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Phenotypic and population differences in the association between CILP and lumbar disc disease

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LETTER TO JMG

Background: Lumbar disc disease (LDD) is one of the leading causes of disability in the working-age population. A functional single-nucleotide polymorphism (SNP), +1184T→C, in exon 8 of the cartilage intermediate layer protein gene (CILP) was recently identified as a risk factor for LDD in the Japanese population (odds ratio (OR) 1.61, 95% CI 1.31 to 1.98), with implications for impaired transforming growth factorβ signalling.

Aim: To validate this finding in two different ethnic cohorts with LDD.

Methods: This SNP and flanking SNPs were analysed in 243 Finnish patients with symptoms of LDD and 259 controls, and in 348 Chinese subjects with MRI-defined LDD and 343 controls.

Results and conclusion: The results showed no evidence of association in the Finnish (OR = 1.35, 95% CI 0.97 to 1.87; p = 0.14) or the Chinese (OR = 1.05, 95% CI 0.77 to 1.43; p = 0.71) samples, suggesting that cartilage intermediate layer protein gene is not a major risk factor for symptoms of LDD in Caucasians or in the general population that included individuals with or without symptoms.

Lumbar disc disease (LDD) is one of the leading causes of disability in the working-age population. Radiological changes indicative of LDD are common, but only a proportion develops complications such as disc herniation and sciatica. Although the aetiology of LDD is not well understood, there is strong evidence for the involvement of both genetic and environmental factors.

A recent study reported an association between LDD and a functional single-nucleotide polymorphism (SNP) (rs2073711), +1184T→C, in exon 8 of the cartilage intermediate layer protein gene (CILP) in a Japanese group (odds ratio (OR) 1.61, 95% CI 1.31 to 1.98). The allelic change resulted in amino acid substitution Ile395Thr. CILP is expressed widely in intervertebral discs and its expression increases as disc degeneration progresses. CILP interacts directly with transforming growth factor (TGF)β1, inhibiting the TGFβ1-mediated induction of extracellular matrix proteins such as aggrecan and collagen II.

Functional studies showed that the C allele (coding for Thr395) increased binding and inhibition of TGFβ1, suggesting that regulation of TGFβ1 signalling by CILP plays a crucial role in the aetiology and pathogenesis of LDD.

Argument for a causal role would be strengthened if the same association could be replicated in a distinct population, and in clinical cases of LDD defined by MRI changes indicative of LDD in general. Therefore, we investigated the association between CILP polymorphisms and LDD in a Finnish sample with symptoms of LDD, and in a Chinese sample with only MRI-defined LDD. These samples were informative in previous studies demonstrating association of LDD with the vitamin D receptor gene and the Gln326Trp (Trp2) allele of COL9A2 in Chinese and the Arg103Trp (Trp3) allele of COL9A3 in Finns. Thus, the Chinese sample is comparable with the Finnish dataset, and a correlation can then be drawn with the Japanese dataset.

Methods

The Finnish cohort

The Finnish patient group consisted of 243 unrelated individuals (146 males, 97 females) with discogenic sciatica, representing an extended set of one published previously. All had unilateral pain radiating from the back to below the knee (sciatica) from 3 weeks to 6 months (mean (SD) duration = 2.5 (1.5) months), which did not respond to non-steroidal anti-inflammatory analgesics. The patients were examined clinically and by MRI of the spine on enrolment and 3 years later. Clinical presentation had to be concordant with MRI findings. Approximately 5% of the cohort did not have a herniated disc on MRI. In all, 29% of the subjects had been operated on for herniated discs by the time of follow-up assessment. The control group consisted of 259 unrelated individuals from the same region of Finland (128 males, 131 females).

The Chinese cohort

In the Chinese sample set, the presence and severity of LDD were assessed using Schneiderman’s classification for 691 individuals recruited from the general population. A score was given for each lumbar level, with 0 indicating no degeneration and 3 indicating a grossly degenerated disc with associated loss of disc height. The MRIs were rated by two spine specialists (KMCC and JK) independently, with good reliability. The sum of the ratings for the five disc levels provides an overall raw LDD score that is positively skewed and tends to increase in mean and variance with age. To obtain standardised, age-adjusted LDD scores, the raw LDD scores were logarithmically transformed to reduce skewness and heteroscedasticity, and then standardised to a mean of 0 and a variance of 1 in each decade of age by subtracting the decade mean and dividing by the decade SD. Finally, the sample was divided into two groups: those with higher median age-adjusted scores were classified as cases (n = 348) and those with lower scores were classified as controls (n = 343).

As this new scoring system (with age adjustment) is different from that used in a previous study, we investigated its validity. In the previous analysis, an association between the Trp2 allele and LDD was significant only after age stratification, suggesting that the association is age-dependent. Using the new scoring system, association was observed using a median split (allelic p = 0.043), and became even more significant when the extreme top and bottom 25% were compared (allelic p = 0.001).

Abbreviations: CILP, cartilage intermediate layer protein; LDD, lumbar disc disease; SNP, single-nucleotide polymorphism; TGF, transforming growth factorβ.
Genotype analysis
For the Finnish cohort, genomic DNA extracted from white blood cells was used as a template for PCR. The recently reported SNP, +1184 T→C (rs2073711) in exon 8, associating with LDD was analysed by sequencing in all the 243 patients and 259 controls. In addition, all exons, exon boundaries and promoter regions of CILP were amplified by PCR and analysed for sequence variations by sequencing. PCR amplifications were typically performed using 20 ng of genomic DNA, 0.25 mM of forward and reverse primers, 1.5 mM MgCl₂, 0.2 mM dNTPs and 1 U of Ampli Taq Gold polymerase (Applied Biosystems, Foster City, California, USA). The PCR conditions included an initial denaturation for 12 min at 95°C, 35 cycles at 95°C for 30 s, at 58–64°C for 30 s and at 72°C for 30 s, followed by 1 cycle at 72°C for 10 min. PCR products were sequenced using an ABI PRISM 3100 sequencer and BigDye Terminator Sequencing Kit (Applied Biosystems) to define the underlying sequence variations.

For the Chinese cohort, the +1184 T→C SNP (rs2073711) and three flanking SNPs (rs1561888, rs3784447 and rs47766680) were genotyped using the Sequenom platform (Sequenom, San Diego, California, USA). The Mass ARRAY AssayDesign software (Sequenom) was used to design amplification and allele-specific extension primers for uniplex or multiplexed assays. The extension primer was designed to hybridise to the amplicon near the SNP site for the extension of a single base or a few bases depending on the genotype of the allele. PCRtreatments of PCR products with alkaline phosphatase and mass extension reactions were all performed according to the manufacturer’s (Sequenom) protocol. The final base extension products were desalted using SpectroClean resin (Sequenom), mixed with 3-hydroxypicolinic acid, and analysed using a modified Brucker Autoflex MALDI-TOF mass spectrometer (Brucker, Billerica, Massachusetts, USA).

Statistical analysis
Genotype data of each SNP were checked for Hardy–Weinberg disequilibrium using standard χ² goodness-of-fit tests. Genotype data were then converted to allele counts in cases (a, b) and allele counts in controls (c, d). These allele counts were used to calculate OR (ad/bc), and 95% CI (using the formula 1/a + 1/b + 1/c + 1/d) for calculating the sampling variance of the natural logarithm of the OR. The allele counts were also subjected to Pearson’s χ² tests for association. These analyses were done by implementing the formulae on an EXCEL spreadsheet. In addition, analysis of variance testing for differences in mean LDD scores between groups was done using the SPSS software. Power calculations of the cohorts were determined using a Genetic Power Calculator, assuming an OR of 1.6 as found in the Japanese cohort. ⁵

RESULTS
The Finnish cohort
Sequence analysis of the CILP SNP (rs2073711), +1184T→C in exon 8, found no significant association, with an OR of 1.35 (95% CI 0.97 to 1.87) and a p value of 0.14 (table 1). Furthermore, the frequencies of the corresponding genotypes did not differ between the two groups (table 1). Sequence analysis of the CILP promoter region, all exons and exon boundaries detected five additional SNPs that are in Hardy–Weinberg equilibrium, but all were present in both patients and controls with similar frequency (data not shown). Four of the variations were intronic at −45C→T (intron 4), −12T→C (intron 6), −19T→C (intron 6) and +19G→A (intron 6), and one was a synonymous change, +3496G→A, in exon 9.

The Chinese cohort
Two SNPs in CILP (rs2073711 and rs1561888) and two in genes flanking CILP, rs3784447 in RASL12 and rs4776680 in PARP16, were genotyped in this sample set. All four SNPs were in Hardy–Weinberg equilibrium in both cases and controls. The functional CILP SNP (rs2073711, +1184T→C in exon 8) had an OR of only 1.05 (95% CI 0.77 to 1.43) and a p value of 0.71 (table 1). The frequencies of the corresponding genotypes did not differ between the two groups (table 1). Furthermore, similar results were obtained when more extreme cut-offs were used for defining cases and controls (table 1), and when the age-adjusted scores for SNP rs2073711 were treated as a continuous variable in a regression analysis using analysis of variance (F = 0.112, with p = 0.74). We also observed no significant allelic association of another SNP (rs1561888) within CILP or of SNPs flanking CILP (rs3784447 and rs4776680) with LDD (data not shown).

Power calculation
Assuming a prevalence of 0.1–0.35 and an OR of 1.6 (per allele, multiplicative risk model), we consider the power to be reasonable with an estimate of over 80% for both the Chinese and Finnish cohorts, for a significance level of 0.05.

DISCUSSION
Many of the candidate genes identified to be associated with LDD are extracellular matrix components (recently reviewed by Chan et al.). CILP is expressed in all structures of the intervertebral discs, and its expression increases with progression in disc degeneration. ⁶ Thus, the finding by Seki et al ⁷ further highlights the importance of extracellular matrix components in the aetiology of LDD, and the role of extracellular matrix in the structural integrity of the tissue and also in regulating signalling molecules in tissue repair and maintenance. This finding opens new ideas for the search for candidate genes for LDD as well as novel therapeutic treatments that target specific pathways such as TGFβ1 signalling. Its significance however needs validation in other populations.

The lack of association in the current study does not imply that the finding in the Japanese cohort is a false-positive result. This is also unlikely, given the strong statistical power of the study with a p value of 0.00002 following correction for multiple testing. ⁴ On the contrary, our findings add important information to the Japanese study. In the Finnish set, the recruitment criteria for disease and control groups were very similar to those in the Japanese study. Thus, the disparity in association with the CILP polymorphism may reflect ethnic differences, suggesting that genetic risk factors for LDD are likely to differ between the Japanese and Northern European populations. This conclusion is supported by previous findings of predisposing collagen IX alleles, Ghn326Trp (Trp2) in the α2 chain, ⁸ Arg103Trp (Trp3) in the α3 chain. Trp2 and Trp3 were found in about 5% and 24% of Finnish patients with LDD, respectively. While Trp2 was found in about 20% of Southern Chinese ⁹ and Japanese δ individuals, Trp3 was absent in Southern Chinese. ⁹ Ethnic variations are further highlighted in the different allele frequency of the rs20073711 SNP in CILP between Japanese and Finnish populations.

Smaller ethnic differences are expected between Japanese and Chinese, and this is reflected in the similar allele frequency for the rs20073711 SNP in CILP. The major difference here is the definition of LDD, wherein the Chinese sample set comprises all individuals with LDD defined by MRI, independently of symptoms. LDD is not always with symptoms, and patients with sciatica represent only a small fraction, and individuals requiring surgical invention are a further subset.
Within the Chinese sample set, we also evaluated a small subset of individuals who are clearly symptomatic versus controls with herniation or sciatica and find no association, with p values of 0.42 and 0.83, respectively. Thus, the lack of association in the Chinese sample suggests that the Japanese sample is not representative of LDD in general but is a subset of individuals with painful disc herniations.

Furthermore, we investigated the possibility that the Japanese sample set represents an even more severe subset of the disease by analysing a subset of 45 Finnish patients with LDD who had undergone surgical treatment for herniated discs, and found no association (data not shown). Given that the criteria for patients with symptoms of LDD and controls are so similar between the Japanese and Finnish samples, the lack of association in Finns may suggest the presence of positive modifiers for CILP or additional disposing factors in the Japanese associated with CILP. However, with an estimated power of around 80% for our cohorts, we cannot exclude the possibility that the lack of association may be a type II error (false negative), especially if the effect size in the Chinese and Finnish populations is different from that reported for the Japanese sample.

To ensure that we have not missed small effects, we combined the data in a meta-analysis of the Finnish and Chinese samples. The ORs from the two samples were combined using an inverse variance weighting method. The OR of the two samples was 1.15, with a 95% CI of 0.93 to 1.43, again providing no evidence for an effect of CILP on LDD. Using Fisher’s method of combining p values, the allele-wise tests of the two samples (p = 0.14 for Finnish and p = 0.71 for Chinese) gave an overall χ² statistic of 4.64 (4 df), and a non-significant p value of 0.33.

A point of interest to note in the Japanese study is the selective criteria of the cohort that consisted of 467 individuals with degenerative disc disease and 654 controls. All of the cases had a history of unilateral pain radiating from the back along the femoral or sciatic nerve to the corresponding dermatome of the nerve root alone cannot be the cause of pain, because over 70% of asymptomatic individuals have disc herniations pressurising the nerve root but with no pain. Therefore, it is noted that degenerative changes are necessary for the disc to herniate. It is also clear that herniation-induced pressure on the nerve root alone cannot be the cause of pain, because over 70% of asymptomatic individuals have disc herniations pressurising the nerve root but with no pain. Thus, the Japanese cohort studied for the association of CILP may not represent LDD in general, but a special extreme subset of individuals with painful disc herniations.

In conclusion, we were unable to replicate the results of an association between CILP and symptoms of lumbar disc herniation found in the Japanese population. Although this may suggest that the CILP association is unique in the Japanese sample, it may be explained by other factors, including differences in ethnicity and phenotype definition.

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Competing interests: None declared.

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REFERENCES


Table 1 Genotype and allele frequencies of the functional cartilage intermediate layer protein (CILP) +1184T→C single-nucleotide polymorphism in the Finnish and Chinese samples

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Finnish</th>
<th>Chinese</th>
<th>Chinese [extreme set]*</th>
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<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
</tr>
<tr>
<td>CC</td>
<td>82 (0.36)</td>
<td>73 (0.28)</td>
<td>4 (0.01)</td>
</tr>
<tr>
<td>CT</td>
<td>116 (0.50)</td>
<td>141 (0.55)</td>
<td>88 (0.26)</td>
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<tr>
<td>TT</td>
<td>33 (0.14)</td>
<td>43 (0.17)</td>
<td>249 (0.73)</td>
</tr>
<tr>
<td>C</td>
<td>280 (0.61)</td>
<td>287 (0.56)</td>
<td>96 (0.14)</td>
</tr>
<tr>
<td>T</td>
<td>182 (0.39)</td>
<td>227 (0.44)</td>
<td>586 (0.86)</td>
</tr>
<tr>
<td>p Value</td>
<td>0.24</td>
<td>0.93</td>
<td>0.14</td>
</tr>
<tr>
<td>p Value (genotype)</td>
<td>0.24</td>
<td>0.93</td>
<td>0.14</td>
</tr>
<tr>
<td>p Value (allele)</td>
<td>0.14</td>
<td>0.71</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*Sample set established by selecting the bottom 30% of the normalised distribution representing a control group of 70 males and 138 females with a mean (SD) age of 45 (4) years, and the top 30% representing a more severe patient group of 93 males, 119 females with lumbar disc disease with a mean (SD) age of 42 (9) years.
The TRP2 allele of COL9A2 is an age-dependent risk factor for the development and severity of intervertebral disc degeneration. *Spine* 2005;30:2735–42.


