

Increased expression of vascular endothelial growth factor is associated with hypertrophic ligamentum flavum in lumbar spinal canal stenosis

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ABSTRACT

Lumbar spinal canal stenosis (LSCS) is the most common spinal disorder in elderly patients, causing low back and leg pain, radiculopathy, and cauda equina syndrome. Vascular endothelial growth factor (VEGF) is a potent regulator of many cellular functions including proliferation, differentiation, wound healing, and angiogenesis. The present study aimed to investigate the pattern of VEGF expression in the ligamentum flavum (LF) of patients with LSCS. 24 patients with LSCS were recruited in this prospective study. We quantified and localized VEGF expression in LF tissues obtained during surgery. VEGF messenger RNA and protein expression in LF were determined using reverse transcription PCR (RT-PCR), and quantitative real-time PCR, immunohistochemistry, and ELISA. VEGF expression was significantly higher in the hypertrophic LF group than in the non-pathological LF group ($p < 0.01$) as quantified by quantitative real-time PCR. Further ELISA analysis showed that the average concentration of VEGF in the hypertrophic LF was significantly elevated compared with that of controls ($p < 0.01$). There was no correlation between the tissue VEGF expression of non-pathological LF and patient age in patients with LSCS. Moreover, the immunohistochemical study revealed that VEGF was positively stained on fibroblasts, inflammatory cells, and endothelial cells representing neovascularization within hypertrophic LF compared to the non-pathological LF of controls. The increased expression of VEGF was associated with the degenerative changes of hypertrophic LF, suggesting that VEGF could contribute to one of the mechanisms of pathogenesis in lumbar spinal stenosis.

INTRODUCTION

Lumbar spinal canal stenosis (LSCS) is a degenerative spinal disorder which mostly occurs in elderly patients. Hypertrophy of the ligamentum flavum (LF) is an important factor in LSCS. Ageing and degeneration of LF induce increased thickness and bulging.¹ The acquired stenosis can be caused by the abnormal spine structure including facet joints, bone spurs,

Significance of this study

What is already known about this subject?

- ▶ Hypertrophy of the ligamentum flavum (LF) contributes to lumbar spinal stenosis.
- ▶ Vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis.
- ▶ Angiogenesis plays a critical role in LF hypertrophy.

What are the new findings?

- ▶ VEGF expression was increased in hypertrophic LF.
- ▶ VEGF expression in non-pathological LF was not correlated with patient age.
- ▶ VEGF was positively stained on fibroblasts as well as on inflammatory and endothelial cells in hypertrophic LF.

How might these results change the focus of research or clinical practice?

- ▶ Increased VEGF expression in hypertrophic LF could contribute to the pathogenesis of lumbar spinal stenosis.

intervertebral disc, epidural fat, and LF.² Spinal canal narrowing can lead to radiculopathy or neurogenic claudication and lower back pain which resulted from various mechanical compression factors.³ Canal narrowing partly results from hypertrophy of LF, which mechanically compresses nerve roots.

A number of environmental, mechanical, and biochemical factors have been recognized as potentially linked to LSCS development. Decreased elastic fiber, increased collagen fiber, calcification, and ossification were demonstrated in the hypertrophied LF.^{4–6} Additionally, cytokines have been hypothesized to play critical roles in fibrosis of the pathological LF. In recent years, an increased expression of TGF- β 1 has been demonstrated in hypertrophied LF.^{7–8} Zhang *et al*⁹ reported



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high platelet-derived growth factor BB (PDGF-BB) expression in pathological lumbar LF. Previous studies have shown fibrosis-like chondroid metaplasia of ligament fibroblasts and calcium pyrophosphate crystal deposition in the hypertrophied LF of patients with LSCS.^{6–10} Several reports have shown that neovascularization was evident in the degenerated LF^{6–11–12} and angiogenesis-related factors have been implicated in the pathology of hypertrophied LF.¹³

Vascular endothelial growth factor (VEGF) is a key regulator of blood vessel growth and is one of the most specific and important mediators of angiogenesis. Angiogenesis is an essential process for growth of new blood vessels during fetal development and tissue repair; however, uncontrolled angiogenesis can promote neoplastic diseases and inflammatory disorders. VEGF-A is the best characterized member of the VEGF family and is commonly referred to simply as VEGF. The VEGF gene in humans is located on the short arm of chromosome 6 (6p21.1).¹⁴ VEGF expression was observed in LF degeneration and was involved with calcium pyrophosphate crystal deposition in the LF of a degenerated lumbar spine.⁶

In this study, we postulated that hypertrophied LF could be associated with VEGF expression in patients with LSCS. VEGF expression could take part in the pathophysiology and pathogenesis of LSCS. To prove this hypothesis, we investigated both the transcription and translation expression of VEGF in the LF of patients with LSCS. The objective of this study was to investigate VEGF expression in hypertrophic LF compared to non-pathological LF of controls.

MATERIALS AND METHODS

Patients

Twenty-four LF samples were prospectively obtained from 24 patients with one-level LSCS at L4/L5 (10 males and 14 females) who underwent decompressive surgery. The mean age of patients was 62.9 ± 1.8 years (range 49–80 years). Patients with degenerative spondylolisthesis and lumbar disc herniation were excluded from this study. Specimens of hypertrophic LF for a pathological LF group were obtained at surgery from patients with LSCS, whose pre-operative MRI showed marked hypertrophy of LF with single level at L4/L5. Non-pathological LF specimens from L3/L4 selected from the same patients served as a control group. The entire layers of the central portion of the LF were carefully obtained for the appropriate specimen. All tissue specimens were stored in liquid nitrogen after harvesting and kept at -80°C until measurement.

This study was approved by the Institutional Review Board on Human Research of the Faculty of Medicine, Chulalongkorn University. This study was conducted in compliance with the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all participants prior to their enrollment in the study.

Laboratory methods

Reverse transcription PCR

Reverse transcription PCR (RT-PCR) analysis of VEGF messenger RNA (mRNA) expression was performed using Mastercycler personal (Eppendorf, New York, USA), and each mRNA expression was normalized to 18s ribosomal

RNA (18s rRNA). Frozen LF tissue samples were crushed with a mortar and pestle under liquid nitrogen and total RNA was extracted with the RNeasy mini kit (Qiagen, Valencia, USA). Total RNA was used in the RT reaction with $1 \times$ Taqman RT buffer, 5.5 mM MgCl_2 , 500 μM dNTPs mixture, 2.5 μM random hexamer, 0.4 U/ μL RNase inhibitor, and 1.25 U/ μL MultiScribe Reverse Transcriptase (TaqMan Universal PCR Master Mix, Applied Biosystems, California, USA) according to the manufacturer's instruction. The final solution was used directly for PCR amplification. Complementary DNA (cDNA) in each sample was run in PerfectTaq Plus MasterMix (5Prime, Maryland, USA). The primer sequences for amplification were as follows:

VEGF forward primer: 5'-CAA-ATG-CTT-TCT-CCG-CTC-TGA-3'

VEGF reverse primer: 5'-CCT-ACA-GCA-CAA-CAA-ATG-TGA-ATG-3'

18s rRNA forward primer: 5'-GCC-CGA-AGC-GTT-TAC-TTT-GA-3'

18s rRNA reverse primer: 5'-TCC-ATT-ATT-CCT-AGC-TGC-GGT-ATC-3'

The thermal cycling protocols were as follows: Reactions were processed using one initial denaturation cycle (94°C for 3 min), then 35 cycles of denaturation (94°C for 30 s), annealing (30 s at variable temperature for the gene), extension (72°C for 1 min), and one final extension cycle (72°C for 10 min). The VEGF mRNA was normalized to the 18s rRNA in every sample. PCR products were run on 2% agarose gel electrophoresis and stained with ethidium bromide. VEGF and 18s rRNA expressions were visualized under ultraviolet light. Band intensity was quantified using ImageQuant software.

Quantitative real-time PCR

Twenty-four cDNA of pathological and non-pathological samples were used for quantitative real-time PCR. The experiment was performed with ThermOne SYBR Green Premix (RBC Bioscience, Taipei, Taiwan) using the StepOnePlus™ Real-time PCR system (Applied Biosystems, California, USA) according to the manufacturer's instructions. Human gene-specific oligonucleotide sequences were as described above in the RT-PCR protocol. Reactions were processed using one pre-denaturation cycle (10 min at 95°C), then 40 cycles of denaturation (15 s at 95°C), and annealing (1 min at variable for gene) followed by a melt curve determination consisting of one denaturation cycle (15 s at 95°C), annealing (one cycle for 1 min at 60°C), and then 80 cycles (15 s at 55°C – 95°C). The 18s rRNA expression served as an internal control. A calibrator sample containing cDNA of interest was run to yield a standard curve for each primer set. The StepOnePlus™ Real-time PCR system calculated a threshold cycle for each sample. Threshold cycles in each sample were used in the quantitation method to yield differences in each mRNA expression as normalized to the 18s rRNA housekeeping gene.

Determination of VEGF protein in the LF

Frozen LF tissue samples (30 mg) were crushed and homogenized with phosphate-buffered saline at 3000 rpm and this was then lysed in lysis buffer. The supernatant was

obtained following centrifugation at 15,000 rpm at 4°C for 30 s. LF protein extracts were obtained using the ProteoExtract Protein Precipitation Kit (Merck KGaA, Darmstadt, Germany). Quantification of protein was performed according to Bradford's method using the bicinchoninic acid protein assay kit (Pierce Chemical Company, Rockford, USA). Bovine serum albumin was used as a standard. The optical density was measured at 570 nm by a spectrophotometer. LF specimen extracts were utilized for quantitative analysis of VEGF. Protein extracts were analyzed for VEGF using a commercially available ELISA kit (Quantikine, R&D Systems, Minneapolis, USA) according to the manufacturer's protocols. Recombinant human VEGF provided by the supplier was utilized for calibration. VEGF values were normalized by the protein content in each well.

Immunohistochemistry of the VEGF-stained LF specimens

The LF specimens were immediately fixed in 10% formaldehyde and embedded in paraffin. Serial sections of paraffin-embedded tissue were cut in 5 µm thickness and processed for VEGF staining. Sections were deparaffinized and rehydrated in Tris-buffered saline. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ for 10 min. For antigen retrieval, tissue sections were microwave heated in 10 mmol/L citrate buffer for 5 min. Non-specific binding was blocked for 20 min with 3% normal horse serum (DAKO, Glostrup, Denmark), followed by incubation with a primary antibody (rabbit polyclonal antihuman VEGF antibody 1:100; Santa Cruz Biotech, Santa Cruz, USA) in Tris-buffered saline containing 2% rabbit serum and 1% bovine serum albumin for 2 h. Tissues were incubated with the same buffer without the antibody to serve as negative controls. Sections were subsequently stained with biotinylated goat anti-rabbit immunoglobulins (1:400; DAKO) and streptavidin/horseradish peroxidase complex (1:400; DAKO) and incubated at room temperature for 30 min. Reaction products were visualized using diaminobenzidine (Sigma, St. Louis, USA) as the chromogen. The sections were subsequently counterstained with Mayer's haematoxylin and mounted onto microscope slides using a permanent medium.

Statistical analysis

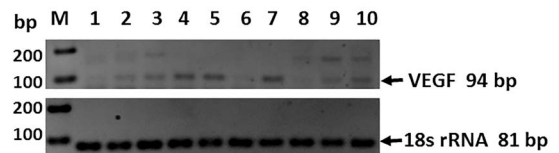
All values were reported as mean±SE of the mean (SEM). Statistical analysis was performed using the statistical package for social sciences (SPSS) software, V22.0 (SPSS Inc, Chicago, Illinois, USA) for Window. Student unpaired t test was used to compare the means of two independent groups. Comparisons between the two groups were made by the Mann-Whitney U test when the distributions were not equal among the groups. Correlation between VEGF in protein extracts and the patient age was assessed using Pearson's correlation coefficient. A p value <0.05 was considered statistically significant.

RESULTS

VEGF mRNA expression by RT-PCR analysis

In the agarose gel, VEGF expression was detected in each patient by the presence of 94 base pair products (figure 1). Band intensity indicated VEGF mRNA expression. The band intensity was analyzed in pathological and non-pathological LF groups. The 18s rRNA band intensity was

Non-pathologic LF



Pathologic LF

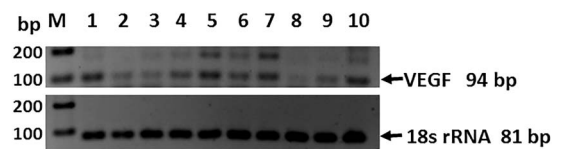


Figure 1 Reverse transcription PCR product of vascular endothelial growth factor (VEGF) in human ligamentum flavum (LF). The 18s ribosomal RNA served as an internal control in each patient. The pathological LF group showed higher VEGF band intensity compared to the non-pathological LF group. M=DNA standard marker.

normalized to the target gene intensity. We found that VEGF mRNA expression was statistically higher in the pathological LF group (0.54 ± 0.08) than in the non-pathological LF group (0.17 ± 0.05 , $p<0.01$).

VEGF mRNA expression by quantitative real-time PCR analysis

In quantitative real-time PCR, we also observed a markedly higher VEGF expression in the pathological LF as compared with non-pathological LF of controls. A significant difference was observed in absolute VEGF mRNA expression between the two groups ($p<0.01$, figure 2).

VEGF protein measurement by ELISA

In the ELISA study, the mean level of VEGF in hypertrophic LF (265.61 ± 39.12 pg/mg) was remarkably

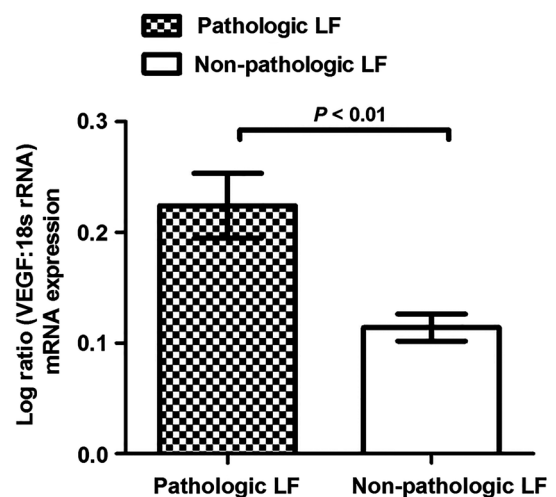


Figure 2 Comparison of the amount of vascular endothelial growth factor (VEGF) messenger RNA normalized by 18s ribosomal RNA between hypertrophic ligamentum flavum (LF) and control using quantitative real-time PCR. Values are shown as mean±SEM.

increased compared with the control (154.59 ± 20.23 pg/mg). A significant difference was observed between the pathological and non-pathological groups ($p < 0.05$, figure 3). Moreover, there was no correlation between the tissue VEGF expression of non-pathological LF and patient age in patients with LSCS ($r = 0.153$, $p = 0.5$).

Immunohistochemical finding of VEGF expression in human LF

An immunohistochemical study showed that VEGF expression was substantially increased in hypertrophic LF compared to non-pathological LF of controls (figure 4). The VEGF positive cell ratio in hypertrophic LF was significantly higher than that in non-pathological LF of controls ($p < 0.01$). Marked VEGF expression was evident in the fibroblasts, inflammatory cells, and endothelial cells representing neovascularization with thickened vessel walls. VEGF was highly expressed in the perivascular area of the extracellular matrix of hypertrophic LF. Hypertrophic LF showed more vascular area with VEGF expression compared to the non-pathological LF. In contrast, slight VEGF expression was observed in the fibroblasts and inflammatory cells of non-pathological LF.

DISCUSSION

LSCS is a degenerative lumbar disorder predominantly affecting the older population. The anatomic changes of degenerative spine disease including degenerated intervertebral disc bulging, overgrowth of the facet joint structure, and hypertrophy of the LF can lead to the progressive narrowing of the spinal canal.^{5 15} It has been reported that a normal LF consists of high elastic fiber. Many studies have shown that the decrease of elastic fibers, proliferation of collagen fiber, ossification, calcium crystal deposition, chondroid-metaplasia of ligament fibroblasts, and inflammatory cytokines were associated with the LF thickening.^{6 9 11 16–18} Despite the contribution of degenerative changes to the hypertrophied LF, the ageing process and

mechanical stress are also involved in the pathological progression of disease.^{19–21}

Sairy *et al*¹¹ suggest that micro-injury in LF tissues can be caused by mechanical stress and repeated micro-injury induces chronic inflammation and ongoing wound healing response, gradually leading to LF fibrosis. During the degenerated LF process, blood vessels invade the degenerated LF tissue, which is known to secrete lysophosphatidic acid (LPA).²² Interestingly, LPA was shown to induce VEGF expression in ovarian cancer cells which implicates involvement in angiogenesis.²³

VEGF has been shown to play a key role in the pathogenesis and progression of a large number of diseases such as cancer, diabetic retinopathy, and rheumatoid arthritis.²⁴ VEGF is a key growth factor participating in reparative angiogenesis after injuries.^{25 26} The outcome from this well-known effect is to induce connective tissue growth factor (CTGF) which stimulates extracellular matrix production and fibrosis.²⁷

In our study, immunohistochemical analysis revealed greater numbers of VEGF positive cells in the pathological LF of patients with LSCS. The fibroblasts showed intense staining at the perivascular area of the pathological LF, suggesting that VEGF is highly produced in the pathological LF. The level of VEGF gene expression, measured by real-time PCR and RT-PCR, was significantly higher in the pathological LF group. The protein level of VEGF was significantly increased in hypertrophied LF compared with the non-pathological LF group. These findings are also in line with previous reports. Yayama *et al*⁶ found that micro-angiogenesis was significant around the degenerated LF area with ruptured elastic fibers and collagen fibrils. Their immunoblot analysis showed increased expression of VEGF and transforming growth factor β (TGF- β) in the ligament with crystal deposition.⁶ In a recent study, Lakemeier *et al*¹² revealed that matrix metalloproteinase (MMP) 1, 3, 9 and VEGF expression were significantly increased in the immunostaining of the hypertrophied LF group. Our results suggest that both VEGF mRNA and protein levels were elevated in hypertrophied LF. VEGF may be involved in the pathogenesis of hypertrophied LF in LSCS.

Yayama *et al*⁶ suggest that mesenchymal fibroblast-like cells infiltrate within the degenerated LF and are eventually transformed to metaplastic chondrocytes. Subsequently, chondrocytes induce angiogenesis and neovascularization through production of VEGF and extension of chondrometaplastic areas. It is possible that hypertrophic chondrocytes could promote crystal formation mediated by secretion of various cytokines and subsequent calcium influx accompanied by the growth of new blood vessels and phosphate influx with increased deposition of calcium pyrophosphate crystal, resulting in mineralization of the matrix. Additionally, Maes *et al*²⁸ reported that chondrocytes induce growth of blood vessels through production of VEGF and extension of chondrometaplastic areas. Angiogenesis is a crucial part of bone formation and endochondral ossification. There was VEGF expression in hypertrophic chondrocyte in vivo.²⁸ Our findings indicated that upregulation of VEGF at both transcription and translation levels could contribute to calcium deposition in the hypertrophic LF.

Considering the roles of VEGF, we hypothesized that VEGF might play an essential role in angiogenesis after the

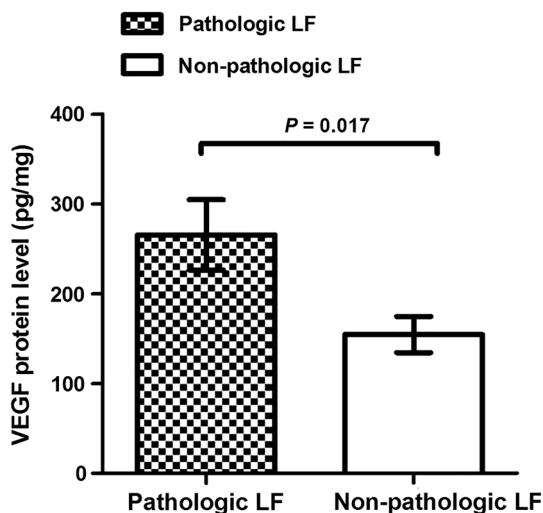


Figure 3 Comparison of vascular endothelial growth factor (VEGF) protein level between pathological and non-pathological ligamentum flavum (LF). Values are shown as mean \pm SEM.

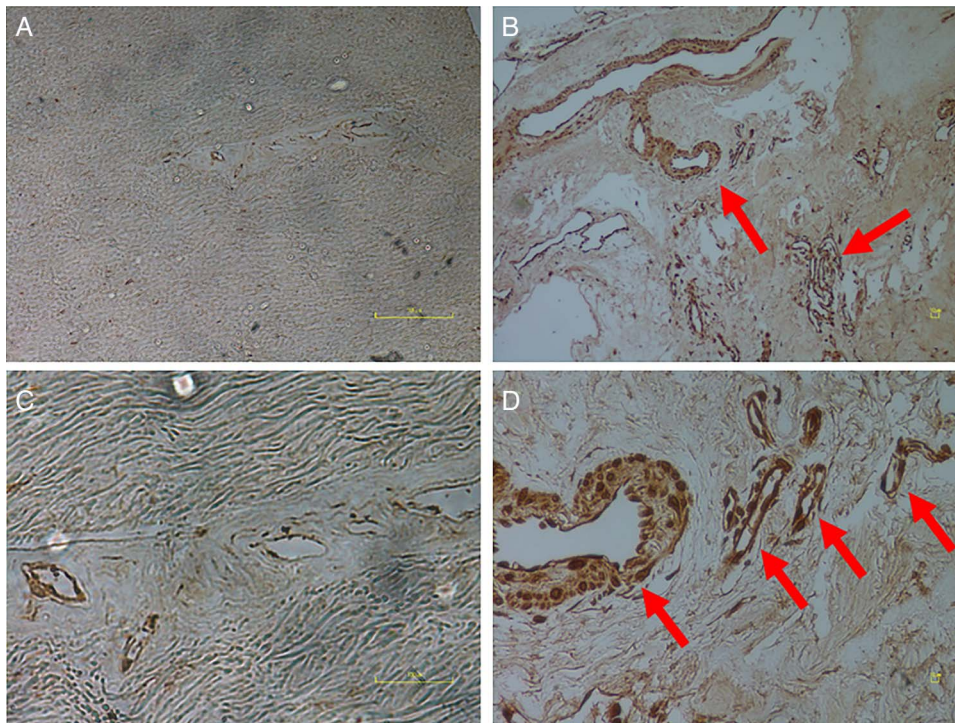


Figure 4 Immunohistochemical staining for vascular endothelial growth factor (VEGF) in hypertrophic ligamentum flavum (LF) (B and D) and control (A and C). Degeneration of the ligament and expression of VEGF are markedly noted in the pathologic LF (B and D) while relatively sparse expression of VEGF was observed in non-pathological LF (A and C) (original magnification $\times 100$ (A and B) and $\times 400$ (C and D)).

LF injuries. Some investigators have hypothesized that activated LF cells can impact the inducement of angiogenesis-related factors, which play a critical role in the hypertrophy of the LF by production of fibrosis and scarring.¹³ Inflammatory reactions are initiated by injury or trauma such as mechanical stress and are followed by the repair process. Mechanical stress-induced tissue damage could be the initial triggering event for an inflammatory reaction and the subsequent development of tissue scarring leading to LF hypertrophy.⁸ Previous studies have shown that upregulation of numerous fibrotic cytokines and chemokines such as TGF- $\beta 1$, CTGF, basic fibroblast growth factor (bFGF), PDGF-BB, and LPA contributed to the fibrotic LF process.^{9 22 29–31} Various kinds of damage including mechanical stress can result in chronic inflammation or wound healing response, then contribute to cell proliferation, migration, and extracellular matrix (ECM) remodeling, which lead to the progression of fibrotic LF.^{11 18} Although the fibrosis process is the main characteristic for the thickening of LF, neovascularization in the matrix is also involved in the pathogenesis of hypertrophied LF. It was found that the excessive production of VEGF and TGF- β induced both abnormal angiogenesis and an epithelial mesenchymal transition, resulting in the development of peritoneal fibrosis.^{32 33} Our findings support that VEGF is associated with the hypertrophy of LF.

Further analysis demonstrated that there was no significant correlation between tissue VEGF expression in the non-pathological LF and patient age. The exact mechanism underlying the relationship remains unclear. However, this finding supports the hypothesis that age is not a direct

contributing factor of angiogenesis. A recent study has illustrated a positive correlation between the thickness of the LF and patient age in patients with spinal stenosis, and that VEGF expression was significantly higher in the pathological LF when compared to the non-pathological LF.³⁴ It was postulated that VEGF might play a potential role in the pathogenesis of LF hypertrophy in patients with LSCS. Further investigations are essential to clarify the relationship between patient age and tissue VEGF expression in patients with LSCS.

The limitations of this study involved the relatively small number of enrolled subjects and the technical difficulty in collecting samples from patients. Since the study was based on a single center, a larger sample size would be needed from multiple centers. Additionally, this study has not investigated whether the upregulated expression of VEGF in hypertrophic LF was a local or systemic phenomenon. To address this issue, further research on circulating VEGF measurement in patients with LSCS will render valuable information. Lastly, this cross-sectional study cannot provide definite information regarding cause-and-effect relationships. The mechanisms underlying increased VEGF expression in the hypertrophic LF remain to be further investigated in the *in vivo* study.

Taken together, the hypertrophied LF shows the increase of VEGF expression in patients with LSCS. These findings reveal that the increased expression of VEGF is associated with hypertrophy of the LF at both mRNA and protein levels. Our study provides potential evidence that the VEGF molecule contributes to the occurrence of degenerative changes in LSCS. Although underlying mechanisms of

this association are not entirely clear, there is abundant room for further studies on the possible role of VEGF in the pathogenesis of degenerative lumbar disease. Modulation of VEGF could be a potential therapeutic target for the future treatment for LSCS.

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Contributors NJ, WL, WY, VP, and SH conceived and designed the experiments. NJ, WL, WY, WS, and SH analyzed the data and provided materials. NJ and SH wrote the paper.

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Competing interests None declared.

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