**Histological and molecular characterization of the femoral attachment of the human ligamentum capitis femoris**

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**Short title:** Ligamentum capitis femoris
Abstract

The ligamentum capitis femoris (LCF) has increased in clinical significance through the development of hip arthroscopy. The histological pathologies and molecular composition of the femoral attachment of the LCF and the degeneration caused by LCF disruption were investigated in the human hip joint. Twenty-four LCFs were retrieved at surgery for femoral neck fracture (age range: 63–87 years). In the “intact” (i.e. intact throughout its length, n=12) group, the attachment consisted of rich fibrocartilage. Fibrocartilage cells were present in the midsubstance. In contrast, the construction of the attachment in the “disrupted” (i.e. ligament no longer attached to the femoral head, n=12) group had disappeared. The attachment in the disrupted group was not labeled for type II collagen or aggrecan, while that in the intact group was labeled for types I, II and III collagen, chondroitin 4-sulfate, chondroitin 6-sulfate, aggrecan and versican. The percentage of single-stranded DNA-positive chondrocytes was significantly higher in the disrupted group than in the intact group. We conclude that the femoral attachment of the LCF has a characteristic fibrocartilaginous structure that is likely to adjust to the mechanical load, and suggest that its degeneration is advanced by disruption and should be regarded as a clinical pathology.

Keywords: enthesis; stability; stress deprivation; ligament rupture.
Introduction

The ligamentum capitis femoris (LCF) is an intracapsular ligament linking the head of the femur to the acetabulum. Although contributions of the LCF to the vascular supply for a small subfoveal zone of the femoral head, joint stability, proprioception, nociception and synovial fluid spreading have been suggested (Crook, 1965; Wertheimer & Fernandes, 1971; Chen et al., 1996; Gray & Villar, 1997; Byrd & Jones, 2004; Martin et al., 2012), they have not been clarified completely.

Although the LCF was previously thought to be nonessential during growth, LCF rupture has been reported to cause hip pain (Gray & Villar, 1997; Byrd & Jones, 2004; Bardakos & Villar, 2009; Haviv & O'Donell, 2011). Moreover, recognition of the LCF has markedly changed through the development of hip arthroscopy (Rao et al., 2001; Byrd, 2003; Byrd & Jones, 2004; Griffiths & Khanduja, 2012). Gray & Villar (1997) classified the causes of LCF rupture as trauma and degeneration. Boster et al. (2011) reported that 51% of cases had an LCF tear in 558 hip arthroscopic surgeries. Byrd (2003) reported that LCF rupture was the third most common problem encountered in athletes with hip pain. Philippon et al. (2007) detected ligamentum teres tears in 65% of 45 athletes with femoroacetabular impingement.

Unfortunately, arthroscopic debridement is currently the standard treatment for an LCF rupture (Haviv & O'Donell, 2011). However, a few authors have claimed the significance of keeping this ligament to prevent the exacerbation of osteoarthritis and tried new procedures such as its re-suture or reconstruction (Wenger et al., 2008; Bardakos & Villar, 2009; Simpson et al., 2011; Philippon et al., 2012).

Despite the increased attention to the LCF, the LCF anatomy was reported in previous studies (Crook, 1965; Wertheimer & Fernandes, 1971), but its histopathology and molecular composition are not fully understood.

In disorders of a tendon/ligament, the attachment to bone (i.e. the enthesis) is
indispensable. The enthesis is a characteristic structure that provides a connection between a soft tendon/ligament tissue and a hard bone tissue. It mostly provides fibrocartilage to adapt to resistance of large mechanical loads from a tendon/ligament (Schneider, 1956; Benjamin et al., 1986; Woo & Buckwalter, 1987). Moreover, since fibrocartilage has a smaller vascular supply and consequently a poorer potential for repair (Benjamin et al., 1986; Benjamin & Ralphs, 1998), similar to hyaline cartilage, assessment of the enthesis is greatly useful when the pathologies and functions of a tendon/ligament are discussed (Kumai et al., 2002; Benjamin et al., 2004). Although degenerative changes are well-known to be implicated in the pathologies of a tendon/ligament and are more conspicuous in a disrupted ligament (Kannus & Jozsa, 1991; Jozsa & Kannus, 1997), no authors have evaluated the variation in the attachment in this condition. Regarding ligaments of the hip joint, Milz et al. (2001) adequately demonstrated the molecular composition of the transverse ligament of the acetabulum, which is another attachment of the LCF. With regard to the femoral attachment of the LCF, it has only been described in one previous report (Ippliito et al., 1980), and we still have insufficient understanding of this feature of the LCF. We consider that such evaluations would be of great help to clarify the characteristics of the LCF and its pathology. The purpose of this study was to investigate the histological and molecular characterizations of the femoral attachment of the LCF. In addition, we compared normal and disrupted LCFs to evaluate the histopathological differences that accompany the disruption of this ligament.

Materials and methods

Subjects

Twenty-four subjects were selected from consecutive patients who underwent prosthetic replacement of the femoral head for a femoral neck fracture by three surgeons between 2001 and 2005. The subjects comprised 12 males and 12 females with an age range of 63–87 years.
(mean ± SD: 80.3±5.64 years). The fracture was on the right side in 14 patients and the left side in 10 patients. The time durations for the surgeries ranged from 48 to 75 minutes (mean ± SD: 61±8.14 minutes). In all 24 patients, it was confirmed by interview that there was no previous history of other hip diseases. Patients with severe coxarthrosis and radiologically visible femoral head necrosis were excluded from the study because we would be unable to distinguish the femoral attachment of the LCF in their hip joints. In this study, severe coxarthrosis was defined as prominent destruction of the bone structure on radiographs that was generally classified as grade four according to Kellgren & Lawrence (1957). The experimental protocol was performed according to the guidelines of the Ethical Committee of Nara Medical University. Informed consent was obtained from all the patients before the operation.

We prepared two different specimens to evaluate the degenerative changes of the attachment after ligament disruption. The “intact” group had an attachment where the LCF was continuous from the acetabulum to the femoral head. The “disrupted” group had an attachment where there was a complete tear, but where the stubs of the ligament were still visible at each insertion. The disruption was easily judged not to be fresh because bleeding in the stubs was macroscopically and microscopically invisible. The sample was removed from the femoral head as a bone-ligament complex, including the attachment of the LCF, the fovea capitis femoris (FCF) and the adjacent articular cartilage. The intact group comprised seven men and five women (age range: 70–87 years; mean age ± SD: 80.1±5.52 years) and the disrupted group comprised five men and seven women (age range: 63–87 years; mean age ± SD: 80.5±6.23 years). The age difference between the two groups was not significant (P=0.86, 5% value=0.27 by the Kolmogorov–Smirnov test).

Histology

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The tissue samples were fixed in 10% neutral-buffered formalin for at least 2 days, decalcified in EDTA and embedded in 58°C paraffin wax. Sections were cut at 8-μm thickness running along the ligament, and then stained with alcian blue, hematoxylin and eosin, Masson's trichrome or toluidine blue (Fig. 1).

Morphometry
The amplitude of the mechanical load in the attachment was assessed using previously described methods (Evans et al., 1990, 1991). The thicknesses of the uncalcified fibrocartilage (UF), calcified fibrocartilage (CF) and subchondral bone were measured at 15 sites in each sample. The irregularity of the interface between the calcified fibrocartilage and bone (CFB) was assessed by expressing the length of the interface relative to the length of the enthesis (CFB:E ratio). All measurements were made three times on a computer connected to a BX41 microscope (Olympus, Tokyo, Japan) using an Image Filing System (Flovel Co., Tokyo, Japan) by two authors (Shinohara, MD, and Kumai, MD, PhD) who performed the measurements according to the methods described in a previous study (Kumai et al., 2002). The two authors had received sufficient training necessary for the present study, and had conducted experiments involving similar measurements and morphometry analyses to those in this study for more than 10 years. Repeatability statistics were obtained to confirm the accuracy of all the data.

Immunohistochemistry
Sections were labeled with antibodies against collagens (types I, II and III), glycosaminoglycans (chondroitin 4-sulfate and chondroitin 6-sulfate), proteoglycans (aggrecan and versican) and single-stranded DNA (ssDNA). The enzyme pretreatments and dilutions of the antibodies are listed in Table 1. An anti-goat immunoglobulin was used for
detecting types I and III collagens. Antibody binding was detected with an LASB2/HRP Streptavidin/biotin/peroxidase Kit (Dako Japan Co., Tokyo, Japan). The localizations of the bound antibodies for the collagens, glycosaminoglycans and proteoglycans were observed by microscopy. In the sections labeled with the anti-ssDNA antibody, the percentages of ssDNA-positive chondrocytes were calculated. We performed comparisons between the intact and disrupted groups for the above evaluations. The tissue preparations and evaluations used in this study were based on our previous paper (Kumai et al., 2002).

Statistical Analysis
All of the data were statistically analyzed using StatMate III software (ATMS Co., Tokyo, Japan). We evaluated the data using a non-parametric equivalent of the t-test (Mann–Whitney test) because they did not exhibit a normal distribution when evaluated by the Kolmogorov–Smirnov test. For the data shown in Table 3, we analyzed the significance of differences by the chi-square test. Differences were considered to be statistically significant for values of $P \leq 0.05$.

Results
Histology
The LCF was attached to the margin of the FCF (enthesis margin), while the opposite site was composed of hyaline articular cartilage (AC margin) (Fig. 1). The attachment of the LCF gradually made a transition from fibrocartilage to hyaline cartilage. A bony prominence was microscopically observed in the deeper part of the attachment of the LCF in all cases, and also noted at the AC margin in 67% of cases in the intact group. In all samples of the intact group, the typical four zones were recognized in the femoral attachment of the LCF, namely fibrous tissue, UF, CF and bone (Fig. 2a). A tidemark separated the UF and CF. The CFB interface
was highly irregular and the subchondral bone was thick (Fig. 2b). Pathologies such as the formation of multiple tidemarks in 75% of all cases and longitudinal fissures in 58% of all cases were observed (Fig. 2c). In 75% of all cases, both fibroblasts and fibrocartilage cells were present in the mids substance (Fig. 2d), which was covered with a rich vascular synovium. The latter cells were surrounded by a characteristic matrix that showed metachromatic staining with toluidine blue. In 25% of all cases, hypoxic degeneration, which included fragmentation and irregular alignment of the collagen fibers, was also noted in the ligament (Fig. 2e). In 92% of all cases, blood vessels in the connective tissue where the LCF was covered intruded into the bone marrow through the subchondral bone of the FCF.

In all samples of the disrupted group, a bony prominence was microscopically observed at each margin of the FCF. However, the four fibrocartilaginous zones were less striking (Fig. 3a). The subchondral bone was thinner and the blood vessels had disappeared. The CFB interface showed less complexity. Lipomatous degeneration, which was not observed in the intact group, was prominent in the remnant attached to the enthesis margin in 67% of all cases. These areas included fewer blood vessels in the synovial tissue around the remnant. Collagen fibers were thin and fragile, and fat tissue infiltrated into the extracellular matrix (Fig. 3b). In 50% of all cases, the degenerative changes at the AC margin of the FCF had proceeded more deeply and extensively than those in the intact group (Fig. 3c).

Morphometry
The morphometric data are summarized in Table 2. The CFB:E ratio was significantly ($P=0.002$, 5% value=0.35 by the Kolmogorov–Smirnov test) greater in the intact group than in the disrupted group. The thicknesses of the fibrocartilage and subchondral bone were also significantly ($P=0.008$, 5% value=0.35 by the Kolmogorov–Smirnov test and $P=0.002$, 5% value=0.35 by the Kolmogorov–Smirnov test, respectively) greater in the intact group than in
the disrupted group.

Immunohistochemistry

In the intact group, types I and III collagen were present throughout the ligament and bone, and the latter was especially obvious in the synovial subintima around the ligament and FCF. Versican was more typically present in the midsubstance than in the attachment. Type II collagen was strongly detected in the fibrocartilage at the attachment (Fig. 4a), as were chondroitin 4-sulfate, chondroitin 6-sulfate and aggrecan (Table 3).

In the disrupted group, the most striking difference at the attachment from the intact group was the absence of type II collagen (Fig. 4b), chondroitin 4-sulfate, chondroitin 6-sulfate and aggrecan (Table 3).

The mean percentage (± SD) of ssDNA-positive chondrocytes at the attachment was 46.3±9.12% in the disrupted group, and significantly ($P=0.002$, 5% value=0.35 by the Kolmogorov–Smirnov test) higher than the value of 9.71±1.95% in the intact group (Fig. 4c, d).

Discussion

A tear of the LCF has come to be recognized as a common cause of hip pain through the development of hip arthroscopy (Rao et al., 2001; Byrd, 2003; Byrd & Jones, 2004). Despite the greater attention, our understanding of this ligament is still insufficient. Although the attachment is generally considered important while debating the pathology of a tendon/ligament, there are surprisingly few reports describing the attachment of the LCF (Ippolito et al., 1980; Bland & Ashhurst, 2001; Milz et al., 2001).

The fibrocartilage present at the femoral attachment of the LCF in the intact group suggests that the attachment takes some of the mechanical load. Previous studies described
that UF is the result of an adaptation to resist compression by bending of the ligament, while CF plays an anchoring role to the subchondral bone (i.e. the CFB interface) to protect against tensile and shear stresses (Schneider, 1956; Benjamin et al., 1986; Evans et al., 1990, 1991). Moreover, this finding is in agreement with a previous study by Ippolito et al. (1980). They described that the femoral attachment of the LCF in two normal specimens from children had an area of fibrocartilaginous metaplasia that was alcian blue-positive, and suggested that this finding arose because the region was subjected to stretching and compressing forces.

In addition, the immunohistochemical profile for the intact group detecting type II collagen, chondroitin 6-sulfate and aggrecan clearly showed that there was some mechanical stress loading on the femoral attachment of the LCF, similar to previous studies (Benjamin & Ralphs, 1998; Waggett et al., 1998; Malaviya et al., 2000; Milz et al., 2001). In contrast, a previous study on the molecular compositions of the femoral attachment of the LCF in rabbits found that types I and IV collagens were strongly bound, while type III collagen was weakly bound and type II collagen was not bound (Bland & Ashhurst, 2001). This result for type II collagen was different from our finding. However, their samples were ligaments of rabbits that walked on four legs from the newborn stage to a young age, while our samples were ligaments from elderly humans. Therefore, it is difficult to directly compare their study with our study. In our case, the mechanical load might make the LCF draw the femoral head toward the acetabulum, in the “ball and chain control” manner described by Michaels & Matles (1970) or the “sling-like” structure described by Kivlan et al. (2012) to describe its role as a stabilizer.

Some previous animal experiments have been performed to address the theory that the LCF prevents the hip joint from dislocating. Smith et al. (1957) found that detachment of the LCF in young dogs significantly increased the incidence of hip dislocation. Wenger et al. (2007) showed that the stiffness of the LCF was similar to that of the anterior cruciate
ligament of the knee (ACL) by examining immature porcine hips. They suggested the possibility that the LCF acts as a stabilizer of the hip joint to prevent dislocation, similar to the ACL. Our findings suggest that the LCF may play a role, to some extent, as a stabilizer of the hip joint, similar to the way in which the bone structure, labrum and extracapsular ligaments function.

Although the midsubstance of a normal tendon/ligament has no fibrocartilage cells (Benjamin et al., 1986; Vogel, 1995; Benjamin & Ralphs, 1998), our series showed fibrocartilage cells in the midsubstance of the LCF in the intact group. Milz et al. (2001) described that the femoral head is sometimes considered to contact the acetabulum even in a normal hip. Our findings suggest that the LCF had undertaken repetitive mechanical stress and that fibrocartilage cells were created in the midsubstance. This change has been thought to involve denaturation of the ligament, like hypoxic degeneration, in some previous studies, and is a serious factor that causes spontaneous wear and tear of the tendon/ligament (Kannus & Jozsa, 1991; Jozsa & Kannus, 1997; Sampatchalit et al., 2009).

The observations in the disrupted group, in which fibrocartilage was inconspicuous at the attachment, suggest that the degeneration would be advanced by a ligament tear. Based on the well-known fact that a mechanical load is indispensable for the maintenance of fibrocartilage (Benjamin et al., 1992; Benjamin & Ralphs, 1998), the disappearance of fibrocartilage would represent loss of the mechanical load. This means that the disruption of the ligament caused loss of the mechanical load and vascular supply, and finally led to arthritic changes. Our immunohistochemical profiles in the disrupted group also supported these irreversible changes. To our knowledge, this might be the first demonstration of the changes in the extracellular matrix of the attachment caused by a ligament rupture. The prominent osteoarthritic changes in the articular cartilage that we frequently observed in the disrupted group could be explained by the characteristics of the hip joint. Owing to its high bone
congruency, a small gap can easily lead to degenerative progression (Eckstein et al., 1997; Hurwitz et al., 2001). Rao et al. (2001) suggested that a complete LCF rupture caused early degenerative arthritis. Consequently, ligament rupture could be a trigger for incongruity in the hip joint, and this incongruity would lead to arthritic changes.

In addition, the low value of the CFB:E ratio in the disrupted group was an interesting difference. This finding is in line with Gao & Messner (1996), who showed that the CFB:E ratio is dependent on the quantity of the mechanical load to the enthesis by examining the rabbit knee joint. Our findings are also thought to indicate a decrease in the mechanical load after the ligament rupture.

In the remnant of the LCF from the disrupted group, fat tissue was dominantly present instead of fibrocartilage cells and collagen fibers, and the blood vessels had disappeared. This is comparable to the fatty degeneration described by Ippolito et al. (1980) and Kannus & Jozsa (1991). They considered that there were four main degenerative types in a tendon/ligament that were respectively formed in response to different stimuli. Among these, fatty degeneration was defined to decrease the tensile force and lead to ligament rupture. However, our result that fatty degeneration was not observed in the intact group could mean that this degeneration developed after the rupture, rather than being a change before the rupture.

The greater percentage of ssDNA-positive cells in the disrupted group suggested that apoptosis caused the advanced degeneration in the ruptured LCF, which was no longer under mechanical loading. This is in line with Hattori et al. (2007) and Mutsuzaki et al. (2007). The former tracked changes in the incidence of apoptotic cells at the tibial insertion of human ACLs at different time points after rupture. The latter compared the tibial cranial cruciate ligament attachment of ruptured and non-ruptured ligaments, and found that the number of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells was significantly larger in the ruptured group than in the
non-ruptured group. Such studies suggest the possibility that the stress deprivation caused by
ligament rupture may induce apoptosis in the fibrocartilage cells at the insertion of the
ligament.

The main limitation of this study was that we could not investigate the LCF in younger
individuals. To evaluate the molecular composition, we obtained the femoral heads from fresh
human specimens that were discarded as waste after prosthetic replacements for femoral neck
fractures. This meant that almost all of the samples were restricted to those from elderly
people. In the present study, it is possible that the patients had some kind of age-related
osteoarthritis in their hips. Since we only excluded patients with osteoarthritis of grade four
according to Kellgren & Lawrence (1957), our subjects probably included patients with
moderate osteoarthritis of grade two or three. Therefore, it is unclear whether our
degenerative findings occurred before or after the disruption. Consequently, it remains unclear
whether the LCF tears in the present study were of the type I (complete traumatic) or type III
(complete degenerative) variety, according to the classification by Gray & Villar (1997).
Furthermore, assuming our subjects had no partial ruptures or avascular necrosis at the early
stage, we may need to evaluate patients with a partial rupture or avascular necrosis. However,
although assessments and comparisons of subjects including younger people would be more
ideal, it is difficult for us to obtain such samples, especially from younger people. In addition,
many studies have already reported that tendon/ligament injuries occur on the basis of
suggested that disruption of the LCF in athletes may be the result of trauma or degeneration,
or a combination of the two. Some authors reported that a spontaneous tear of the LCF
without trauma (e.g., joint dislocation or fracture) is occasionally observed during
arthroscopic surgeries (Rao et al., 2001; Byrd, 2003; Bardakos & Villar, 2009). Consequently,
we may be able to speculate about the pathologies of younger people or athletes based on the
pathologies of the elderly people examined in this study. Although there may be a lack of
repeatability statistics with regard to the measurements in the present study, we believe that
our data warrant acknowledgment because our methodology was performed in accordance
with previous studies (Evans et al., 1990, 1991; Kumai et al., 2002).

Perspectives

We have demonstrated that a fibrocartilaginous structure and degenerative pathologies are
present at the femoral attachment of the LCF, similar to the case for other tendons/ligaments.
These findings show that this insertion is subjected to mechanical force and suggest that the
LCF may function as a stabilizer to maintain joint congruity. The characterization of the
histological and molecular differences between the intact and disrupted LCFs provides us
with missing basic knowledge that could help in the future understanding of clinical
pathologies in the hip joint.

Acknowledgements

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regarding this manuscript. This study was not supported by any financial grants or funding.
The authors declare that they have no competing interests.
References


### Table 1. Primary antibodies used for immunohistochemical labeling

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<th>Antigen</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Enzyme pretreatment</th>
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<td>Pepsin (0.4%)</td>
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<td>Collagen II</td>
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<td>Chondroitinase (0.2IU/ml)</td>
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<tr>
<td>Collagen III</td>
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<td>Chondroitin 4-sulfate</td>
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<td>Hyaluronidase (1.5IU/ml)</td>
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<td>Single-stranded DNA</td>
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<td>1:100</td>
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Table 2. Morphometric data comparisons between the intact and disrupted groups

<table>
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<tr>
<th></th>
<th>CFB length (mm)</th>
<th>CFB:E ratio</th>
<th>Fibrocartilage thickness (mm)</th>
<th>Subchondral bone thickness (mm)</th>
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<td><strong>Intact (n=12)</strong></td>
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<tr>
<td>Mean±SD</td>
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<td>1.79±0.27</td>
<td>1.04±0.40</td>
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<td><strong>Disrupted (n=12)</strong></td>
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<tr>
<td>Mean±SD</td>
<td>3.00±0.18</td>
<td>1.13±0.05</td>
<td>0.30±0.46</td>
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<td><strong>P value</strong></td>
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</table>

CFB, interface between the zone of calcified fibrocartilage and bone; E, enthesis; Fibrocartilage thickness, includes uncalcified and calcified fibrocartilage.
Table 3. Immunohistochemical labeling in the intact and disrupted groups. The numbers of positive cases are shown for a total of 12 patients in the intact group and 12 patients in the disrupted group. Statistical comparisons between the two groups were made by the chi-square test.

<table>
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<tr>
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<th>Disrupted</th>
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<th>P value</th>
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<td>Aggrecan</td>
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<td>0</td>
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Table 3. Immunohistochemical labeling in the intact and disrupted groups. The numbers of positive cases are shown for a total of 12 patients in the intact group and 12 patients in the disrupted group. Statistical comparisons between the two groups were made by the chi-square test.
Fig. 1. Section stained with toluidine blue in the intact group. A bony prominence (arrow) is microscopically identified at the enthesis margin. The bony prominence is 5 mm in height and 7 mm in length. L, ligamentum capitis femoris; B, bone; F, fovea capitis femoris; AC, articular cartilage.

82x38mm (300 x 300 DPI)
Fig. 2. Histological findings in the intact group. a, The femoral attachment is composed of fibrocartilaginous tissue in which a tidemark separates CF from UF. Stain: toluidine blue. b, The CF is thick, and strongly interlocks bone like a jigsaw (yellow line). Stain: toluidine blue. c, A longitudinal fissure (arrow) is observed. Stain: toluidine blue. d, Fibrocartilage cells are occasionally present. Stain: hematoxylin and eosin with alcian blue. e, The ligament is denatured (red color). The collagen fibers are thin and rough. Stain: Masson trichrome. UF, uncalcified fibrocartilage; TM, tidemark; CF, calcified fibrocartilage; B, bone.

162x240mm (300 x 300 DPI)
Fig. 3. Histological findings in the disrupted group. a, The four-zone structure of the enthesis is indistinguishable. Stain: hematoxylin and eosin with alcian blue. b, Fat tissue is often present, and the collagen fibers have disintegrated in the ligament. Stain: hematoxylin and eosin with alcian blue. c, Osteoarthritic changes are present at the AC margin of the FCF. Stain: toluidine blue. B, bone; AC, articular cartilage.

162x162mm (300 x 300 DPI)
Fig. 4. Immunochemical staining at the enthesis in both groups. 
a, Type II collagen is detected in the intact group. 
b, In the disrupted group, type II collagen is not detected. 
c, Few cells are labeled for ssDNA in the intact group. 
d, Many apoptotic cells are detected in the disrupted group.

162x162mm (300 x 300 DPI)