Alteration of Elastic Fiber Proteins in Ligamentum Flavum from Patients with Lumbar Spinal Canal Stenosis.

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Disclosures:

Introduction: Lumbar spinal canal stenosis (LCS) is among the most common spinal disorder in the elderly. LCS patients suffer limited motility characterized by intermittent claudication and impair their activities of daily living. Whereas LCS has been suggested to result in part from narrowing the spinal canals by hypertrophy of ligamentum flavum, the molecular pathogenesis of LCS is not fully elucidated yet. This study was attempted to identify molecules involved in degeneration of the ligamentum flavum using proteomic approach and to investigate the underlying mechanisms on the degeneration of ligamentum flavum in LCS patients.

Methods: The study was approved by the institutional ethical committee of National Center for Geriatrics and Gerontology. All participants signed informed consent statements prior to participation. Ligamentum flavum were obtained from LCS and lumbar disc herniation (LDH) patients as cases and controls, respectively. Surgically removed ligaments were trimmed with scissors, crushed using a tissue disrupter with stainless beads, and fixed by methanol. The extracts were enzymatically treated in sodium dodecyl cholate solutions. The resulting peptides were extracted in the water fraction using the water/ethyl acetate partition system, and then analyzed by nanoLC-MS/MS coupled by 2-Dimensional Image Converted Analysis of LC-MS (2DICAL). 2DICAL enables to perform large-scale quantitative proteomics by multidimensional LC separation and MS/MS analysis because of its comprehensiveness, reproducibility, and accurate quantification with a wide dynamic range. For immunohistological approaches, ligamentum flavum tissues were fixed by formalin, dehydrated by ethanol, embedded by paraffin, and serial sections were made using microtome. Immunohistochemistry was performed using primary antibodies against proteins interested with the peroxidase-conjugated secondary antibodies.

Results: By 2DICAL-based proteomic analysis, 24 and 3 proteins were identified as the molecules increased and decreased, respectively, with more than 3-fold difference in their abundance. The majority of the differentially expressed proteins were extracellular matrix components. To assess the expression and localization of proteins, of which expression were markedly altered in LCS patients identified by the proteomic analysis, immunohistological staining was performed. The staining patterns of these proteins exhibited the altered expression patterns in ligamentum flavum of LCS, in comparison with controls. Microfibril associated protein 4 (MFAP4) and fibulin 5 (FBLN5), which have been demonstrated as important regulators of microfibril assembly and coacervation of elastin molecules in elastic fiber formation, respectively, were detected as proteins significantly decreased in ligamentum flavum from LCS patients. On the other hands, tenascin C and fibronectin were increased, suggesting that the change of extracellular matrix protein levels well reflect the degeneration of elastic fibers and progression of tissue fibrosis in the ligamentum flavum. Several plasma proteins, such as apolipoproteins and fibrinogens, were also detected in ligamentum flavum from LCS patients, suggesting the increased vascularization in hypertrophied ligamentum flavum. Interestingly, several proteins, recognized as cartilage elements such as PRELP, aggrecan, and chondroadherin, were also detected as molecules increased in the ligamentum flavum; consistent with pathological observation that chondrometaplasia is often occurred in the hypertrophied ligaments of LCS. Furthermore, the expression level of extracellular protease HTRA1 was markedly increased in ligamentum flavum from LCS patients, comparing to controls. HTRA1 has been reported that its expression was upregulated in osteoarthritic joints and related to intervertebral disc degeneration. To determine whether HTRA1 degrade MFAP4 and FBLN5, co-transfection experiments were employed. HTRA1 dose-dependently decreased the expression levels of MFAP4 and FBLN5. Wild-type HTRA1, but not a mutant carrying a mutation on Ser-328 that is responsible for catalytic activity of the enzyme, cleaved FBLN5, and to a lesser degree, MFAP4.

Discussion: While most tendons and ligaments mainly consist of collagen fibers, the ligamentum flavum is primarily composed of elastic fibers. It has been demonstrated that both MFAP4 and FBLN5 play critical roles in elastic fiber formation, especially in the steps of microfibril association and coacervation, respectively. The decrease in these proteins suggested that the elastic fiber formation in the degenerated ligaments may be impaired. On the other hand, it has been described that loss-of-function and gain-of-function mutation caused elastic tissue degeneration including age-related macular degeneration and cerebral
autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy. Therefore, the protease may be involved in the alteration of extracellular matrix on degeneration of ligamentum flavum, through the degradation of elastic fiber proteins.

**Significance:** Shotgun proteomics of hypertrophic ligamentum flavum from patients with lumbar spinal canal stenosis

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**References:**
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- Clouet et al. (2009) Rheumatology

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