

# Cartilage Intermediate Layer Protein and Asporin Polymorphisms Are Independent Risk Factors of Lumbar Disc Degeneration in Male Collegiate Athletes

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## Abstract

**Objective:** Lumbar disc degeneration (LDDG), recently reported to have strong genetic determinants, is a major cause of discopathy and lower back pain. However, most studies have only evaluated the effects of a single susceptibility polymorphism. Our purpose was to examine the effect of two susceptibility polymorphism for LDDG in Japanese collegiate athletes. **Design:** We investigated two susceptibility genes for LDDG—cartilage intermediate layer protein (CILP) and asporin (ASPN)—in 516 collegiate athletes and genotyped the risk allele of CILP (I184T/C) and ASPN (D14). LDDG was evaluated using T2-weighted magnetic resonance imaging. **Results:** By using logistic regression analysis, we found that the ASPN D14 allele and CILP genotype were associated with an increased risk of LDDG in male but not female athletes (CILP CT: odds ratios [OR] = 1.77, 95% confidence interval [CI] = 1.07-2.93; CILP CC: OR = 4.38, 95% CI = 1.42-13.54; ASPN D14: OR = 2.17, 95% CI = 1.10-4.28]. We also found that CILP C and ASPN D14 were independent variables. The ORs with more than two risk alleles were largely increased. **Conclusions:** The CILP and ASPN polymorphisms are independent genetic risk factors for LDDG in male but not female Japanese collegiate athletes.

## Keywords

disc degeneration, polymorphisms, athlete, CILP, ASPN

## Introduction

Lumbar disc degeneration (LDDG) is a degenerative change in the lumbar disc that occurs with aging, affecting from 30% in people younger than 30 years to 50% in those 30 years and older.<sup>1</sup> LDDG also occurs in competitive athletes<sup>2-5</sup> and is one of the causes of lumbar disc diseases, including lower back pain and discopathy. LDDG is caused by genetic and environmental factors. Cartilage layer intermediate protein (CILP) is abundantly expressed in the intervertebral discs.<sup>6</sup> CILP interacts directly with transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), controlling the TGF- $\beta$ 1-mediated construction of extracellular matrix proteins. Several recent reports have shown that the CILP gene polymorphism (I184T/C) is significantly associated with lumbar disc diseases in Japanese subjects.<sup>4-6</sup> Mutated C allele was reported to increase the binding of TGF- $\beta$ 1 and to disturb the regulation of TGF- $\beta$ 1 signaling.

Previous studies have also shown that asporin (ASPN) alleles with 14 aspartic acid repeats (designated D14) in the N-terminal region are associated with osteoarthritis (OA) of the knee.<sup>7</sup> Recently, ASPN D14 was also shown to be associated with LDDG in the Asian population.<sup>8</sup> ASPN mRNA

is abundantly expressed in the articular cartilage, heart, and liver.<sup>9</sup> High ASPN mRNA and protein expression were also observed in degenerated lumbar discs, especially in the outer annulus.<sup>7,10</sup> Nakajima *et al.* reported that ASPN inhibits TGF- $\beta$ 1/smad signaling by co-localization with TGF- $\beta$  and blocking the interaction between TGF- $\beta$ 1 and its receptors.<sup>11</sup> Kizawa *et al.* showed that the ASPN D14 allele results in greater inhibition of TGF- $\beta$  activity in ATDC5 cells.<sup>7</sup> As both CILP and ASPN proteins in some way affect TGF- $\beta$  signaling, these two susceptible polymorphisms for LDDG may mutually or independently affect the inhibition of TGF- $\beta$  signaling and LDDG occurrence.

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Recently, we showed that there was a significant association between the CILP 1184T/C polymorphism and LDDG occurrence in Japanese collegiate male athletes.<sup>4,5</sup> In this study, we first examined whether ASPN D14 is a significant risk factor for LDDG occurrence in collegiate athletes. As a result, we found that ASPN D14 and LDDG occurrence were significantly associated. We then hypothesized that the evaluation of multiple risk alleles of ASPN and CILP shows higher statistical predictability in comparison to only one risk allele, because these two susceptible polymorphisms have similar inhibitory effects on TGF- $\beta$  signaling.

## Materials and Methods

### Subjects

We analyzed the relationship between genetic factors and LDDG in 516 (male, 341; female, 175) collegiate athletes from seven types of sports (wrestling, judo, gymnastics, swimming, soccer, American football, and track and field), with an average of more than 5 years of experience. We defined wrestling, judo, soccer, and American football as collision sports and gymnastics, swimming, and track and field as noncollision sports. We obtained written informed consent from all subjects by ethical committees (Permission No. 008-G04).

### MRI Examination

The analysis of the distribution of LDDG was based on our previous study.<sup>4</sup> We modified the definitions of LDDG presented by Pfirrmann's classification.<sup>12</sup> MRI evidence of LDDG was defined as decreased signal intensity of the intervertebral discs at L1/L2 and L5/S1 in the T-2 weighted midsagittal fast spin-echo images (repetition time, 5,000 ms; echo time, 125 ms). All MRI scans were analyzed and graded independently by two experienced orthopedic surgeons who were blinded to the subject's status. The grading system for the assessment of LDDG was based on the study of Pfirrmann *et al.*<sup>12</sup> We defined grades 3, 4, and 5 as the LDDG group and grades 1 and 2 as the CONTROL group, as reported by Kaneoka *et al.*<sup>13</sup> If the athlete had multiple degenerations, the highest grade and its location were determined. In the case of any discrepancy in the grading of LDDG between the two orthopedic surgeons, the disagreement was resolved by consensus. The frequency of LDDG in these athletes was 37% (191/516).

### Genotyping

DNA was extracted from the buccal cells of the athletes using cotton swabs. The CILP polymorphism was performed as described in our previous study.<sup>4</sup> The ASPN

D-repeat polymorphism was genotyped by fragment size. The D-repeat region was amplified using the forward primer 5'-TCCTAGACTGGTCTTCTACACT-3' and the reverse primer 5'-TCTGAGCAATGTACAACCTCGTG-3'. The forward primer was 5' labeled with FAM. The polymerase chain reaction (PCR) cycling reactions, using a thermal cycler (MJ Mini, BioRad, Hercules, CA), were performed at 94°C for 10 minutes, 30 cycles at 94°C for 30 seconds, at 64°C for 30 seconds, and at 72°C for 30 seconds, followed by one cycle at 72°C for 10 minutes. After amplification, the alleles were discriminated using the ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA) and the fragment analysis GeneMapper V4.0 software (Applied Biosystems).

### Statistical Analysis

We used the software R (version 2.8.0) for statistical analyses. The genotype-specific risks were estimated as odds ratios (ORs) and 95% confidence intervals (CIs). Logistic regression analysis was performed to investigate the relationships between LDDG and the two susceptibility genes, as well as with body weight, gender, and collision sport. *P* values less than 0.05 were considered statistically significant.

## Results

### Characteristics of the Subjects

The characteristics of the subjects are shown in **Table 1**. In male athletes, the height, weight, and body mass index of the LDDG group were significantly higher than that of the CONTROL group. There was no significant difference between the LDDG and CONTROL groups in female athletes.

### Effect of ASPN D14 Polymorphism on LDDG Occurrence

The association between the ASPN D14 allele and LDDG is shown in **Table 2**. The frequencies of the genotypes were similar to those of the previous study.<sup>8</sup> The frequency of the ASPN D14 allele in the LDDG group was higher than that in the CONTROL group. We found a significant association between the ASPN D14 allele and LDDG ( $P < 0.01$ ) with an OR of 2.02 (95% CI = 1.21-3.36) in all athletes (**Table 2A**). By using logistic regression analysis concomitant with the interaction term and the Wald test, we found that weight (OR = 1.03; 95% CI = 1.01-1.05) and ASPN D14 allele (OR = 2.14; 95% CI = 1.1-4.18) were significant risk factors for LDDG (**Table 2B**). We also confirmed that the effect of D14 was significant only for male athletes (**Table 2C**).

**Table 1.** Characteristics of the Subjects.

Variable	LDDG		CONTROL	
	Male	Female	Male	Female
Age (years)	19.9 ± 1.2	19.9 ± 1.3	19.8 ± 1.2	19.8 ± 1.1
Height (cm)	172.7 ± 7.0**	159.8 ± 6.0	170.5 ± 6.6**	160.3 ± 5.5
Weight (kg)	74.0 ± 15.6***	54.5 ± 6.6	68.01 ± 12.2***	53.9 ± 5.9
BMI (kg/m <sup>2</sup> )	24.7 ± 4.3**	21.3 ± 2.1	23.3 ± 3.6**	21.0 ± 1.8
Sport experience (years)	8.4 ± 3.6	9.5 ± 3.9	7.7 ± 3.1	9.6 ± 3.6

LDDG = subjects with lumbar disc degeneration; CONTROL = subjects without lumbar disc degeneration; BMI = body mass index. \*\*Unpaired t-test,  $P < 0.01$ . \*\*\*Unpaired t-test,  $P < 0.001$ .

**Table 2.** Association between the Presence of the ASPN D14 Allele and LDDG in 516 Athletes.

A. Frequency of LDDG and ASPN D14 Allele in 516 Athletes.

	LDDG (%)	CONTROL (%)
D14+	34 (8.9)	30 (4.6)
D14-	348 (91.1)	620 (95.4)
P value	0.006**	
OR (95% CI)	2.02 (1.21-3.36)	

OR = odds ratio; CI = confidence interval. \*\* $P < 0.01$ .

B. Logistic Regression Analysis.

Group	Crude		Adjusted		P Value
	OR	95% CI	OR	95% CI	
Weight	1.02	1.01-1.04	1.03	1.01-1.05	<0.001***
Gender	0.87	0.59-1.27	1.48	0.89-2.44	.129
Collision	1.33	0.93-1.9	0.94	0.62-1.42	.755
D14	2.05	1.2-3.52	2.14	1.1-4.18	.025*
Sex:D14			0.9	0.28-2.87	.864

OR = odds ratio; CI = confidence interval; D14 = allele of ASPN gene. The formulated logistic model is as follows:  
 $LDDG = \exp(\beta_0 + \beta_1 W + \beta_2 G + \beta_3 COL + \beta_4 D14 + \beta_5 G \times D14)$ ,  
 where W, G, COL, and D14 denote weight, gender, collision, and ASPN D14 allele, respectively.  $\beta_j$  ( $j = 1-6$ ) are parameters to be estimated. \* $P < 0.05$ . \*\*\* $P < 0.001$ .

C. Wald Test.

	Test Statistics	P Value
D14 + D14:sex	1.884	0.17

The null hypothesis is composed of the following restrictions:  $D14:\beta_5 + D14:gender:\beta_6 = 0$ .  $\beta_5$  and  $\beta_6$  are coefficients in the multinomial logistic model as shown in **Table 2B**.

### Logistic Regression Analysis on LDDG Occurrence in CILP and ASPN Genotypes

We further evaluated the clinical and genetic risk factors for LDDG. The logistic regression model was constructed with six variables, as shown in **Table 3**. LDDG was significantly correlated with body weight, gender, ASPN D14, CILP CT, and CC. However, collision sports were not correlated with LDDG (**Table 3A**). Furthermore, the two genotypes had no effect on LDDG occurrence in female athletes (**Table 3B**).

We also calculated the ORs of the susceptibility genes by using logistic regression analysis from the result of each genotype. The OR of LDDG occurrence was increased by the risk alleles of the two susceptibility genes (**Table 4**).

### Interaction Curve of Susceptibility Genes

The interaction curve of the two susceptibility genes is presented in **Figure 1**. Although the proportion of LDDG occurrence increased with the number of risk alleles, there was no significant interaction between these two susceptibility genes ( $P > 0.05$ ; **Fig. 1**).

### Discussion

We previously reported that CILP (1184T/C) was significantly associated with LDDG in collegiate male athletes. In this study, we also found a significant association between the ASPN D14 allele and LDDG in collegiate male athletes. Except for gender difference, this finding is consistent with the results in the Asian population as reported by Song *et al.*<sup>8</sup> Although significant interactions of these two susceptibility genes have not been observed, we also found that the addition of the risk alleles CILP 1184T/C and ASPN D14 increased the odds ratio.

The D14 allele of ASPN has been reported as a susceptibility gene for OA of the knee.<sup>7</sup> Recently, Song *et al.* found that ASPN D14 was also associated with LDDG in a Chinese and Japanese cohort.<sup>8</sup> We also found that ASPN D14 was significantly associated with LDDG occurrence in

**Table 3.** Logistic Regression Analysis for LDDG in Two Susceptible Genes

## A. Logistic Regression Analysis.

Group	Crude		Adjusted		P Value
	OR	95% CI	OR	95% CI	
Weight	1.02	1.01-1.04	1.03	1.02-1.05	<0.001***
Gender	0.87	0.59-1.27	2.1	1.14-3.87	0.018*
Collision	1.33	0.93-1.9	0.93	0.61-1.42	0.723
D14	2.05	1.2-3.52	2.17	1.10-4.28	0.026*
TC	1.07	0.73-1.57	1.77	1.07-2.93	0.027*
CC	2.79	1.28-6.06	4.38	1.42-13.54	0.010*
D14:gender			0.89	0.28-2.86	0.842
TC:gender			0.39	0.17-0.92	0.032*
CC:gender			0.4	0.08-2.11	0.281

OR = odds ratio; CI = confidence interval; D14 = allele of ASPN gene; TC = genotype of CILP gene; CC = genotype of CILP gene.

The formulated logistic model is as follows:

$$\text{LDDG} = \exp(\beta_0 + \beta_1 W + \beta_2 G + \beta_3 \text{COL} + \beta_4 \text{D14} + \beta_5 \text{TC} + \beta_6 \text{CC} + \beta_7 \text{D14} \times G + \beta_8 \text{TC} \times G + \beta_9 \text{CC} \times G),$$

where W, G, COL, D14, TC, and CC denote weight, gender, collision, ASPN D14 allele, CILP TC genotype, and CILP CC genotype, respectively.  $\beta_j$  ( $j = 1-9$ ) are parameters to be estimated.

\* $p < 0.05$ . \*\*\* $p < 0.001$ .

## B. Wald Test.

	Test Statistics	P Value
D14 + D14:gender = 0	4.21	0.24
CILP CT + CILP CT:gender = 0		
CILP CC + CILP CC:gender = 0		

The null hypothesis is composed of the following restrictions:

$$\text{D14:}\beta_4 + \text{D14:gender:}\beta_7 = 0$$

$$\text{TC:}\beta_5 + \text{TC:gender:}\beta_8 = 0$$

$$\text{CC:}\beta_6 + \text{CC:gender:}\beta_9 = 0$$

$\beta_4$  to  $\beta_9$  are coefficients in the multinomial logistic model as shown in

**Table 3A.**

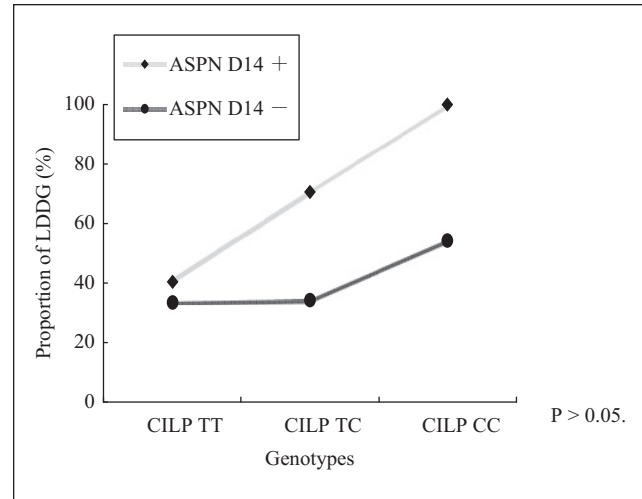
**Table 4.** Odds Ratios by Logistic Regression Analysis.

Risk Alleles	OR	95% CI	P Value
CILP CT + D14	3.84	1.61-9.15	<0.01**
CILP CC + D14	9.51	2.54-35.64	<0.001***

OR = odds ratio; CI = confidence interval.

\*\* $p < 0.01$ . \*\*\* $p < 0.001$ .

collegiate athletes. Song *et al.* also showed that there was a trend for an association between ASPN D14 and severe LDDG.<sup>8</sup> In their study, the Chinese cohort was defined only by radiological abnormality, but the Japanese cohort was dependent on painful disc herniations. In this study, the LDDG group was defined by the MRI findings and the definition was independent of pain. Thus, we think that the

**Figure 1.** Interaction curve of CILP and ASPN polymorphisms. There was no significant interaction between these two susceptibility genes.

ASPN D14 allele is associated with LDDG, independent of symptoms such as pain or discomfort.

It is very important to evaluate whether different susceptibility genes produce a combination effect. We previously found that CILP 1184T/C was significantly associated with LDDG occurrence in collegiate athletes.<sup>4,5</sup> In this study, we also found that there was a significant association between ASPN D14 and LDDG. As both these genes are related to TGF- $\beta$ 1 signaling and their high expression was observed in degenerated lumbar discs, we hypothesized that there might be a significant association between them. Surprisingly, we found that CILP C and ASPN D14 were independent variables. Thus, we believe that these two genes affect different portions of the TGF- $\beta$  signaling pathway. In addition, there is no evidence suggesting that these two molecules interact with each other. As we further confirmed that each additional risk allele increased the odds of LDDG, we think that these two risk alleles have a cumulative effect on the occurrence of LDDG.

We previously reported that CILP 1184T/C was a LDDG-susceptible gene for men, but not women.<sup>5</sup> In this report, we also found that ASPN D14 is only a significant risk factor for men. Even in the logit model concomitant with two susceptibility genes, genetic variance was only a significant risk factor for men. As these two proteins interact with the TGF- $\beta$  pathway, a similar sex dependency might be observed. Valdes *et al.* found that a haplotype of the CILP gene gave the strongest association in men with knee OA.<sup>14</sup> Song *et al.* reported that there is a significant difference in the allele frequency of ASPN D13 between female OA patients and female controls.<sup>15</sup> Although the molecular mechanisms of this phenomenon are ambiguous, we think that the effect of the CILP and ASPN polymorphisms is gender dependent.

We evaluated not only genetic factors but also intrinsic and extrinsic factors, such as gender, weight, and collision. As a result, we found that weight and gender were significant risk factors for LDDG. We previously reported that high body weight is a risk factor for LDDG occurrence in collegiate athletes.<sup>4,5,16</sup> We also reported that women are at risk for LDDG occurrence.<sup>5</sup> As high body weight causes greater stress on the lumbar disc, it is reasonable that high body weight is significantly associated with LDDG occurrence. On the contrary, it is difficult to fathom why gender difference is a risk factor for LDDG. There are several reports showing that more women complained of lower back pain and hip joint stress than men.<sup>17,18</sup> Although the molecular mechanisms are still unclear, we think that the occurrence of LDDG might be gender-dependent after adjusting for body weight, collision, and several genetic factors, as discussed before.

The predictive power of tests can be evaluated by the area under the receiver operating characteristic curve (AUC).<sup>19</sup> In this study, we calculated AUC for our logit model. A perfect model would have an AUC of 1, whereas a model with no discriminative power has an AUC of 0.5. The AUC of our model was 0.63, indicative of a poor predictive power. Cauchi *et al.* evaluated 15 polymorphisms associated with type 2 diabetes and found that a good predictive model would have an AUC of 0.98.<sup>20</sup> As in type 2 diabetes, LDDG is a polygenic disease caused by genetic and environmental factors. Additional genetic and clinical information is needed to obtain a highly predictive model.

## Conclusion

The present study demonstrated that the CILP and ASPN polymorphisms are independent genetic factors for LDDG in Japanese male collegiate athletes. We also raise the possibility that the relationship between these genetic factors and LDDG occurrence is a gender-dependent phenomenon.

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## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Ethical Approval

This study does not require institutional review board approval.

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