Identification of a Novel Common Genetic Risk Factor for Lumbar Disk Disease

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Lumbar disk disease (LDD) is one of the most common musculoskeletal disorders worldwide. Although Finland may be the only country where the prevalence of sciatica has been studied through nationwide screening and clinical examination, there is no evidence that Finland would be substantially different in this regard from other industrialized countries. A prevalence study of persons by Heliovaara et al involving a sample of 8000 persons found 5.1% of men and 3.7% of women met the diagnosis of lumbar disk syndrome, half of whom were deemed to be in need of medical care. Results of different surveys performed in the United States indicate that the prevalence of sciatic pain in adult

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GENETIC RISK FACTOR FOR LUMBAR DISK DISEASE

populations is between 1% and 40%. Clinically significant sciatica occurs in 4% to 6% of the US population. The frequency of disk surgery performed due to sciatic pain in Finland is approximately equal to that in most other western countries, but lower than in the United States. These data indicate that disk disease is a significant problem in the western world.

Lumbar disk disease often results in physical impairment requiring surgery, and it contributes significantly to health care costs and work disability. A number of environmental and anthropometric risk factors such as driving, torsional stress, smoking, and height have been implicated in the pathogenesis of LDD. There are a number of studies to suggest, however, that disk disease consisting of disk herniation, sciatica, and disk degeneration may be explained to a large degree by genetic factors, and this is supported by findings of a considerable genetic predisposition to early onset sciatica and lumbar disk herniation in some families.

Our recent finding that a Gln326→Trp (glutamine→tryptophan) substitution in the α2 chain of collagen IX (Trp2 allele) is associated with dominantly inherited LDD also supports this hypothesis. The Trp2 allele was found in 6 out of 157 (4%) Finnish patients with LDD but in none of a series of 174 individuals without the disease. Linkage analysis indicated that the allele cosegregated with the phenotype in a dominant fashion in the families of the patients carrying it. The Trp2 allele (COL9A2) is associated with dominantly inherited LDD because it was not found in the controls; also, families of 4 of the 6 probands were evaluated and the results showed that all persons (n=23) in these families with the Trp2 allele had LDD.

Collagen IX is a heterotrimERIC protein consisting of 3 genetically distinct chains, α1(IX), α2(IX), and α3(IX), encoded by the COL9A1, COL9A2, and COL9A3 genes. The chromosomal locations of the genes are 6q12-q13 (COL9A1), 1p33-p32.3 (COL9A2), and 20q13.3 (COL9A3). Collagen IX is an attractive candidate for LDD because it serves as a minor component in both main structures of the intervertebral disk, the annulus fibrosus and the nucleus pulposus, in addition to being present in cartilage and the vitreous body of the eye. The findings that the Trp2 allele of the α2(IX) chain is associated with dominantly inherited LDD and that transgenic mice harboring a large internal deletion in the Col9a1 gene for the α1(IX) chain develop intervertebral disk herniation and degeneration, in addition to degenerative joint disease, also confirms a role for collagen IX in LDD.

Herein, we report an analysis of patients with LDD and controls without LDD for sequence variations in the collagen IX genes, undertaken to estimate the overall significance of collagen IX alleles in the pathogenesis of LDD. All subjects were residents of Finland. The analysis identified an Arg103→Trp (arginine→tryptophan) substitution in the α3(IX) chain (Trp3 allele), which was shown to be the first common genetic risk factor for LDD.

METHODS

Subjects

The case sample consisted of 171 unrelated Finnish patients with discogenic sciatica. All of them had a history of unilateral discogenic pain radiating from the back to below the knee (dermatomes L4, L5, and S1) with a duration of at least 1 month. The main exclusion criteria were an application for early retirement and rare causes of sciatica such as synovial cysts and non-degenerative spondylolisthesis. Patients with severe back injury were also excluded. The age of the patients ranged from 19 to 78 (mean [SD], 45 [13]) years. All the patients were evaluated clinically. The clinical examination included the straight-leg-raising test, assessment of lumbar flexion by the modified Schober measure, tendon reflexes, and evaluation of motor and sensory deficits. Leg pain was assessed using 100-mm visual analog scales (VASs), disability was evaluated using the Oswestry Low Back Disability Questionnaire, and quality of life was measured using the Nottingham Health Profile (NHP). In the absence of neurologic deficits, the dermatomal distribution of pain was used as the basis for the identification of the affected nerve root.

The clinical diagnosis was supplemented with electroneuromyography and periradicular infiltration of the suspected nerve root. In the infiltration, the nerve root was first identified with contrast medium (Gd-DTPA, Schering, Berlin, Germany) and possible pain provocation was recorded. Thereafter, the treatment agent (either 0.9% saline or a combination of methylprednisolone and bupivacaine [Soluét c bupivacain, Orion, Espoo, Finland]) was injected and possible alleviation of leg pain was recorded. In addition, 152 of the patients were evaluated by magnetic resonance imaging (MRI) and the remaining 19 by computed tomography (CT). Three of the patients also had osteoarthritis (OA) and none of them had rheumatoid arthritis (RA) or chondrodysplasia (CD). Because of the close relation between abnormalities in the lumbar intervertebral disks and sciatic pain, these 2 entities are jointly referred to as lumbar disk disease or lumbar disk syndrome.

The control sample consisted of 321 unrelated Finnish individuals without LDD. Of these, 186 were healthy individuals aged 21 to 75 (mean [SD], 38 [11]) years; 83 were individuals with primary knee and/or hip OA, aged 21 to 66 (55 [7]) years; 31 were individuals with RA, aged 29 to 76 (60 [12]) years; and 21 were individuals (all <20 years of age) with various CDs. The CD diagnoses were based on the 1992 international classification for constitutional diseases of bones, and the patient data were reevaluated according to the present classification established in 1997. The diagnosis of RA was based on American Rheumatism Association criteria. Healthy individuals were mainly hospital and laboratory employees. None of the controls had a history of discogenic pain or had been operated on for a herniated disk.
The participation rate was 99% for the healthy individuals and 98% for the other controls. All the patients with LDD and all the controls except for some of the CD cases were from the same geographical region of Finland.

After informed consent, which consisted of verbal communication regarding the study and a subsequent explanatory form that was signed by participants, blood samples were collected from all the subjects to obtain genomic DNA. The institutional review board of the University of Oulu, Oulu, Finland, approved the study.

MRI and CT
The MRI scans, obtained with a 1.5-T imaging system, consisted of sagittal images with a repetition time/echo time of 4000/95 milliseconds and axial images with a repetition time/echo time of 640/14 milliseconds. The CT images consisted of scans through the L2-L3 to L5-S1 interspaces. Findings of MRI or CT were considered positive if they indicated disk extrusion, at least 2-level disk herniation, or at least 4-level disk bulging, or endplate degeneration at 1 or more levels in patients younger than 30 years of age, at 2 or more levels in patients aged 30 to 50 years, or at 4 or more levels in patients older than 50 years.

Analysis of the Collagen Genes
Polymerase chain reaction (PCR) primers were designed to amplify all possible exons of the human COL9A1 (38 exons), COL9A2 (32 exons), and COL9A3 (32 exons) genes. The PCR products varied in length between 200 and 450 base pairs (bp). The primers were designed so that each PCR product contained at least 60 bp of the exon flanking sequences at each end of the products. The PCR conditions consisted of initial denaturation at 94.5°C for 2.5 minutes, followed by 35 cycles at 94.5°C for 50 seconds, 54°C to 65°C for 45 seconds, and 72°C for 50 seconds, and a final extension at 72°C for 10 minutes. The PCR samples were denatured at 95°C for 5 minutes and annealed at 68°C for 30 minutes to generate heteroduplexes for analysis by conformation-sensitive gel electrophoresis (CSGE).

Approximately 50 to 100 ng of the products was used for heteroduplex analysis by CSGE. The conditions for CSGE analysis were similar to those previously described. We have extensively tested the sensitivity of the CSGE/PCR method in detecting sequence variations in collagen genes (COL1A1, COL1A2, COL2A1, COL3A1, COL9A1, COL9A2). The results indicated that the method detected all 75 previously identified sequence variations. Also, the analysis detected 223 new sequence variations that were confirmed by nucleotide sequencing. Based on these results the sensitivity and specificity of the method would seem to be 100%.

However, this is not likely to be true since it is impossible to test all sequence variations in all possible sequence contexts. Sequence variations observed as heteroduplexes in CSGE analysis were identified by automated sequencing (ABI PRISM 377 Sequencer and dRhod Dye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, Calif). Prior to sequencing, the PCR products were treated with exonuclease I to degrade the residual PCR primers and with shrimp alkaline phosphatase to dephosphorylate the residual nucleotides. Controls were analyzed only for those sequence variations identified in cases except for an initial assessment of 95 controls (see “Results”).

Statistical Analysis
The Fisher-Irwin exact test was used to test for equality in allele frequencies between the LDD patients and the non-LDD controls, using 2 × 2 tables of allele counts.

RESULTS
Analysis of the Collagen IX Genes for Sequence Variations
We previously analyzed 157 patients for sequence variations in exon 19 of the COL9A2 gene. Six of the patients were found to have a sequence variation leading to a Gln326→Trp substitution, the Trp2 allele. Fourteen additional patients with LDD were included in this series. The new patients were first analyzed for the presence of the Trp2 allele, and 1 additional occurrence was found. We did not examine the family members of this new case. The remaining 31 exons of the COL9A2 gene and all 38 exons of the COL9A1 gene were also analyzed for polymorphisms. The analysis of these genes did not identify any other potentially disease-causing alleles, such as those containing glycine substitutions, premature translation termination codons, or changes in the splicing consensus sequences. Using CSGE and by sequencing for polymorphisms, we then analyzed all 32 exons and exon boundaries of the COL9A3 gene in 86 patients with LDD and in 95 controls (consisting of 30 healthy controls and 65 controls with primary OA). Electrophoresis (Figure 1) and sequencing (Figure 2) of PCR products for exon 5 identified 2 nucleotide variations in the same codon and thus 3 alleles: CGG (Arg), CAG (Gln), and TGG (Trp). The polymorphic site was at amino acid position 103, located in the extreme N-terminal collagenous domain, COL3. The remaining patients and controls were subsequently analyzed for this polymorphism. Because some individuals were homozygous for the CGG codon or for the TGG codon, which gave similar patterns in CSGE analysis (Figure 1) and were thus in-
distinguishable, all samples were also analyzed by sequencing (Figure 2 and TABLE 1). The frequency of the Trp allele in the α3(IX) chain was much higher among the LDD patients than among the controls, being 12.2% (ie, 40 of 328 alleles [excluding the 7 patients with the Trp2 allele to avoid confounding]) in the patients with LDD, but only 4.7% (ie, 30 of 642 alleles) in the controls (4.1% in the healthy controls, 6.0% in the controls with primary OA, 4.4% in the RA controls, and 4.8% in the CD controls).

**Statistical Analysis**

To evaluate whether the frequency of the Trp3 allele differed significantly between the 171 LDD patients and the 321 non-LDD controls, Fisher-Irwin exact tests were performed on tables of allele counts. Since the goal of this investigation was to identify potential collagen-related risk factors for LDD, all individuals without LDD, including healthy individuals and those with OA,
RA, and CD, constituted the joint control sample of individuals without the disease. To simplify the analysis, the CAG allele coding for Gln was omitted, as it was found only 3 times in the whole data set (once in cases and twice in controls).

Allele counts were thus obtained for LDD cases (CGG [Arg], 299; TGG [Trp], 42) and controls (CGG [Arg], 610; TGG [Trp], 30). The 2-sided Fisher-Irwin P value for these data is .000028, demonstrating that the frequency of the Trp3 allele is significantly higher in the LDD patients than in the controls without LDD. This finding is in agreement with our previously published observation that another Trp allele (Trp2), located in the COL9A2 gene, is a strong risk factor for the disease.11

In fact, that prior study suggested that the Trp2 allele has a high penetrance in heterozygous carriers, who may therefore provide no evidence of additional risk factors (eg, potential predisposing alleles in the COL9A3 gene, as investigated herein). The elimination of individuals carrying the Trp2 allele (7 out of the 171 patients) provided allele counts for LDD cases (CGG [Arg], 285; TGG [Trp], 42) and controls (CGG [Arg], 610; TGG [Trp], 30). The 2-sided Fisher-Irwin P value for these data is .000013.

It is impossible to accurately determine from our data the proportion of affected individuals directly or indirectly caused by the Trp3 allele. Assuming that Trp3 acts in a dominant fashion (most individuals with Trp3 are heterozygous), an upper bound for its “etiologic fraction” can be estimated to be about 23%, as only 40 out of 171 LDD cases carry Trp3. The disease in the remaining LDD cases must obviously be caused by other factors, either genetic or environmental. Given an estimated LDD prevalence of 5% in Finland, the disease risk attributable to Trp3 in the population is estimated as about 15%, compared by [40/171 × 0.05/(40/171 × 0.05 + 30/321 × 0.95)] − [131/171 × 0.05/(131/171 × 0.05 + 291/321 × 0.95)] × (40/171 × 0.05 + 30/321 × 0.95)/0.95.

In any case, it is clear that the presence of Trp3 does not cause disease outright, but merely increases the risk of disease. The probability of disease given the presence of at least 1 Trp3 allele is 11.6%, computed by 40/171 × 0.05/(40/171 × 0.05 + 30/321 × 0.95). Conversely, the probability of disease without Trp3 is 4.3%, computed by 131/171 × 0.05/(131/171 × 0.05 + 291/321 × 0.95). For an individual with at least 1 Trp3 allele, the risk of disease is thus increased nearly 3-fold (11.6%/4.3% = 2.7) relative to an individual without Trp3.

Analysis of the COL9A1, COL9A2, and COL9A3 genes also identified additional polymorphisms that changed the encoded amino acid (Table 2, Table 3, and Table 4). Unlike tryptophan substitutions, these amino acid changes are generally considered neutral changes in collagens. 31,33,34 Nonetheless, many of the patients and controls were analyzed for the presence of these variations. Since they do not differ significantly in frequency between the groups (L.A.-K., unpublished data, 2000), it is likely that, as expected, they have no effect on the LDD phenotype. The analysis also identified several sequence variations presumed to be neutral because they did not alter the amino acid sequence of the protein or the consensus sequences for splicing. In any case, the frequencies of the observed sequence variations did not differ statistically between the patients and the control group (L.A.-K., unpublished data, 2000).

### Table 2. Amino Acid Changes and Corresponding Allele Counts in the COL9A1 Gene

<table>
<thead>
<tr>
<th>Subjects</th>
<th>GTA (Val154)/CTA (Leu154)</th>
<th>TCA (Ser339)/CCA (Pro339)</th>
<th>GAA (Glu450)/GGA (Gly450)</th>
<th>CAG (Gln621)/CGG (Arg621)</th>
<th>ATG (Met767)/GTG (Val767)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDD</td>
<td>259/1 (130)</td>
<td>257/3 (130)</td>
<td>257/3 (130)</td>
<td>207/53 (130)</td>
<td>259/1 (130)</td>
</tr>
<tr>
<td>Healthy</td>
<td>162/0 (81)</td>
<td>166/0 (83)</td>
<td>176/2 (89)</td>
<td>99/25 (62)</td>
<td>176/0 (88)</td>
</tr>
<tr>
<td>OA</td>
<td>110/0 (55)</td>
<td>110/0 (55)</td>
<td>110/0 (55)</td>
<td>78/32 (55)</td>
<td>110/0 (55)</td>
</tr>
</tbody>
</table>

*Allele frequencies were estimated from heteroduplex analysis. The standard for evaluation of polymorphisms is analysis of 50 individuals (100 alleles) and if no substantial differences are identified, this amount suffices. Number of readings varied due to logistics of performing the assays. Because the frequency of these variations did not differ statistically, it was not necessary to analyze all the control groups. Val indicates valine; Leu, leucine; Ser, serine; Pro, proline; Glu, glutamic acid; Gly, glycine; Gln, glutamine; Arg, arginine; Met, methionine; LDD, lumbar disk disease; and OA, osteoarthritis.

### Table 3. Amino Acid Changes and Corresponding Allele Counts in the COL9A2 Gene

<table>
<thead>
<tr>
<th>Subjects</th>
<th>ACG (Thr246)/ATG (Met246)</th>
<th>CTA (Leu335)/GTA (Val335)</th>
<th>CGG (Arg628)/CAG (Gln628)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDD</td>
<td>232/10 (121)</td>
<td>290/22 (156)</td>
<td>197/3 (100)</td>
</tr>
<tr>
<td>Healthy</td>
<td>174/6 (90)</td>
<td>177/15 (96)</td>
<td>177/3 (90)</td>
</tr>
<tr>
<td>OA</td>
<td>138/4 (71)</td>
<td>135/7 (71)</td>
<td>142/0 (71)</td>
</tr>
</tbody>
</table>

*Allele frequencies were estimated from heteroduplex analysis. For further explanation of data, see Table 2 footnote. Thr indicates threonine; Met, methionine; Leu, leucine; Val, valine; Arg, arginine; Gln, glutamine. LDD, lumbar disk disease; and OA, osteoarthritis.
tients evaluated by CT had disk herniations, and all of them had been operated on before the study was initiated. At the time the study was initiated none of the patients evaluated by MRI had been operated on for herniated disks, but during the follow-up time of 1 year, 27% of those with the Trp3 allele and 20% without it had undergone such a procedure (P = .38). All except 4 patients with negative MRI findings showed electroneuromyography abnormalities or experienced pain provocation with periradicular contrast medium or instant leg pain alleviation after infiltration of the suspected nerve root, indicating the discogenic nature of sciatica.

COMMENT

The difference in the frequency of the Trp3 allele between the 164 patients with LDD (12.2%, excluding the 7 patients with the Trp2 allele) and the 321 controls (4.7%) was statistically significant (P = .000013; 2-sided). Since neither the healthy controls nor the controls with cartilage diseases were evaluated clinically or radiologically for LDD, it is possible that some control individuals also had LDD but were asymptomatic. About 5% of asymptomatic individuals have been reported to have positive MRI findings using the same criteria as were used in this study. If some of the control individuals truly had LDD, the statistical significance of the Trp3 allele frequency difference between cases and controls is likely to be understated rather than overstated. It is unlikely that our findings are caused by poor matching of case and control groups because all the individuals were Finnish and, except for some of the CD controls, all were from the same geographical region of Finland.

The results also indicated that the Trp3 allele does not itself cause LDD but increases the risk of LDD, and thus it represents the first common genetic risk factor for musculoskeletal diseases. It has been speculated that common genetic risk factors may play a role in LDD 11 and many other common diseases, such as Alzheimer disease, breast cancer, and diabetes. However, only a few such factors have been identified, most likely because identification of such factors is extremely difficult. The best example of such a factor is the apolipoprotein E type 4 allele that increases the risk of late-onset Alzheimer disease in a dose-dependent fashion, while the type 2 allele is protective.

The findings that the Trp3 and Trp2 alleles are associated with LDD were somewhat surprising because mouse studies have indicated that collagen IX gene defects may result in joint cartilage degeneration and phenotypes resembling human OA in addition to disk disease. Six collagen IX mutations have also been identified in humans—namely, splicing mutations in the COL9A2 and COL9A3 genes in families with multiple epiphyseal dysplasia (MED), a phenotype that resembles OA but is more severe. Surprisingly, all the MED mutations lead to skipping of exon 3 and thus to a deletion of 12 amino acids in the N-terminal collagenous domain of the α2 (IX) or α3(IX) chains, suggesting a role for the particular sequences in MED. Thus, the splicing mutations and the Trp alleles seem to be associated with different phenotypes. It is very likely, of course, that different mutations or similar mutations in different locations in the molecule cause different phenotypes and even affect different tissues, as is the case with collagen II, which is expressed in the same tissues as collagen IX. Collagen II haploinsufficiency causes Stickler syndrome, and a Gly67→Asp (glycine→asparagine) substitution in this collagen leads to Wagner syndrome, which is characterized by severe eye findings (eg, vitreoretinal degeneration, retinal detachment, myopia, and cataract) but has no cartilage involvement. Other Gly substitutions in collagen II usually lead to severe cartilage diseases with or without eye involvement.

Collagen IX is a structural component of the annulus fibrosus and nucleus pulposus and also of the endplates, ie, the hyaline cartilage structures of the vertebral bodies adjacent to the intervertebral disks. The defects in collagen IX may thus play a role in intervertebral disk pathology. The mechanism by which Trp alleles may cause LDD or predispose individuals to it is not clear, but they may play a role in intervertebral disk pathology, since Trp is the most hydrophobic amino acid, and is not normally found in collagen IX. This is supported by our recent results showing that wild-type recombinant human collagen IX contains a Gly326→Trp substitution in the α2 chain (the Trp2 allele) bind recombinant collagen II differently (S. S. R., A. Fertala, L. A.-K., unpublished data, 2000).

Since the analysis failed to identify any disease-associated changes in collagen IX genes in about two thirds of the patients, it is likely that sequence variations in other genes coding for intervertebral disk diseases may play a role in the development of LDD. It is also possible that the Trp2 allele increases the risk of LDD, and thus it plays a role in intervertebral disk pathology.

**Table 4.** Amino Acid Changes and Corresponding Allele Counts in the COL9A3 Gene

<table>
<thead>
<tr>
<th>Subjects</th>
<th>CCG (Pro70)/CTG (Leu70)</th>
<th>CGA (Arg252)/CTA (Gln252)</th>
<th>CCA (Pro296)/CTA (Leu296)</th>
<th>GCA (Ala435)/GAA (Glu435)</th>
<th>GCC (Ala606)/ACG (Thr606)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDD</td>
<td>192/8 (100)</td>
<td>200/0 (100)</td>
<td>300/18 (159)</td>
<td>264/52 (158)</td>
<td>312/6 (159)</td>
</tr>
<tr>
<td>Healthy</td>
<td>207/11 (109)</td>
<td>236/0 (118)</td>
<td>226/8 (117)</td>
<td>203/35 (118)</td>
<td>235/1 (118)</td>
</tr>
<tr>
<td>OA</td>
<td>121/7 (64)</td>
<td>129/1 (65)</td>
<td>124/6 (65)</td>
<td>108/22 (65)</td>
<td>126/4 (65)</td>
</tr>
</tbody>
</table>

* Allele frequencies were estimated from heteroduplex analysis. For further explanation of data, see Table 2 footnote. Pro indicates proline; Leu, leucine; Arg, arginine; Gin, glutamine; Ala, alanine; Glu, glutamic acid; Thr, threonine; LDD, lumbar disk disease; and OA, osteoarthritis. For other data pertaining to COL9A3, see Table 1.
tervertebral disk proteins or variations in the regulatory regions of the collagen IX genes may also lead to predisposition to LDD.22


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Obtained funding: Kröger, Vanharanta, Ala-Kokko.

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Study supervision: Kröger, Vanharanta, Ala-Kokko. Study contacts, physical examinations: Karpipinen, Hakala, Palm, Kröger, Katilla.

Clinical expertise: Karpipinen, Hakala, Kröger, Katilla.

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Clinical expertise: Karpipinen, Hakala, Kröger, Katilla.

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