Association study of *COL9A2* with lumbar disc disease in the Japanese population

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Abstract Lumbar disc disease (LDD) is a common musculo-skeletal disease with strong genetic determinants. In a Finnish population, a single nucleotide polymorphism (SNP) causing an amino-acid substitution (*Trp2* allele) in *COL9A2*, which encodes the $\alpha 2$ (IX) chain of type IX collagen, has been reported to associate with LDD. However, replication studies in different populations have produced controversial results. To further investigate the association of *COL9A2* with LDD in Japanese, we examined SNPs in

COL9A2, including *Trp2*, in 470 LDD patients (mean age 35) along with 658 controls (mean age 48). We identified a total of 43 sequence variations in *COL9A2*. Nine SNPs, including *Trp2*, were selected and genotyped. After Bonferroni's correction, none of these SNPs showed association. Unlike observations in the Finnish population, *Trp2* was common in Japanese, and no association with LDD was apparent. However, we did see association of a *COL9A2* specific haplotype with LDD ($P=0.025$; permutation test); this association is more significant in patients with severe lumbar disc degeneration ($P=0.011$). Thus, the association of *Trp2* with LDD was not replicated, but *COL9A2* susceptibility allele(s) other than *Trp2* may be present in Japanese LDD.

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Introduction

Lumbar disc disease (LDD) consists chiefly of disc degeneration and disc herniation, contributing to the development of low back pain and unilateral leg pain. Low back pain affects 70–85% of all people during their lifetimes, and LDD is the most common cause of activity limitation in individuals younger than 45 years of age (Andersson 1999). Lumbar disc degeneration causes low back pain and discogenic pain (Ito et al. 1998), and herniation is a common cause of radiculopathy manifesting as unilateral leg pain. Studies show that 20% of patients with lumbar disc herniation require surgical treatment during follow-up due to prolonged or aggravated leg pain (Saal and Saal 1989;

Komori et al. 2002). Thus, LDD is a significant health issue for individuals and society.

The etiology and pathogenesis of LDD are still largely unknown. LDD has a strong familial component (Varlotta et al. 1991; Matsui et al. 1998), as evidenced by the finding that a positive family history increases the risk for juvenile lumbar disc herniation up to 5.6-fold (Matsui et al. 1992). Twin studies have demonstrated the genetic contribution to degeneration of intervertebral discs of the lumbar spine, observed through magnetic resonance imaging (MRI) (Battie et al. 1995; Sambrook et al. 1999). Recently, several genes have been reported to associate with LDD, most of which encode matrix proteins in the intervertebral disc.

COL9A2, which encodes the $\alpha 2$ (IX) chain of type IX collagen, has been reported to associate with LDD in a Finnish population. *Trp2*, a rare *COL9A2* allele that replaces the wild-type arginine with tryptophan at codon 326 (R326W), was found in 6 of 157 patients (allelic frequency: 1.9%) but was absent in 174 controls (Annunen et al. 1999). A subsequent study of Finnish families revealed that family members who carry the *Trp2* allele have a greater degree of degeneration in the vertebral disc and end-plate (Karppinen et al. 2002). However, the *Trp2* association was not replicated in a German study of 250 surgically treated patients who experienced disc prolapse of the cervical and lumbar spine (Wrocklage et al. 2000).

In this study, we examined the association of *COL9A2* with LDD in the Japanese population. In contrast with the Finnish population, *Trp2* is common in Japanese individuals and does not associate with LDD. However, we have identified a haplotype of *COL9A2* that associates specifically with severe disc degeneration in LDD.

Materials and methods

Subjects

The study examined 470 patients with LDD and 658 controls, all of whom were Japanese (Seki et al. 2005). The mean ages (range) of the patient and control groups were 35 (13–75) and 48 (13–86) years, respectively. All patients had received radiographic evaluation, including plain radiographs and MRI, and had been treated and monitored for at least 1 year by orthopedic surgeons specializing in spinal diseases. Patients had reported unilateral pain radiating from the back along the femoral or sciatic nerve to the corresponding dermatome of the nerve root for longer than 3 months. The grade of disc degeneration was

determined according to Schneiderman's classification for MRI (Schneiderman et al. 1987), using exclusion criteria as described (Seki et al. 2005). The study protocol was approved by ethical committees at each participating institution, and written informed consent was obtained from all participants prior to joining the study.

SNP discovery and genotyping

Through assembly of GenBank sequences (AF019406 and AL050341), we generated a reference sequence for a ~24-kb genomic region containing *COL9A2*. The sequence included 3-kb upstream and downstream regions of the *COL9A2* transcribed region. Sequence variations in *COL9A2* were identified in public databases or by sequencing the region using 48 genomic DNA samples from the non-affected Japanese population.

Genomic DNA was extracted from peripheral blood using standard procedures, and *Trp2* was genotyped by polymerase chain reaction (PCR) and direct sequencing. Other *COL9A2* polymorphisms were genotyped using the TaqMan assay (Livak 1999) using a Prism 7700 sequence detector (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Conditions for the TaqMan assay were as follows: 50°C for 2 min; 95°C for 10 min; 95°C for 15 s; and 62°C for 1 min, for 40 cycles.

Statistical analysis

For each SNP, a χ^2 test was performed to assess Hardy–Weinberg equilibrium and association between the LDD and control groups based on allelic and genotypic frequencies. The odds ratio (OR) and 95% confidence interval (CI) were calculated with respect to the minor allele compared with the major allele. Pairwise linkage disequilibrium (LD) was estimated as previously described (Seki et al. 2005). Haplotype frequencies and permutation tests were estimated by the expectation-maximization algorithm using Haploview software (<http://www.broad.mit.edu/mpg/haploview/>).

Results

Association analysis

Our association analysis found that *Trp2* was under-represented in the Japanese LDD population and showed no association with LDD (Table 1). In contrast with the Finnish population, *Trp2* was common in the Japanese population. Given that genotypic risk ratios

Table 1 Sequence variations in COL9A2 and association analysis with lumbar disc disease (LDD). The A nucleotide in the translation initiation codon (ATG) is denoted as + 1. χ^2 tests on the sequence variations were calculated using a 2×3 table for the genotype frequency and a 2×2 table for the allele frequency. OR Odds ratio, 95% CI 95% confidence interval, IVS intervening sequence

Location	Nucleotide change	Amino acid change	Genotype		Allelic frequency		χ^2 test (P value)		OR (95% CI)	Reported allelic frequency	rs ID
			Case	Control	Case	Control	Allele	Genotype			
Promoter	−2066C>A	(−)	256/182/32	393/234/25	0.262	0.217	0.015	0.031	1.27 (1.05–1.55)	(−)	rs209923
IVS 5	IVS5+26G>A	(−)	230/207/32	368/245/43	0.289	0.252	0.053	0.057	1.20 (1.00–1.45)	(−)	rs1983658
Exon 19	c.976C>T	R326W ^a	370/91/9	504/136/14	0.116	0.125	0.500	0.801	0.92 (0.71–1.18)	0.019 ^b	rs12077871
Exon 19	c.977A>G	R326Q	217/221/32	327/277/50	0.303	0.288	0.443	0.306	1.08 (0.89–1.29)	0.255 ^c	rs7533552
Exon 19	c.982G>A	G328R	457/12/0	636/18/0	0.012	0.013	0.844	(−)	0.93 (0.45–1.94)	(−)	Novel
Exon 19	c.1003C>G	L335V	414/54/1	576/76/2	0.060	0.061	0.886	0.955	0.97 (0.69–1.39)	0.076 ^d	rs12722877
IVS 19	IVS19+1147G>C	(−)	177/222/70	232/327/86	0.386	0.387	0.966	0.510	1.00 (0.84–1.18)	(−)	rs2076696
Exon 21	c.1062G>A	P354P	290/77/8	291/75/8	0.124	0.121	0.890	0.987	0.98 (0.72–1.33)	(−)	rs6676013
Exon 30	c.1741G>A	V581I	423/45/2	597/58/1	0.052	0.046	0.674	0.619	1.15 (0.78–1.69)	0.037 ^e	rs3737821

^a Trp2, which shows positive association with LDD in the Finnish population^b Annunen et al. (1999); Wrocklage et al. (2000)^c Annunen et al. (1999)^d Paasilta et al. (2001)^e IMS-JST SNP (<http://www.snp.ims.u-tokyo.ac.jp>)

for *Trp2* were calculated to be 1.6-, 2- or 4-fold, the false negative ratios (β) would be 0.05, 0.0075 and 0.0000033, respectively. To examine the possibility that *Trp2* is associated with a specific sub-population of LDD, subjects were grouped according to various phenotypes, including age, sex and the severity of disc degeneration. Even after phenotypic sub-grouping, however, *Trp2* showed no association.

We identified a total of 43 *COL9A2* polymorphisms in addition to *Trp2*. Of these, we selected and genotyped nine SNPs, including *Trp2*, which were in the coding region or had minor allele frequencies greater than 20%. One SNP (−2066C>A) showed a positive association ($P<0.05$); however, after Bonferroni's correction for multiple testing, no SNP showed significant association with LDD (Table 1).

Haplotype analysis

To search for hidden susceptibility alleles in *COL9A2*, we examined the haplotype structure of the gene. The LD structure was determined using SNPs with minor allele frequencies equal to or greater than 20% (Table 2). The entire *COL9A2* gene was contained in a 13-kb LD block. Three SNPs (−2066C>A, c.977A>G and IVS19+1147G>C) were selected for the haplotype study by SNP tagger (<http://www.ihg.gsf.de/ihg/databases.html>); they were not in complete LD with each other, as estimated using the Δ measure. We estimated haplotype frequencies for case and control groups, and calculated P value based on 1,000,000 permutations.

Table 2 Pairwise linkage disequilibrium (LD) analysis on *COL9A2* single nucleotide polymorphisms (SNPs). Pairwise LD between all pairs was evaluated by the D' and D^2 statistics. Three SNPs in *COL9A2* (SNP1: −2066C>A, SNP2: c.977A>G, SNP3: IVS19 +1147G>C) with minor allele frequencies more than 20% were selected for LD analysis

SNP	LDD			Control		
	1	2	3	1	2	3
			D'			D'
1		0.851	0.877		0.891	1.000
2	0.604		1.000	0.566		0.976
3	0.178	0.276		0.163	0.230	
	Δ^2			Δ^2		

Through this analysis, we found that the “221” haplotype was over-represented in LDD ($P=0.025$) (Table 3). Further, this association was more significant when tested for the group with severe (Schneiderman's grade 10–20) disc degeneration ($P=0.011$). The *Trp2* allele was linked to the “111” haplotype rather than to the “221” haplotype.

Discussion

We were unable to replicate the previously reported association of *Trp2* with LDD (Annuen et al. 1999), despite examining more than three times the number of study subjects. Our results may be due to a lack of power in the study; the estimated false negative rate is considerable when the genotypic risk ratio is lower than 1.6, indicating that we might have missed a causal

Table 3 Haplotype analysis of *COL9A2* with LDD. Common (>2%) haplotypes of the *COL9A2* locus are shown. The markers are, from left to right, -2066C>A, c.977A>G, IVS19 + 1147G>C. 1, 2 Major and minor alleles of the markers, respectively

Haplotype	Overall	Case	Control	<i>P</i> value
LDD (470) versus control (658)				
H1 (1/1/2)	0.382	0.378	0.385	0.995
H2 (1/1/1)	0.312	0.295	0.324	0.449
H3 (2/2/1)	0.211	0.238	0.191	0.025
H4 (1/2/1)	0.075	0.065	0.083	0.378
Severe LDD (161) versus control (658)				
H1 (1/1/2)	0.386	0.382	0.387	1.000
H2 (1/1/1)	0.317	0.293	0.323	0.757
H3 (2/2/1)	0.206	0.265	0.191	0.011
H4 (1/2/1)	0.076	0.048	0.083	0.144

variant with a weak effect. Alternatively, the discrepant results may arise from population differences, as evidenced by the very different allelic frequencies of *Trp2* and other SNPs in control groups. Both the Finnish and the Japanese populations are genetically isolated (de la Chapelle 1993; Wright et al. 1999).

It is not surprising that a genetic variant is associated in some populations but not in others. Such inter-study heterogeneity has been reported for many complex common diseases (Wang et al. 2005), and significant heterogeneity between studies on the same topic has been reported in 14 of 36 cases (39%) (Ioannidis et al. 2001). Humans are significantly outbred, with each collection of samples representing an unquantifiable mixture of genetic strains and environments. Therefore, the phenotypic effects of genetic variants will differ considerably among diverse environmental and genetic contexts. The lack of replication in this study does not diminish the value of the Finnish pioneer study. Even if genetic variants are population-specific, *COL9A2* remains the gene responsible for LDD, a common disease that affects millions of people. Such variants can help shed light on molecular pathways relating to the gene, providing important clues for identifying additional disease genes in distinct populations.

Type IX collagen is a fibrillar collagen that cross-links with type II collagen on the surface of cartilage collagen fibrils (Nishimura et al. 1989). A heterotrimeric protein, type IX collagen is comprised of $\alpha 1$, $\alpha 2$ and $\alpha 3$ (IX) chains, which are encoded by the *COL9A1*, *COL9A2*, and *COL9A3* genes, respectively (Perala et al. 1993). In mice, abnormal type IX collagen causes LDD, and the skeletal phenotypes of collagen IX-deficient mice suggest that this protein is essential for the functional longevity of the intervertebral disc (Fassler et al. 1994; Kimura et al. 1996). Transgenic mice expressing mutant $\alpha 1$ (IX) collagen also develop disc degeneration and

progressive joint degeneration with age (Nakata et al. 1993); at 20 months, these mice show protrusion of the disc with impingement on the spinal cord, mimicking human herniation (Kimura et al. 1996). These findings, along with the observed association in the Finnish population (Annunen et al. 1999), justify consideration of *COL9A2* as a susceptibility gene.

To date, functional data supporting the pathogenesis and causality of *Trp2* are lacking. Cartilage samples from individuals homozygous for *Trp2* yielded normal amounts of collagen IX that include the $\alpha 2$ chain. The profile of cross-linked peptides and incorporation of the *Trp2* product into the cross-linked fibrillar network also were normal in these samples (Matsui et al. 2003). In the Finnish study, linkage was identified more than 10 cM from *Trp2* (Annunen et al. 1999). A German study did not replicate the association; *Trp2* was found only in three older patients (61–77 years of age) (Wrocklage et al. 2000). Therefore, further study is necessary to confirm the association of *Trp2* with LDD and its specific functionality in disease pathogenesis. Meta-analysis, the quantitative assessment of combined results from these studies, would be useful in estimating and explaining their diversity.

We have identified a specific *COL9A2* haplotype associated with LDD in the Japanese population. This association also awaits replication. The haplotype itself may confer susceptibility to LDD, or a hidden susceptibility allele may exist within the haplotype. Continued genetic analysis of *COL9A2* will help define the molecular mechanisms and pathogenesis of LDD, leading to improved methods for screening, diagnosis, and treatment.

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