The Notch1/Jagged1 Pathway is Involved in the Development of Rheumatic Heart Disease

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ABSTRACT
Objective: To investigate the role of Notch1/Jagged1 in the development of rheumatic heart disease (RHD). Methods: 35 RHD patients who underwent mitral valve replacement, 35 healthy volunteers, and 10 dead persons by traffic accident were selected in the present study. The morphological changes of mitral valve in the RHD patients were observed. The mRNA and protein levels of Notch1/Jagged1 in the mitral valves were assayed using real-time PCR and western blot, respectively. The location and distribution of Notch1/Jagged1 proteins in the mitral valves were analyzed by immunohistochemistry. Flow cytometry (FCM) was used to measure the positive ratios of Notch 1/Jagged1 in isolated peripheral mononuclear cells. Results: Histomorphology data showed that the fusion of commissures, cusps, and chordae tendinea resulted in thickened, shortened and inflexible alterations of the mitral valves. Real-time PCR and western blot assays revealed that the mRNA and protein levels of Notch1/Jagged1 were significantly higher in the impaired mitral valves of the RHD patients compared to controls. Immunohistochemistry revealed that Notch1/Jagged1 proteins were mainly located in the cytoplasm of fibroblasts in the mitral valves. The positive ratio of Jagged1 in peripheral mononuclear cells in the RHD patients was significantly elevated compared to the controls. Conclusion: Notch1/Jagged1 expression in the damaged mitral valves of the RHD patients is significantly increased compared to controls. Meanwhile, the positive expression ratio of Jagged1 in the peripheral mononuclear cells of the RHD patients is markedly up regulated. In summary, the Notch1/Jagged1 pathway correlates with the development of RHD.

Key words: Rheumatic heart disease, Notch1, Jagged1, Mitral valve, Peripheral mononuclear cell.

INTRODUCTION
The Notch signaling pathway is a highly conserved cell signaling system in biological evolution. It has been reported that this ancient signaling system plays critical roles in various types of proliferation including arterial and endothelial growth, and myocardial development.1-3 Notch signaling pathway also widely affect cellular functions, including apoptosis,4-5 differentiation6-9 and developmental lineage choices.10-11 In humans, there are 4 Notch receptors, including Notch1, 2, 3, 4, as well as 5 Notch ligands (Jagged1 and 2, Deltalike ligand 1, 3 and 4), have been found in different types of tissues and cells.12 The binding of Jagged 1 or 2 will produce a series of proteolytic reactions. As a result, the cleaved Notch releases an intracellular domain of Notch (NICD), which translocates into the nucleus and functions as a transcriptional activator of downstream genes.13-14

Some studies have revealed that Notch signaling significantly promote T-cell development in the hematopoietic system,15-16 and regulate differentiation and functions of Th1/Th2 whose out of balance is associated with permanent impairment of cardiac valves in peripheral blood cells.17 More recent researches have demonstrated Notch signaling to be involved in tissue fibrosis and functions.18-20 Furthermore, Notch signaling might be important to control the maintenance and commitment of a cardiac stem cell compartment to renew cardiac tissue.21 One study has demonstrated that cardiac developmental defects emerged when Notch1 intracellular domain (NICD1) was over expressed, or Notch signaling in the embryonic cardiomyocyte compartment was selectively silenced.22 To date, there are plenty of studies identified that perturbed Notch-Jagged signaling system accounts for some forms of congenital and adult cardiac disease (including aortic valve disease).23-25 Endothelial deletion of Jagged1 leads to valve calcification, which is one of important evidences for Jagged 1 in valve morphogenesis.26 Insufficiency of Notch1 receptor is also indicated in aortic valve disease in humans.

Valvular heart disease which originates from rheumatic heart disease (RHD) is one of main causes for heart failure in West China. Despite there are many studies to prevent and treat RHD, the morbidity and mortality are still very high in this region. As we know, the main alterations of valvular disease derives from RHD are immune and autoimmune responses. Actually, details of the mechanism to the pathological variations in RHD are unclear. To characterize the role of Notch signaling, which maybe the link between immune cell and cardiac valve, specifically in the development and evolution of RHD, in the present study herein, we investigated the expression of Notch1/Jagged1 in the mitral valves and peripheral mononuclear cells of the RHD patients.

METHODS
Participants
There were 35 RHD patients, including 14 male and 21 female, who underwent mitral valve replacements in the Affiliated Hospital of Luzhou Medical College (Luzhou, Sichuan Province, China) from August 2009 to March 2010 were selected for the present study. The age was 50.8±11.7 years. The RHD diagnosis was confirmed by echocardiography and biopsy tests. The exclusion criteria included congenital heart disease, senile valvular disease, syphilitic valvular disease, infective endocarditis, insufficiency of the papillary muscle, rupture of chordae tendineae and mucoid degeneration and other similar diseases. Only one patient had family history and her sister also died of RHD. All patients who were preoperative given the routine administration had grading II cardiac function (New York Heart Function Assessment). Meanwhile, 35 healthy volunteers (16 male and 19 female) who had no valvular disease, and 10 persons (6 male and 4 female) who died by traffic accidents served as controls. A positive diagnosis also was confirmed by echocardiography. Each patients and volunteers (including the dead persons by accidents) had a result of electrocardiogram (ECG) test or been found results in their physical examination file. In the present study herein, the RHD
patients, the healthy volunteers, and the relatives of the dead persons by accidents read, understood, and signed the informed consent. This study was also approved by the Committee of the Affiliated Hospital of Luzhou Medical College (Luzhou, China). The detail is listed in Table 1. It should be noted that both the healthy and dead controls had no heart disease or any complications. Echocardiography results showed that 35 (100%) RHD patients had mitral stenosis, 21 (60%) had mitral insufficiency, 13 (37.1%) had aortic stenosis/insufficiency, and 27 (77.1%) had tricuspid insufficiency.

Histology and morphology of the mitral valves

The separated mitral valves, which acquired from RHD patients and dead persons timely, were cut into a size of 3.0×1.0 mm. After then the samples were stained by hematoxylin and eosin (H.E.) and observed under CX31 optical microscope (Olympus Co., Tokyo, Japan). The cardiac mitral valve tissues for real-time PCR, western blot, and immunohistochemistry assays were from the same valves that were used for the histology and morphology assays. The pathological changes of the mitral valves tissues were observed and assessed by two independent pathologists. Any discrepancy between the two reviewers was resolved by reexamination to achieve an agreement.

Real-time PCR assay of Notch1/Jagged1 mRNAs in the mitral valves

The total RNA was extracted from 50-100 mg of the separated mitral valves, in accordance with the manufacturer’s instructions, respectively. After DNase I treatment, 2 μg of RNA was reverse transcribed to cDNA using AMV reverse transcriptase. Standard reaction system consisted real-time PCR Master Mix SYBR Green I, forward primer, reverse primer, cDNA, and ddH2O. The reaction conditions included denaturation at 95°C for 4.5 min and annealing at 58°C for 40 sec. The data were analyzed using IQ5 software of the Gene express module (Bio-Rad, CA, USA). There were three replicate reactions were performed and values were normalized to the housekeeping gene β-actin, CT values were determined by using the 7500 System SDS Software (version.1.2.3; Applied Biosystems, USA), and ΔΔCT values were computed using the housekeeping gene β-actin; CT values as internal controls. Expression ratios were finally calculated in accordance with 2 ΔΔCT method.27

Western blotting assay of Notch1/Jagged1 proteins in the mitral valves

The cardiac mitral valve tissues (about 50 mg) were lysed with 1mL of pre-cooled RIPA lysate. Subsequently, the cells were disrupted using pre-cooled RIPA lysate. Subsequently, the cells were disrupted using pre-cooled RIPA lysate. The mitochondria were centrifuged, the supernatants were collected. The concentration of the total protein was quantified by the Bradford method. The proteins were denatured at 95°C for 4.5 min and incubated at 105°C for another 2 h. The membrane was stained by enhanced chemiluminescence (ECL) reagent (Pierce, USA), and imaged on X-ray film (Fuji film, Tokyo, Japan) by autoradiography. Quantity one Imagine System and analysis software (Bio-Rad, CA, USA) were used to analyze the specific straps quantitatively.

Immunohistochemistry assay of Notch1/Jagged1 in the mitral valves

Specimens from 35 RHD patients and 10 died controls were used for this study. The tissue sections were suffered from degrading and dehydration, and then processed using the streptavidin immunoperoxidase method. Briefly, the sections were submitted to antigen retrieval at 95°C and incubated in 10% normal serum, followed by an overnight incubation at 4°C with the diluted goat anti-human Notch1 (1:100, eBioscience, USA)/Jagged1 (1:200, Abcam, USA). After that, the samples were incubated with biotinylated anti-goat immunoglobulins for 15 min at 37°C, followed by streptavidin peroxidase complexes. After hematoxylin counterstaining, immunostaining was quantificationally scored by a CM-2000B imaging analysis system (Beijing University of Aeronautics and Astronautics, Beijing, China). Identification of immunohistochemistry results was according to the criteria proposed by Maruyama. The membrane, cytoplasm, and nucleus were observed. If gray yellow or claybank particles were more than 10% in the cytoplasm and nucleus, it was regarded as positive.

Flow cytometry assays of positive Notch1/Jagged1 expressions in peripheral mononuclear cells

Anticoagulant venous blood, 1 mL, was collected from each of RHD patients and healthy controls. Then the mononuclear cells were separated and harvested by a gradient separation method in accordance with the manufacturer’s instructions of a human mononuclear cells separation medium kit (Lenqton Biotech. Inc., Shanghai, China). The cells were resuspended and adjusted to a concentration of 1×106/mL. Notch1-PE and Jagged1-FITC, 100 μl respectively, added to co-culture with the treated cells for 10-15 min at room temperature, and without light. Then material was centrifuged and washed with PBS twice. The positive ratios of Notch1/Jagged1 in the cells were detected using a FACScan Flow Cytometer (Becton Dickinson, USA). The data were analyzed by Cell Quest 3.0 software (Becton Dickinson, USA).

Data presentation and statistic analysis

Data are presented as mean± standard deviation, and Student-Newman-Keuls (SNK) test was employed to compare the difference between groups using SPSS11.0 statistics software (SPSS Inc., Chicago, IL, USA). A p value of less than 0.05 was considered significant.

RESULTS

General information of participants

There were no significant differences in baseline values, including age, gender, and nationality among groups (Table 1). Meanwhile, no other complications including diabetes mellitus, hyperthyroidism, and immunologic diseases were found in these participants.

Morphological changes of mitral valve in RHD patients

Fusion of the mitral valve apparatus including commissures, cusps, and chordae tendinae resulted in the shortened, thickened (Maximum thickness=0.6 cm) aspects and inflexibility of these structures. Normal structures of mitral valves were severely destroyed and replaced by a mass of fibrous tissues, leaving cicatrization and hyalinosis. The new micro vessels arose in stenotic valves around the lymphocytes (Figure 1).

The mRNA and protein levels of Notch1/Jagged1 were upregulated in the mitral valves of the RHD patients

The results showed that both Notch1 and Jagged1 mRNAs in the mitral valves of the RHD patients were significantly upregulated compared to controls (Both P<0.01) (Figure 2a). Furthermore, western blot data also
Positive ratio of Jagged1 was elevated in the peripheral mononuclear cells of the RHD patients

In order to assess the role of Notch1/Jagged1 in the development of RHD comprehensively, we employed FCM to assay positive ratios of Notch1/Jagged1 in peripheral mononuclear cells. The FCM results showed that the positive ratio of Jagged1 in the peripheral mononuclear cells of the RHD patients was strikingly higher than that observed for the controls ((17.63±5.28) vs. (3.17±1.23)%,

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Figure 2: The mRNA and protein expression of Notch1/Jagged1 in the mitral valves. (a), QRT-PCR assay of Notch1/Jagged1 mRNAs in the mitral valves. *P<0.01 vs. Control; (b), Western blotting assay of Notch1/Jagged1 proteins in the mitral valves. *P<0.01 vs. Control; (c), The protein expression of Notch1/Jagged1 in the mitral valves. *P<0.01 vs. Control. (QRT-PCR: quantitative real-time polymerase chain reaction)

Immunohistochemistry assay of Notch1/Jagged1 in the mitral valves

To identify the location and distribution of Notch1/Jagged1 in the mitral valves, we used an immunohistochemistry assay in this part of the experiment. The result revealed that the amount of fibroblasts was significantly increased and their arrangement became disordered, compared to the normal control (Figure 3). Gray yellow or clay bank particles, which represented positive staining of Notch1/Jagged1, were found located in the cytoplasm of fibroblast. Positive cells spread all over the field or distributed in the focal field (Figure 3b and 3e). The average integral optical density (IOD) of Notch1 in the mitral valve in the RHD patients was markedly elevated compared with that in the control (0.4231±0.0539 vs. 0.1528±0.0331, *P<0.05). Similarly, the IOD of Jagged1 was significantly higher than the control (0.4305±0.0421 vs. 0.1027±0.0203, *P<0.05) (Figure 3g).

DISCUSSION

Rheumatic heart disease (RHD) is mainly caused by rheumatic fever, and the most common damage is mitral stenosis, usually accompanied by mitral insufficiency or aortic stenosis and/or insufficiency. During the last few years, there are remarkable changes in the diagnosis, evaluation

Figure 3: Immunohistochemistry assays of Notch1/Jagged1 in the mitral valves (Streptavidin-perosidase ×400). Compared with normal control (a), more fibroblasts are presented in RHD mitral valve and there was lots of clay bank particles (Shown by red arrow) which shows that Jagged1 expresses in the cytoplasm of fibroblast (b). Abundant of similar particles are found in positive control (colon cancer cells) (c).
and management of RHD, but high morbidity and mortality still exists, especially in west China. The conspicuous characteristic of RHD is that it is closely associated with group A streptococci infection. In some degrees, it is deemed as an autoimmune disease. There have antigen driven immune responses in cardiac lesions of RHD patients after a common initial bacterial challenge. The autoantibodies, cytokines, activated T lymphocytes (CD4+/CD8+ T cells), mononuclear cell and other important molecules participant in this process. Recent studies demonstrated that the persisting penetration of T lymphocytes implies the damage of the valve is continuous and developing. More advanced studies revealed that the penetrating T lymphocytes are directly involved in the occurrence and development of RHD, especially CD4+ T cells which can differentiate into Th1 and Th2. Actually, the balance between Th1/Th2 is of great importance to autoimmune diseases. Roberts et al. found several antibodies to valvar endothelium and found a plenty of CD4+ T cells’ infiltration around the complex. Similarly, Ellis showed that clonal T cells from the RHD patients could react with streptococci M6 protein, cardiac myosin, valvular protein and laminin.

Notch signaling is a key pathway in evolution, which can modulate cellular and tissue differentiation accurately. A Notch signaling pathway is comprised of Notch receptors such as Notch1, 2, 3 and 4, Notch ligands including Jagged1, Jagged2, Delta1, Delta3 and Delta4, intracellular DNA binding proteins, and Notch modulators. These receptors and ligands form the DSL (Delta/Serrate/Lag-2) family in which the members are trans membrane proteins and hydrolyzed by interaction. After that, they penetrate into the nucleus and control the target genes. Notch1 presents in most tissues including brain, heart, liver, and thymus gland. Jagged1 is abundantly expressed in T cells and antigen-presenting cells. The Notch- Jagged signaling pathway is activated by the Jagged1 which combined to the Notch intracellular domain, which exists in the surface of cells nearby, mediating the target genes by a series of transcription factors such as Hes-1, pre-Tα, Nrarp, Nur77, and others. This signaling pathway plays a crucial role in the development of the heart valve. In rat embryonic heart, it is essential to activate the Notch signaling pathway, which can induce epithelial cells to be the mesenchymal transition (EMT) that forms valve eventually. Many studies have demonstrated that mutations of Notch genes were associated with congenital heart diseases, such as bicuspid aortic valve, calcification of valve, and ventricular septal defects. Timmerman et al. found that it would generate a hypertrophic valve when the Notch signaling pathway was continuously activated by Notch ICD gene. Meanwhile, a study also showed that mutation of the Jagged1 gene exists in 94% of Alagille syndrome patients whom commonly had pulmonary stenosis. In our study herein, the expressions of Notch1 and Jagged1 in the damaged valves of the RHD patients were significantly higher than those in the normal controls. Also, we found that there was significant proliferation of connective tissue and a mass of lymphocytes infiltration in the damaged valves. Thus, we speculated that the high expression of Notch1/Jagged1 might induce the differentiation and activation of T cells, thereby aggravating the impairment of the valves and promoting excessive proliferation of fibroblasts, which led to thickening and inflexibility of the valves. It was found that Notch1 played an essential role in lymphoid stem cells’ differentiation into T cells, and Jagged1 could restrain thymocytes transformation to B cells. Furthermore, Notch1/Jagged1 had a great influence on the development of CD4+ and CD8+ T cells. Additionally, Notch-Delta also could impel CD4+ T cells to transform into Th1 cells, while the balance between Th1 and Th2 is a critical factor for lesions of heart valves. It was also found that blockage of Notch1 signaling of peripheral blood mononuclear cells (PBMCs) could regulate the immune balance of Th1/Th2. In the early study, researchers reported that PBMCs from rheumatic fever (RF) patients react with cell wall and membrane streptococcal antigens, which similar to the action of T-cell lines derived from heart valve specimens, and that from RHD patients display a vigorous recall response to previous streptococcal infection. The advanced studies demonstrated that peripheral and heart-infiltrating mononuclear cells produced a series of cytokines which are the mediators of myocardium and valvular inflammation and drive the autoimmune response and further lead to permanent and progressive valvular damage.

In the present study, we found that it was Jagged1 but not Notch1 in the PBMCs of the RHD patients was significantly higher than that found in the healthy controls. It is well known that Jagged1 is one kind of ligands that can link the receptors of Notch, not only Notch1, but also Notch2 or 3. This, we thought that in the development of RHD, Jagged1 was more susceptible to be activated than Notch1.

**CONCLUSION**

It suggested that there was a close correlation between Notch1/Jagged1 pathways and the development of RHD. Actually, a larger sample is needed to clarify the role of Notch1/Jagged1 in the development of RHD more deeply. In our future work, we would like to explore the relationships more particularly between other Notch receptors and ligands to illustrate the more details about how the Notch signaling pathway works in RHD, which may give beneficial clinic significance for the therapy of RHD.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

REFERENCES