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SnoN Is Involved in Regulation of the TGFβ/Smad Signaling Pathway in Patients with Degenerative Lumbar Canal Stenosis

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Abstract

Degenerative lumbar canal stenosis is characterized by fibrosis-driven thickening of the ligamentum flavum, which is a key factor in the pathology of the disease and is responsible for clinical symptoms. Given the central role of transforming growth factor-β/Small mothers against decapentaplegic (Smad) signaling in fibrosis, this study aimed to investigate its role in ligamentum flavum remodeling in patients with degenerative lumbar canal stenosis. Ligamentum flavum tissue samples were obtained from 27 patients undergoing surgery for either degenerative lumbar canal stenosis (n = 18) or lumbar disc herniation (n = 9). The lumbar disc herniation patients served as the control group. The severity of the fibrosis was quantified using Masson trichrome and hematoxylin-eosin staining, and radiological measurement of ligamentum flavum thickness. Quantitative reverse transcription-polymerase chain reaction was used to analyze gene expression levels of transforming growth factor-β, transforming growth factor-β receptor, Smad2, Smad3, and Smad4. Changes at the protein level were verified using Western blot analysis. All statistical analyses were conducted post-normalization. Based on histological evaluation, the degenerative lumbar canal stenosis group showed a significantly higher degree of fibrosis compared to the control group. Radiological measurements also revealed increased ligamentum flavum thickness in the degenerative lumbar canal stenosis group ($p < 0.0001$). Significant upregulation ($p < 0.05$) of the Ski, transforming growth factor-β, transforming growth factor-β receptor, Smad2, Smad3, and Smad4 genes was detected, as confirmed by Western blot results. Collective analysis indicated that reduced SnoN gene expression in degenerative lumbar canal stenosis undermined the inhibition of target proteins on Smad2 and Smad3, resulting in elevated levels of phosphorylated Smad2/3. These findings suggest that downregulating SnoN may enhance the activation of the transforming growth factor-β/transforming growth factor-β receptor/Smad2/3 signaling pathway, thereby promoting ligamentum flavum fibrosis in patients with degenerative lumbar canal stenosis.

Keywords: ligamentum flavum, fibrosis, lumbar spinal canal stenosis, TGFβ/Smad signaling

Introduction

Ligamentum flavum (LF) fibrosis, which leads to hypertrophy of the LF, is known as the main histopathological feature of degenerative lumbar canal stenosis (DLCS) and is widely considered to arise secondary to degenerative changes in the intervertebral discs and facet joints, and associated biomechanical alterations.[1,2] It is a highly dynamic molecular process that involves increased collagen fibers and decreased elastic fibers.[3,4] Previous studies have shown that several cytokines are associated with this fibrotic process in patients with DLCS.[1,3,5,6] Notably, transforming growth factor-β (TGFβ), a member of the cytokine superfamily, plays a pivotal role in the pathogenesis of numerous fibrotic diseases, including LF fibrosis.[7,8]

The TGFβ/Smad signaling pathway is known as the canonical signaling pathway of TGFβ.[9] Smad2/3, along with Smad4, are well-established downstream mediators of this

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signaling cascade. Upon activation of the TGFβ receptor, Smad2/3 are phosphorylated and subsequently form a complex with Smad4, which then translocates to the nucleus to regulate target gene expression. Wang et al.[10] reported the significance of Smad2 in TGFβ-induced hypertrophy of the LF in cultured LF fibroblasts. Moreover, the TGFβ/Smad pathway, particularly the roles of Smad2 and Smad4, has been well documented in renal fibrosis and has also been implicated in hepatic, pulmonary, and cardiac fibrosis.[11-15]

The Sloan-Kettering Institute proto-oncogene (Ski) and the related gene, Ski-related novel protein N (SnoN), have recently been identified as pivotal transcriptional corepressors that modulate Smad protein activity. These genes are nuclear residents and play a regulatory role in the TGFβ/Smad signaling pathway, which is essential for maintaining tissue homeostasis.[16] Moreover, the Ski and SnoN genes are negative regulators of the TGFβ/Smad signaling pathway.[17]

In light of these findings, it is crucial to elucidate the regulatory role of SnoN in the TGFβ/Smad signaling pathway in order to understand the molecular mechanisms underlying LF fibrosis and thickening in DLCS. A clearer understanding of this relationship may contribute to the development of novel nonsurgical therapeutic strategies for DLCS. To our knowledge, no comprehensive gene analysis of this signaling axis in LF fibrosis has been reported to date. Therefore, in this study, we aimed to analyzed in detail the gene expression profile of the TGFβ/Smad signaling axis in LF samples obtained from DLCS patients.

Materials and Methods

Study population

A total of 27 LF samples were collected from patients who underwent surgery for degenerative lumbar canal stenosis (DLCS; n = 18) or lumbar disc herniation (LDH; n = 9). In the control group, LDH patients were clinically and radiologically confirmed to be devoid of DLCS. In terms of clinical presentation, neurogenic intermittent claudication was confirmed in patients with DLCS. In the study population, none of the patients had a history of any of the following: tumor, lumbar surgery, trauma, or epidural steroid injection.

Measurement of LF thickness

The LF thickness was measured at the operated lumbar segment level using axial T2-weighted imaging, by 2 independent experienced surgeons, following the methodology described in our previous study.[18] The measurement results were converted to numerical values by the device software and are given in millimeters (mm).

Histological analysis

LF tissue samples were fixed in 10% formalin solution for histological evaluation. Paraffin blocks were obtained by completing routine tissue follow-up steps. Five-micron sections were taken from LF paraffin blocks and stained with hematoxylin & eosin (H&E) to investigate the histological features of the LF tissue. In addition, the slides were stained Masson's trichrome to detect changes in elastic and collagen fiber and to assess the degree of LF tissue fibrosis. The H&E and Masson trichrome-stained slides were analyzed under a light microscope (Olympus BX51, Tokyo, Japan) and photographed at ×200 magnification (Olympus DP20 microscopic digital camera system). The degree of fibrosis was determined using Masson's trichrome-stained photographs at ×200 magnification via an image analysis system (version 2.11.5.1; Camera-Argenit, Istanbul, Turkey). Three different measurements were made by the investigator, and the mean percentage of fibrosis was determined. The degree of LF fibrosis was scored according to a system based on collagen density.[4,18]

Tissue preparations

Approximately 10-20 mg of formalin-fixed LF tissue was rapidly frozen in liquid nitrogen for molecular analysis. After 3 cycles of disruption for 2 minutes each using tungsten carbide beads in 2 mL cryo-grinding tubes, the frozen tissue was pulverized into a fine powder using a TissueLyser LT system (Qiagen, Hilden, Germany). The resulting tissue powder was divided into equal halves and stored at -80°C in cryo-vials for subsequent gene expression and protein analyses.

Total RNA isolation, reverse transcription, and real-time polymerase chain reaction (qRT-PCR) assays

Total RNA was extracted using a combination of the TRIzol method (Invitrogen, Carlsbad, CA, USA) and the PureLink™ RNA Mini Kit (Invitrogen, USA), in accordance with the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, USA). Quantitative reverse transcription-polymerase chain reaction (qRT − PCR) was performed using Power SYBR™ Green PCR Master Mix (Life Technologies, USA) on a Quant Studio 5 real-time PCR system (Life Technologies, USA) to evaluate changes in target gene expression. The specific primers and PCR conditions are listed in Supplementary Table 1. Relative gene expression levels were calculated using the 2-ΔΔCt method, with β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serving as dual-endogenous controls.

Immunoblot assays

For protein analyses, total protein was extracted using the Radioimmunoprecipitation Assay (RIPA) Lysis Buffer System (Santa Cruz, USA) according to the manufacturer's instructions. Protein concentrations were determined prior to electrophoresis. Western blot analysis was performed using NuPAGE™ 4%-12% Bis-Tris, 1.0 mm Mini Protein Gel

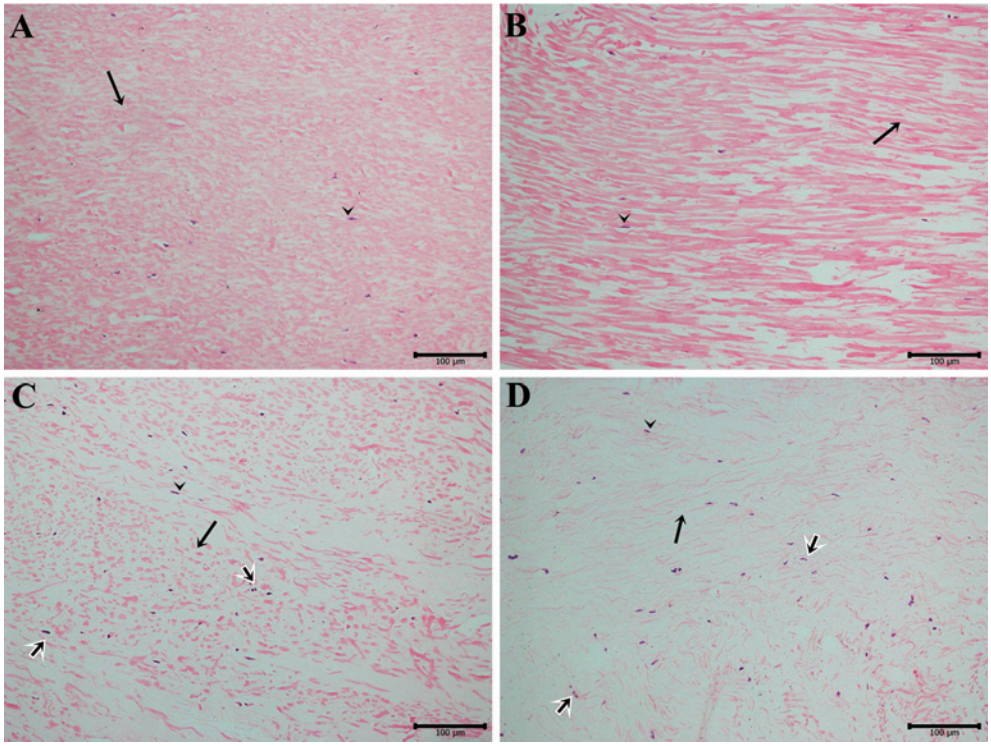


Figure 1 Histopathological features of ligamentum flavum in control and hypertrophy groups. (A, B) In the control group, well-organized, parallel alignment of elastic fibers (arrows) interspersed with collagen fibers and fibroblasts (arrowheads) are shown. (C, D) The hypertrophy group demonstrated marked fibrotic changes, including reduced elastic fibers, disorganized fiber architecture, and fibrocartilaginous changes (short arrows), along with inflammatory cell infiltration (H&E, ×200, Scale bar = 100 μm). H&E: hematoxylin and eosin

(NP0321, Thermo Fisher Scientific, USA) in combination with the WesternBreeze™ Chemiluminescent Kit (Thermo Fisher Scientific, USA), following the protocol described by Delen et al.[19] A set of 6 primary antibodies was used: TGFβ (AbmGood, Canada, Y058105), TGFβ (AbmGood, Canada, Y413545), phospho-Smad2/3 (AbmGood, Canada, Y413249), Smad2 (Novus Biologicals, USA, NBP2-88916), Smad3 (Novus Biologicals, USA, NB100-56479), and β-actin (Novus Biologicals, USA, NB600-503). Protein expression levels were quantified by normalizing band intensities to β-actin, as described in our previous study.[18]

Statistical analysis

The statistical analyses were performed using SPSS software, version 20 (IBM Corp., Armonk, NY, USA). The data are presented as the means ± standard deviations, and $p \leq 0.05$ was considered statistically significant. The Shapiro-Wilk test was used to determine the distribution normality for each variable. Comparative analyses of the relative gene expression and protein levels between the LDH and DLCS groups were performed using the Mann-Whitney U test. Correlations between variables were assessed using Pearson's correlation coefficients. In addition, heatmap analysis was conducted using the R-based MetaboAnalyst software to visualize of the multidimensional data patterns.

Results

Histological examination of LF hypertrophy

Histopathological analysis of the LF revealed well-organized, parallel-alignment of the elastic fibers in the control group specimens as indicated by H&E staining. These fibers were interspersed with collagen fibers and fibroblasts (Figure 1A and B). Conversely, LF samples from the hypertrophy group demonstrated notable fibrotic alterations, characterized by a loss of elastic fibers and an accumulation of collagen fibers. In addition, fibrocartilaginous changes and the infiltration of inflammatory cells were observed. Furthermore, the hypertrophic samples exhibited various morphological changes in the elastic fibers, including variations in fiber diameter, disorganized fiber alignment, fragmentation, and an overall decrease in fiber density (Figure 1C and D).

In order to histologically assess fibrosis, LF tissue sections were stained using Masson's trichrome, which highlights collagen fibers in green and cell nuclei in dark brown (Figure 2). Tissue samples were categorized according to a standardized grading system. Grade 0 represented normal tissue without fibrosis (Figure 2A), while Grade 1 indicated fibrosis encompassing 25% or less of the tissue area, attributable to a reduction in elastic fibers (Fig-

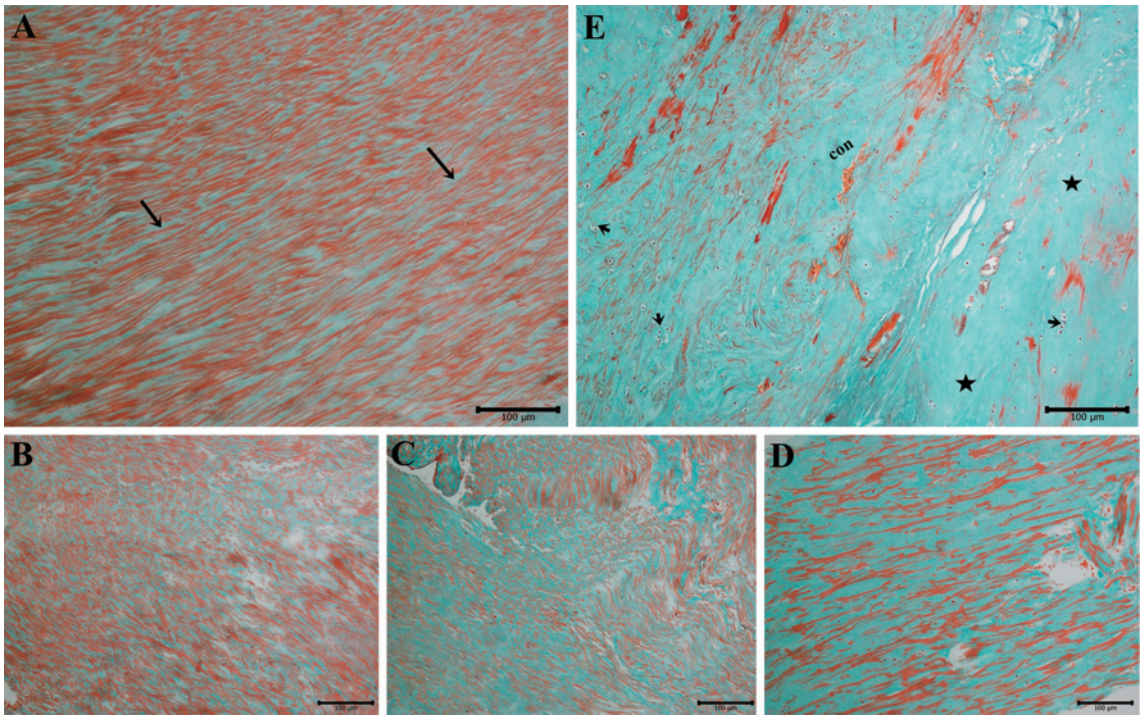


Figure 2 Histological grading of fibrosis in ligamentum flavum using Masson's trichrome staining. Collagen fibers are stained green, while cell nuclei appear brown. In ligamentum flavum tissue sections, a gradual increase in collagen fibers is shown, which is associated with an increase in the fibrosis score. (A) Grade 0: Normal tissue without fibrosis. (B) Grade 1: fibrosis ≤25%. (C) Grade 2: fibrosis 25%-50%. (D) Grade 3: fibrosis 50%-75%. (E) Grade 4: fibrosis >75% Elastic fibers are indicated by arrows, collagen-rich areas by (★), fibrocartilaginous changes by short arrows, and congested regions by "con" (Masson Trichrome, ×200, Scale bar = 100 μm).

ure 2B). Grade 2 exhibited fibrosis spanning 25% to 50% of the area, associated with elevated collagen levels and a lower elastic-to-collagen ratio (Figure 2C). Grade 3 exhibited a range of fibrosis levels between 50% and 75% (Figure 2D). Grade 4 was characterized by the presence of fibrosis in excess of 75%, accompanied by disorganized structure, congestion (con), focal disruptions of elastic fibers, and marked collagen proliferation (Figure 2E). As demonstrated in the micrographs of Figure 2A to E, a progressive increase in green-stained areas was observed, indicating a decrease in elastic fibers and a corresponding increase in collagen fibers, consistent with advancing fibrosis.

Relationship between LF thickness and transcriptional regulators Ski and SnoN mRNA expressions

In this study, we assessed the correlation between LF thickness and the mRNA expression levels of the transcriptional regulators Ski and SnoN in patients with DLCS. The analysis revealed that LF thickness was significantly increased in the tissues from DLCS patients as compared to those from the LDH patients ($p < 0.0001$; Figure 3A). However, when examining Ski mRNA expression in DLCS LF samples, only a marginal elevation was observed relative to samples from LDH patients, with this difference failing to achieve statistical significance ($p = 0.56$). No significant correlation was also found between LF thickness and Ski mRNA expression ($R_Ski = 0.23$; $p = 0.24$). Conversely, there was a significant downregulation of SnoN mRNA expression in DLCS LF samples compared to those from LDH samples ($p = 0.005$). Additionally, a significant negative correlation was established between LF thickness and SnoN mRNA expression ($R_SnoN = -0.78$; $p < 0.001$) (Figure 3B).

TGFβ/TGFβ-R/Smad2-Smad3 axis

The TGFβ/TGFβ-R/Smad2-Smad3 signaling axis was evaluated in patients with DLCS as compared to those with LDH. Initial analysis using heatmap visualization revealed distinct expression patterns of the TGFβ/TGFβ-R/Smad signaling axis between DLCS and LDH samples. Although partial clustering between the groups was observed, some degree of overlap and heterogeneity was present within the DLCS cohort (Figure 4A). qRT-PCR results showed significantly increased mRNA expression levels of TGFβ and TGFβ-R in the DLCS group compared to the LDH group ($p < 0.001$). Although there was observed an increase in Smad2 expression in DLCS samples, this increase was not statistically significant ($p = 0.1418$). In contrast, Smad3 and Smad4, the downstream components of the TGFβ signaling pathway, were significantly upregulated

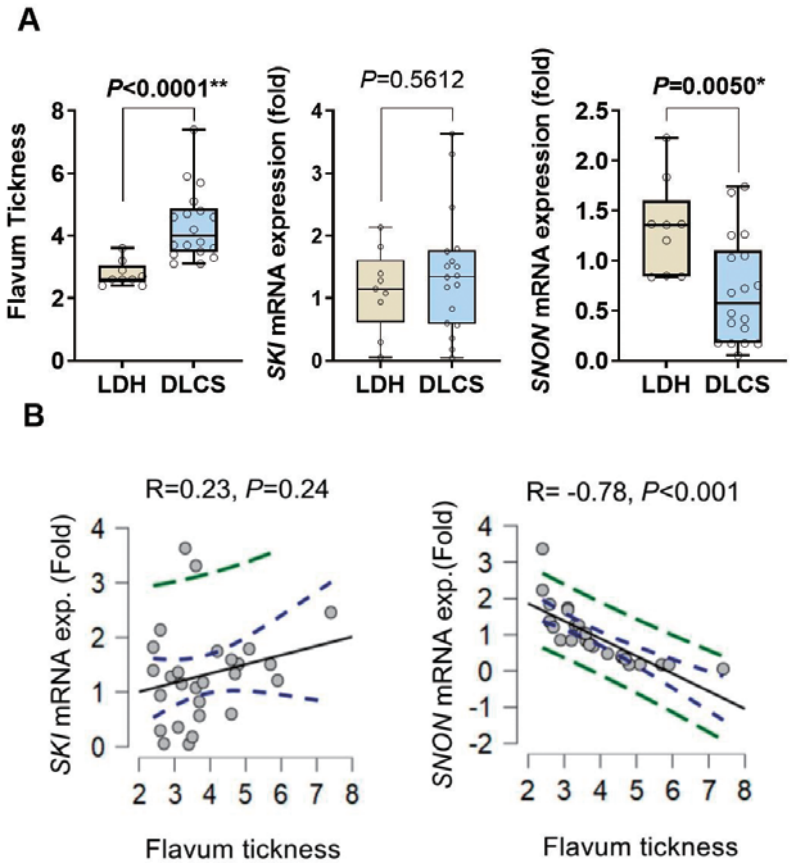


Figure 3 Association between ligamentum flavum thickness and Ski/SnoN exp. in DLCS and LDH groups. (A) LF thickness and relative mRNA expression levels of Ski and SnoN in DLCS and LDH groups. (B) Correlation analysis between LF thickness and mRNA exp. levels of Ski and SnoN. All gene exp. data were normalized to β-actin and GAPDH (Mann-Whitney U test) (*p < 0.05, ** p < 0.0001).

DLCS: degenerative lumbar canal stenosis; exp.: expression; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; LDH: lumbar disc herniation; LF: ligamentum flavum; mRNA: messenger RNA

compared to the LDH group ($p \leq 0.05$, Figure 4B).

Western blot analyses were performed to validate the qRT-PCR findings. The results confirmed increased activity of the TGFβ signaling pathway in DLCS patients, particularly within the TGFβ/TGFβ-R/Smad2-Smad3 axis. Notably, p-Smad2/3 levels were significantly elevated in the DLCS group, showing a 2.54-fold increase compared to the LDH group (Figure 5).

Discussion

Degenerative lumbar spinal stenosis is becoming a major indication for spine surgery with the ageing population. However, surgical treatment is often associated with increased complication rates due to the advanced age and comorbidities of patients. Therefore, elucidating the molecular mechanisms underlying DLCS is essential for the development of effective nonsurgical therapeutic strategies. Despite extensive research, there are currently no established pharmacological treatments for DLCS in routine clinical practice. In this context, targeting of TGFβ pathway has emerged as a promising approach for the development of novel medical therapies.[20]

In the present study, we demonstrated marked histopathological alterations in the LF, characterized by disorganized fiber alignment and fibrosis, including decreased elastic and increased collagen fibers. This type of extracellular matrix remodeling is widely recognized as one of the most common histopathological changes related with DLCS.[21,22] Increased LF thickness resulting from fibrotic deposition is a major contributor to spinal canal narrowing and plays a key role in the development of clinical symptoms in DLCS. Consistent with previous reports, LF thickness was significantly increased in DLCS group, supporting the role of fibrotic remodeling in spinal canal narrowing.[5,18,21,22] The TGFβ/Smad signaling axis, along with its coregulatory Ski and SnoN, is intricately modulated through a series of feedback loops consisting of both positive and negative regulatory mechanisms. These interactions not only govern the cross-talk within the signaling network but also modu-

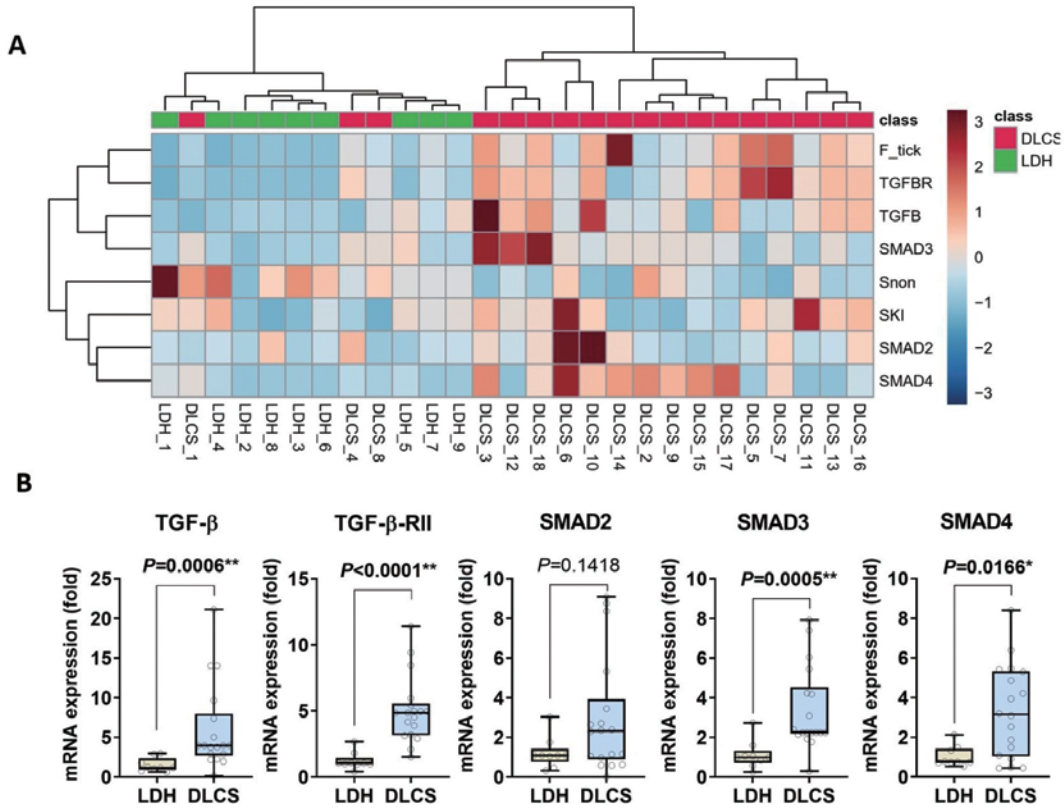


Figure 4 Expression profile of the TGFβ/Smad signaling axis in DLCS and LDH groups. (A) Heatmap of gene expression patterns of the TGFβ/TGFβ-R/Smad signaling pathway in DLCS and LDH samples, demonstrating partial clustering and heterogenity within the DLCS group. (B) Relative mRNA expression levels of TGFβ, TGFβ-R-II, Smad2, Smad3, and Smad4 in DLCS and LDH groups as determined by qRT-PCR. All data were normalized to β-actin and GAPDH (*p < 0.05, **p < 0.01).

DLCS: degenerative lumbar canal stenosis; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; LDH: lumbar disc herniation; mRNA: messenger RNA; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; TGFβ: transforming growth factor-β; TGFβ-R: transforming growth factor-β receptor

late the intensity and persistence of TGFβ-mediated signals.[23] In the light of this information, we investigated whether Ski and SnoN have a regulatory role on TGFβ signaling in DLCS patients by determining their relationship with Smad2/3 phosphorylation, which remains the focal point for these transcriptional repressors. The analysis, phosphorylated Smad 2 and 3 positively correlated both LF thickness and TGFβ or its receptor gene expression and protein levels. Conversely, SnoN expressions displayed a considerable inverse relationship with LF thickness. These findings suggest that a decrease in SnoN expression could diminish its inhibitory impact on Smad2 and Smad3, potentially leading to enhanced Smad2/3 phosphorylation and the activation of gene expressions associated with LF hypertrophy in patients with DLCS. The differential regulation of SnoN and Ski observed in this study may be associated with distinct regulatory mechanisms governing these 2 transcriptional corepressors. Although both proteins function as negative regulators of TGFβ/Smad signaling, previous studies suggest that SnoN is more susceptible to ubiquitin-mediated degradation in response to TGFβ stimulation, whereas Ski may exhibit greater stability under similar conditions. Additionally, differential transcriptional or post-transcriptional modifications may preferentially affect SnoN expression.[16,17] This difference may contribute to the selective downregulation of SnoN and persistent activation of TGFβ/Smad signaling.

LF thickening, attributable to fibrosis, is precipitated by multiple etiologies, including age-related degenerative changes and inflammation induced by mechanical stress.[5,18] The previous studies indicated that the pivotal role of TGFβ in inflammation-related LF fibrosis.[4,5,24] Sairyo et al.[4] revealed that TGFβ triggered by mechanical stress in LF fibrosis. Furthermore, the noted overexpression of collagen fibers within LF tissues of DLCS patients is consistent with the hypothesis of mechanical stress contributing to such alterations.[8] P-Smad2/3 are well-established downstream intracellular mediators of the TGFβ β signaling pathway. Chen et al.[25] revealed that the overexpression of p-Smad 2/3 associated with decreased elastic and increased collagen fibers in DLCS patients. SIS3, a selective Smad3 inhibitor, suppresses of the TGFβ1-induced type I

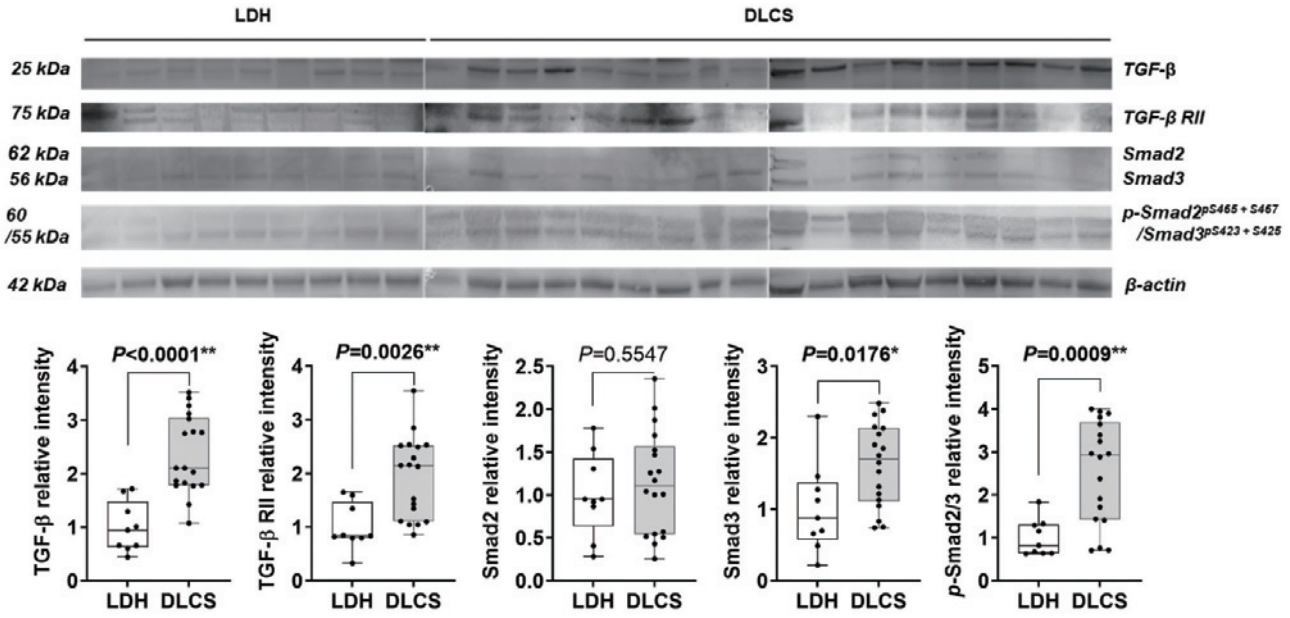


Figure 5 Western blot analysis of TGFβ/Smad signaling pathway in DLCS and LDH groups. Representative Western blot images showing protein expression levels of TGFβ, TGFβ-R-II, Smad2, Smad3, and p-Smad2/3 (p-Smad2 [Ser465/467] and Smad3 [Ser423/425]) in LF tissues. Densitometric analysis of band intensities is presented as relative protein expression normalized to β-actin (*p < 0.05, **p < 0.01).

DLCS: degenerative lumbar canal stenosis; LDH: lumbar disc herniation; LF: ligamentum flavum; p-Smad2: phosphorylated Smad2; p-Smad3: phosphorylated Smad3; TGFβ: transforming growth factor-β; TGFβ-R-II: transforming growth factor-β receptor II

procollagen upregulation in human dermal fibroblasts.[26] Smad signaling is a major part of TGFβ signaling in fibrotic diseases including LF fibrosis.

The comprehensive analysis in the present study, utilizing heatmap, qRT-PCR and Western blot methodologies, investigated the TGFβ/TGFβ-R/Smad2-Smad3 signaling in LF tissues from both the DLCS and LDH groups. Our findings revealed that DLCS group exhibited increased fibrosis and significantly elevated levels of p-Smad2/3 compared to LDH group. Additionally, our heatmap analysis also showed that increased p-Smad2/3 levels accompanied by high level of TGFβ and TGFβ-R expression patterns. These results collectively supported that the activation of p-Smad 2/3 signaling via TGFβ may have an essential role in the progression of LF fibrosis.

Furthermore, the negative regulatory impact of Ski and SnoN on the TGFβ/Smad signaling pathway has been documented. Ski and SnoN antagonize fibrotic processes by disrupting Smad complexes, thereby attenuating TGFβ-driven profibrotic signaling.[27,28] In this way, the effect of these proteins limits tissue fibrosis caused by TGFβ/Smad signaling pathway. Yang et al.[2] revealed that the overexpression of the Ski and SnoN genes caused the inhibition of the TGFβ/Smad profibrotic effect in renal cells. Our study shows that the downregulation of SnoN, rather than Ski, genes is related to LF fibrosis caused by TGFβ/Smad signaling. Similarly, Chi et al.[29] reported that SnoN overexpression may be effective against liver fibrosis by inhibiting the expression of fibrogenesis-related proteins dependent on TGFβ1.

Turning our attention to bone morphogenetic proteins (BMPs), which belong to the TGFβ superfamily and typically act in opposition to TGFβ-induced fibrosis, the study by Ertugrul et al.[30] reported no significant differences in BMP immunoreactivity between patients with DLCS and LDH. While BMPs are expected to influence LF fibrosis, Ertugrul et al.'s[30] observations suggest a complex interplay that is not yet fully understood. To our knowledge, there is a minority of studies investigating the cross-talk between BMP and TGFβ signaling specifically within the scope of LF fibrosis. This gap suggests that interactions between BMP and TGFβ signaling in LF fibrosis could be further explored in future studies.

This study has several limitations. First, the relatively small sample size may limit the generalizability of our findings; however, this is a common limitation in studies involving human LF tissue, and similar sample sizes have been reported in numerous molecular studies using human LF tissue. Second, although the expression levels of SnoN and Ski were primarily evaluated at the mRNA level, the absence of complementary protein-level validation may limit the interpretation of their functional roles in the regulation of TGFβ/Smad signaling. In addition, although LF fibrosis is widely regarded as the principal histopa-

thological feature of DLCS, it is generally considered to develop secondary to intervertebral disc degeneration and facet joint arthropathy. Nevertheless, LF fibrosis and the associated LF thickening represent the direct pathological processes responsible for neural compression and the resulting clinical symptoms in DLCS.

Conclusions

To our knowledge, this study provides the novel evidence that SnoN expression within the TGFβ signaling pathway is associated with LF fibrosis. These findings advance the current understanding of the molecular mechanisms underlying LF hypertrophy and highlight the involvement of the TGFβ/Smad axis in this process; however, the observed associations should be interpreted with caution. Further investigations are required to confirm these observations and to define their clinical relevance.

Conflicts of Interest Disclosure

All authors have no conflict of interest.

Funding

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Ethics Approval

Approval for our study was granted by the Human Ethics Review Committee of Trakya University (TUTF-BAEK 2020/161). Written informed consent was obtained from all individual participants included in the study.

This manuscript was published as an oral presentation in XIV. International Turkish Spine Congress.

Consent to Participate

Written informed consent was obtained from all individual participants included in the study.

Consent to Publish

This is not applicable.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code Availability

This is not applicable.

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