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The $\alpha 2$ type IX collagen tryptophan polymorphism is associated with the severity of disc degeneration in younger patients with herniated nucleus pulposus of the lumbar spine

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Abstract Tryptophan alleles in *COL9A2* (Trp2) and *COL9A3* (Trp3) have been linked to lumbar disc diseases in the Finnish population. Although such diseases consist of various pathogenetically different conditions, detailed analysis of each has not been well documented. The aim of this study was to clarify whether the collagen IX tryptophan alleles influence the symptomatic degeneration of the lumbar disc in Japanese patients with herniated nucleus pulposus. We performed a prospective study of 84 patients who underwent lumbar discectomy. The degree of disc degeneration was evaluated by magnetic resonance images in relation to the collagen IX genotype. Twenty patients (21.4%) had the Trp2 allele and no patients had the Trp3 allele. Patients under 40 years with the Trp2 allele showed more severe disc degeneration at the surgical level than did those without the Trp2 allele (odds ratio 6.00, $P=0.043$). In contrast, patients aged 40 years or over did not show significant association between disc degeneration and collagen IX genotype. Our results suggest that the Trp2 allele is an age-dependent risk factor for the severity of disc degeneration in younger patients with symptomatic herniated nucleus pulposus of the lumbar spine.

Résumé Les allèles tryptophane *COL9A2* (Trp2) et *COL9A3* (Trp3) ont été chaînés à la pathologie discale dans la population finlandaise. Bien que cette pathologie reconnaisse des pathogénies bien différentes, l'analyse détaillée de chacune n'a pas été faite. Le but de cette étude était de clarifier si l'allèle tryptophane du collagène IX influençait la dégénérescence du disque lombaire chez les patients japonais porteurs de hernie discale. Le degré de dégénérescence discale était apprécié par IRM en relation avec leur génotype du collagène IX. Vingt patients (21,4%) avaient l'allèle Trp2 et aucun n'avait le Trp3. Les patients de moins de quarante ans avec le Trp2 avaient une dégénérescence discale plus marquée au niveau opéré que ceux sans l'allèle (odds ratio 6.00, $p=0,043$). En revanche les patients de plus de quarante ans ne montraient pas d'association entre la dégénérescence et le génotype du collagène IX. Nos résultats suggèrent que l'allèle Trp2 est un facteur de risque âge-dépendant pour l'importance de la dégénérescence discale chez les jeunes patients avec un hernie discale lombaire symptomatique.

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Introduction

The aetiology of lumbar disc diseases is complicated, with various environmental risk factors such as age, gender, occupation (lifting heavy loads), cigarette smoking, height, weight, and exposure to vehicular vibration [3, 21]. Previous studies have also suggested a significant contribution of genetic factors, such as collagen IX, aggrecan and MMP3 [1].

Collagen IX is a relatively minor component of the nucleus pulposus, which is integral to the proper formation of the collagen types II/IX/XI heteropolymer [6]. Transgenic mice expressing mutant $\alpha 1$ (IX) collagen were reported to have accelerated disc degeneration [10]. Ala-Kokko and others reported the association between allelic variants in the collagen IX genes, *COL9A2* and *COL9A3*, and degenerative lumbar disc diseases [2, 9, 11, 13, 14, 17]. Both of the variants predict introduction of a bulky tryptophan residue into the triple-helical region of

COL9A2 (Trp2) and COL9A3 (Trp3). It is well known that genetic variations are influenced by the population examined. For example, the frequency of the Trp alleles in patients with intervertebral disc diseases was different among the Finnish, the Greeks and the Chinese [7, 8]. Although degenerative disc diseases (DDD) consist of aetiologically and pathologically different conditions, detailed analysis of each has not been established. Therefore, we aimed to clarify whether collagen IX tryptophan alleles influence the symptomatic degeneration of the lumbar discs and studied Japanese patients with herniated nucleus pulposus who underwent discectomy.

Materials and methods

Patients

Between June 2004 and October 2005, 84 consecutive patients who underwent lumbar disc nucleotomy for lumbar nucleus pulposus at Takamatsu Red Cross Hospital were included in this study. Seventy-nine patients underwent micro-endoscopic discectomy according to the procedure described by Perez-Cruet et al. [15], while five patients underwent conventional discectomy. All patients were Japanese and had low back pain and/or sciatica after a series of conservative treatments. The study protocol was approved by the Ethics Committees of Takamatsu Red Cross Hospital and The University of Tokushima Graduate School. Written informed consent was obtained from all patients before participation in the study.

Genetic analysis

Blood samples were collected from the patients for analysis of *COL9A2* and *COL9A3*. Genomic DNA was extracted from 2 ml of peripheral blood using gene trapping by liquid extraction (GenTLE; TaKaRa, Shiga, Japan). Primers for DNA amplification and sequencing were designed manually, according to the genomic sequence flanking exon 19 of the *COL9A2* or exon 3 of the *COL9A3* [12]. The 20 µl of reaction mixture contained genomic DNA (10 ng), standard polymerase chain reaction (PCR) buffer, dNTPs (2 nM each), Ampli Taq Gold (0.1 µl), and the primer pair (10 µM each). PCRs were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, Calif., USA) with an initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 30 s, 56°C for 10 s and 72°C for 30 s, and a final step at 72°C for 5 min. Five microliters of PCR products were treated with two units of shrimp alkaline phosphatase and 10 units of exonuclease I (Amersham, Buckinghamshire, UK) at 37°C for 15 min, followed by incubation at 80°C for 15 min for enzyme inactivation. Sequencing reactions were performed using ABI Prism BigDye Terminator kit (Applied Biosystems) in a GeneAmp PCR system 9700 with an initial denaturation at 96°C for 5 min, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. Products were purified

using Sephadex G-50 Fine (Amersham) and MultiScreen-PCR filter plate (Millipore, Bedford, Mass., USA). Purified products were analysed on an ABI Prism 3100 multi-capillary sequencer (Applied Biosystems).

Clinical assessment

The severity of the clinical symptoms was calculated using a scoring system for lumbar disease, which contains three categories: (1) subjective symptoms (low back pain, leg pain and gait); (2) clinical signs (straight-leg-raising test, sensory disturbance and motor disturbance); (3) urinary bladder function. The intensity of low back pain before surgery was assessed by 100-mm visual analog scale (VAS). We also evaluated environmental risk factors of disc degeneration, including occupation (lifting heavy loads), obesity [body mass index (BMI) over 25] and smoking.

Magnetic resonance imaging equipment and protocol

Magnetic resonance (MR) images of the lumbar spine were obtained with a 1.5-T instrument (Gyrosan ACS-NT, Philips, Holland) with a surface coil. T2-weighted sagittal images were obtained for evaluation, with repetition/echo times of 2,000/110 ms, an acquisition matrix of 256×256 pixels and a slice thickness/inter-slice gap of 5/0.5 mm.

Assessment of the magnetic resonance images and plain radiographs

The degree of the intervertebral disc degeneration was evaluated by MR images in all patients according to the classification described by Schneiderman et al. [16]. In this, signal intensity of the discs on the midline sagittal section was scored as follows: grade 1 (normal), normal height and signal intensity; grade 2 (intermediate), speckled pattern or heterogeneous, decreased, signal intensity; grade 3 (marked), diffuse loss of signal; grade 4 (absent), signal void (Fig. 1). All the MR images were analysed by experienced orthopaedic physicians with no information about the genetic analysis.

Statistical analysis

The subjects were divided into two groups: Trp2(+) and Trp2(-). The statistical difference between two genotypes was analysed using the Mann-Whitney U test. The statistical differences of other assessments were analysed using the χ^2 test. Fisher's exact test was also used when any expected cell count was less than 5. A *P* value of less than 0.05 was considered significant.

Fig. 1 T2-weighted MR images of the lumbar spine. **a** A 54-year-old Trp2(-) patient showing grade 2 disc degeneration at the level of L4/5. **b** A 29-year-old Trp2(+) patient showing grade 4 disc degeneration at the level of L4/L5

Results

Study population and comparison of patients with and without the Trp2 allele

Eighteen of the 84 patients (21.4%) had the Trp2 allele, and no patient had the Trp3 allele (Table 1). Clinical characteristics were analysed in relation to the type IX collagen tryptophan allele. No significant differences were detectable between the presence of the Trp2 allele and age, gender, scoring system for lumbar disease, VAS, obesity (BMI over 25), occupation (lifting heavy loads), or smoking.

Comparison of patients based on the disc degeneration at the surgical level

Environmental risk factors including obesity (BMI over 25), occupation (lifting heavy loads), smoking, and a genetic risk factor, the tryptophan allele, were analysed in

relation to the grading of degenerative disc findings on MR images at the surgical level. Specifically, patients with grade 2 or 3 degeneration were compared with patients with grade 4 degeneration. As a total, no significant differences were observed (data not shown). However, patients aged under 40 years with the Trp2 allele had more severe disc degeneration at the surgical level (odds ratio 6.00, $P=0.043$) (Table 2). In contrast, patients aged 40 years or over showed no significant differences (Table 3).

Discussion

In this study significant difference of the severity of disc degeneration at the surgical level was observed in the younger patients (aged under 40 years) when analysed for the Trp2 allele. Having the Trp2 allele resulted in a six-fold increase in the risk of severe disc degeneration in the Japanese population. In contrast, no patients had the Trp3 allele in our study, suggesting an ethnic difference of the polymorphism.

There are numerous qualitative methods of evaluating disc degeneration from MR images, such as disc space narrowing and reduction of signal intensity. MR findings of degenerative discs are clearly associated with age [4, 20]. Single-leveled disc space narrowing is more likely to reflect a traumatic or biomechanical origin than a systemic origin. Disc signal intensity seems to be one of the representative degenerative findings, on which Schneiderman's classification stands, with a reduced signal intensity most highly associated with age. In this context our results, showing significant association between the Trp2 allele and reduction in signal intensity of the disc at the surgical level in younger patients, implying that Trp2 is an age-dependent risk factor for the severity of disc degeneration.

Our results, showing that the Trp2 allele predisposes carriers to develop severe disc degeneration under 40 years of age, contradict the Chinese study, which suggests that

Table 1 Distribution of the Trp2 allele according to selected characteristics of the study subjects

Characteristics	Total	Trp2(+)	Trp2(-)	<i>P</i>
Patients (%)	84	18 (21.4%)	66 (78.6%)	
Age (years)				
Mean	43.4	40.6	44.2	0.426
Gender				
Female (%)	19 (22.6%)	5 (27.8%)	14 (21.2%)	0.540
Male (%)	65 (77.4%)	13 (72.2%)	52 (78.8%)	
BMI				
Mean	23.8	22.6	24.2	
<25 (%)	26 (31.0%)	4 (22.2%)	22 (33.3%)	0.566
VAS (1~100 mm)				
Mean	68.8	85.3	64.4	0.059
Occupation				
Lifting heavy loads (%)	30 (35.7%)	4 (22.2%)	26 (39.4%)	0.268
Smoking (%)	44 (52.4%)	9 (50.0%)	35 (53.0%)	>0.999

Table 2 Grading of degenerative disc findings on MR images at the surgical level in patients aged under 40 years

Characteristics	Total	Grade 2 or 3	Grade 4	Odds ratio ^a	<i>P</i>
BMI					
25< (%)	13 (35.1%)	9 (60.2%)	4 (30.8%)	0.74	0.734
≥25 (%)	24 (64.9%)	15 (62.5%)	9 (37.5%)		
Occupation					
Lifting heavy loads (%)	13 (35.1%)	7 (53.8%)	6 (46.2%)	2.08	0.302
Desk workers (%)	24 (64.9%)	17 (70.8%)	7 (29.2%)		
Smoking					
Smokers (%)	21 (56.8%)	13 (61.9%)	8 (38.1%)	1.35	0.739
Non-smokers (%)	16 (43.2%)	11 (68.7%)	5 (31.3%)		
Trp2					
Trp2(+), (%)	9 (24.3%)	3 (33.3%)	6 (66.7%)	6.00	0.043 ^b
Trp2(-), (%)	28 (75.7%)	21 (75.0%)	7 (25.0%)		

^aCalculated for 2×2 table for each risk factor (exposure) vs grading of degenerative disc findings, alpha=0.05

^bFisher's exact probability test, alpha=0.05

the predisposition relates to those over 40 years of age [7]. This difference might reflect the different subjects analysed in each study, i.e., Japanese patients with herniated discs who underwent discectomy in our study and Southern China volunteers (the general Chinese population of Hong Kong) in their study.

Despite many reports on an association between MRI-based disc degeneration and environmental risk factors, our study did not detect any significance for obesity (BMI over 25), occupation (lifting heavy loads) or smoking. These results may reflect the multifactorial nature of DDDs, in which various environmental factors and many genes are expected to act together to determine the degenerative phenotype. In some populations, the contribution of each factor for DDDs may not be recognised.

Since we exclusively analysed patients who underwent lumbar discectomy for herniated nucleus pulposus, we cannot tell whether the Trp2 allele is associated with an increased risk of the herniated nucleus pulposus in general. It is possible that the risk of herniated nucleus pulposus is much higher in Trp2(+) individuals than in Trp2(-) individuals, or that Trp2(+) patients are more likely to

develop symptoms that require surgical treatment. The latter possibility is less likely, because the frequency of Trp2(+) patients in the study (21.4%) was similar to the population frequency in the Chinese (19.9%) [7]. Nevertheless, our findings that patients aged under 40 years with the Trp2 allele had more severe disc degeneration suggest that having Trp2 allele may accelerate the onset of the disc degeneration. The premature disc degeneration may lead to spondylolisthesis, which is linked to the tryptophan polymorphisms [11].

A number of limitations in the current study need to be considered, such as the small series of patients and the marginal significance in statistics. It is important to see whether our results are reproducible in a large series or in a different population.

The nucleus pulposus has a collagen network similar to that of hyaline cartilage. The collagen network is a cross-linked co-polymer of collagens type II, IX and XI [22]. Type IX collagen, a heterotrimer of genetically distinct α1 (IX), α2 (IX) and α3 (IX) chains, consists of three triple-helical domains, COL1, COL2 and COL3, and four non-helical domains, NC1–NC4. Human mutations of

Table 3 Grading of degenerative disc findings on MR images at the surgical level in patients aged 40 years and over

Characteristics	Total	Grade 2 or 3	Grade 4	Odds ratio ^a	<i>P</i>
BMI					
<25 (%)	13 (27.7%)	8 (61.5%)	5 (38.5%)	0.89	>0.999
≥25 (%)	34 (72.3%)	20 (58.8%)	14 (41.2%)		
Occupation					
Lifting heavy loads (%)	17 (36.2%)	9 (53.9%)	8 (47.1%)	1.54	0.486
Desk workers (%)	30 (63.8%)	19 (63.3%)	11 (36.7%)		
Smoking					
Smokers (%)	23 (48.9%)	15 (65.2%)	8 (34.8%)	0.63	0.440
Non-smokers (%)	24 (51.1%)	13 (54.2%)	11 (45.8%)		
Trp2					
Trp2(+), (%)	9 (19.1%)	5 (55.6%)	4 (44.4%)	1.23	>0.999
Trp2(-), (%)	38 (80.9%)	23 (60.5%)	15 (39.5%)		

^aCalculated for 2×2 table for each risk factor (exposure) vs grading of degenerative disc findings, alpha=0.05

collagen IX genes, which predict in-frame deletion of a part of the COL3 domain, produce a chondrodysplasia phenotype, multiple epiphyseal dysplasias, featuring early onset osteoarthritis [18]. In contrast, type IX collagen tryptophan polymorphisms are not linked to the degenerative joint disorders such as osteoarthritis and rheumatoid arthritis [19]. Although the reason for this gap is currently unknown, we have recently shown that Trp2-containing collagen IX is present in the cartilage matrix [12]. The Trp2 locates in the middle of $\alpha 2(\text{IX})$ COL2, within a few residues of the site of cross-linking between the $\alpha 3(\text{IX})$ chain and the type II collagen [5, 23], possibly interfering with this molecular interaction. Therefore, individuals with Trp2 allele may have a less cross-linked collagen network and may allow more enzymatic cleavage of the disc matrix.

In conclusion, our study demonstrates a novel association between the type IX collagen Trp2 allele and severe disc degeneration in younger patients with symptomatic herniated nucleus pulposus of the lumbar spine. Biochemical analysis of collagen IX containing disc matrix in relation to Trp2 allele may clarify the pathological bases of this genetic risk factor.

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