Table 3. Polymorphisms in *COL11A1* and Their Association with LDH

Location in *COL11A1* and Nucleotide Sequence Change	Amino Acid Change	dbSNP	No. in the Three Genotype Groups[a]		Allelic Frequency		*P*[b]		OR (95% CI)[c]
			Case	Control	Case	Control	Allele	Genotype[d]	
IVS1:									
9284T→C	...	*rs1415359*	423/63/1	422/42/1	.07	.05	.068	.16	.69 (.47–1.03)
IVS6:									
82274A→C	...	...	437/49/1	424/38/1	.05	.04	.35	.61	.82 (.53–1.25)
IVS10:									
90221G→A	...	*rs945748*	426/62/1	414/48/1	.07	.05	.29	.54	.82 (.56–1.19)
IVS11:									
90406A→G	...	*rs3767272*	396/76/3	401/55/0	.09	.06	.032	.049	1.47 (1.03–2.10)
IVS20:									
104122A→T	...	*rs2622877*	438/47/2	400/46/0	.05	.05	.94	.38	.98 (.65–1.48)
IVS26:									
111262T→C	...	*rs2786125*	428/49/1	429/33/1	.05	.04	.11	.24	.70 (.45–1.08)
IVS41:									
146354T→C	...	*rs1012282*	425/62/1	415/47/1	.07	.05	.24	.47	1.26 (.86–1.84)
IVS42:									
165864A→C	...	*rs1841838*	381/104/3	374/84/6	.11	.1	.52	.27	1.10 (.82–1.47)
IVS44:									
169351A→G	...	*rs2126643*	378/100/3	373/79/6	.11	.1	.44	.23	1.12 (.84–1.51)
172702C→G	...	*rs3767273*	382/103/3	372/84/4	.11	.1	.41	.5	.88 (.66–1.19)
IVS50:									
192606G→A	...	*rs4908273*	231/211/43	271/167/23	.31	.23	.00023	.001	1.47 (1.20–1.80)
Exon 52:									
193817(c.3968)T→C	L1323P	*rs3753841*	193/230/65	238/187/38	.37	.28	.000081	.00041	1.47 (1.21–1.79)
IVS52:									
194187T→C	...	...	218/214/48	258/178/26	.32	.25	.00038	.0016	.69 (.57–0.85)
IVS54:									
200918A→G	...	*rs3767274*	399/73/4	367/86/5	.09	.1	.15	.34	.79 (.58–1.08)
206255G→T	...	*rs3767275*	457/30/0	442/15/1	.03	.02	.088	.068	.60 (.33–1.09)
208970T→A	...	*rs1676500*	443/45/1	425/33/1	.05	.04	.29	.53	1.27 (.81–1.99)
IVS58:									
218282C→G	...	...	431/46/1	430/32/1	.05	.04	.15	.32	.72 (.46–1.13)
Exon 62:									
219597(c.4603)C→T	P1535S	*rs1676486*	204/223/62	252/177/33	.35	.26	.000015	.000099	1.54 (1.27–1.88)
Exon 63:									
221284(c.4770)C→T	I1590I	*rs2229783*	169/236/83	214/201/47	.41	.32	.000028	.00017	1.49 (1.24–1.80)
IVS63:									
221659G→A	...	*rs1463048*	169/235/83	212/199/50	.41	.32	.000081	.00047	1.46 (1.21–1.76)
IVS65:									
225275T→A	...	*rs3753844*	207/223/55	239/186/33	.34	.28	.0014	.0056	1.38 (1.13–1.68)
Exon 67 (3′ UTR):									
230265C→T	...	*rs1031820*	443/45/1	430/33/1	.05	.04	.27	.5	.78 (.50–1.21)
230461A→G	...	...	439/45/1	429/33/0	.05	.04	.17	.3	.73 (.46–1.15)

NOTE.—The cDNA (accession number NM001854.2) and genomic DNA (accession numbers AC093150.4, AL627203.7, and AC099567.2) sequences of *COL11A1* are based on data from GenBank. The A of the ATG translation initiation codon in the reference sequence corresponds to position +1.

[a] Homozygote of the major allele/heterozygote/homozygote of the minor allele.

[b] By the χ^2 test.

[c] Calculated for the alleles.

[d] Calculated for the homozygotes of the major allele versus the heterozygotes and the homozygotes of the minor allele.

plied Biosystems). We performed quantitative real-time PCR using the ABI PRISM 7700 (Applied Biosystems) and QuantiTect SYBR Green PCR (QIAGEN) according to the manufacturer's instructions.

RNA Stability Assay

We amplified by PCR ~1,700-bp of *COL11A1* cDNA that contained the entire ORF. We cloned the *COL11A1* cDNA containing the associated SNP c.4603C→T into pCR-Blunt II-TOPO (Invitrogen) and confirmed the sequence of the inserts. Vectors were

Table 4. Correlation between Age and Genotype at c.4603C→T (*rs1676486*) in *COL11A1*

Population	Mean ± SD Age (in years) for Genotype			*P*[a]
	CC	CT	TT	
Case	36.8 ± 15.0	36.9 ± 14.5	36.8 ± 14.5	.58
Control	64.8 ± 12.1	63.9 ± 11.1	63.1 ± 13.1	.54

[a] *P* value was calculated using the Kruskal-Wallis test.

Table 5. Genotype at c.4603C→T (*rs1676486*) in *COL11A1*, Stratified by Sex

Measure	Male			Female		
	Case	Control	Total	Case	Control	Total
No. of subjects:						
All	298	177	475	191	285	476
CC	116	98	214	88	154	242
CT	144	65	209	79	112	191
TT	38	14	52	24	19	43
T allele frequency (%)	.37	.26	.33	.33	.26	.29
P value[a]	...	...	.00074	...	...	.021

[a] *P* value for allelic difference between the patients with LDH and the control groups for each sex, by the χ^2 test.

digested using *Hin*dIII, and *COL11A1* mRNAs were transcribed using RiboMax Large Scale RNA Production System-T7 (Promega) and were purified by SV Total RNA Isolation System (Promega). The whole-cell extract was prepared by washing OUMS-27 cells in PBS and resuspending them in an extraction buffer. After incubation on ice for 30 min and microcentrifugation at 4°C, we transferred supernatants to new tubes and stored them at −80°C until use. We mixed and incubated each 5 μg of synthesized RNA and the diluted (1:1,000) whole-cell extract at room temperature for the tested time (5 or 10 min). We stopped the reaction with addition of a formamide dye. The samples were then heated at 95°C for 5 min and were placed on ice immediately. We detected *COL11A1* mRNAs of the samples by northern-blot analysis and quantified their signal intensities, using the Esper-Scanner (Epson) and Adobe Photoshop 6.0.

Immunohistochemistry for Type XI Collagen

We processed and embedded tissue samples in paraffin by the AMeX method. We predigested the tissue sections with 500 U/ml of testicular hyaluronidase (Sigma) for 30 min at 37°C. For immunofluorescent visualization, we blocked nonspecific labeling with blocking reagent (DakoCytomation) for 10 min at room temperature and then incubated the sections with the rabbit polyclonal antibody against bovine type XI collagen (1:500) at 4°C overnight. For the staining of the negative control, we applied nonimmune rabbit IgG (DakoCytomation) to the section instead of primary antibody. After washing them with Tris-buffered saline, we incubated the sections with secondary antibody conjugated to horseradish peroxidase–labeled polymer (Envision+ [DakoCytomation]) for 30 min at room temperature. We visualized the immunoreactive products using a diaminobenzidine reagent and counterstained them with hematoxylin.

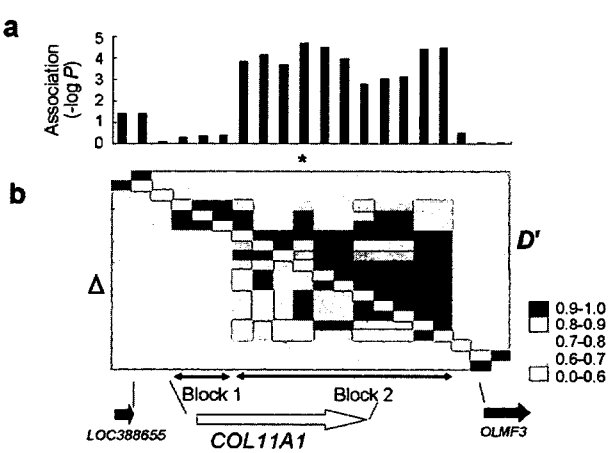

Figure 1. Case-control association study and linkage-disequilibrium mapping. *a*, Association of *COL11A1* with LDH. The $-\log_{10}$ transformation of the corrected *P* value (allele 1 vs. allele 2) was plotted on the *Y*-axis. The asterisk (*) indicates c.4603C→T. *b*, Pairwise linkage disequilibrium between SNPs in and around *COL11A1* measured by *D'* and Δ in 465 controls. The *COL11A1* region is divided into two linkage-disequilibrium blocks.

Results

We first examined the association of the type XI collagen genes (*COL11A1*, *COL11A2*, and *COL2A1*) with LDD, which included patients with and without LDH. We tested tag SNPs that were selected from the JSNP Database and the International HapMap Project database. A comparison of 188 LDD cases and 179 controls revealed no association with any of the SNPs; however, there was a significant association with *COL11A1* when cases were stratified on the basis of the presence or absence of LDH (table 2). In a comparison of 130 patients with LDH with 179 controls, one SNP (c.4603C→T [*rs1676486*]) had a significant association. To confirm the association, we examined another 359 LDH cases and 286 controls for the *COL11A1* SNP. Again, we identified the significant association between the SNP and LDH (table 2). Adjusted $P = .00030$ was obtained by 10^7 permutations

To identify the disease-causing sequence variation, we examined sequence variations in *COL11A1* exons and their flanking regions from a public database and by resequencing 24 patients with LDH. A total of 23 sequence variations were identified and were tested for association. SNP c.4603C→T had the most significant association (table 3), which remained significant after Bonferroni correction for multiple testing. We examined whether confounding effects, such as age and sex, affect the associations with LDH and found no relationship between the genotype and

Table 6. Haplotype Association Analysis of *COL11A1* with LDH

Haplotype	Frequency		*P*[a]
	Case	Control	
H1	.527	.616	.000154
H2	.302	.222	.000150
H3	.038	.039	.90
H4	.041	.037	.63
H5	.045	.034	.27
H6	.014	.014	.91
H7	.011	.008	.50

NOTE.—Results are for the haplotypes of block 2 that contained the susceptibility SNP, c.4603C→T.

[a] By the χ^2 test.

Table 7. Association between Genotype at c.4603C→T (*rs1676486*) in *COL11A1* and LDH in the Japanese Population

Group	No. with Genotype			Allelic Frequency	*P*	OR (95% CI)
	CC	CT	TT			
Case	360	367	96	.34	.0000033	1.42 (1.23–1.65)
Control	453	325	60	.265		

these factors (table 4). The association was positive in both sexes (table 5).

Using the 20 SNPs in and around *COL11A1* that had a minor-allele frequency >10%, we analyzed the linkage-disequilibrium structure of the region and found highly structured linkage-disequilibrium blocks (fig. 1). *COL11A1* was covered by two blocks, and the SNP with a significant association (c.4603C→T) was contained in block 2. We further analyzed the haplotype structure of block 2 and identified seven haplotypes with frequencies >0.01 that covered >97% of both the case and control groups (table 6). The association was weaker than that of c.4603C→T alone, suggesting the absence of a hidden causal SNP. We further examined the association of the SNP, using an additional 334 patients with LDH and 376 controls. Our findings of the association between this SNP and LDH were replicated ($P = .044$; OR 1.27 [95% CI 1.01–1.59]. Therefore, this SNP is strongly associated with LDH (combined $P = 3.3 \times 10^{-6}$ in allelic frequency) (table 7).

To clarify the functional impact of c.4603C→T, we quantified the allelic difference of the mRNA expression by real-time RT-PCR. The expression level of the susceptibility allele c.4603T was significantly lower than that of the c.4603C allele (fig. 2*a*). We hypothesized that this SNP affects *COL11A1* transcription by altering mRNA stability and examined the stability of *COL11A1* mRNA containing the SNP. We mixed mRNAs produced by in vitro transcription with cell lysate and assessed mRNA degradation by endogenous components of the cells, using northern-blot analysis. The transcript containing the susceptible allele degraded faster (fig. 2*b* and 2*c*).

To gain insight into the role of type XI collagen in LDH, we examined *COL11A1* expression in tissues and cells by quantitative real-time PCR. *COL11A1* mRNA was predominantly expressed in IVD (fig. 3*a*). We investigated the correlation between the *COL11A1* mRNA expression level and a variety of LDH phenotypes and found that severity of disc degeneration evaluated by MRI was inversely correlated with *COL11A1* expression in IVDs of patients with LDH (fig. 3*b*). We further analyzed the expression and localization of type XI collagen in IVD by immunohistochemistry. Normal discs had a highly uniform ECM structure, with intense immunostaining of type XI collagen in the nucleus pulposus cells and ECM (fig. 3*c*). In degenerative discs, however, we observed weak immunostaining of type XI collagen around the nucleus pulposus cells (fig. 3*d*). These findings implicate a decrease of type XI collagen in the pathogenesis of LDH.

Discussion

Through a case-control association study focusing on type XI collagen, we identified *COL11A1* as a susceptibility gene for LDH. *COL11A1* mRNA was substantially ex-

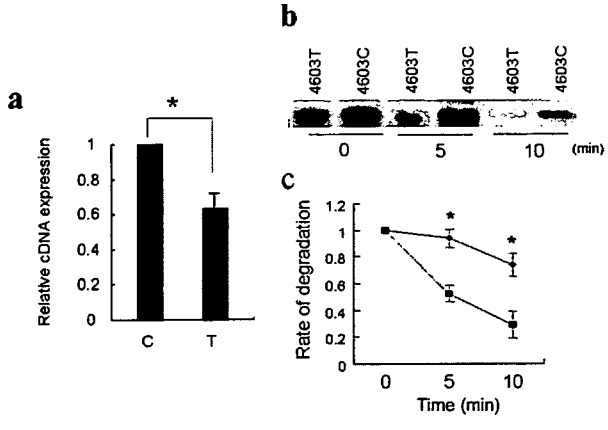

Figure 2. Difference in transcription and stability of *COL11A1* mRNA containing the LDH-associated SNP. *a*, Relative cDNA expression of c.4603C→T evaluated by real-time PCR. Data represent the ratios of cDNA to genomic DNA, and expression of the C allele is converted to 1 (an asterisk [*] indicates $P < .05$, by Student's *t* test). Data represent the mean ± SD in triplicate assays. *b*, Sequential change of *COL11A1* mRNA analyzed by northern blotting. "4603C" and "4603T" indicate *COL11A1* mRNA produced by in vitro transcription with c.4603C and c.4603T, respectively. *c*, Rate of degradation of the transcripts. Diamonds indicate the transcript with c.4603C; squares indicate the transcript with c.4603T. The difference of the rate of degradation was significant at both 5 min and 10 min after the reaction (an asterisk (*) indicates $P < .05$, by Student's *t* test). Data represent the mean ± SD in triplicate assays.

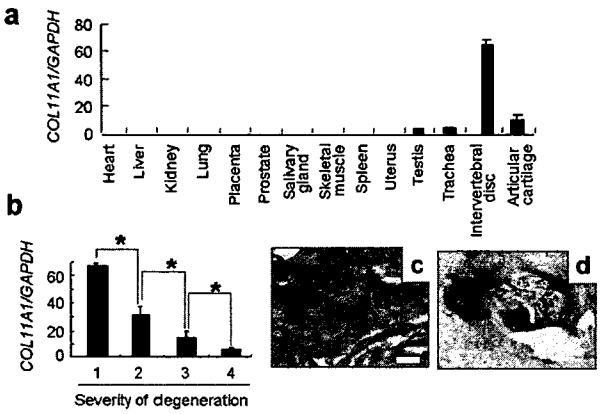

Figure 3. Type XI collagen expression in human. *a, COL11A1* expression in different tissues. *COL11A1* mRNA was predominantly expressed in IVD. *b,* Inverse correlation between *COL11A1* expression and severity of degeneration of IVD in patients with LDH (an asterisk [*] indicates $P < .05$, by Student's *t* test). The degree of disc degeneration is evaluated by MRI and is scored according to the classification of Schneiderman. *c* and *d,* Immunostaining of type XI collagen in IVDs from an unaffected individual (*c*) and a patient with LDH (Schneiderman's grade 3) (*d*). Ubiquitous and intense staining was found in the normal disc. In contrast, the staining was found only in and around the territorial matrices of clustered cells in the degenerative disc. The white scale bar indicates 50 nm.

pressed in IVD, and the expression in patients with LDH was decreased according to the severity of degeneration. Our findings further indicate that the susceptibility SNP produces unstable *COL11A1* transcripts. A few *cis*-elements have been implicated in mRNA stabilization.[25] The 4856–4865 nucleotides (caaaaaatct) in *COL11A1* mRNA closely match the consensus for a mRNA stability motif, "g/tanaaaag/tcc/t."[26] The sequence variation might affect the mRNA stability motif and disrupt the *cis*-element critical for mRNA stability, although they are >200 bp apart. Alternatively, the sequence variation might induce a conformational change in the mRNA that would decrease mRNA stability or increase the sensitivity to RNase. The decrease of the *COL11A1* transcript would lead to a decrease in type XI collagen in the ECM of IVD.

IVD has a highly structured ECM to resist mechanical forces. The highly oriented network of the fibrillar collagens offers tensile strength,[27,28] and highly hydrated aggregating PG resists comprehensive forces. They form a mesh suited to holding water molecules, which further increases their ability to withstand mechanical forces. Therefore, the structural integrity of ECM and the physiologic balance of its components are critical to IVD function. Perturbation of ECM metabolism would increase the mechanical load of the IVD, leading to its degeneration. The reduction in type XI collagen, the critical organizer of ECM, ultimately causes disintegration of ECM and hence IVD degeneration, although it could occur as a secondary event of LDH. The present study underscores the importance of ECM proteins in the pathogenesis of common bone and joint diseases, including LDH. Our results should lead to a better understanding of the pathogenic mechanisms of LDH and suggest promising targets for a novel treatment strategy for LDH.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

Applied Biosystems, http://www.appliedbiosystems.com/index.cfm

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *COL11A1* sequences [accession numbers NM001854.2, AC093150.4, AL627203.7, and AC099567.2])

International HapMap Project, http://hapmap.org/

JSNP Database, http://snp.ims.u-tokyo.ac.jp/index.html

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for Stickler syndrome type II, Marshall syndrome, and oto-spondylo-mega-epiphyseal dysplasia)

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Age-Related Changes in Expression of Tissue Inhibitor of Metalloproteinases-3 Associated With Transition From the Notochordal Nucleus Pulposus to the Fibrocartilaginous Nucleus Pulposus in Rabbit Intervertebral Disc

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Study Design. Experimental study on age-related changes in expression of tissue inhibitor of metalloproteinases-3 (TIMP-3) associated with transition from notochordal nucleus pulposus (NP) to fibrocartilaginous NP in rabbit intervertebral disc (IVD).

Objectives. To identify roles of notochordal NP in extracellular matrix (ECM) metabolism of IVD.

Summary of Background Data. One of most interesting properties of TIMP-3 is to inhibit aggrecanases in addition to matrix metalloproteinases. Balance of aggrecanase/TIMP-3 is critical to maintain homeostasis of ECM metabolism.

Methods. Four-week-old and 160-week-old male Japanese white rabbits were used. Age-related changes in IVDs were evaluated histologically using previously established grading system. Immunohistochemistry of TIMP-3 and semiquantitative reverse transcriptase-polymerase reaction (RT-PCR) of TIMP-3, a disintegrin and metalloproteinases with thrombospondin motifs (ADAMTS) 4, 5, and transforming growth factor-β1 (TGF-β1), were conducted.

Results. Semiquantitative assessment of histologic changes indicated that 4-week-old rabbit was equivalent to fetus to 2-year-old human and 160-week-old rabbit was equivalent to 11- to 30-year-old human, particularly 11- to 16-year-old, which corresponds to transition period from notochordal to fibrocartilaginous NP. Immunohistochemistry revealed that TIMP-3 was positive in 4-week-old rabbit only. Semiquantitative RT-PCR revealed that levels of expressions of TGF-β1 and TIMP-3 mRNAs in 4-week-old were significantly higher than those in 160-week-old rabbits. There was no significant difference in expression of ADAMTS4 mRNA. ADAMTS5 mRNA was not detected or extremely low in both groups. Expression of TIMP-3 mRNA in NP was upregulated by TGF-β1 but was not affected by IL-1β. On the contrary, expression of ADAMTS4 mRNA was not upregulated by TGF-β1 but was upregulated by IL-1β.

Conclusions. Levels of expression of TIMP-3 in notochordal NP were significantly lower in 160-week-old rabbits than those in 4-week-old rabbits. Decrease in expression of TIMP-3, possibly mediated in part by TGF-β1, may cause imbalance of ADAMTS4/TIMP-3 ratio at transition period from notochordal to fibrocartilaginous NP.

Key words: notochordal nucleus pulposus, tissue inhibitor of metalloproteinases-3, a disintegrin and metalloproteinases with thrombospondin motifs 4, transforming growth factor-β1, intervertebral disc. **Spine 2007;32: 849–856**

The intervertebral disc (IVD) is a specialized fibrocartilaginous connective tissue whose function is load-bearing while resisting the external stresses exerted on the vertebral column. The IVD is able to perform these functions because of its unique design. It is a complex structure consisting of 3 distinct components: the nucleus pulposus (NP), a centrally located gelatinous tissue, the anulus fibrosus (AF) consisting of concentric lamellas rich in collagen fibrils, and the endplate, which separates the NP and AF from the adjacent vertebral body.[1]

Unlike other musculoskeletal tissues, the IVD undergoes early and extensive degenerative changes with aging.[2] The IVD consists of a relatively small number of cells and an abundant extracellular matrix (ECM). The major components of the ECM are aggregating aggrecan and collagens.[3,4] The abnormal degradation of ECM molecules, especially aggrecan and collagens, is thought to contribute significantly to the progressive degeneration of the IVD. In the normal IVD, turnover and synthesis of ECM molecules is at equilibrium, but in the degenerated IVD, loss of ECM components exceeds new synthesis. One of the primary causes of this imbalance is elevated activity of proteinases that degrade aggrecan and collagens. The major enzymes that are involved in these processes are members of the matrix metalloproteinase (MMP) family.[4] Recent studies have indicated that members of a family called a disintegrin and metalloproteinases (ADAMs) also participate.[5,6] The activities of these MMPs and ADAMs must therefore be exactly controlled under normal physiologic conditions. While the regulation of the activities of ADAMs is less understood at the present time, the activities of MMPs are strictly controlled by endogenous inhibitors called tissue inhibitors of metalloproteinases (TIMPs).[7]

Four TIMPs, which are homologous proteins of 21 to 29 kDa consisting of 2 domains, are currently identified

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in humans.[7] Among them, TIMP-3 has several features that distinguish it from other TIMPs. This is the only TIMP that binds to the ECM[8,9] and inhibits members of the ADAM family, including ADAM10,[10] ADAM12,[11] ADAM17 (TNF-α converting enzyme [TACE]),[12] and ADAM with thrombospondin motifs (ADAMTS) 4 and 5 (also called aggrecanase-1 and -2, respectively),[13,14] which are known to be potent initiators of cartilage matrix degradation. However, the expression of TIMP-3 and aggrecanase in the IVD and their roles in the metabolism of the IVD matrix are not well understood.

In human, the notochordal cells in the NP typically disappear and undergo a chronologic transition from the notochordal to the fibrocartilaginous NP by the second decade, the time that coincides with the development of morphologic signs of disc degeneration.[2] In other species, the notochordal cells either disappear very early in life or persist throughout their lives. For example, in chondrodystrophoid dogs such as beagles, the notochordal cells vanish during growth; but in nonchondrodystrophoid dogs, they persist throughout their lives.[15] Interestingly, the proteoglycan content is reported to be higher in nonchondrodystrophoid dogs than that in chondrodystrophoid dogs.[15,16] A coculture study of notochordal NP cells with AF cells has demonstrated that notochordal NP cells activate the metabolism of AF cells, and retard disc degeneration when they are reinserted into the disc.[17] Therefore, we hypothesized that the notochordal NP, which is defined as the NP that contains notochordal cells, play an important role to preserve the IVD homeostasis and the disappearance of the notochordal cells from the NP, *i.e.*, the transition from the notochordal to the fibrocartilaginous NP, has an influence on the ECM metabolism.

This study was undertaken to identify the roles of the notochordal NP on the ECM metabolism of the IVD by investigating age-related changes in the expression of TIMP-3 associated with a transition from the notochordal to the fibrocartilaginous NP.

■ Materials and Methods

Animals. Four-week-old (immature, weighing 0.5–0.6 kg) and 160-week-old (mature, weighing 3.5–3.8 kg) male Japanese white rabbits (Kitayama Labes, Nagano, Japan) were used in the present study. The IVDs were removed aseptically from the rabbits of different ages after euthanasia by an excess dose of sodium pentobarbiturates (University Experimental Animal Committee approval no. 001096).

Semiquantitative Assessment of Age-Related Histologic Changes in Intervertebral Disc. Fresh L4–L5 IVD tissues from different age rabbits (4-week-old, n = 7; and 160-week-old, n = 7) were used to evaluate temporal variations in histologic features. Each IVD tissue was fixed with periodate-lysine-paraformaldehyde fixative for 24 hours at 4°C and embedded in paraffin. Thin sagittal sections of the whole IVD were prepared and stained with hematoxylin and eosin or Alcian blue.

The age-related histologic changes of the IVD were evaluated in 5 distinct regions (*i.e.*, anterior outer AF, anterior inner AF, NP, posterior inner AF, and posterior outer AF) using the modified grading system reported by Boos *et al*[2] (Table 1), which is one of the most meticulous classification systems for age-related changes of the human IVD. In the original classification system by Boos, there are 5 parameters (*i.e.*, cells, mucous degeneration, cell death, tear and cleft formation, granular changes); however, we excluded a parameter on cell death in the present study because it was difficult to correctly evaluate cell death with hematoxylin and eosin staining. Each value for these parameters of the rabbits was compared with those of human, which has been reported previously.[2]

Table 1. Classification System for Grading Age-Related Histologic Change in Intervertebral Disc

Cells (chondrocyte proliferation)
0 = no proliferation
1 = increased cell density
2 = connection of two chondrocytes
3 = small-size clones (*i.e.*, 2–7 cells)
4 = moderate-size clones (*i.e.*, >8 cells)
5 = huge clones (*i.e.*, 15 cells)
6 = scar/tissue defect
Mucus degeneration
0 = absent
1 = rarely present
2 = present in intermediate amounts
3 = abundantly present
4 = scar/tissue defects
Tear and cleft formation
0 = absent
1 = rarely present
2 = present in intermediate amounts
3 = abundantly present
4 = scar/tissue defects
Granular changes
0 = absent
1 = rarely present
2 = present in intermediate amounts
3 = abundantly present
4 = scar/tissue defects

Furthermore, the presence of notochordal cells in the NP was assessed. The number and the proportion of the notochordal cells were calculated at random sampling of the 3 distinct areas in the NP using a microscope under ×200 magnifications.

Immunohistochemistry. Fresh L4–L5 IVD tissues from different age rabbits (4-week-old, n = 3; and 160-week-old, n = 3) were used for immunostaining. The IVDs were immunostained using a monoclonal antibody against TIMP-3 (clone 136-13H4; 10 μg/mL Daiichi Fine Chemical, Toyama, Japan) by ENVISION kit (Dako, Kyoto, Japan). No immunostaining was obtained with nonimmune mouse IgG to exclude the artifacts (negative controls).

Isolation of Total RNA. A total of 30 mg of NP and AF tissues from 4 consecutive (L2–L3, L3–L4, L4–L5, L5–L6) IVDs in the rabbits (4-week-old, n = 5; and 160-week-old, n = 5) were used for the extraction of total RNA by RNeasy mini kit (Qiagen, Tokyo, Japan) as directed by the manufacturer. Concentration and quality of RNA in each sample were evaluated by a spectrophotometer (Amersham Biosciences Corp., Piscataway, NJ) with absorbances at 260 and 280 nm. All samples were stored at −80°C until analyzed.

Table 2. Rabbit Specific Primer Sequences

Gene	Product (bp)	Primer Sequence	Temperature (C)	Cycle No.
TGF-β1	271	CGGCAGCTGTACATTGACTT AGCGCACGATCATGTTGGAC	55	26
ADAMTS4	133	GACCTTCCGTGAAGAGCAGTGT CCTGGCAGGTGAGTTTGCAT	55	28
ADAMTS5	111	ATGACCATGAGGAGCACTACGA GGAGAACATATGGTCCCAACGA	55	28
TIMP-1	326	GCAACTCCGACCTTGTCATC AGCGTAGGTCTTGGTGAAGC	55	26
TIMP-2	416	GTAGTGATCAGGGCCAAG TTCTCTGTGACCCAGTCCAT	55	26
TIMP-3	454	TCTGCAACTCCGACATCGTG CGGATGCAGGCGTAGTGTT	55	26
GAPDH	293	TCACCATCTTCCAGGAGCGA CACAATGCCGAAGTGGTCGT	55	26 or 28

Semiquantitative Reverse Transcriptase-Polymerase Chain Reaction. Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using a one-step RT-PCR kit (Qiagen) with 20 ng of the total extracted RNA. The levels of mRNA expression of transforming growth factor-β1 (TGF-β1), TIMP-1, -2, and -3 in the NP and the AF of 4-week-old rabbits were assessed. Furthermore, age-related changes in mRNA expression of TGF-β1, ADAMTS4, ADAMTS5, and TIMP-3 between the NP of 4-week-old and 160-week-old rabbits were compared. The rabbit-specific primer sequences and the RT-PCR conditions were depicted in Table 2.[18,19] Ten microliters of PCR products were separated in 2% agarose gels at 50 V/cm in Tris-acetate buffer. The agarose gels were stained with ethidium bromide for 25 minutes and then the intensity of the experimental bands was calculated using Electrophoresis Documentation and Analysis System 290 (EDAS290; Invitrogen, Tokyo, Japan). The amounts of RT-PCR products were normalized using glyceraldehyde phosphate dehydrogenase mRNA expression as an internal control, which was constantly expressed regardless of age and sites (data not shown). For all reported experiments, the cycle numbers of PCR condition were determined within the linear range of amplification, which ranged from 26 to 28 cycles. (Table 2).

Effects of Cytokines on the Expression of TIMP-3 and ADAMTS4 mRNA. A total of 30 mg NP tissues isolated from 4-week-old rabbits (n = 5) was cultured directly in a serum-free minimum essential medium (MEM; Invitrogen, Tokyo, Japan) supplemented with 25 μg/mL ascorbate in the presence of increasing concentrations (0, 0.1, 1, or 10 ng/mL) of TGF-β1 prepared from fresh human platelets (σ-Aldrich Co., St. Louis, MO) and recombinant human Intereukin-1β (IL-1β) (Strathmann Biotec AG, Hamburg, Germany) for 24 hours, and the levels of mRNA expression of TIMP-3 and ADAMTS4 were assessed using the semiquantitative RT-PCR method as described above.

Statistical Analysis. Statistical significance was assessed using a software package, Statcel (OMS publishing, Saitama, Japan). Data were expressed as the mean ± standard deviation (SD). Comparisons between the 2 groups and among the different culture conditions were made by Student *t* test, Mann-Whitney *U* test, or 1-way factorial analysis of variance. *P* value of less than 0.05 was considered significant.

■ Results

Semiquantitative Assessment of Age-Related Histologic Changes in Intervertebral Disc

The histologic changes of the IVDs from different age rabbits stained with hematoxylin and eosin or Alcian blue were shown in Figure 1. In 4-week-old rabbits, proteoglycans were most intensely stained with Alcian blue in the inner AF, which directly surrounds the NP, thereby suggesting a gradual increase in the content of proteoglycans from the outer AF toward the NP. On the other hand, the concentric lamellar structure of the AF became indistinct and the intensity of proteoglycan staining decreased in the NP and the inner AF surrounding the NP in 160-week-old rabbit.

The mean values for all histologic parameters and the number of disc with histologically confirmed notochordal cells were presented in Table 3. The mean value of each histologic parameter corresponded with that of human, which was reported by Boos *et al*,[2] as follows: cells (0–1 month), mucous degeneration (fetus), tear and cleft formation (1 month to 2 years), granular changes (fetus or 0–1 month) in 4-week-old rabbits; cells (11–16 years or 17–20 years), mucous degeneration (11–16 years), tear and cleft formation (21–30 years), granular changes (11–16 years) in 160-week-old rabbits, respectively.

The notochordal cells, which form cell clusters with large vacuoles, were observed abundantly with a clear demarcation from the AF in all specimens from 4-week-old rabbits. On the other hand, only a small number of notochordal cells were observed in the NP in 4 of 7 specimens from 160-week-old rabbits (Figure 1C, F). Although all NP cells were notochordal cells in 4-week-old rabbit, the number and the proportion of the notochordal cells decreased significantly in 160-week-old rabbits ($P < 0.01$) (Table 3).

Immunohistochemistry

Immunohistochemistry revealed that, in 4-week-old rabbits, TIMP-3 was stained positively in the cells of the NP and the AF (Figure 2A, B). On the other hand, the stain-

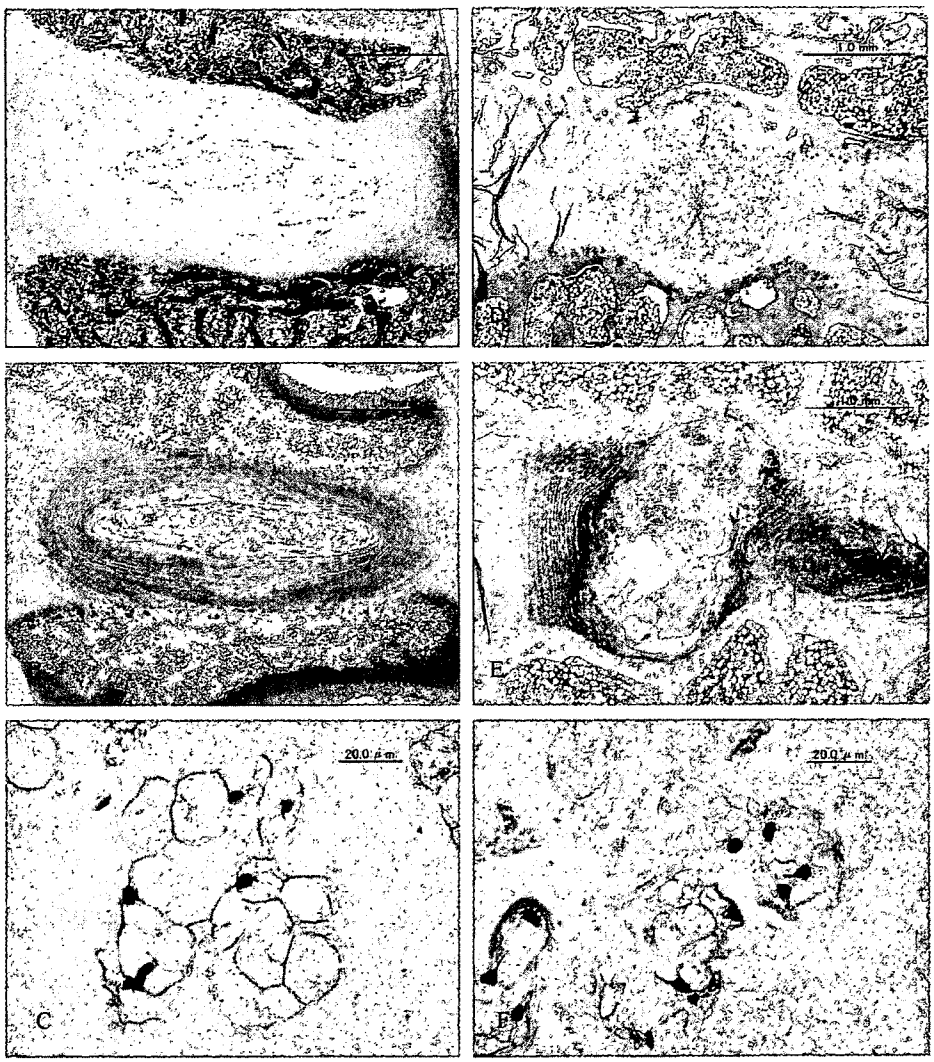

Figure 1. Histology of the different age rabbit IVDs. Left column (**A–C**): 4-week-old rabbit. Right column (**D–F**): 160-week-old rabbit. A, C, D, and F, hematoxylin and eosin; B and E, Alcian blue staining. Note that proteoglycans are stained most intensely in the inner AF surrounding the NP in the 4-week-old rabbit (B), while the intensity of proteoglycan staining decreased in 160-week-old rabbit (E). The cells that form clusters with large vacuoles were defined as the notochordal cell. In 4-week-old rabbits, the notochordal cells were observed abundantly at the center of the IVDs with a clear demarcation from the inner AF (C). A small number of notochordal cells were still present in some 160-week-old rabbits (F).

ing was negative in both the NP and the AF cells of 160-week-old rabbits (Figure 2C, D). Nonspecific staining was negative with nonimmune mouse IgG in all samples (negative control).

Semiquantitative Analysis of Gene Expression

When the mRNA expression levels of TGF-β1 and TIMP-1, -2, and -3 in the NP and the AF of the 4-week-old rabbits were compared, the levels in the NP were significantly higher than those in the AF ($P < 0.05$) (Figure 3).

In the NP, the expression levels changed dramatically with aging. Semiquantitative RT-PCR showed that the levels of TGF-β1 and TIMP- 3 mRNA in the NP of 160-week-old rabbits were significantly lower than those of 4-week-old rabbits ($P < 0.05$). On the other hand, the levels of ADAMTS4 mRNA were not significantly different between 4-week-old and 160-week-old rabbits (Figure 4). ADAMTS5 mRNA was not detected or extremely low in samples from both age groups.

Table 3. Age-Related Histologic Change in Intervertebral Disc

Criteria	4-Week-Old (n = 7)	160-Week-Old (n = 7)	Statistical Significance (*P*)
Cells (chondrocyte proliferation)	0.22 ± 0.42	1.23 ± 0.46	<0.01
Mucus dgeneration	0	0.80 ± 0.92	<0.01
Tear and cleft formation	0.13 ± 0.34	1.45 ± 0.81	<0.01
Granular changes	0	0.55 ± 0.75	<0.01
Presence of notochordal cell	7/7	4/7	NS
Notochordal cell/field	35.8 ± 11.2	3.8 ± 3.3	<0.01
% of notochordal cell in the NP cell	100% (751/751)	66% (80/121)	<0.01

NS, not significant.

Effects of Cytokines on the Gene Expressions of TIMP-3 and ADAMTS4

In the organ culture of the NP, the expression levels of TIMP-3 mRNA were upregulated by the treatment with TGF-β1 in a dose-dependent manner (Figure 5A) but were not affected by the treatment with IL-1β (Figure 5B). On the other hand, the expression levels of ADAMTS4 mRNA were not upregulated by the treatment with TGF-β1 (Figure 5A) and were upregulated by the treatment with IL-1β (Figure 5B).

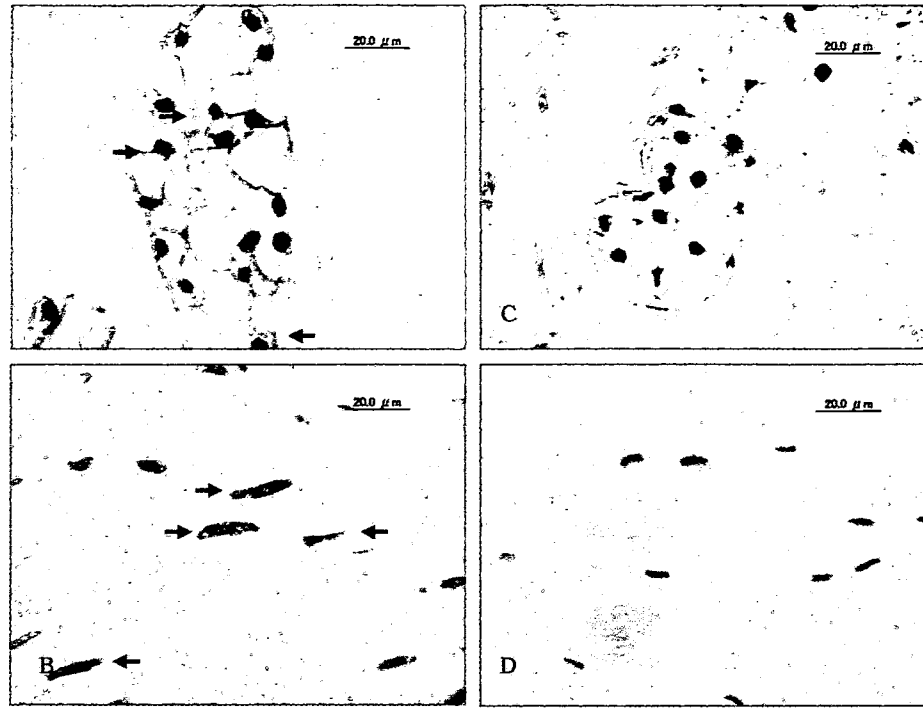

Figure 2. Immunohistochemistry using anti-TIMP-3 antibody. Left column (**A, B**): 4-week-old rabbit. Right column (**C, D**): 160-week-old rabbit. A and C are the specimens from the NP. B and D are the specimens from the AF. TIMP-3 staining (arrow) is positive only in the NP and AF cells of the 4-week-old rabbit (A, B). The intracellular staining (arrow) was observed using antibody against TIMP-3 but was not observed using nonimmune IgG.

■ Discussion

Although an increasing number of studies that focused on the possible novel treatments for IVD degeneration, including gene therapy[20,21] and tissue engineering[22] have emerged, there still is a lack of definition of what change actually represents this specific pathologic condition, especially in the early stage. One of the key features at the second decade in the human IVD is a transition from the notochordal NP to the fibrocartilaginous NP.[2] Clarification of the mechanism of transition from the notochordal to the fibrocartilaginous NP should therefore be useful for the elucidation of the etiology of disc degeneration and for the development of a novel treatment strategy for degenerative disc diseases.

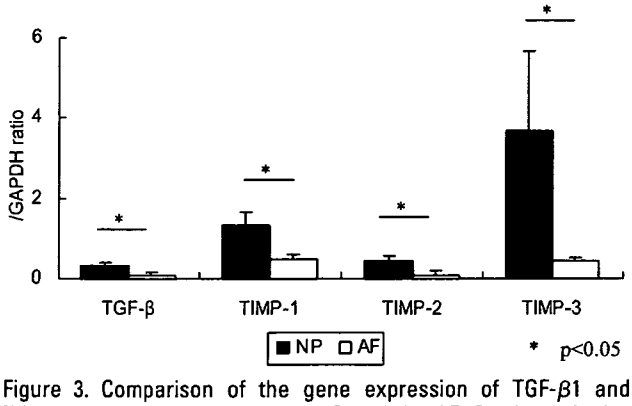

Figure 3. Comparison of the gene expression of TGF-β1 and TIMP-1, -2, and -3 between the NP and the AF. Semiquantitative RT-PCR was performed to examine the expression levels of TGF-β1, TIMP-1, -2, and -3 in the NP and the AF of 4-week-old rabbit as described in Materials and Methods. Note that the expression levels of all mRNAs are significantly higher in the NP than in the AF.

In the present study, we used rabbits from different age groups: 4 weeks and 160 weeks. Although the results of our study cannot be translated directly into human, the age-related histologic changes, which were reported in human (*i.e.*, chondrocyte proliferation, mucous degeneration, tear and cleft formation, granular changes), were also observed in the rabbit. Semiquantitative assessments of the histologic changes indicated that the 4-week-old rabbit is equivalent to human of fetus to 2-year-old and the 160-week-old rabbit is equivalent to human of 11- to 30-year-old, particularly to 11- to 16-year-old.

The timing of the loss of notochordal cells in the rabbit IVDs is poorly defined and the descriptions in previous reports were inconsistent. Recently, cytomorphologic study demonstrated that notochordal cells persist even in 1-year-old rabbit IVDs.[23] The present study demonstrated that the notochordal cells persist in 4 of 7 rabbits in the 160-week-old group. Therefore, it is reasonable to presume that the NP of 160-week-old rabbit is equivalent to the end stage of notochordal NP in human, which implies the transition period from the notochordal to the fibrocartilaginous NP.

The present study gives certain insights on the rabbit IVDs, which is increasingly used as a model of human diseases and their treatments. The rabbit models of disc degeneration and *in vitro* studies using rabbit IVD cells are widely used to study the biology of the NP cells.[17,20,22,24] However, the majority of the NP cells, especially in immature rabbits, are notochordal cells and the number and/or proportion of the notochordal cells in the NP exhibit significant chronological changes. It should therefore be reminded that the presence of the

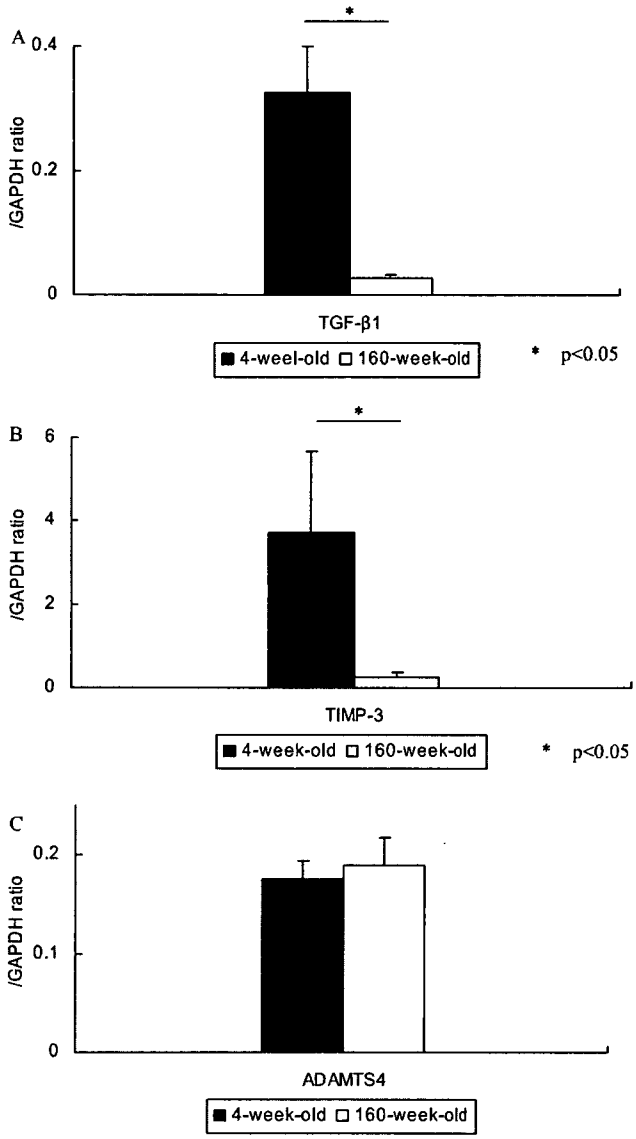

Figure 4. Age-related changes in the mRNA expressions of TGF-β1 (**A**), TIMP-3 (**B**), and ADAMTS4 (**C**) in the NP are shown in the bar graphs. Note that the gene expressions of TGF-β1 and TIMP-3 are significantly lower in the 160-week-old rabbits than in the 4-week-old rabbits. On the other hand, there was no significant difference in the level of expression of ADAMTS4 between 4- and 16-week-old rabbits.

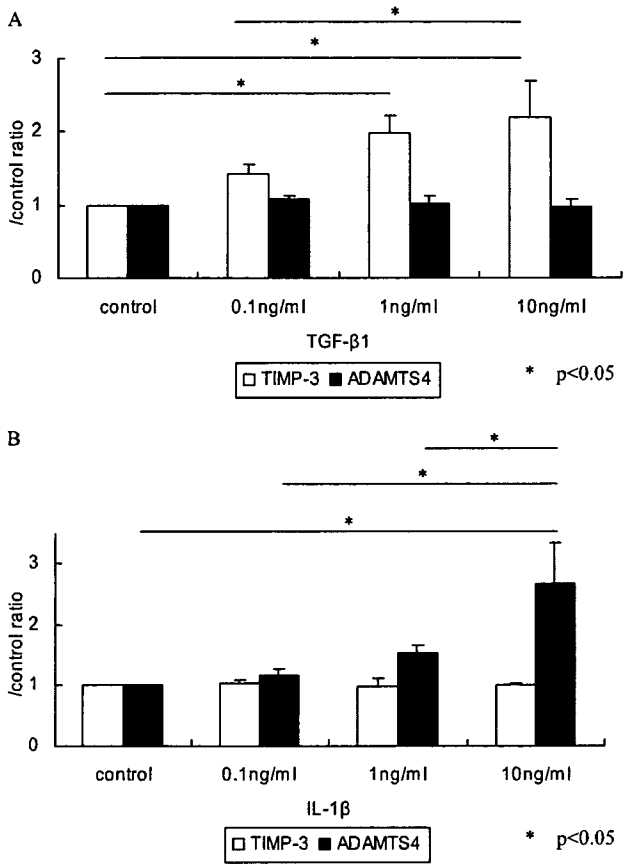

Figure 5. Effects of TGF-β1 (**A**) and IL-1β (**B**) on the mRNA expression of TIMP-3 and ADAMTS4 in an organ culture. The NP tissues from 4-week-old rabbit were cultured in the presence of TGF-β1 or IL-1β (0, 0.1, 1, or 10 ng/mL), and the expression levels of TIMP-3 or ADAMTS4 mRNA were examined as described in Materials and Methods. There is a significant upregulation of TIMP-3 mRNA expression by the administration of increasing concentration of TGF-β1; however, no such upregulation was observed by the administration of IL-1β. On the other hand, the levels of ADAMTS4 mRNA expression were upregulated by the treatment of IL-1β but were not upregulated by the treatment of TGF-β1.

large amount of notochordal cells might make the rabbit NP fundamentally different from the mature or degenerated human NP.

Semiquantitative gene expression studies demonstrated that, in the 4-week-old notochordal NP, the mRNA expression of TGF-β1 was higher than those in the AF. Since TGF-β1 is known to upregulate ECM synthesis in the IVD,[21] the notochordal NP may promote ECM synthesis within the NP by expressing TGF-β1. However, one of the authors has already demonstrated that rabbit notochordal NP cells produce significantly less proteoglycans and collagens than AF cells.[24] Other studies have also indicated that the major center of matrix synthesis is the inner AF.[25] Our histologic study revealed that the proteoglycan contents were the highest in the inner AF surrounding the notochordal NP. All these data suggest that the notochordal NP may promote ECM synthesis of the inner AF. It is therefore reasonable to presume that a role of the notochordal NP is not only the synthesis of the ECM molecules by itself but also the regulation of the ECM metabolism of the whole IVD by expressing cytokines including TGF-β1.

The ECM of the IVD consists of a complex of aggrecan, hyaluronan, link protein, and other molecules embedded in the framework of collagens.[4] The ECM holds cells and provides optimal cellular microenvironment. Such cell–matrix interaction is considered to have an important role in the regulation of chondrocyte function.[26] Timely synthesis and degradation of the ECM are crucial for controlling proper cellular behaviors and is required during the skeletal development and tissue remodeling, which are associated with cell differentiation, growth,

maturation, and apoptosis. One of the most important components of IVD matrix is aggrecan, which provides the ability to withstand compression. The loss of aggrecan leads to the decrease of glycosaminoglycan and water, consequently to dysfunction of the IVD.[27] There are many modulators on the loss of aggrecan, such as mechanical overload, cell apoptosis,[28] cytokines, and proteinases. Recently, 2 newly discovered members of the ADAMTS families, which can cleave aggrecan at the specific interglobular domain site (Glu373-Ala374), have been cloned and named as ADAMTS4 (aggrecanase-1) and ADAMTS5 (aggrecanase-2).[29,30] Furthermore, inhibition kinetic studies demonstrated, that among the 4 endogenous TIMPs, TIMP-3 inhibits the aggrecanase activity most effectively.[13,14]

Although there are many studies examining the cleavage of aggrecan at aggrecanase site in the articular cartilage, limited information is available for their contribution to aggrecan degradation in the IVD. Several previous studies have suggested that the aggrecan depletion by aggrecanase in the NP occurs earlier in life than that mediated by MMPs.[5] In the degenerated human fibrocartilaginous IVD, an increase in the number of ADAMTS4 immunopositive cells is observed with increasing degeneration, which is not paralleled by a rise in its inhibitor TIMP-3.[31] However, little is known about the expression of aggrecanase and TIMP-3 in the notochordal NP.

The results of the present study demonstrated that all TIMP-1, -2, and -3 gene expression in the notochordal NP of the 4-week-old rabbit was higher than those in the AF. Among the 3 TIMPs, TIMP-3 is said to effectively inhibit the activity of aggrecanases in addition to the inhibition of MMPs.[13,14] Therefore, we focused on TIMP-3 among the 3 TIMPs and proceeded to investigate the age-related changes of TIMP-3 and aggrecanase.

In the present study, we provided evidence for the first time that TIMP-3 and ADAMTS4 mRNA were expressed in the rabbit notochordal NP; however, ADAMTS5 mRNA was not detected in both age groups. Therefore, we particularly investigated the expression of TIMP-3 and ADAMTS4. The expression of TIMP-3 decreased at the end stage of the notochordal NP, whereas there was no significant difference in the expression of ADAMTS4 mRNA between 4-week-old and 160-week-old rabbits. According to these findings, it is possible that the decrease of the TIMP-3 expression in the notochordal NP causes an imbalance of ADAMTS4/TIMP-3 ratio, which may lead to depletion of aggrecans in the IVD. However, the actual enzyme synthesis and activation of ADAMTS4 were not examined in the present study, and further investigations are necessary to fully support this evidence.

Little is known about the mechanism of the regulation on the expression of TIMP-3 and aggrecanases in the notochordal NP. The results of the present study demonstrated that TGF-β1, which is one of the most potent anabolic regulatory factors in cartilage,[32] upregulated TIMP-3 mRNA but not ADAMTS4 mRNA *in vitro*. Since the mRNA expression of both TGF-β1 and TIMP-3 decreased with aging in the notochordal NP, the age-related increasing changes of ADAMTS4/TIMP-3 mRNA ratio can be mediated, at least in part, by decrease of TGF-β1. Therefore, all these data suggest that notochordal NP may play a key role to preserve the IVD rich in ECM molecule, especially aggrecan, by expressing TGF-β1 and TIMP-3.

The results of the present study also revealed that ADAMTS5 mRNAs could not be detected even in 160-week-old rabbits. On the other hand, ADAMTS4 mRNA expression was present at and was not significantly different between both time points, suggesting a role for ADAMTS4 in the physiologic matrix remodeling in the IVD. The present *in vitro* study demonstrated the upregulation of the expression of AMAMTS4 mRNAs after treatment with IL-1β. Previous studies have also demonstrated the presence of aggrecanases in conjunction with catabolic cytokines, such as IL-1 in the extracts from the degenerated human IVDs and/or in the cultured disc tissues.[4,32,33] Taken all these findings together, it may therefore be speculated that the decrease in the expression of TGF-β1 and TIMP-3 may occur in the transition period from the notochordal to the fibrocartilaginous NP before the upregulation of aggrecanases, which may play a role in the later degenerative stage. However, the possibility that other MMPs might be involved in the rabbit IVD degeneration process cannot be ruled out, and this needs to be clarified in the future studies.

We also have to keep in mind that the 4-week-old rabbit is still in a developing and growing stage, in which the ECM can be remodeled for development and/or growth.[34] Thus, the results of our study cannot be translated directly to the aging or degenerative processes. Future investigations on the interactions between TIMP-3 and aggrecanases are necessary to clarify the precise mechanism of IVD degeneration, thereby developing potential therapeutic strategies to prevent the IVD breakdown.

■ Conclusion

The levels of expression of TIMP-3 in the notochordal NP were significantly lower in 160-week-old rabbits than those in 4-week-old rabbits. Decrease in the expressions of TIMP-3, mediated in part by TGF-β1, may cause imbalance of ADAMTS4/TIMP-3 ratio at the end stage of the notochordal NP. Notochordal NP may play a key role in the homeostasis of the intervertebral disc metabolism, by expressing TGF-β1 and TIMP-3.

■ Key Points

- Semi-quantitative assessment of histologic changes indicated that the 4-week-old rabbit was equivalent to fetus to the 2-year-old human and the 160-week-old rabbit was equivalent to the 11- to 30-year-old human, particularly 11- to 16-year-olds.

- A decrease in the expressions of TIMP-3 mRNA mediated in part by TGF-β1 cause an imbalance of ADAMTS4/TIMP-3 mRNA ratio at the end stage of notochordal NP.
- Notochordal NP may play a role in the maintenance of the homeostasis of intervertebral disc metabolism by expressing TIMP-3.

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Original article

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Japanese Orthopaedic Association Back Pain Evaluation Questionnaire. Part 2. Verification of its reliability

The Subcommittee on Low Back Pain and Cervical Myelopathy Evaluation of the Clinical Outcome Committee of the Japanese Orthopaedic Association

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Abstract

Background. The project to develop a new Japanese Orthopaedic Association (JOA) score rating system for low back disorders, the JOA Back Pain Evaluation Questionnaire (JOABPEQ), is currently in progress. Part 1 of the study selected 25 "candidate" items for use on the JOABPEQ. The purpose of this current Part 2 of the study was to verify the reliability of the questionnaire.

Methods. A total of 161 patients with low-back disorders of any type participated in the study. Each patient was interviewed twice at an interval of 2 weeks using the same questionnaire. The reliability of the questionnaire was evaluated by determining the extension of the kappa and weighted kappa coefficients.

Results. Both kappa and weighted kappa were more than 0.50 for all but one item, which was 0.48. The lower 95% confidence interval exceeded 0.4 in all but two items, which was 0.39. This implied that the test–retest reliability of JOABPEQ was acceptable as a measure of outcome.

Conclusions. The tentative questionnaire of the JOABPEQ with 25 items was confirmed to be reliable enough to describe the quality of life of patients who suffer low back disorders.

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Introduction

Measurement of the outcome is critical for any decision-making and results of evaluations in all medical circumstances. This should be applicable when managing patients who have lumbar spine-related problems. The Japan Orthopaedic Association (JOA) developed and published a specific instrument to measure outcomes for patients with low back problems in 1986.[1] It was called the JOA score rating system for low back pain, with a full score being 29 points. Since then, the instrument has been widely utilized to evaluate the functional results of many types of intervention for patients with such problems. It has been referred to not only in articles by Japanese investigators[2] but also in those by non-Japanese-speaking investigators.[3,4] One of the major criticisms of this specific instrument, however, is that it is not a patient-oriented measurement but a physician-based one. It is now widely accepted that a patient's perspective is essential for making medical decisions and for evaluating the results of interventions.[5] Based on the current needs for measuring outcome, the JOA was urged to revise its original score rating system and to develop a new one. In 2002, a Subcommittee on Evaluation of Back Pain and Cervical Myelopathy was organized in the Clinical Outcome Committee of JOA,

and work began on revising the original JOA scoring system.

This revision process consisted of four steps: Parts 1 to 4. As described in the previous literature concerning Part 1, the original JOA scoring system was revised and a new scoring system (the JOA Back Pain Evaluation Questionnaire — JOABPEQ) was developed.[6] The key points of this revision were to make the original JOA score more patient-oriented. For the survey in the Part 1 study, we first created a preliminary questionnaire consisting of 60 items. The questionnaire was a self-administered, disease-specific measure that was created with reference to the Japanese editions of the short form health survey with 36 questions (SF-36)[7] and the Roland-Morris Disability Questionnaire (RDQ)[8] to assess health-related quality of life. From the survey, a total of 25 items were selected for tentative use on a draft of the JOABPEQ (Table 1).

Table 1. Items (n = 25) selected for the draft of the JOABPEQ evaluated in this study

With regard to your health condition during the last week, please circle the item number of the answer for the following questions that best applies. If your condition varies depending on the day or time, circle the item number when your condition is at its worst.

Q1-1. To alleviate low back pain, you often change your posture.
1) Yes
2) No

Q1-2. Because of low back pain, you do not do any routine housework these days.
1) No
2) Yes

Q1-3. Because of low back pain, you lie down more often than usual.
1) Yes
2) No

Q1-4. Because of low back pain, you sometimes ask someone to help you when you do something.
1) Yes
2) No

Q1-5. Because of low back pain, you refrain from bending forward or kneeling down.
1) Yes
2) No

Q1-6. Because of low back pain, you have difficulty standing up from a chair.
1) Yes
2) No

Q1-7. Your lower back aches most of the time.
1) Yes
2) No

Q1-8. Because of low back pain, turning over in bed is difficult.
1) Yes
2) No

Q1-9. Because of low back pain, you have difficulty putting on socks or stockings.
1) Yes
2) No

Q1-10. Because of low back pain, you walk only short distances.
1) Yes
2) No

Q1-11. Because of low back pain, you cannot sleep well. (If you take sleeping pills because of the pain, select "No.")
1) No
2) Yes

Q1-12. Because of low back pain, you stay seated most of the day.
1) Yes
2) No

Q1-13. Because of low back pain, you become irritated or angry at other persons more often than usual.
1) Yes
2) No

Q1-14. Because of low back pain, you go up stairs more slowly than usual.
1) Yes
2) No

Table 1. *Continued*

Q2-1. How is your present health condition?
1) Excellent
2) Very good
3) Good
4) Fair
5) Poor

Q2-2. Do you have difficulty in climbing stairs?
1) I have great difficulty.
2) I have some difficulty.
3) I have no difficulty.

Q2-3. Do you have difficulty in any one of the following motions: bending forward, kneeling, or stooping?
1) I have great difficulty.
2) I have some difficulty.
3) I have no difficulty.

Q2-4. Do you have difficulty walking more than 15 minutes?
1) I have great difficulty.
2) I have some difficulty.
3) I have no difficulty.

Q2-5. Have you been unable to do your work or ordinary activities as well as you would like?
1) I have not been able to do them at all.
2) I have been unable to do them most of the time.
3) I have sometimes been unable to do them.
4) I have been able to do them most of the time.
5) I have always been able to do them.

Q2-6. Has your work routine been hindered because of the pain?
1) Greatly
2) Moderately
3) Slightly (somewhat)
4) Little (minimally)
5) Not at all

Q2-7. Have you been discouraged or depressed?
1) Always
2) Frequently
3) Sometimes
4) Rarely
5) Never

Q2-8. Do you feel exhausted?
1) Always
2) Frequently
3) Sometimes
4) Rarely
5) Never

Q2-9. Do you feel happy?
1) Always
2) Almost always
3) Sometimes
4) Rarely
5) Never

Q2-10. Do you think you are in reasonable health?
1) Yes (I am healthy.)
2) Fairly (my health is better than average)
3) Not (very much)/particularly (my health is average)
4) Barely (my health is poor)
5) Not at all (my health is very poor)

Q2-11. Do you feel your health will get worse?
1) Very much so
2) A little at a time
3) Sometimes yes and sometimes no
4) Not very much
5) Not at all

The purpose of the Part 2 study in this project was to evaluate the reliability of the 25 items selected for the draft JOABPEQ; for this, test–retest reliability was ascertained.

Materials and methods

Recruitment of patients

Altogether, 460 of the 829 Japanese board-certified spine surgeons were randomly selected, and each was asked to recruit two patients to evaluate the JOABPEQ between January and June 2004. The recruited patients were scheduled to reply to the questionnaire twice at a 2-week interval. Patient criteria were as follows: (1) patients could be any age of either sex; (2) patients had any lumbar spine disorder and were currently visiting an outpatient clinic; (3) the severity of the symptoms was expected to be at the same level between the two interviews. Exclusion criteria were the presence of: (1) other musculoskeletal diseases requiring medical treatment; (2) psychiatric disease (e.g., dementia), potentially leading to inappropriate answers; (3) a postoperative condition; (4) having participated in previous surveys of the related study.

Testing the questionnaire

Each patient was asked to complete the same questionnaire twice at an interval of 2 weeks (±3 days). The attending surgeon filled out the patient information on the diagnosis and the presence or absence of concomitant diseases, followed by judging the severity of symptoms using a three-step rating scale (mild, moderate, severe). Symptom severity was determined subjectively by the attending surgeon, who was asked not to select a similar patient solely on the basis of severity. Patients who had the same level of severity as judged by all surgeons were then selected and analyzed to verify the reliability of the questionnaire.

This study was approved by the Ethics Committee of the Japanese Society for Spine Surgery and Related Research. Informed consent was obtained from each patient.

The reliability of the questionnaire was evaluated by determining the extension of the kappa coefficients. The weighted kappa coefficient was calculated in the items with three choices or more. The kappa and weighted kappa coefficients were calculated based on a formula using Microsoft Office Excel 2003. Kappa and weighted kappa coefficients of 0.4 or above were judged to be reliable.[9] The 95% confidence intervals (95% CI) were calculated for all reliability coefficients using the bootstrap method.

Results

Patient characteristics

A total of 350 patients participated in this study and completed the questionnaire twice following the project's plan. However, 135 patients were excluded because the severity of their symptoms had changed between the two interviews or they violated the interval period. Of the remaining 215 patients, 54 were ineligible because of other musculoskeletal diseases, such as knee and hip osteoarthrosis. As a result, a total of 161 patients were available for the analysis in this study: 86 men and 75 women with a mean age of 57.7 years (SD 16.3 years). The clinical diagnosis included degenerative lumbar canal stenosis in 49 patients, lumbar disc herniation in 44, spondylolisthesis in 20, spondylosis in 16, degenerative disc disease in 13, mechanical low back pain in 11, and miscellaneous in 8. The patients' age varied from their twenties to their eighties, and symptom severity varied from mild to severe (Table 2). Neurological and physical status was evaluated for each patient using the current JOA score rating system and finger–floor distance (Table 3). Neurological deficits varied from mild to severe, and trunk flexibility varied among the subjects as well.

Face validity

Face validity was checked in terms of the completion rate for filling out the questionnaire. The distribution of

Table 2. Distribution of age and severity of symptoms in the patients analyzed (n = 161)

	No. of patients, by severity of symptoms			
Age (years)	Mild	Moderate	Severe	Total
Men				
20–	2	2	3	7
30–	4	4	1	9
40–	2	2	0	4
50–	7	9	1	17
60–	13	8	3	24
70–	6	18	0	24
80–	0	0	1	1
Total	34	43	9	86
Women				
20–	2	3	0	5
30–	4	5	0	9
40–	6	1	1	8
50–	9	4	1	14
60–	7	11	1	19
70–	8	9	1	18
80–	1	1	0	2
Total	37	34	4	75
Total no.	71	77	13	161

Table 3. Current Japanese Orthopaedic Association score rating system and finger to floor distance for the patients analyzed ($n = 161$)

Parameter	No.
SLR test	
Normal	124
30°–70°	35
<30°	2
Motor function	
Normal	113
Slight weakness (MMT: good)	38
Severe weakness (MMT: less than good)	10
Sensory function	
Normal	80
Slight disturbance	59
Severe disturbance	22
Bladder function	
Normal	147
Mild dysuria	12
Severe dysuria	2
Finger to floor distance (cm)	
—15	1
−14–−5	17
−4–−4	41
5–14	40
15–24	32
25–34	9
35–44	7
45–54	7
55–64	4
65–74	1
Immeasurable	2
Total no.	161

SLR, straight leg raising; MMT, manual muscle testing

the answers for all question items was then checked to ensure that there were no biased answers. Items remaining unanswered accounted for less than 5% in the first test, and there was no skewed distribution, such as "floor and ceiling" effects (Table 4).

Reliability

The test–retest reliability was confirmed by calculating the kappa and weighted kappa coefficients for each item (Tables 5A, 5B). Both kappa and weighted kappa were more than 0.50 in all items, except in one item with 0.48. The lower 95% CI exceeded 0.4 in all items, except in two items with 0.39. This implied that the test–retest reliability of JOABPEQ was acceptable as a measurement of outcome.

Discussion

Measurement of the outcome is generally divided into two categories: generic and disease-specific measures.[5,10] SF-36 has been commonly used as representative of a measurement of generic health status.[5,7,10] The RDQ and the Oswestry Disability Index are widely used as disease-specific measurements for back pain.[8,11] The JOA score rating system for low back pain, developed in 1986, was also a disease-specific measuring instrument for back disorders and injuries and has been widely utilized in clinical research and the decision-making process in Japan. However, this is not a patient-based outcome measure reliable enough to describe the objective status of the function and quality of life (QOL) of patients with low-back disorders. There has, to date, been insufficient psychometric analysis to confirm the validity and reliability of this JOA score rating system.

The project for developing the new questionnaire, JOABPEQ, was initiated to create a self-administered, disease-specific method for measuring low back pain. This instrument should include functions of the lumbar spine as well as health-related QOL. The reliability of the questionnaire that includes the 25 suggested items was evaluated using psychometric analysis as Part 2 of this project. Kappa and weighted kappa coefficient were utilized to verify the test–retest reliability.[12,13]

In terms of external validity, biased data were inevitable because one criterion that was included was that the severity of the symptoms was expected to be at the same level between the two interviews. However, there was no bias on the choices of answer to each question. This implies that test–retest reliability was acceptable even if the subjects had symptoms of different severity. The older the patients were, the worse was the interpretation of each question. There were small numbers of patients of younger generations, such as those in their thirties and forties, in this study. Thus, the reliability would not deteriorate even if the number of young people were to increase.

In terms of English expression, there is a possibility of ambiguity in questions 1-2 and 1-11, where double negatives (two "no's" in the answer) may be confusing. It is necessary to reconsider and revise the English expression so it is more easily understood by native English-language users. The number of choices for the answer in all questions varied from two to five, which is also a point to be reconsidered in the future.

The current study demonstrated that the 25 items had enough reliability to describe the QOL in patients suffering low back disorders. However, further studies are needed to complete the project, including a factor analysis to determine the underlying cluster of the questionnaire items, a formula for calculating the severity score, and confirmation of the responsiveness to the questionnaire.

Table 4. Reproducibility of each item (n = 161)

Item	Choices for answer					No answer
	1	2	3	4	5	
Q1-1	117	43				1
	72.7%	26.7%				0.6%
Q1-2	32	127				2
	19.9%	78.9%				1.2%
Q1-3	76	83				2
	47.2%	51.6%				1.2%
Q1-4	42	119				
	26.1%	73.9%				
Q1-5	77	84				
	47.8%	52.2%				
Q1-6	31	130				
	19.3%	80.7%				
Q1-7	68	93				
	42.2%	57.8%				
Q1-8	65	95				1
	40.4%	59.0%				0.6%
Q1-9	72	89				
	44.7%	55.3%				
Q1-10	87	74				
	54.0%	46.0%				
Q1-11	35	122				4
	21.7%	75.8%				2.5%
Q1-12	41	119				1
	25.5%	73.9%				0.6%
Q1-13	36	125				
	22.4%	77.6%				
Q1-14	115	45				1
	71.4%	28.0%				0.6%
Q2-1		18	59	66	16	2
		11.2%	36.6%	41.0%	9.9%	1.2%
Q2-2	15	92	52			2
	9.3%	57.1%	32.3%			1.2%
Q2-3	25	93	38			5
	15.5%	57.8%	23.6%			3.1%
Q2-4	35	70	55			1
	21.7%	43.5%	34.2%			0.6%
Q2-5	15	12	85	35	13	1
	9.3%	7.5%	52.8%	21.7%	8.1%	0.6%
Q2-6	13	36	66	32	11	3
	8.1%	22.4%	41.0%	19.9%	6.8%	1.9%
Q2-7	12	8	79	39	22	1
	7.5%	5.0%	49.1%	24.2%	13.7%	0.6%
Q2-8	8	27	88	24	12	2
	5.0%	16.8%	54.7%	14.9%	7.5%	1.2%
Q2-9	8	42	74	31	5	1
	5.0%	26.1%	46.0%	19.3%	3.1%	0.6%
Q2-10	13	59	42	34	12	1
	8.1%	36.6%	26.1%	21.1%	7.5%	0.6%
Q2-11	17	48	56	28	11	1
	10.6%	29.8%	34.8%	17.4%	6.8%	0.6%

Table 5A. Kappa coefficient with 95% CI for items Q1-1 to Q1-14

Item	κ	95% CI
Q1-1	0.69	0.60–0.77
Q1-2	0.62	0.51–0.73
Q1-3	0.67	0.60–0.75
Q1-4	0.65	0.56–0.75
Q1-5	0.48	0.39–0.57
Q1-6	0.55	0.43–0.66
Q1-7	0.65	0.57–0.73
Q1-8	0.55	0.47–0.64
Q1-9	0.71	0.64–0.78
Q1-10	0.63	0.55–0.72
Q1-11	0.50	0.39–0.61
Q1-12	0.56	0.46–0.65
Q1-13	0.65	0.55–0.74
Q1-14	0.72	0.64–0.80

CI, confidence interval

Table 5B. Weighted kappa coefficient with 95% CI for items Q2-1 to Q2-11

Item	Weighted κ	95% CI
Q2-1	0.51	0.43–0.57
Q2-2	0.61	0.52–0.68
Q2-3	0.57	0.49–0.64
Q2-4	0.73	0.68–0.78
Q2-5	0.54	0.47–0.60
Q2-6	0.61	0.55–0.67
Q2-7	0.53	0.46–0.59
Q2-8	0.55	0.48–0.61
Q2-9	0.54	0.46–0.60
Q2-10	0.54	0.47–0.61
Q2-11	0.53	0.46–0.60

Conclusions

The tentative JOABPEQ with 25 items was confirmed to be reliable enough to describe the QOL of patients suffering low back disorders.

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