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ORIGINAL ARTICLE

Absence of the mutated Trp2 allele but a common polymorphism of the COL9A2 collagen gene is associated with early recurrence after lumbar discectomy in a German population

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Abstract Genetic factors seem to play a role in symptomatic lumbar disc disease (LDD). It has been shown previously that a tryptophan mutation of the COL9A2 gene is a major risk factor for LDD in a Finish population. The impact of collagen gene variations on the relapse rate after lumbar discectomy, however, has not been studied so far. Here, we conducted a cross-sectional genotyping study of patients who underwent lumbar discectomy to determine the influence of a COL9A2 mutation on the recurrence rates. Biopsy samples from 288 patients suffering from LDD with and without relapse were analyzed by PCR restriction fragment analysis and direct sequencing. The mutated Trp2 allele was not detected in the patients' samples of the present study. However, nine patients with recurrent LDD, but only two without recurrence were homozygous for the Arg allele. Homozygosity for the Arg allele of Col9A2 seems to be more frequent in the patient group with early recurrence although the differences in the allele frequencies were statistically not significant. In contrast, the Trp2 mutation seems not to be a major susceptibility factor for LDD in a German population.

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Introduction

Lumbar disc disease (LDD) leading to disc degeneration and concomitant disc herniation is a primary cause of low back pain and sciatica. Low back pain affects 70–85% of all people during their lifetimes in Western civilizations and lumbar disc herniation is the most common cause for activity limitation in individuals younger than 45 years of age [1]. Lumbar discectomy is the standard treatment for the herniated disc with neurological deficits or resistance to conservative therapy with recurrence rates between 5 and 15% [16]. Not only adequate surgical technique and rehabilitation strategy but also the mechanical stability and the level of degeneration of the remaining discs play a major role in recurrent disease.

Increasing evidence suggests that genetic factors play an important role besides other risk factors leading to symptomatic intervertebral disc disease [1, 3, 11]. To analyze the influence of genetic alterations and polymorphisms on lumbar disc disease, many studies have focused on genes encoding structural cartilage proteins that are thought to be of importance for the mechanical stability. Mutations and functional polymorphisms were found in aggrecan, the vitamin D receptor and collagen genes affecting collagen type IX. In animal models mutations of the collagen types IX and II were associated with clinical phenotypes [11]. Recent studies focused on the collagen genes COL9A2 and COL9A3, encoding the α_2 und α_3-chain of type IX collagen [2, 5, 10, 14, 15, 17]. In 1999 Annunen and co-workers [2] were the first to associate a mutant allele of the COL9A2

gene with degenerative disc disease. A single nucleotide polymorphism in exon 19, resulting in a substitution of glutamine (Gln) or arginine (Arg) with tryptophan (Trp) at amino acid 326 was found in 6 of 157 (3.8%) patients of a Finnish population with degenerative disc disease but in none of the 174 healthy control patients. Previously, Kimura et al. [7] showed that transgenic mice deficient in collagen IX developed disc degeneration followed by herniation .

Here, we investigate the influence of the COL9A2 gene polymorphism on the recurrence rate after lumbar discectomy and found that homozygosity for the Arg allele is associated with a higher risk of early recurrence.

Materials and methods

Patients

In the present study formalin-fixed and paraffin-embedded biopsy samples from 288 patients (131 female, 157 male) from a Bavarian population suffering from LDD who had been surgically treated for intervertebral disc herniation at the Neurosurgical Clinic, Technical University of Munich, Germany, were analyzed. Inclusion criteria were surgical intervention due to neurological deficits or intolerable pain resistant to non-operative treatment and an uncomplicated lumbar disc herniation demonstrated by magnetic resonance imaging and CT-scan. The standardized operative technique included laminotomy, sequesterectomy and dichotomy. Two groups of patients were investigated: 144 patients (66 female, 78 male, mean age 49.7 years) experienced recurrent disease within 2 years after first operation and underwent additional surgical treatment, and 144 patients (65 female, 79 male, mean age 49.7 years) were free of disease with a minimal disease-free interval of 10 years. A strict definition of recurrent disc herniation was used, including disc herniation at the same level (ipsi- or contralateral) and a pain-free interval of at least 6 months since initial surgery [16]. The clinical data of the patients are summarized in Table 1.

Table 1 Patients data

	Relapse free	Relapse
N	144	144
Male	79	78
Female	65	66
Mean age	49.7 years (m 50.7, f 48.5)	49.7 years (m 50.5, f 48.6)
Range	26–68 years	26–67 years
L2/L3	4	2
L3/L4	16	8
L4/L5	86	86
L5/S1	38	48

DNA isolation and PCR amplification

Genomic DNA was extracted from formalin-fixed, paraffin-embedded biopsy samples. Three 20-µm thick unstained sections were de-paraffinized in xylol and digested by proteinase K at 55°C for 36 h followed by 10 min at 98°C heat inactivation of proteinase K. PCR amplification was performed in a 50 µl reaction containing 50–100 ng template DNA, 20 pM each primer (Col9α2 F1: 5′-TTA TCT GTC TAT CCC AGG GCA GTG-3′, Col9α2 R1: 5′-CTA ACC TCA TCA GCC ACT AGC C-3′), 200 µM each dNTP, 2.5 U Taq polymerase (Amersham Biosciences, Braunschweig, Germany) and 10 µl Q-solution (Qiagen, Hilden, Germany). PCR conditions included an initial denaturation at 94°C for 4 min, followed by 40 cycles of amplification (94°C, 30 s; 60°C, 30 s; 72°C, 90 s) and a final extension at 72°C for 7 min. PCR results were confirmed by agarose gel electrophoresis of 5 µl of the PCR reaction.

BsmFI and BceAI restriction digest

For the detection of the Trp allele a *Bsm*FI digest according to Wrocklage et al. [17] was performed. Briefly, purified PCR fragments from 20 µl of the initial PCR were digested in a 25 µl reaction containing 2 U *Bsm*FI, 2 µg BSA at 65°C for 12 h. The Gln and the Arg allele of the Col9A2 gene were discriminated by a *Bce*AI digest. Therefore, purified PCR fragments from 20 µl of the initial PCR were digested in a 25 µl reaction containing 2 U *Bce*AI (New England Biolabs, Beverly, MA), 2 µg BSA at 37°C for 12 h. For details see Tables 2, 3. Results were confirmed by direct sequencing of the PCR results of four different probes.

Statistical analysis

Differences in the frequency distribution of the genotypes and allelotypes between the patient groups were tested using the Chi-square test.

Results

A 128 bp PCR fragment was amplified from all samples included in the present study (Fig. 1a, lane 2). Using the two restriction enzymes *Bsm*FI and *Bce*AI it became

Table 2 PCR restriction length polymorphisms

Allele	Sequence	*Bsm*FI site	*Bce*AI site	Fragment size after *Bsm*FI digest	Fragment size after *Bce*AI digest
Gln	5′-ggga***cag***cccg-3′	1	None	79 bp+ 49 bp	128 bp
Arg	5′-ggga***cgg***cccg-3′	1	1	79 bp+ 49 bp	74 bp+ 54 bp
Trp	5′-ggga***tgg***cccg-3′	none	none	128 bp	128 bp

Table 3 Cleavage sites

*Bce*AI	*Bms*FI
5′…ACG GC(N)12…3′	5′…GGG AC(N)10…3′
3′…TGC CG(N)14…5′	3′…CCC TG(N)14…5′

possible to discriminate the three Col9A2 alleles Trp, Arg and Gln by their restriction fragment lengths. The Trp codon does not present a restriction site for *Bsm*FI, whereas the restriction site created by the Arg and Gln codons would result in a 79 and a 49 bp restriction fragment. A *Bce*AI restriction site is present only in the Arg allele giving a 74 and a 54 bp restriction fragment (Tables 2, 3).

Using this approach, all samples showed a complete digestion resulting in a 79 and a 49 bp fragment after treatment with the *Bsm*FI restriction enzyme (Fig. 1a, lanes 3–5). These data indicate the absence of the Trp allele in the biopsy samples of the present study.

The *Bce*AI restriction enzyme was used to discriminate between the Gln and the Arg allele. The *Bce*AI digest gave different results: In the group of patients with recurrent LDD nine PCR fragments were completely digested resulting in a 54 bp and a 74 bp fragment (Arg), 112 samples showed an undigested 128 bp fragment (Gln) and 23 probes showed both an undigested 128 bp fragment and the two fragments of 74 bp and 54 bp resulting from cutting with *Bce*AI (heterozygous Arg/Gln). In contrast, in the group without recurrent disease only two samples were completely digested, 117 PCR fragments were undigested and 25 samples showed three restriction fragments indicating a heterozygous situation (Fig. 1b).

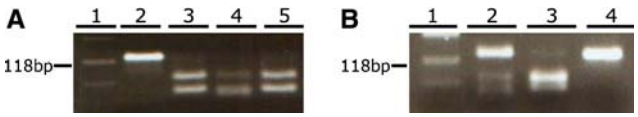

Fig. 1 **a** *Bsm*FI restriction length polymorphism. PCR products were separated on 2% agarose gels before (*lane 2*) and after *Bsm*FI digest (*lanes 3–5*, *lane 1*=size marker). A 128 bp undigested fragment was present in all probes from patients with and without relapse (*lane 2*). All PCR products showed a complete *Bsm*FI digest (*lanes 3–5*) indicating the presence of the restriction site in both alleles and consequently the absence of the tryptophan allele. **b** *Bce*AI restriction length polymorphism. PCR products were separated on 2% agarose gels after *Bce*AI digest. Three different results were obtained: A 128 bp undigested fragment was present in patients homozygous for the Gln allele (*lane 4*), a 74 bp and a 54 bp completely digested fragment were present in patients homozygous for the Arg allele (*lane 3*), and a 128 bp, a 74 bp and a 54 bp fragment were present in heterozygous patients (*lane 2*). *Lane 1*=Marker

The results were confirmed by sequencing of four representative PCR fragments which exhibited a homozygous Arg/Arg, a homozygous Gln/Gln and heterozygous Arg/Gln genotype (Fig. 2). The *Bsm*FI assay was confirmed by digestion of a cloned Col9A2 fragment containing the Trp codon and therefore, the *Bsm*FI restriction site (data not shown).

By statistical analysis, homozygosity for Arg could more often significantly been observed in the relapse group when compared with the other two genotypes (heterozygotes and homozygotes for Gln) together ($p = 0.0313$). However, there was no significant difference in the distribution of the two alleles Arg versus Gln ($p = 0.1248$) or in the distribution of the three possible genotypes ($p = 0.0978$, Table 4)

Discussion

Although various environmental, ergonomic and biometric risk factors are associated with symptomatic LDD, increasing evidence suggests that genetic factors also play an important role [15]. Recently, a mutation of the COL9A2 gene was found to be associated with a higher risk for disc degeneration. In the present study we found an

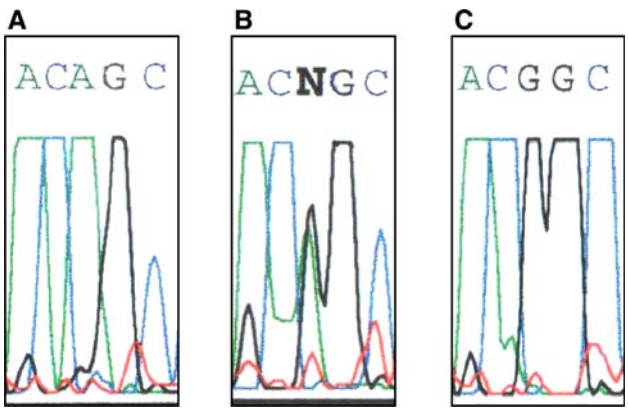

Fig. 2 Sequences of the Col9A2 alleles [**a**, homozygous Gln (CAG); **b**, heterozygous; **c**, homozygous Arg (CGG)]

Table 4 Genetic data

A. Distribution of the allele

	Gln	Arg	
Relapse-free	259	29	
Relapse	247	41	$p = 0.1248$
	506	70	

B. Arg homozygosity compared with heterozygotes and Gln homozygotes

	Gln/Gln or Gln/Arg	Arg/Arg	
Relapse-free	142(Gln/Gln 117) (Gln/Arg 25)	2	
Relapse	135(Gln/Gln 112) (Gln/Arg 23)	9	$p = 0.0313$
	277(Gln/Gln 229) (Gln/Arg 48)	11	

association of a common polymorphism in the COL9A2 gene and early recurrence after discectomy. However, none of our patients exhibited the mutant Trp2 allele.

Annunen et al. [2] demonstrated the Trp2 mutation of COL9A2 in 6 of 157 (3.8%) patients in a Finnish population with symptomatic LDD but not in the 174 healthy control persons. These data have been corroborated by Paassilta et al. [10] who found a frequency of 4% of the mutant allele in 171 Patients with clinical and radiological signs of LDD. Finally, Karppinen and co-workers [6] observed a prevalence of 3.8% (6 of 159) for the Trp2 allele in their study of a Finnish population. They investigated the impact of the mutation on the disease phenotype and reported a higher flexibility in the affected group, but found no additional clinical differences when compared with the control group. A part of the patients' groups was investigated by magnetic resonance imaging. Three of six patients with the Trp2 allele, but none of the 18 control subjects had a radial tear in one of the non-herniated discs. In summary the three studies investigated Finnish patients and showed a prevalence of the Trp2 mutant allele of ca. 4% in the Finnish population.

These data are in contrast to the results of the present study. We could not detect the mutant Trp2 allele of COL9A2 in the 288 patients of the present study. Our results are in accordance with data obtained by Stefanos et al. [15] who examined 105 symptomatic patients with intervertebral disc disease and 102 age-matched control persons from a Greek population for the presence of the tryptophan mutation in the COL9A2 (Trp2) and COL9A3 (Trp3) gene, respectively. They could not detect these mutations in any of their patients. Wrocklage et al. [17] used the same approach as in the present study and screened disc tissue samples from 250 patients who underwent lumbar discectomy. They also found a lower frequency of the Trp2 allele of 1.2% in a German population.

These differences seem to be most likely due to variations in the allele frequencies between the Finnish and the Middle and South European populations since clinical reasons could be largely excluded. A misclassification of patients seems to be unlikely. In all studies the patients meet the criteria for LDD, defined by clinical symptoms, physical examination or magnetic resonance imaging (MRI) findings [2, 5, 10, 15, 17]. In our study we included only patients with operative therapy, therefore, all patients fulfilled the criteria for severe LDD. The mean age of the patients was in the same range in all studies (present study 49.7 years, Wrocklage 47 years, Annunen 44 years, Karpinen 45 years, Stefanos 39 years [2, 5, 10, 15, 17]). From the results of our study that investigated the largest collection so far of 288 patients with LDD we assume that the Trp2 mutation seems not to be a consistent genetic marker for the predisposition of LDD or for recurrent LDD after discectomy in a Middle European population.

Recently, two studies investigated the relevance of sequence variations of COL9A2 for LDD in Japan and found that the Trp2 allele is common in the Japanese population. However, the impact on LDD was controversial since one study did not detect any association [13] whereas, the other found an association but only in younger patients under 40 years of age [5].

The codon 326 in exon 19 that is affected by the Trp mutation of the COL9A2 gene normally codes for Gln or Arg. Interestingly, we found an impact of this polymorphism on the probability for early recurrence after discectomy. Nine patients homozygous for the Arg allele (6.25%) experienced early recurrence of LDD within 2 years after initial discectomy. In the group with a disease-free interval of 10 years or more only two patients (1.38%) were homozygous for the Arg allele. Homozygosity for Arg was significantly more often present in the recurrent group when compared with the two other genotypes together ($p < 0.05$). However, statistical analysis of all three genotypes or for the allelotypes revealed no significant differences ($p > 0.05$). Therefore, our data might implicate a trend towards a higher risk of the Arg allele for early recurrence.

It has been speculated that differences in the amino acid sequences of the collagens might influence the mechanical stability of the proteins [8]. A disruption of the collagen triple helix has been discussed as a possible mechanism by which the mutant Trp substitution could contribute to the disease phenotype. Since Trp is a highly hydrophobic amino acid an interference with the binding to collagens IX and II or by prevention of lysyl oxidase could lead to a reduced cross linking and a lower mechanical stability of the collagen [4, 9]. Transgenic mice expressing mutant alpha 1 collagen IX developed progressive joint degeneration with age, and accelerated intervertebral disc degeneration. Radiological and histological studies showed

that cervical and lumbar disc degeneration was more advanced in the transgenic mice than in the littermate controls. The changes included shrinkage or disappearance of the nucleus pulposus, and fissures in the fibrous annulus that eventually lead to disc herniation. These findings suggest that mutations of the type IX collagens may cause certain forms of degenerative disease in the spine and in the joints. Recently, Seki et al. [12] associated a higher susceptibility to lumbar disc disease with a functional SNP in the CILP gene encoding the Cartilage Intermediate Layer Protein. These data might also implicate a role for polymorphisms in the normal amino acid sequences for the overall stability of the intervertebral disc and could therefore, explain its impact on the disease course of LDD. The data of the present study showing an allelic imbalance in the amino acid sequence of the type IX collagen are in accordance with this point of view.

Recurrence rates after lumbar discectomy, however, depend on various factors. Operative technique, perioperative treatment and degenerative status of the disc are the most important factors for the post-operative clinical course of the patients [16]. Clinical differences have been excluded in our study since all patients underwent surgery in the same hospital and under the same clinical regimen. The establishment of a set of prognostic biological markers would be of great clinical interest for an optimized individual post-operative rehabilitation program.

In conclusion, the demonstration of a higher percentage of homozygosity for the Arg allele in the recurrence group of the present study might indicate that the polymorphic Col9A2 codon could be a marker for an increased risk for early recurrence after lumbar disc surgery. In contrast, the mutant Trp2 allele previously described as contributing to the development of LDD in the Finnish population is not likely to be a major susceptibility factor in the Middle European population.

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