Progression of lumbar spinal stenosis is influenced by polymorphism of thrombospondin 2 gene in the Korean population

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Abstract

Purpose The aim of this study is to determine the contribution of thrombospondin 2 (THBS2) polymorphisms to the development and progression of lumbar spinal stenosis (LSS) in the Korean population.

Methods We studied 148 symptomatic patients with radiographically proven LSS and 157 volunteers with no history of back problems from our institution. Magnetic resonance images were obtained for all the patients and controls. Quantitative image evaluation for LSS was performed to evaluate the severity of LSS. All patients and controls were genotyped for THBS2 allele variations using a polymerase chain reaction-based technique.

Results We found no causal single nucleotide polymorphism (SNPs) in THBS2 that were significantly associated

with LSS. Two SNPs (rs6422747, rs6422748) were over-represented in controls [$P = 0.042$, odds ratio [OR] = 0.55 and $P = 0.042$, OR = 0.55, respectively]. Haplotype analysis showed that the “AGAGACG” haplotype (HAP4) and “AAGGACG” haplotype (HAP5) were over-represented in severe LSS patients ($P = 0.0147$, OR = 2.02 and $P = 0.0137$, OR = 2.48, respectively). In addition, the “AAAGGGG” haplotype (HAP1) was over-represented in controls ($P = 0.0068$, OR = 0.30).

Conclusions Although no SNPs in THBS2 were associated with LSS, haplotypes (HAP4 and HAP5) were significantly associated with progression of LSS in the Korean population, whereas another haplotype (HAP1) may play a protective role against LSS development.

Keywords Genetic study · Haplotype · Lumbar spinal stenosis · Polymorphism · Thrombospondin gene

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Abbreviations

DSCSA	Dural sac cross-sectional area
ECM	Extracellular matrix
LRP	Lipoprotein receptor-related protein
LSS	Lumbar spinal stenosis
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
PCT	Polymerase chain reaction
SNP	Single nucleotide polymorphism
THBS	Thrombospondin

Introduction

Lumbar spinal stenosis (LSS) typically affects patients over 50 years of age, with a prevalence of 1.7–10 % [24].

Degenerative LSS is a common and debilitating spinal disease, the frequency of which continues to increase, because of the increasing number of individuals aged >65 years. Spinal stenosis is defined as any type of narrowing of the vertebral canal, the lateral recesses, and/or the intervertebral foramina [24, 26]. Spinal stenosis may be congenital or acquired [26, 29]. Congenital stenosis is attributable to idiopathic developmental narrowing of the vertebral canal, typically associated with achondroplasia and hypochondroplasia [26, 29]. In contrast, acquired or degenerative stenosis is usually caused by disk herniation or degenerative changes in the intervertebral disks, facet joints, and/or ligamentum flavum, but may also arise because of spondylolisthesis, tumor development, concurrent Paget's disease, or ossification of the posterior longitudinal ligament of the spine [17, 26, 29]. Although congenital stenosis is rare, degenerative stenosis is one of the most common clinically important spinal disorders in aging populations. Because congenital spinal stenosis is typically associated with chondrodysplasias, which are genetic disorders [29], genetic factors may also play roles in the etiology of degenerative LSS [16, 21].

The thrombospondins (THBSs) are a family of five secreted, modular glycoproteins whose functions in the extracellular matrix (ECM) are diverse [6, 9, 10]. Both THBS1 and THBS2 interact with matrix metalloproteinase 2 (MMP2) and MMP9 and regulate their effective levels in the pericellular ECM [5, 30] (Fig. 1). A recent Japanese study reported a functional polymorphism in THBS2 that affects alternative splicing and MMP binding is associated with lumbar disk herniation [15]. However, few studies on the genetic association of single nucleotide polymorphisms (SNPs) or haplotypes with progression of LSS have appeared. To determine the contribution of THBS2 polymorphisms to the development and progression of LSS in the Korean population, we therefore prospectively genotyped LSS patients and controls. In addition, we have identified polymorphisms, including SNPs and haplotypes, in THBS2.

Materials and methods

Patient population

We recruited 148 LSS patients [82 (55.4 %) female; mean $\pm$ SD age = 62.1 $\pm$ 7.8 years), diagnosed at our

institution between June 2009 and December 2010. All LSS patients met the following three criteria: (1) diagnosis of lumbar spinal canal stenosis by magnetic resonance imaging (MRI): regardless of the presence of foraminal stenosis; (2) treatment and monitoring for more than 3 months by spine surgeons; and (3) a history of typical LSS symptoms, consisting of self-reported intermittent neurogenic claudication, stenotic symptoms extending to the lower extremities upon extension of the lumbar spine, or numbness or weakness of the lower extremities. All patients were examined both clinically and radiologically to confirm the degenerative nature of the stenosis. Exclusion criteria included the presence of a synovial cyst, epidural lipomatosis or a spinal tumor; spondylolisthesis; presentation with trauma; and diagnosis with an infectious disease. Peripheral pulse was evaluated to exclude vascular claudication.

The control group consisted of 157 volunteers [86 (54.7 %) female; mean $\pm$ SD age = 56.7 $\pm$ 9.2 years] with no history of back problems. All controls were examined by MRI to exclude individuals with asymptomatic LSS and baseline demographic characteristics were adjusted to be similar to those of the patient group. This study protocol was approved by the Ethics Committee of our institution, and written informed consent was obtained from each participant.

Grading of LSS

MRI was performed using a 1.5 T scanner (Gyrosan Intera Achieva; Philips Healthcare, Amsterdam) and a Synergy Spine Coil (Philips Healthcare). Each subject was placed in the supine position with a cushion under both knees. T1-weighted spin-echo sagittal and axial images and T2-weighted fast spin-echo sagittal and axial images were obtained (TR/TE: 500/15 for T1-weighted images and 3,600/120 for T2-weighted images; slice thickness, 4 mm; slice gap, 0.4 mm; field of view, 32 cm for sagittal images and 16 cm for axial images; matrix, 512 $\times$ 512; flip angle, 90°; and excitations, 3). The degree of LSS was assessed by quantitative image evaluation [25]. The cross-sectional area of the dural sac was measured on transverse angled sections through the central part of the disk as shown on conventional MR images. The central dural sac cross-sectional area (DSCSA) was calculated using the DICOM (version 3.0; CompuMed, Los Angeles, CA) picture-archiving and communication system. The central DSCSA >100 mm²

Fig. 1 Intron–exon organization, related ligands and receptors of the thrombospondin 2 gene

was considered to be normal; those 76–100 mm² in area was considered moderately stenotic, and those <76 mm² in area was classified as severely stenotic. All MR images were examined by two experienced clinicians who were unaware of the results of genetic analysis and of the clinical history and physical status of patients/subjects. Professionals responsible for clinical assessment were also unaware of the results of genetic analysis.

Genotyping

Genomic DNA for sequencing was isolated from peripheral blood leukocytes of affected individuals and controls. Genotypes of bi-allelic polymorphisms were determined by polymerase chain reaction (PCR) using sequence-specific primers designed employing the Primer 3 program to amplify fragments of approximately 500 bp and with melting temperatures ranging from 59 to 62 °C. PCR reactions were performed using 20 ng genomic DNA as template in 30 µL reaction mixtures containing AmpliTaq Gold (Applied Biosystems, Foster City, CA). The amplification protocol consisted of 1 cycle of Taq polymerase activation at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 30 s, annealing at 55–68 °C for 30 s, and extension at 72 °C for 45 s, concluded by a 10-min extension at 72 °C. For automated direct sequencing, PCR products were amplified using internal forward and reverse primers and a BigDye Terminator v3.1 Cycle Sequencing Kit. DNA samples containing extension products were mixed with Hi-Di formamide (Applied Biosystems), incubated at 95 °C for 5 min, placed on ice for 5 min, sequenced using the ABI Prism 3730 DNA Analyzer (Applied Biosystems), and analyzed employing DNASTar software (<http://www.dnastar.com>) and the PolyPhred program (<http://droog.gs.washington.edu/PolyPhred.html>).

Statistical analysis

All statistical analyses were performed using SPSS software (version 12.0, 2003; SPSS, Inc., Chicago, IL). Associations and Hardy–Weinberg equilibriums were assessed using the Chi square (χ^2) test. Differences in allele frequencies between LSS patients and controls were examined using χ^2 tests on 2×2 tables of allele counts. Linkage disequilibrium coefficients (D' and r^2 values) were calculated as previously described [23]. Haplotype frequencies for THBS2 were estimated using HapAnalyzer software, which applies a maximum likelihood estimation method by means of an expectation–maximization algorithm [12]. The algorithm equates each genotype frequency to the sum of the probabilities of all possible haplotype configurations resulting in that genotype. The relationship between LSS severity and genotype information was

assessed using the χ^2 test. The level of significance was 0.05 (a probability value) and all tests were two tailed.

Results

SNP discovery and association analysis

We identified THBS2 polymorphisms in addition to an intronic SNP (IVS10; rs9406328) associated with lumbar disk herniation in a Japanese population [15]. Of these polymorphisms, we selected and genotyped seven SNPs, which were in the coding region or showed minor allele frequencies greater than 20 % based on information in the HapMap phase II database (International HapMap Consortium). These selected SNPs covered most haplotypes and more than 80 % of the THBS2 alleles with r^2 values of 0.8 or greater. We genotyped these SNPs in 148 individuals with LSS and 157 controls, followed by χ^2 tests for dominant, co-dominant, recessive, and allele-frequency models. Although we attempted to identify any THBS2 SNP showing a nominal P value <0.05 in any of the four genetic models, we found no causal SNPs that were significantly associated with LSS (Table 1), whereas two SNPs (rs6422747, rs6422748) were over-represented in controls ($P = 0.042$, [OR] = 0.55 and $P = 0.042$, OR = 0.55, respectively) (Fig. 2).

To examine whether the examined SNPs were associated with a specific sub-population of LSS patients, subjects were grouped according to the various phenotypes, including age, gender, severity of LSS and levels (single-level versus multi-level LSS). However, even after phenotypic sub-grouping, the SNPs of THBS2 showed no association with LSS. Our sample size had an 80 % power to detect an LSS-relevant SNP at an OR >1.25, and at a significance level of 0.05.

Haplotype analysis

To search for hidden LSS susceptibility alleles in THBS2, we examined the haplotype structure of the gene. The linkage disequilibrium structure was determined using SNPs in the coding region or with minor allele frequencies of 20 % or more. Seven SNPs (rs13192849 A>C, rs9283850 A>G, rs9406328 A>G, rs6940420 G>A, rs6422747 A>G, rs6422748 C>G and rs9505891 G>C) were selected for the haplotype study (Table 2). We estimated haplotype frequencies in the LSS and control groups, and calculated P values based on 1,000,000 permutations. We found that five haplotypes with frequencies more than 5 % covered more than 95 % of both the LSS and control populations.

In the course of this analysis, we found that the “AAG-GACG” haplotype (HAP5) was over-represented in LSS

Table 1 Association of SNPs in THBS2 with lumbar spinal stenosis

db SNP ID	Location	LSS				Control				Test for allele frequency	
		11	12	22	Sum	11	12	22	Sum	<i>P</i>	Odd ratio (95 % CI)
rs13192849	IVS6	97	44	7	148	103	49	5	157	0.490	1.51 (0.47–4.86)
rs9283850	IVS9	109	38	1	148	123	33	1	157	0.966	1.06 (0.07–17.12)
rs9406328	IVS10	39	72	37	148	49	72	36	157	0.672	1.12 (0.66–1.90)
rs6940420	IVS14	94	46	8	148	100	49	8	157	0.903	1.06 (0.39–2.91)
rs6422747	IVS14	58	68	22	148	50	69	38	157	0.042	0.55 (0.31–0.98)
rs6422748	IVS14	57	69	22	148	50	69	38	157	0.042	0.55 (0.31–0.98)
rs9505891	IVS14	59	67	22	148	63	71	23	157	0.957	1.02 (0.54–1.92)

Allele 1 and allele 2 indicate the major and minor allele, respectively. Odds ratio for allele 1 versus allele 2

LSS lumbar spinal stenosis, IVS intervening sequence, CI confidence interval

Fig. 2 Representative data showing SNPs (rs6422747; A>G and rs6422748; C>G at IVS14) in the thrombospondin 2 gene (red arrow). These SNPs (rs6422747, rs6422748) were over-represented in controls ($P = 0.002$, OR = 0.29 and $P = 0.002$, [OR] = 0.29, respectively)

patients ($P = 0.0465$, OR = 2.00), particularly in those with severe stenosis (DSCSA $< 76 \text{ mm}^2$; $P = 0.0137$, OR = 2.48) (Table 3). In addition, the “AGAGACG” haplotype (HAP4) was over-represented in severe LSS patients ($P = 0.0147$, OR = 2.02). We also found that the “AAAGGGG” haplotype (HAP1) was over-represented in controls comparing with severe LSS ($P = 0.0068$, OR = 0.30).

Discussion

Lumbar spinal stenosis is a frequent, clinically important, spinal disorder. Because genetic factors such as the gene encoding type IX collagen, MMP have been implicated in disk herniation and degeneration [2, 16, 17, 19, 21], which

are the leading causes of degenerative stenosis, we investigated possible associations between the THBS2 gene, which regulates MMP2 and MMP9 levels in the pericellular ECM [5, 30], and degenerative LSS, as well as analyzing polymorphisms, including SNPs and haplotypes, in THBS2. Screening of all exons and the boundaries including intervening sequences of THBS2 for mutations in patients with LSS did not identify any causative SNPs. Our sample size had an 80 % power to detect an LSS-relevant SNP at an OR > 1.25 , and at a significance level of 0.05. However, if the true effect size was lower, the association between an SNP and LSS would not be statistically significant. In contrast, we found that the haplotypes (HAP4 and HAP5) of THBS2 were significantly associated with LSS in the Korean population, whereas the HAP1

Table 2 Association of haplotypes of the THBS2 region with lumbar spinal stenosis

SNP							Haplotype frequency		<i>P</i>
rs13192849	rs9283850	rs9406328	rs6940420	rs6422747	rs6422748	rs9505891	LSS	Control	
A	A	A	G	G	G	G	0.37	0.43	0.1507
C	A	G	G	A	C	C	0.19	0.18	0.6602
A	A	G	A	A	C	C	0.18	0.18	0.8153
A	G	A	G	A	C	G	0.13	0.09	0.1452
A	A	G	G	A	C	G	0.08	0.04	0.0465

LSS lumbar spinal stenosis

Table 3 Haplotype analysis of THBS2 with LSS according to severity of LSS

Haplotype	Haplotype frequency		<i>P</i>
	Case	Control	
LSS (148) versus control (157)			
HAP1 (1/1/1/1/2/2/1)	0.37	0.43	0.1507
HAP2 (2/1/2/1/1/1/2)	0.19	0.18	0.6602
HAP3 (1/1/2/2/1/1/2)	0.18	0.18	0.8153
HAP4 (1/2/1/1/1/1/1)	0.13	0.09	0.1452
HAP5 (1/1/2/1/1/1/1)	0.08	0.04	0.0465
Severe LSS (98) versus control (157)			
HAP1 (1/1/1/1/2/2/1)	0.18	0.43	0.0068
HAP2 (2/1/2/1/1/1/2)	0.19	0.18	0.7072
HAP3 (1/1/2/2/1/1/2)	0.15	0.18	0.4283
HAP4 (1/2/1/1/1/1/1)	0.16	0.09	0.0147
HAP5 (1/1/2/1/1/1/1)	0.10	0.04	0.0137

The makers are, from left to right, rs13192849 A>C, rs9283850 A>G, rs9406328 A>G, rs6940420 G>A, rs6422747 A>G, rs6422748 C>G and rs9505891 G>C. 1, 2 major and minor alleles of the markers, respectively

LSS lumbar spinal stenosis

haplotype appeared to significantly protect against LSS development. Our results further strengthen the hypothesis that THBS2 plays a role in spinal stenosis, but the mechanism of this involvement is not yet clear.

Thrombospondins are a potent inhibitor of angiogenesis by CD36 and form a family of multidomain ECM proteins with related sequences but diverse tissue distributions [3, 8, 11]. They are involved in a wide range of processes in the vessel wall, including smooth muscle cell proliferation, endothelial cell proliferation, and migration, and they bind to various ECM glycoproteins [3, 6, 7, 10]. Especially, THBS2 plays an anti-inflammatory role in an in vivo study [22, 27, 28]. Therefore, variations in the genes coding for these proteins are potential risk factors for cardiovascular disease [4], neoplasm development such as gastric cancer [20] and spinal disk disease [15].

Mice with deficiencies in either THBS1 or THBS2 show abnormal spine curvature [18]. THBS2 knockout mice also

exhibit increased levels of MMP2 after injury [1]. A more recent study has shown immunolocalization of THBSs in the human intervertebral disk [13]. Our findings are in agreement with those of several human and animal studies that support a role for THBSs in spinal health [1, 13, 15, 18]. Because lumbar disk disease is a common cause of LSS, particular THBS2 alleles also predispose carriers to stenosis. Although alterations in THBS2 alone likely do not lead directly to LSS development, such changes may predispose to the disease.

One possible cause of LSS is aberrant MMPs regulation of the THBS2. MMP2 and MMP9 activities are mainly regulated by endocytosis, a general clearance mechanism for ECM. Endocytic clearance of MMPs is mediated by the scavenger receptor, low-density lipoprotein receptor-related protein (LRP) [14, 31]. THBS2 promotes LRP-mediated endocytosis of MMP2 and its subsequent lysosomal degradation [31]. Thus, THBS2 may play a major role in intervertebral disk and ligament flavum ECM homeostasis as a modulator of MMP2 and MMP9 endocytosis. A recent Japanese study reported a functional polymorphism in THBS2 that affects alternative splicing and MMP binding is associated with lumbar disk herniation [15]. These findings, along with the observed association between the THBS2 gene and LSS in a Japanese population, suggest that THBS2 is an LSS susceptibility gene.

We found that a specific THBS2 haplotype was associated with LSS in the Korean population. However, several limitations of our study should be considered. Our samples were limited in their power to detect risk alleles of small effect. In particular, the LSS and control group consisted of only 148 and 157 volunteers, respectively. While this sample had adequate power to detect a substantial OR of 2.0, it had 80 % power to detect a risk allele at an OR of 1.25. Therefore, our findings should not be overestimated. Despite our small sample size, the findings provide evidence that genetic factors are important in the etiology of LSS. Studies with larger populations are needed to confirm our findings. Another weakness of this study is that the age of the groups was not matched, which may have caused selection bias, although baseline demographic

characteristics were adjusted to be similar among each groups. The future studies need to focus on younger patients with LSS, because the elderly volunteers were more frequently diagnosed of LSS by MR imaging.

The haplotype per se may confer susceptibility to LSS, or a susceptibility allele may be hidden within the haplotype. Continued genetic analysis of THBS2 will help define the molecular mechanism and mode of pathogenesis of LSS, leading to improved methods for LSS screening, diagnosis, and treatment [16].

Conclusion

Although no causal SNPs in THBS2 were associated with LSS, haplotypes (HAP4 and HAP5) were significantly associated with progression of LSS in the Korean population, whereas another haplotype (HAP1) and two SNPs (rs6422747, rs6422748) may play a protective role against LSS development. However, the genetic functions of THBS2 haplotypes in LSS remain to be determined.

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Conflict of interest None.

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