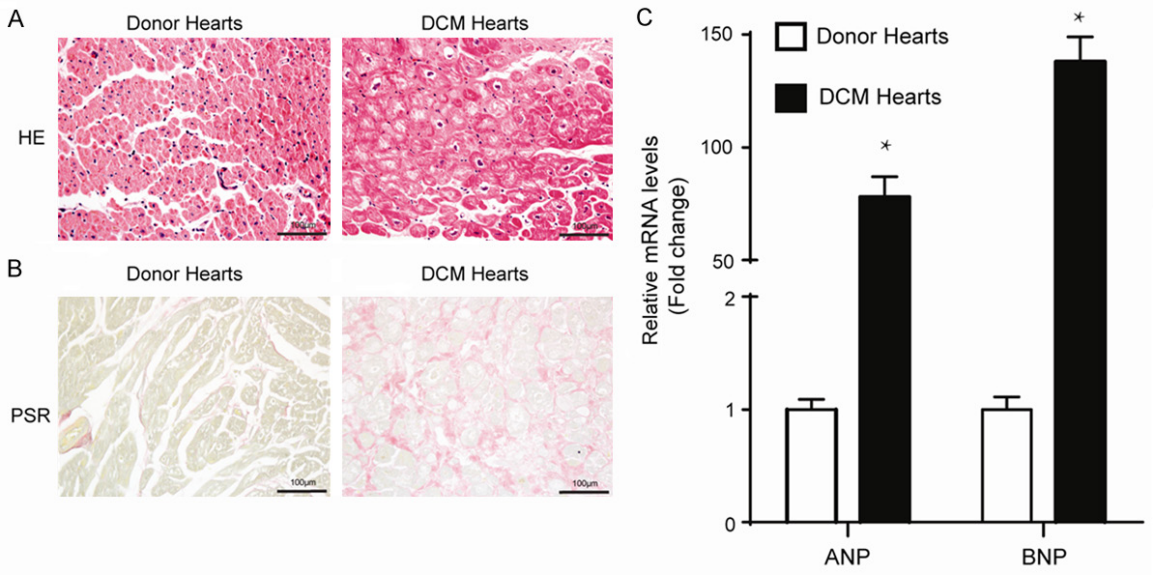


**Figure 1.** The pathology and molecular properties of human dilated cardiomyopathy (DCM) hearts. A. The HE staining of left ventricle of donor hearts (n=4) and DCM hearts (n=10) (scale bar, 100 μm); B. The PSR staining of left ventricle of human hearts (n=4 in donor hearts and n=10 in DCM hearts, scale bar, 100 μm); C. Real-time PCR detected the mRNA levels of heart failure biomarkers atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in the human hearts (n=4 in donor hearts and n=10 in DCM hearts).

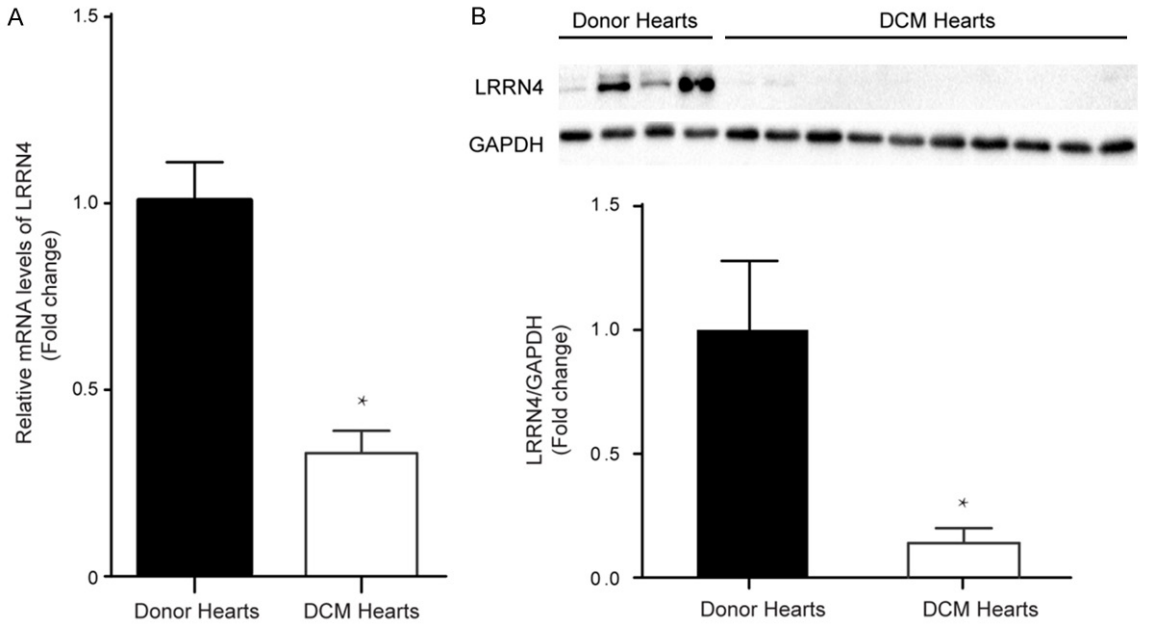


**Figure 2.** LRRN4 was expressed in human left ventricular and the LRRN4 level was dramatically decreased in the DCM hearts. A. Real-time PCR result of LRRN4 in the human donor and DCM hearts (n=6 in donor hearts and n=14 in DCM hearts). B. The protein level of LRRN4 was detected via western blot (n=4 in donor hearts and n=10 in DCM hearts). *P<0.05 vs normal hearts.

uncover in which type of cell is LRRN4 expressed. Since LRRN4 mRNA and protein were detected in left ventricle, we next analyzed the cellular localization of LRRN4 in the ventricle myocardium. We performed immunohistochemistry in slices of human donor hearts

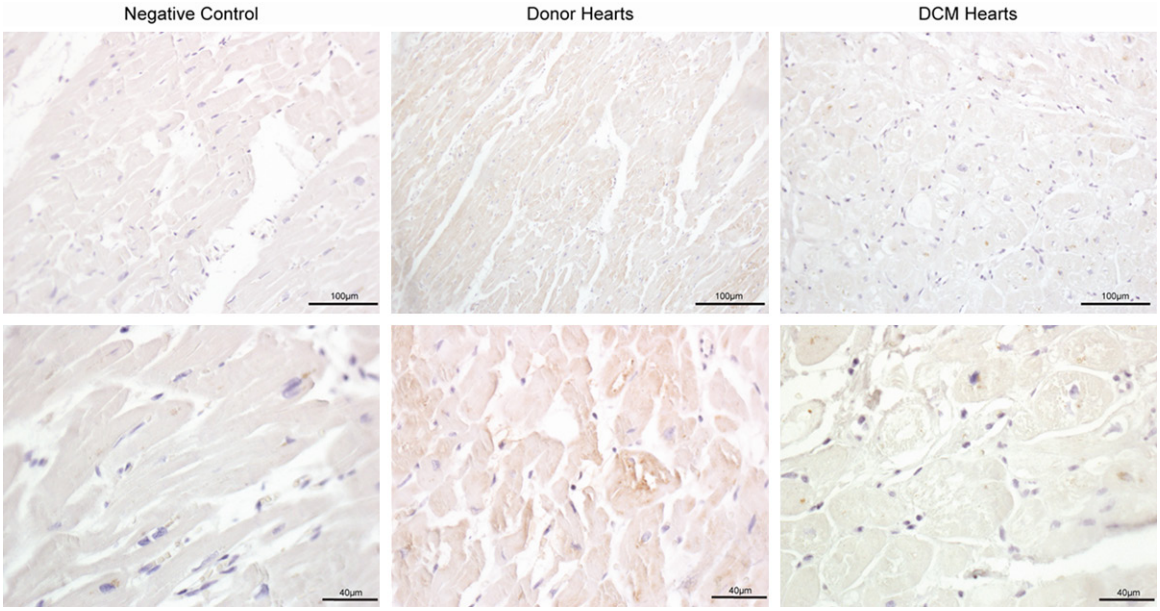


**Figure 3.** Distribution of LRRN4 in human left ventricular. The immunohistochemistry assay showed LRRN4 (brown) expression and localization specially on ventricular cardiomyocytes (n=6 in donor hearts and n=10 in DCM hearts; first panel, scale bar, 100 μm; second panel, scale bar, 40 μm).

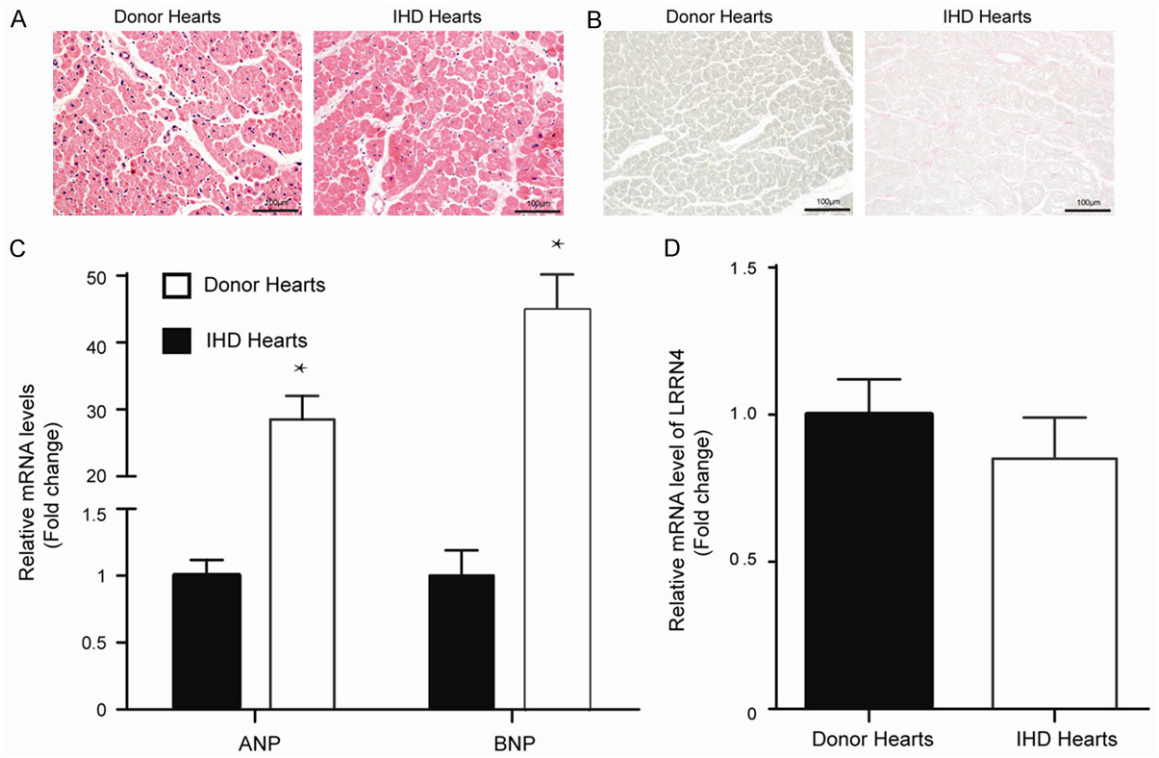


**Figure 4.** The pathology and molecular properties of human ischemia heart disease (IHD) hearts and the mRNA level of LRRN4 has no significant change in the IHD hearts. A. The HE staining of left ventricle of donor hearts (n=6) and IHD hearts (n=14) (scale bar, 100 μm). B. The PSR staining of left ventricle of human hearts ((n=6 in donor hearts and n=14 in IHD hearts, scale bar, 100 μm). C. Real-time PCR detected the mRNA levels of heart failure biomarkers brain natriuretic peptide (BNP) and atrial natriuretic peptide (ANP) in the human hearts (n=6 in donor hearts and n=14 in IHD hearts. D. Real-time PCR results of LRRN4 in the human donor and IHD hearts (n=6 in donor hearts and n=14 in IHD hearts).

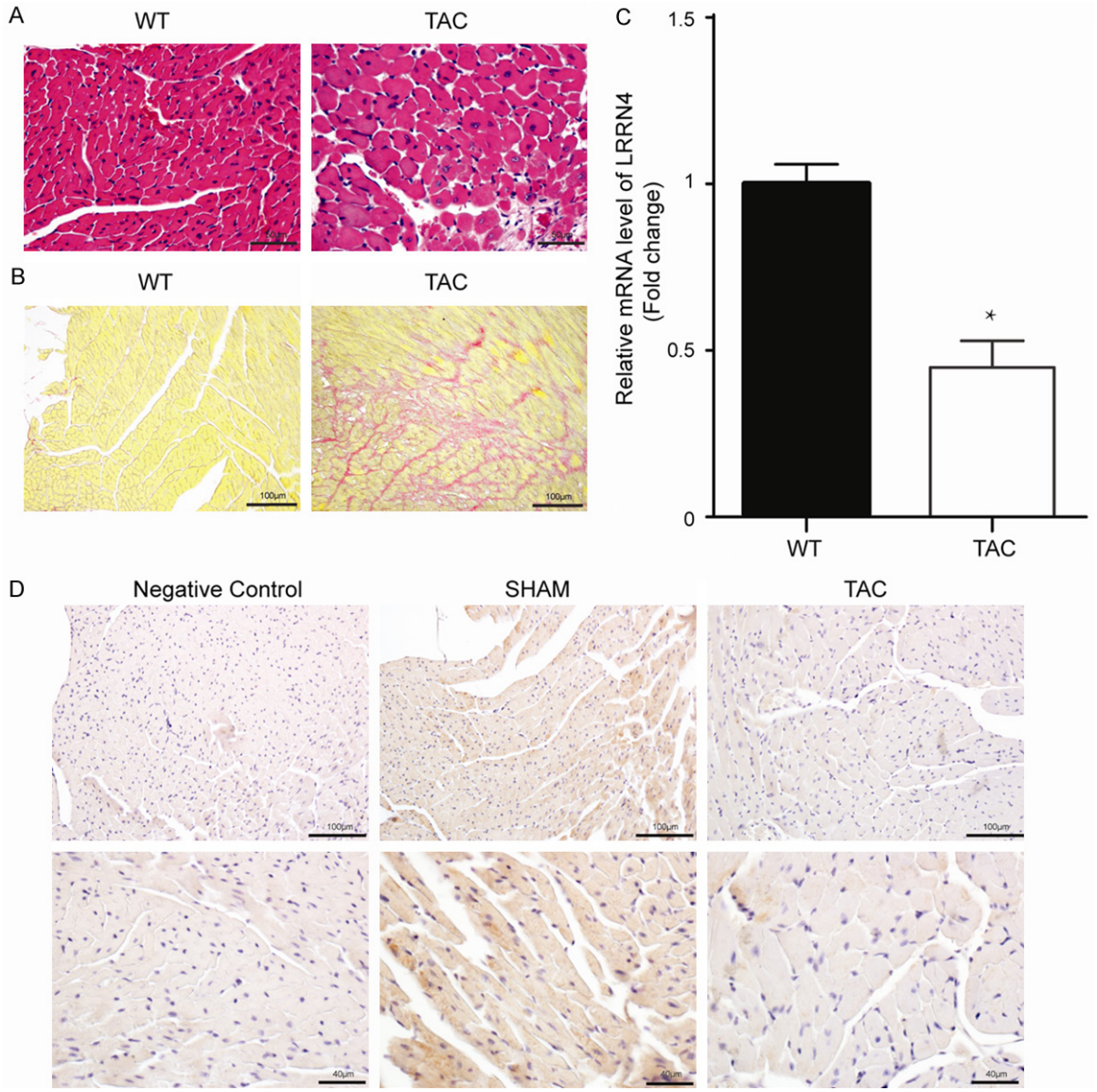


**Figure 5.** After 8 weeks transverse aortic constriction (TAC), wild type (WT, C57BL/6 background) mice appeared significant cardiac hypertrophy. A. The HE staining of left ventricle of mice with sham and TAC treatment for 8 weeks (n=8 per experimental group) (scale bar, 50 μm). B. The PSR staining of left ventricle in the indicated groups (n=8 per experimental group) (scale bar, 100 μm). C. Real-time PCR results of LRRN4 of left ventricle in the indicated groups (n=8 experimental group). D. The immunohistochemistry assay shows LRRN4 (brown) expression and localization specially on ventricular cardiomyocytes (n=8 per experimental group; first panel, scale bar, 100 μm; second panel, scale bar, 40 μm).

and DCM hearts. The result showed that LRRN4 expressed in cardiomyocytes mostly (**Figure 3**). In agree with the aforementioned results, the level of LRRN4 was notably decreased in DCM hearts.

*Expression level of LRRN4 has no significant change in IHD patients*

It was recognized that after the acute phase of myocardial ischemia, a chronic maladaptive phase of ventricular remodeling occurs [18]. Both DCM hearts and IHD hearts displayed cardiac remodeling, as evidenced by cardiomyocytes hypertrophy and cardiac fibrosis (**Figure 4A** and **4B**). Thus, it was of great interest to determine whether the level of LRRN4 changed in IHD hearts. Similar to DCM hearts, ANP and BNP were significant increased (**Figure 4C**). However, the mRNA level of LRRN4 had no significantly difference between donor hearts and IHD hearts, although there was a trend toward decreased in IHD hears (**Figure 4D**).

*LRRN4 expression level was reduced in the pressure overload-induced hypertrophic mouse hearts*

Given the results in human hearts, we verified LRRN4 expression pattern in normal mice and pressure overload-induced hypertrophic mice model. The wild type (WT, C57BL/6 background) mice were subjected to TAC treatment to produce pressure overload-induced cardiac remodeling and heart failure model. As shown in our previous study [15], the results showed that the heart weight (HW)/body weight (BW), lung weight/BW and HW/tibia length (TL) ratios were increased after 8 weeks of TAC treatment. Echocardiographic results demonstrated that the WT mice exhibited decreased cardiac function, which indicated that WT mice developed remarkable cardiac hypertrophy. As shown in the **Figure 5A** and **5B**, histological tests showed that cardiomyocytes cross-sectional area and aberrant collagen deposition were apparently increased after 8 weeks of TAC treatment. Next, we utilized immunohistochemistry analysis to detect the expression level and cellular localization of LRRN4 in the heart of mice with sham or TAC surgery. Our results presented that LRRN4 expression was sharply decreased in cardiomyocytes of mice subjected to TAC surgery compared to sham mice (**Figure 5C**).

## Discussion

According to previous studies, it was discovered that a plenty of LRR proteins played an important role in heart disease [10, 19, 20]. However, the expression pattern of LRRN4 during the heart pathologic process has remained unclear. Our results demonstrated that LRRN4 expression significantly decreased in DCM hearts but not IHD hearts, while LRRN4 highly expressed in normal cardiomyocytes. Consisting with the results in human hearts, the expression level of LRRN4 sharply decreased in hypertrophic mice hearts. As far as we know, this is the first study to discover the expression pattern of LRRN4 in human heart and the potential role of LRRN4 in pathological process of DCM.

Dilated cardiomyopathy was described as the final response of myocardium when face to diverse genetic and environmental insults, such as biomechanical stretch or strain that is sensed by mechanosensory machinery embedded in the Z-disc cytoskeleton and sarcolemma [1, 21]. Pathological stimuli subsequently activated cellular signaling pathway like protein PI3K/Akt signaling pathway [22], stretch-activated channel [23], etc., which acted as bridge between environment stimulation and intracellular gene expression changing [14, 24]. Several LRR proteins have been discovered functioned not only in response to pathological stimuli but also in various typical signaling pathways in DCM [25]. In regard to the molecular mechanisms of cardiac hypertrophy, LRR proteins are the focus of much attention. Previous study showed that leucine-rich repeat protein played an important role in cardiac remodeling [10] and cardiac contractile function maintaining [26]. It has been identified that dynamic interaction between leucine-rich repeat containing protein 10 (LRRC10) and actin thin filaments is reduced in response to pressure load [25]. Their results suggested an integral role for LRRC10 in the response of the heart to mechanical stress [25]. *Purcell NH. et al.* showed that PH domain leucine-rich repeat protein phosphatase-1 (PHLPP1), a member of leucine-rich repeat protein family, knockout mice showed increased Akt Ser473 phosphorylation and Akt targeted gene expression elevated which is responsible for the attenuation of cardiac hypertrophy and fibrosis 2 weeks after TAC treatment [10].

LRRN4 is a new member of the leucine-rich repeat protein family. *Atsushi Miyajima et al.* first described the identification and characterization of LRRN4 [11]. Their results showed that LRRN4 expressed in mice hippocampus and LRRN4$^{-/-}$ mice displayed the ability of hippocampus-dependent learning and failed to retain long lasting memory [11]. LRRN4 was identified as type I transmembrane protein with leucine-rich repeat domain with a fibronectin type III repeat (FN-III) domain [11, 27]. FN-III domain is a dimer which is composed of two similar polypeptides and involved in the process of cell adhesion [28]. *Yoshihiro Morikawa et al.* showed that LRRN4 expressed in a subset of nociceptive neurons of dorsal root ganglia (DRGs) and LRRN4 expression was reduced in injured side of DRGs after sciatic nerve axotomy [16]. These results suggested that LRRN4 might function as a synaptic adhesion molecule to keep nociceptive circuits [16]. As we know, cell-surface adhesion molecule, such as the integ-

rin family, regulated cardiac hypertrophy through mechanical stimulation mediating and signal transduction resulting from protein interaction [29, 30]. Considering the crucial role of LRR protein played in DCM and the protein feature of LRRN4, our research focused on its expression pattern and potential role in DCM. Innovatively, our present study showed that lower LRRN4 expression was detected in DCM hearts while its highly expression in normal hearts. It indicated that LRRN4 was associated with DCM. However, the exactly role of LRRN4 on DCM was needed future study.

In conclusion, our present study discovered the expression pattern of LRRN4 in human hearts and proved that LRRN4 level decreased in DCM hearts but not IHD hearts. But the specific mechanism underlying decreased LRRN4 expression level in DCM hearts is still unclear. As LRRN4 is a transmembrane protein with protein-protein interaction motifs, we prudently hypothesized that LRRN4 functioned as cellular adhesion molecule and played a potential role in DCM. It will be of great interest to perform further study to reveal the specific role of LRRN4 in DCM and the underlying mechanism. LRRN4 may be a novel therapeutic target for DCM with cardiac remodeling.

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## Disclosure of conflict of interest

None.

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