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Original Article

Increased expression of WISP-1 (CCN4) contributes to fibrosis in the hypertrophied lumber ligamentum flavum

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Abstract: The molecular mechanism of the tissue fibrosis of ligamentum flavum (LF) in patients with lumbar spinal canal stenosis (LSCS) has not been ascertained. Wnt-induced secreted protein-1 (WISP-1) is a critical growth factor regulating cell growth, proliferation, and extracellular matrix expression and involving in fibrotic process. The present study aimed to explore the possible relationship between WISP-1 expression and LF fibrosis in subjects with LSCS. Twenty-one patients with LSCS scheduled to surgical treatment were recruited in this study, while eleven patients with lumbar disc herniation as the control group. The thickness of the LF at the level of the facet joint was measured on MRI. LF samples were harvested from the posterior surgery. Then, fibrosis score was assessed by Masson's trichrome staining. mRNA and protein expression of WISP-1 in LF cells was assayed by RT-PCR and western blotting, respectively. Results were compared between the two groups. Correlation among LF thickness, fibrosis score, and WISP-1 expression was analyzed. In addition, human LF cells were cultured and exposed to recombinant human WISP-1. Types I and III collagen expression were analyzed using PCR. The patients in both the groups showed similar outcomes with regards to age, gender, level of LF tissues. The thickness and fibrosis score of LF in the LSCS group were significantly greater than those in the control group (all $P < 0.05$). mRNA and protein expression of WISP-1 in LF cells was substantially higher in the LSCS group than in the control group ($P < 0.001$). Furthermore, the WISP-1 concentration exhibited a positive correlation with the LF thickness ($r=0.917$, $P < 0.001$) and fibrosis score ($r=0.898$, $P < 0.001$). Different concentrations of rhWISP-1 treated LF cells upregulated the mRNA expression of types I and III collagen ($P < 0.001$). We concluded that the increased expression of WISP-1 in human LF cells from patients with LSCS may play an important role in the pathogenesis of the fibrosis of LF.

Keywords: Ligamentum flavum, lumbar spinal canal stenosis, hypertrophy, fibrosis, Wnt-induced secreted protein-1

Introduction

Lumbar spinal canal stenosis (LSCS) is one of the most common spinal disorders in the aging population. Neurologically, LSCS is mostly associated with low back pain, neurogenic claudication, and cauda equina syndrome [1]. The reasons for LSCS include abnormal growth of osteophytes, disc protrusion, and fibrosis and hypertrophy of the ligamentum flavum (LF). Among them, fibrosis and hypertrophy of the LF has been identified as a significant pathogenetic factor contributing to the development of LSCS [2, 3].

Numerous studies regarding the LF hypertrophy have been performed to explore the pathogenesis; however, the precise mechanism remains unknown. To date, it has been accepted that LF hypertrophy is a multifactorial disease, which is often associated with age [1], mechanical stress [4], inflammatory cytokine [5], and herniated intervertebral disk [6]. Histologically, LF hypertrophy is characterized by the loss of elastic fibers and an increase in collagen fibers, ultimately resulting tissue fibrosis [7]. Furthermore, previous studies suggested that fibrosis was the main cause contributing to LF hypertrophy [8]. However, the molecular mechanisms underlying the tissue fibrosis in LF has not been fully elucidated.

Connective tissue growth factor (CTGF) is a member of the CCN family growth factors. A previous study has demonstrated that CTGF expression was evident in sample of LF and

Table 1. Demographic and characteristics of patients

Index	LSCS group	LDH group
Number of patients	21	11
Mean age (years)	55.67±9.5	51.86±5.8
Gender (male/female)	7/14	3/8
Level of LF tissues	L4/5	L4/5

LSCS, Lumbar spinal canal stenosis; LDH, lumbar disc herniation.

was involved in the fibrosis and hypertrophy of the LF in patients with lumbar spinal stenosis [9]. Wnt-induced secreted protein-1 (WISP-1), also termed CNN4, is another member of the CCN family growth factors. Briefly, the cellular targets of WISP-1 are specific and include myocytes, fibroblasts, endothelial cells, and some tumor cells [10]. The WISP-1 proteins have been shown to exert important effects on fibrotic process such as cell growth, proliferation, and extracellular matrix expression [9, 10]. It has been reported that WISP-1 is involved in the fibrotic process in multiple organ systems, including liver, lung and heart [10-12]. Surprisingly, despite the importance of WISP-1 in the pathogenesis of fibrotic process, whether WISP-1 also plays a role in fibrotic process of the LF in LSCS patients is still unknown. The aim of this study was to investigate the expression of WISP-1 in human LF cells from patients with and without LSCS and shed light on the possible relationship between WISP-1 expression and LF fibrosis in these patients.

Materials and methods

Study population

This study was approved by the Ethical Committee of Nanjing Medical University and an informed consent form was obtained from each patient prior to their participation in the study. In the study group, patients undergoing decompressive laminectomy due to LSCS were enrolled from January 2014 to December 2015. The inclusion criteria were as follows: age 50-65 years, one level at L4/L5, degenerative LSCS. Exclusion criteria were patients with diseases as follows: concurrent lumbar disc herniation, degenerative spondylolisthesis, scoliosis, or fractures. Finally, a total of twenty-one patients with one-level LSCS at L4/L5, including 9 males and 12 females, were enrolled in this study.

As a control group, we selected eleven age- and gender-matched patients with lumbar disc herniation (LDH) being operatively managed for that disorder. Briefly, all the patients in this group were confirmed with no evidence of hypertrophy on magnetic resonance imaging (MRI). The mean age was 51.86 years (range: 48-60 years), and the male-female ratio was 3:8. The characteristics of patients in each group are shown in **Table 1**. LF samples were collected during the surgery.

LF thickness measurement

All patients had preoperative magnetic resonance images (MRI). The LF thickness was measured in the patient's preoperative MRI as previously described [13]. The images of the patients were spread to the PACS system (a picture analyzing system, Nanjing Medical University, Nanjing 211100, China) and measured automatically.

Histologic analysis

Masson's trichrome staining was used to determine the degree of fibrosis. Samples were fixed in 4% formaldehyde and embedded in paraffin blocks. All specimens were equally processed. 4-micrometer paraffin sections were stained by Masson's trichrome staining kit (Maxim Biotech, Nanjing, China) according to the manufacturer's instructions. Evaluation criterion of LF fibrosis was as follows: Grade 0 indicated normal tissue showing collagen in < 20% of the entire area; grade 1 indicated fibrosis at ≤ 25% of the entire area; grade 2 represented fibrosis involving 25%-50% of the area; grade 3 represented fibrosis involving 50%-75% of the area; and grade 4 indicated > 75% fibrosis [8]. Typical cases were shown in **Figure 1**.

Culture of human LF cells

LF samples were harvested from the patients with LSCS during the surgery. The ligaments were cut into smaller pieces and washed with PBS (Invitrogen, Canada) several times. Then, they were digested for 1 h in serum-free medium (Gibco) containing 0.2% type I collagenase (Sigma), followed by washing with serum-containing medium (DMEM, Canada). Finally, The specimens were incubated at 37°C in air humidified incubator containing 5% CO_2, in the presence of DMEM supplemented with 10%

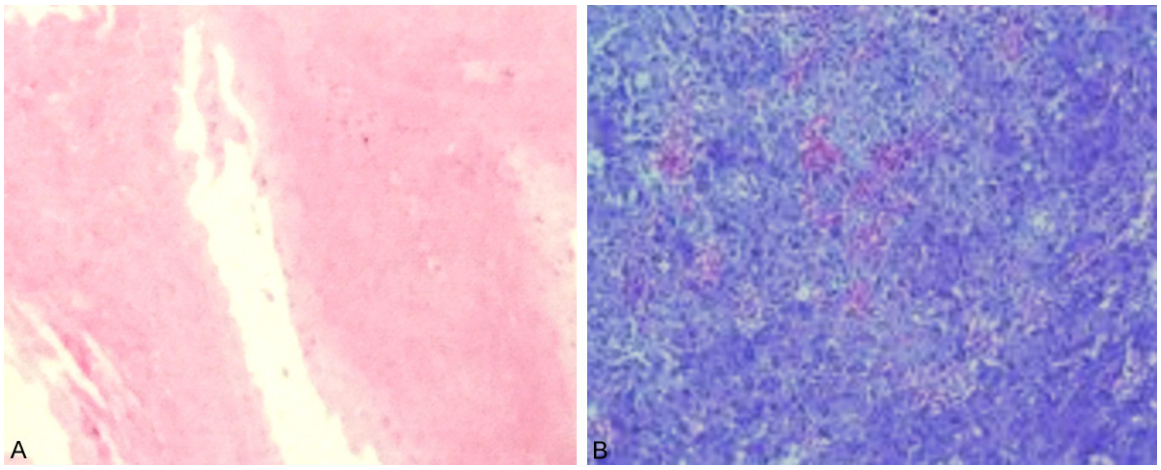

Figure 1. Grading of the LF fibrosis by Masson's trichrome staining. A. Grade 0, most of the area of the LF was stained a pink color of elastic fiber, indicating a normal tissue (× 200). B. Grade 4, most of the area was stained a blue color of collagen fiber, indicating fibrosis (× 200).

Table 2. Sequences of RT-PCR primers used in this study

Primer	Sequence	Size (bp)
WISP-1	5'-CCAGCCAGAGGCATCCATGA-3'	332
	5'-AGGGCTGACTGCTTCACAGG-3'	
Type I collagen	5'-CCTGTCTGCTTCCTGTTAAC-3'	177
	5'-AGAGATGAATGCAAAGGAAA-3'	
Type III collagen	5'-CTGCCATCCTGAACTCAAGAGTGG-3'	447
	5'-CTGCCATCCTGAACTCAAGAGTGG-3'	
β-actin	5'-GGCATCCTCACCCTGAAGTA-3'	200
	5'-GGGGTGTTGAAGGTCTCAAA-3'	

WISP-1, Wnt-induced secreted protein-1.

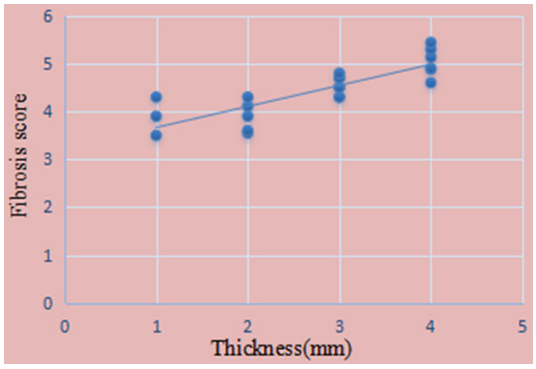

Figure 2. Correlation between fibrosis score and thickness of the ligamentum flavum. These scores have a positive linear correlation with ligamentum flavum thickness.

fetal bovine serum (Sigma, USA), 100 U/ml penicillin and 100 pg/ml streptomycin (Sigma, USA). The culture medium was changed twice weekly [14, 15]. The third passage LF cells were treated with different concentrations of rhWISP-1 (Invitrogen) ranging from 0 to 200 ng/ml for 24 h.

Reverse transcription polymerase chain reaction (PCR)

To ascertain total RNA, the third passage LF Cells were isolated using TRI reagent (Invitrogen). The concentration of extracted RNA was determined from absorption at 260 nm. Then 4 μL of total RNA was reversibly converted to cDNA using PrimeScript RT Master Mix (Takara, China), followed by real-time polymerase chain reaction (PCR) using a Thermal Cycler Dice Real-Time system (Takara). The primers used for real-time PCR of WISP-1, types I and III collagen, and β-actin are listed in **Table 2**. The PCR products were electrophoresed on 2% agarose gel and stained by ethidium bromide. All the real-time PCR reactions were performed in triplicate. The gene expression levels were normalized by dividing the resulting mRNA values by the value for β-actin mRNA isolated at the same time-point. Finally, the electrophoresis strips were analyzed with Smart-view 2001 software. Quantitative real-time PCR was performed to determine the mRNA expression of WISP-1, types I and III collagen.

Western blotting

The third passage LF cells were lysed using lysis buffer (Beyotime, China). After 30 min

Table 3. Comparison of data between two groups

Index	LSCS group	LDH group	*P* value
LF thickness	4.4±0.6	2.1±0.4	< 0.001
Fibrosis score	2.8±1.0	1.1±0.9	< 0.001
WISP-1 expression			
mRNA	0.62±0.18	0.41±0.18	< 0.05
Protein	0.078±0.03	0.051±0.01	< 0.05

LF, ligamentum flavum; WISP-1, Wnt-induced secreted protein-1; LSCS, Lumbar spinal canal stenosis; LDH, lumbar disc herniation.

incubation at 4°C, lysates were sonicated for 30 min and centrifuged at 12,000 × g for 10 min at 4°C. Supernatant was taken and denaturated at 100°C for 5 min before being subjected to a gel. Proteins (200 μg/well) were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then converted to a nitrocellulose membrane (Sigma, USA) by electroblotting. The membranes were blocked for 1 h at room temperature by 5% skim milk in TBS with 0.1% Tween-20 (Sigma, USA). Subsequently, membranes were incubated overnight at 4°C with 1:5000 rabbit polyclonal anti-WISP-1 (Bioworld, USA), and 1:5000 mouse anti-GAPDH antibody (Bioworld, USA). Following incubation with the primary antibody, membranes were washed carefully and re-incubated for 2 hours at room temperature with 1:5000 goat anti-rabbit or anti-mouse secondary antibodies (Bioworld, USA). After careful washing, reactive bands were detected using western blotting chemiluminescence kit (Bioworld, USA) according to the manufacturer's specifications and analyzed by Amersham Imaging System and software (Biosciences).

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (version 17.0; SPSS, Chicago, IL, USA). Data were expressed as mean ± standard deviation. Different groups were compared by using Student's t-test. Pearson correlation analysis was conducted to determine the correlations among LF thickness, fibrosis score and WISP-1 concentration. In this study, the level of statistical significance was set at $P < 0.05$.

Results

Anthropometric data

Twenty-one patients with LSCS and eleven patients with LDH were recruited into this study. Their physical characteristics are shown in **Table 1**. Student's t-test showed these variables did not differ significantly between the two groups ($P > 0.05$). The mean thickness in the hypertrophic LF, as measured on the T1-weighted axial image of the facet joint level, was 4.4 mm (range, 3.5 to 5.4 mm). While in the controls, it was 2.2 mm (range, 1.8 to 3.0 mm). A significant difference was observed in the LF thickness between the two groups ($P < 0.001$).

Histologic study

As shown in **Figure 1**, each sample of hypertrophied LF showed different degrees of fibrotic changes by manifesting a loss of elastic fibers and an increase of collagen fibers. The mean score along the dorsal side in the LSCS group was 2.8 and 1.1 in the LDH group. A significant difference was observed in the fibrosis score between the two groups ($P < 0.05$). Pearson correlation analysis showed that the LF thickness exhibited a strong positive correlation with fibrosis score in the LCSS group ($r=0.833$, $P=0.000$) (**Figure 2**).

Expressions of WISP-1 in LF cells

The mean amount of WISP-1 mRNA was 0.62±0.18 in the LCSS group and 0.41±0.18 in the LDH group, whereas the mean amounts of protein were 0.078±0.03 and 0.051±0.01, respectively (**Table 3** and **Figure 3**). Expression of both mRNA and protein of WISP-1 in LF cells of patients with LSCS was significantly higher than in LF cells of patients with LDH ($P < 0.05$). Furthermore, the Pearson correlation test showed that the WISP-1 concentration exhibited a strong positive correlation with the LF thickness ($r=0.917$, $P < 0.001$) and fibrosis score ($r=0.898$, $P < 0.001$).

Characterization of LF cells and the effect of WISP-1 on LF cells

After 8-10 days culture, a few irregular or spindle shaped cells that had migrated from LF samples were observed by inverted microscope (**Figure 4A**). Once the cells had reached full confluence after 4 weeks culture, their morphology displayed mainly short spindle shapes (**Figure 4B**).

In order to determine the effect of WISP-1 on LF cells, we plated the third passage LF cells into

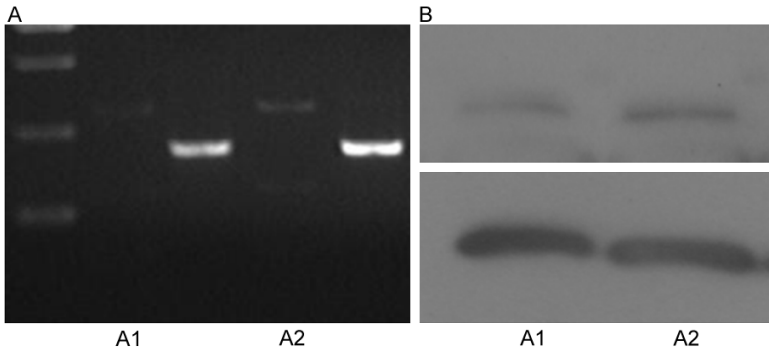

Figure 3. The mRNA and protein expression of WISP-1. The mRNA expression (A) was determined by PCR while the protein expression (B) was assessed by western blotting. The expression of WISP-1 of LF cells from a patient (56 years, female) with LSCS (A2) was higher than that in patient (54 years, female) with LDH (A1).

96-well plates at 3,000 cells per well. Next, cells were continuously stimulated with different concentrations of hrWISP-1 (ranged from 0 to 200 ng/ml) for 24 h. Then, we collected the LF cells and measured the mRNA expression of types I and III collagen. As shown in **Figure 5**, addition of different concentrations of rhWISP-1 in the culture medium upregulated mRNA expression of types I and III collagen in the LF cells as compared with untreated cells (all $P < 0.05$).

Discussion

Since the first report by Elsberg [16] in 1913, hypertrophy of LF in patients with LSCS has been widely documented in previous reports [1-4, 6-9]. As the most of the posterior and lateral part of the spinal canal, the hypertrophy of LF plays an important role in narrowing the lumbar spinal canal [17, 18]. To date, numerous studies have investigated the mechanisms underlying LF hypertrophy. However, the exact mechanism has not been fully elucidated. It has been well accepted that the causes of LF hypertrophy are multifactorial in nature, including activity levels, age, and mechanical stress [1, 4]. Histologically, fibrosis is an important mechanism contributing to LF hypertrophy [8, 18]. Briefly, the LF fibrotic changes showed an increase of collagen fibers and a decrease of elastic fibers [7]. In the current study, the thickness of LF in the LSCS group was significantly more evident than that in the control group. Also, we found that the LF tissue showed a high fibrosis score with an increase of collagen fibers and a decrease of elastic fibers, which was similar to those reported by previous literatures [7, 8, 18].

Many possible molecular mechanisms regarding the fibrosis of LF in patients with LSCS have been put forward. Several inflammatory cytokines, such as transforming growth factor (TGF)-b1, IL-1a, IL-6, TNF-a, PGE 2, NO, and matrix metalloproteinase (MMP) [5, 6, 19, 20], participate in the pathological processes. Also, the increasing expression and activity of another fibrosis-related factors, including platelet-derived growth factor-BB (PDGF-BB) [21], fibroblast growth factor (bFGF) [22], and lysophosphatidic acid (LPA) [23], were demonstrated to be associated with the fibrosis and hypertrophy of the LF in patients with LSS.

In a study by Zhong et al [9], they reported that the up-regulation of connective tissue growth factor (CTGF) expression was associated with fibrosis and hypertrophy of the LF in patients with LSCS and that rhCTGF could induce the increase in collagen synthesis and deposition of collagen types I and III. CTGF is a member of the CCN family growth factors. Considering these findings, we hypothesized that another members of the CCN family growth factors might also play a role on the fibrotic changes that occur during the LF hypertrophy. We chose to examine WISP-1, which most prominently possessed the ability to stimulate fibroblast proliferation and collagen expression. Recent studies have revealed an important role of WISP-1 in the pathogenesis of fibrotic diseases, including liver, lung and heart [10-12]. However, very little is known about the role of WISP-1 in LF fibrosis. Here, in this study, we found that WISP-1 was significantly upregulated in LF cells of LSCS patients compared with that in LDH patients. Correlation analysis showed that WISP-1 expression was correlated with the thickness and fibrotic score. Furthermore, we isolated the LF cells and observed that WISP-1 could upregulate the expression of type I and type III collagen in these cells, indicating the important role of WISP-1 in the fibrosis process of LF.

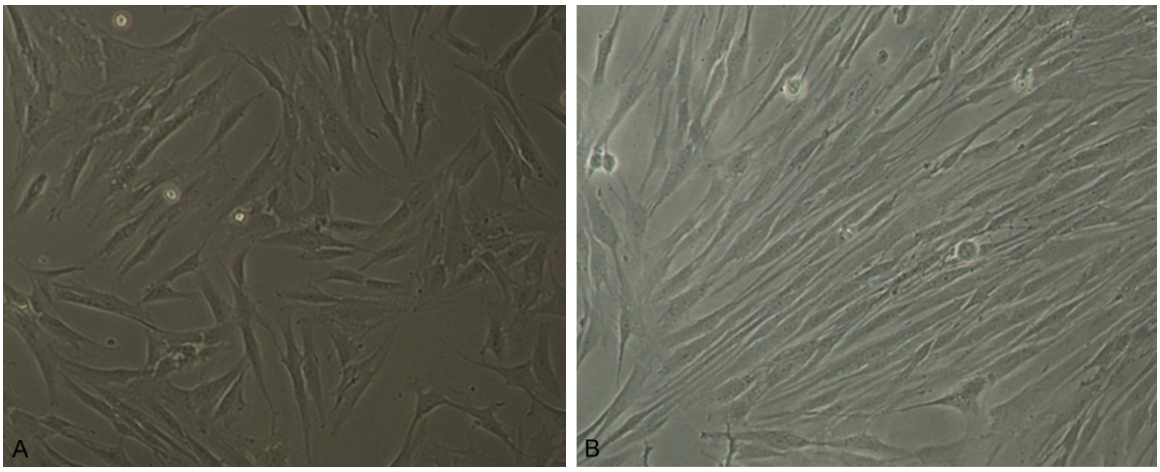

Figure 4. Characterization of LF cells. A. The morphology of LF cells displayed multiform shape for the first 8-10 days culture (× 200). B. After 4 weeks culture, their morphology displayed mainly short spindle shapes (× 200).

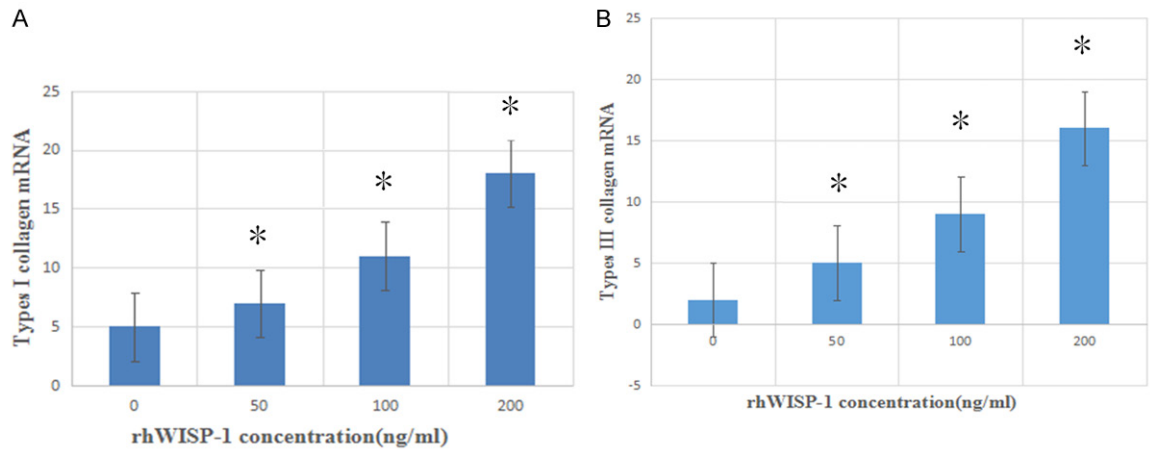

Figure 5. The effect of WISP-1 on LF cells. A, B. Real-time PCR analysis showed that the relative mRNA levels of collagen I and collagen III in hrWISP-1-treated cells were significantly higher as compared with untreated cells (*P < 0.05).

Fibroblasts are the predominant cells involved in fibrosis process. WISP-1, one of the CCN family growth factor, is important for fibroblasts differentiation, proliferation and collagen expression [10, 11]. Based on the above analysis, it can be theoretically inferred that the upregulate the expression of WISP-1 in fibroblasts of LSCS patients may lead to a relative increase in fibroblasts differentiation and proliferation, which might promote collagen synthesis, thus decisively result in LF fibrosis in these patients. Additionally, in hypertrophic LF, there may be an ongoing wound healing response and chronic inflammation [5, 6]. Accumulating evidence has indicated that progressive LF degeneration results from over-expression of various cytokines [5-7]. Also, WISP-1 has important roles in inflammation process [10]. All these data lend support to the postulation that WISP-1 may at least partly participate in LF fibrosis by mediating the inflammatory response. In conclusion, our results offer new insights into the potentially important role of WISP-1 in LF fibrosis. However, the precise mechanism of WISP-1 in LF fibrosis remains unclear and it requires further investigations into the role of this potentially important factor in LF fibrosis.

Previous study has showed that LF thickness and degeneration increased with age [24]. Thus, we assured that the two groups were age-matched, thereby excluding the interference

factor of age, which underlined the results of this study more believable. As with any study, our study had a few shortcomings. As previously stated, fibrosis process is very complicated processes. During this process, many proinflammatory cytokines, molecules, and proteins are involved in their regulation, whether other factors are involved in this process and interrelate with each other is not known. Further studies about other related factors and their mutual interactions may help better understand the molecular mechanism of fibrosis in LF. The another limitation of our study lies in the small size samples in the control group, which could make it difficult to stratify the patients with distinct purposes.

Despite of these limitations, to the best of our knowledge, this is the first study regarding an association between expression of WISP-1 in LF cells and the fibrosis and hypertrophy of LF in patients with LSCS. Our findings suggest that abnormal expression of WISP-1 in LF cells is a possible cause and pathogenetic factor in the fibrosis and hypertrophy of LF in patients with LSCS.

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Disclosure of conflict of interest

None.

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