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Expression of chondro-osteogenic BMPs in clinical samples of patellar tendinopathy

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Abstract
Purpose The pathogenesis of patellar tendinopathy remains unclear. Expression of BMP-2/-4/-7 was reported in an ossified failed tendon healing animal model of patellar tendinopathy. This study aimed to investigate the expression of these chondro-osteogenic BMPs in clinical samples of patellar tendinopathy.
Methods Patellar tendon samples were collected from 16 consecutive patients with patellar tendinopathy and 16 consecutive controls undergoing anterior cruciate ligament reconstruction with bone-patellar tendon-bone autograft in the authors' hospital after getting their consent. The expression of BMP-2/-4/-7 was examined in all samples using immunohistochemistry. Ossification observed in two tendinopathy samples was characterized by histology, alizarin red S staining, alcian blue staining, TRAP staining and immunohistochemical staining of Sox9, osteopontin (OPN) and osteocalcin (OCN).
Results Regions of hypo- and hyper-cellularity and vascularity, with loss of crimp structure of collagen matrix, were observed in patellar tendinopathy samples. Round cells and in some cases, cells with typical chondrocyte phenotype were observed. For the ossified tendinopathy samples with positive alizarin red S staining, OPN-positive and Sox9-positive chondrocyte-like cells in alcian blue-stained extracellular matrix, OCN-positive osteoblast-like cells and TRAP-positive multi-nucleated cells were observed around the ossified deposits. No expression of BMP-2/-4/-7 was observed in healthy patellar tendons. However, the expression of BMP-2/-4/-7 was observed in all patellar tendinopathy samples with or without ossification.
Conclusions Clinical samples of patellar tendinopathy showed ectopic expression of BMP-2/-4/-7. This was not evident in control samples from healthy patellar tendons.
Level of evidence Prognostic studies, Level III.

Keywords Patellar tendinopathy · Endochondral ossification · Bone morphogenetic protein 2 · Bone morphogenetic protein 4 · Bone morphogenetic protein 7 · Pathogenesis

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Introduction

Patellar tendinopathy is a tendon disorder characterized by activity-related, anterior knee pain and local tenderness [29]. It is a common clinical problem in athletes especially in those

participating in sports characterized by high demands on speed and power of the leg extensors [17]. Despite the high morbidity of patellar tendinopathy, evidence-based management for this condition is lacking [28]. Better understanding of the pathogenesis of patellar tendinopathy is essential both for its prevention and for its treatment.

Histopathologically, patellar tendinopathy is characterized by progressive tissue degeneration, yet with the absence of inflammatory cells [8, 11]. Failed tendon healing due to the accumulation of micro-injuries in overused patellar tendon has been suggested to contribute to the pathogenesis of patellar tendinopathy [14]. Tissue metaplasia, including hyaline metaplasia [31], fibrocartilaginous metaplasia [7, 12], tendolipomatosis [10] and bony metaplasia [6, 7, 10, 11], were reported within the patellar tendon tissues in patients with patellar tendinopathy.

In a previous study, chondrocyte phenotype and ossified deposits were observed in a collagenase-induced failed tendon healing animal model of tendinopathy [19]. The expression of bone morphogenetic protein (BMP) -2, -4 and -7 in the healing tendon fibroblast-like cells and later around the chondrocyte-like cells and ossified deposits was also reported in the same animal model [30], suggesting that these chondro-osteogenic BMPs might contribute to the pathogenesis. Recently, another study reported that repetitive cyclic loading increased the expression of BMP-2 in rat tendon–derived stem cells (rTDSCs) and BMP-2 could induce the osteogenic and chondrogenic differentiation of rTDSCs in vitro [23, unpublished results]. These observations suggested that ectopic expression of these chondro-osteogenic BMPs during change of tendon loading, such as overuse, might induce the formation of ectopic bone/cartilage, promote structural degeneration and resulted in failed tendon healing [18]. We hypothesized that chondro-osteogenic BMP-2, BMP-4 and BMP-7 could be observed in clinical samples of patellar tendinopathy, the results of which would further support the roles of chondro-osteogenic BMPs in the pathogenesis. This study therefore aimed to investigate the expression of these chondro-osteogenic BMPs in clinical samples of patellar tendinopathy.

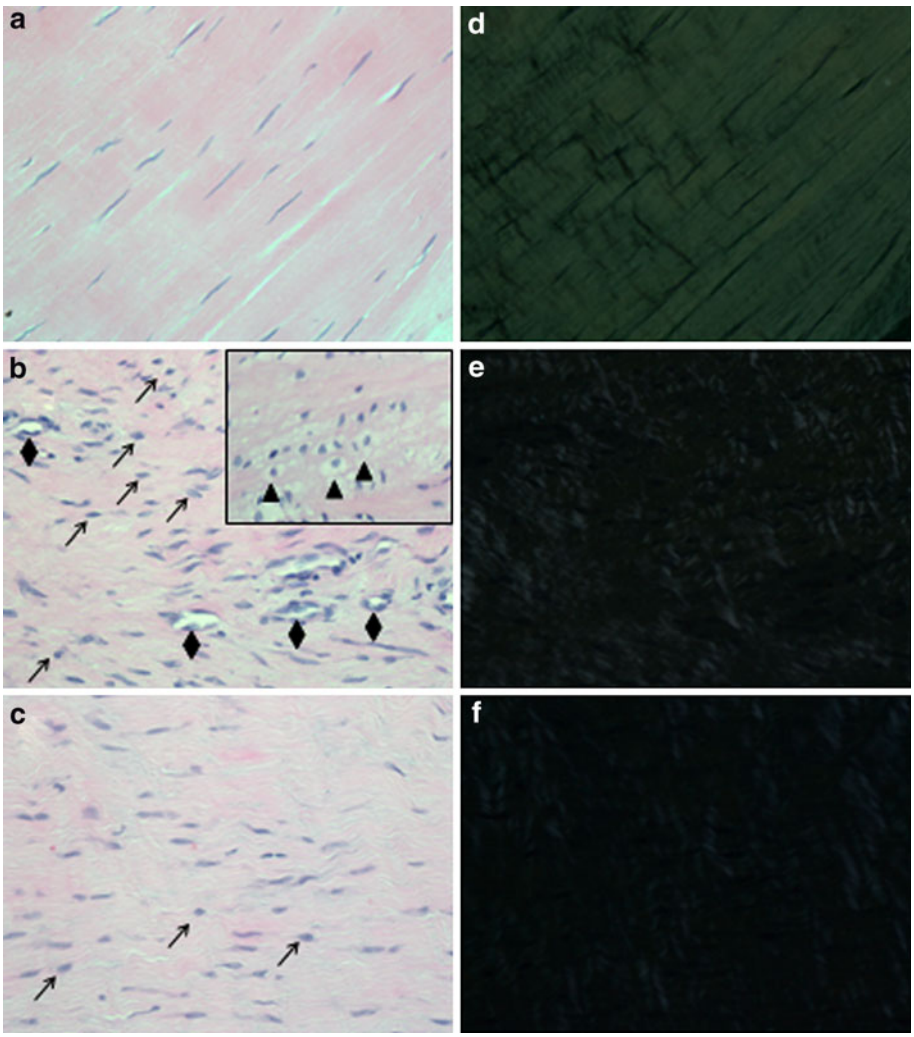

Fig. 1 Photomicrographs showing the histopathology of the un-ossified patellar tendinopathy samples (**b–c**, **e–f**). Samples from the healthy patellar tendons (**a**, **d**) were used as controls. **a–c** H&E staining; **d–f** polarizing microscopy. *Arrow* healing tendon cells; *filled triangle* chondrocyte-like cells; *filled diamond* blood vessels; magnification: ×400

Materials and methods

The study was approved by the Clinical Research Ethics Committee of the authors' institution. All subjects were recruited from the Prince of Wales Hospital, Hong Kong SAR, China, after obtaining their consent. Sixteen consecutive patients diagnosed as having patellar tendinopathy, 14 men and 2 women, with an average age of 30 ± 8 years, were included in the current study. All subjects fulfilled the diagnostic criteria of patellar tendinopathy, with well-defined clinical features, and were verified by ultrasound or magnetic resonance imaging (MRI). They all had more than 6 months of ineffective non-operative treatment including physiotherapeutic modalities. Sixteen consecutive control subjects, 12 men and 4 women, with an average age of 25 ± 7 years, were patients undergoing anterior cruciate ligament (ACL) reconstruction with bone-patellar tendon-bone (BPTP) autograft. The control subjects had no previous history or clinical signs of patellar tendon injury and tendinopathy. There was no significant difference in age and gender between the two groups ($P = 0.077$ and 0.654, respectively).

Guided by clinical findings and ultrasound or MRI scans, the pathological patellar tendon tissue was identified in the subjects with patellar tendinopathy. The macroscopically abnormal region of tendon was then removed with a surgical blade, and a 0.5×1.5 cm piece of tissue was preserved for analysis. In the control subjects, a 0.2×0.5 cm piece of healthy patellar tendon was removed from the remnant of the BPTP autograft during ACL reconstruction.

The samples were used for general histology and immunohistochemical staining of BMP-2, -4 and -7. Two tendinopathy samples with ossified deposits were incidentally identified. The ossified deposits in these two samples were additionally subjected to alizarin red S staining, alcian blue staining and immunohistochemical/histochemical staining of chondrocytic (Sox9), osteoblastic [osteocalcin (OCN), osteopontin (OPN)] and osteoclastic [tartrate-resistant acid phosphatase (TRAP)] markers. One healthy sample from the control group was used as control in this experiment for comparison.

General histology and immunohistochemistry

The patellar tendon was washed in phosphate buffer saline (PBS), fixed in buffered formalin and 70% ethanol, embedded in paraffin, cut longitudinally to 5-μm thick sections and mounted on 3-aminopropyl-triethoxy-silane (Sigma-Aldrich, St Louis, MO, USA)-coated slides. After deparaffination, the sections were stained with hematoxylin and eosin. Immunohistochemistry staining was done as described previously [19]. Primary antibodies against BMP-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-6895; 1:100), BMP-4 (Abcam, Cambridge, USA; ab39973; 1:100), BMP-7 (Abcam, Cambridge, USA; ab56023; 1:200), Sox9 (Santa Cruz Biotechnology, Santa Cruz, CA; sc-20095; 1:30), OPN (Novus Biological, LLC, USA; NB110-89062; 1:100) or OCN (Abcam, Cambridge, USA; ab13420; 1:100) were used. Donkey anti-goat- (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-2020; 1:100), goat anti-rabbit- (Chemicon, Temecula, CA; AP132P; 1:100) or goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (Millipore, Billerica, MA; AP124P; 1:100), together with 3,3′ diaminobenzidine tetrahydrochloride (DAKO, Glostrup, Denmark), were used for signal detection. Primary antibodies were replaced with blocking solution in the controls. The incubation times and conditions were strictly controlled. The sections from the control and tendinopathy groups were stained in the same batch. The sections were examined under light and polarized microscopies (DMRXA2 and DMRB, Leica Microsystems Wetzlar GmbH, Germany). The assessors were blinded to the grouping of the samples.

Alizarin red S staining assay

The sections were deparaffinized and rehydrated with xylene and graded ethanol. They were then stained with 0.5% alizarin red S (pH 4.1, Sigma, St. Louis, MO) for 30 min. Finally, the stained slides were dehydrated and mounted with *p*-xlyene-bis-pyridinium bromide (DPX, Sigma-Aldrich, St Louis, MO, USA). The ossified deposits appeared red.

Alcian blue staining assay

The sections were deparaffinized and rehydrated the same as above. They were incubated with 3% acetic acid (pH 2.5) for 3 min and then incubated with 1% alcian blue solution (alcian blue 8GX 1 g/3% acetic acid 100 ml, pH 2.5; Sigma-Aldrich, St Louis, MO, USA; A3157) for 30 min at room temperature. After washing, the slides were counterstained with 0.2% nuclear fast red solution, dehydrated and mounted with DPX. The accumulated glycosaminoglycans appeared blue, and the nuclei of the cells appeared reddish pink.

Tartrate-resistant acid phosphatase (TRAP) staining assay

The sections were deparaffinized and rehydrated the same as above. They were fixed in citrate/acetone solution, rinsed in deionized water and air-dried. The slides were then incubated with the TRAP staining solution for 1 h at 37°C in the dark according to the instruction of the acid

phosphatise, leukocyte (TRAP) kit (Sigma-Aldrich, St Louis, MO; 386A). After washing, the slides were stained in acid hematoxylin solution, washed, air-dried and mounted with nail polish. The acid phosphatase activity appeared as purple to dark red granules in the cytoplasm of the multi-nucleated cells.

Statistical analysis

Demographic information was presented as mean ± SD or frequency. The age and gender difference between the two groups was compared by 2-sample T test and Fisher's exact test, respectively, using SPSS analysis software (SPSS Inc, Chicago, IL, version 16.0). $P < 0.05$ was regarded as statistically significant. The histological and immunohistochemical data were reported qualitatively.

Results

Histopathology of the patellar tendinopathy samples

The cellularity and vascularity were low in the healthy patellar tendons (Fig. 1a). Tendon cells in slender shape were well-aligned within the tightly packed and longitudinally arranged collagen fibrils (Fig. 1a), with the typical collagen birefringence of tendon tissue (Fig. 1d).

The patellar tendinopathy samples showed characteristic histopathological changes including regions of hypercellularity and hypervascularity (Fig. 1b, arrows, filled diamond) as well as regions of hypocellularity and hypovascularity (Fig. 1c). The crimp structure of collagen matrix and the collagen birefringence was lost (Fig. 1e, f). The healing tendon cells became round (Fig. 1b, c, arrows). Cells separated from the pericellular matrix by lacunar space, resembling chondrocytes, were observed in 3 un-ossified samples (3/14) (Fig. 1b insert, filled triangle). Ossification was observed in two samples (2/16).

For these two ossified patellar tendinopathy samples, ossified deposits with marrow-like cells (Fig. 2d, g, OR, open square), as indicated by alizarin red S staining (Fig. 2e, h, OR), were observed. The cellularity and vascularity were high around the ossified regions (Fig. 2d, arrows, filled diamond). Glycosaminoglycans expression as indicated by alcian blue staining was observed around and in the ossified regions (Fig. 2f, i). Chondrocyte-like cells as indicated by morphology (Fig. 2g, filled triangle) and expression of Sox9 (Fig. 3i, filled triangle) were seen around and embedded inside the ossified regions. Interestingly, strong expression of Sox9 (Fig. 3e, i) was

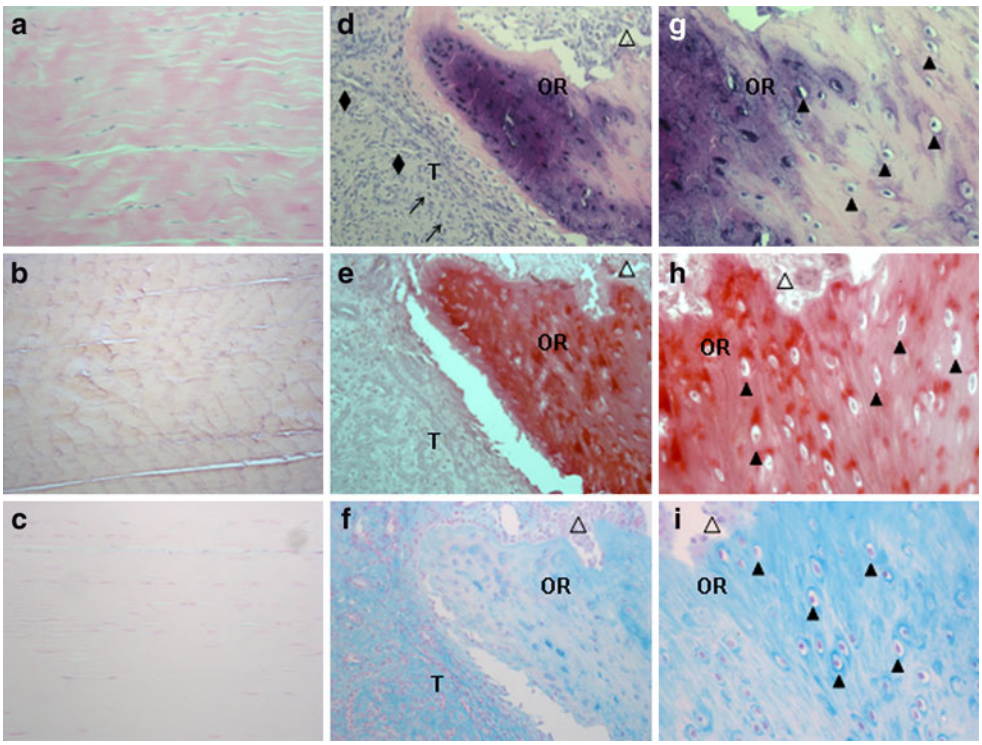

Fig. 2 Photomicrographs showing the histopathology of the ossified patellar tendinopathy samples (**d–i**). Samples from the healthy patellar tendons were used as controls (**a–c**). **a**, **d**, **g** H&E; **b**, **e**, **h** alizarin red S staining; **c**, **f**, **i** alcian blue staining. *Arrow* healing tendon cells; *filled triangle* chondrocyte-like cells; *open triangle* marrow-like cells; *filled diamond* blood vessels; *OR* ossified region, *T* tendon; magnification: ×200 (**a–f**); ×400 (**g–i**)

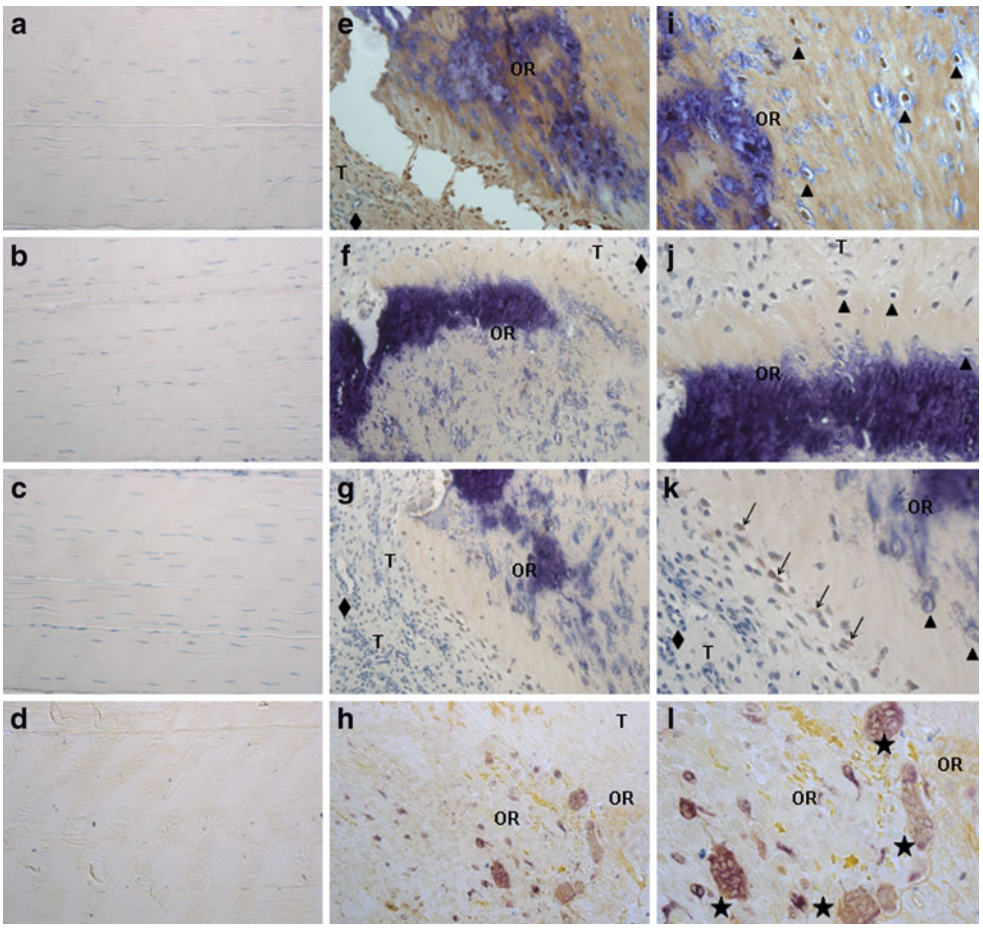

Fig. 3 Photomicrographs showing the immunohistochemical staining of chondrocytic markers, osteoblastic markers and histochemical staining of osteoclastic markers in the ossified patellar tendinopathy samples (**e–l**). A sample from the healthy patellar tendon was used as control (**a–d**). **a**, **e**, **i** Sox 9 expression; **b**, **f**, **j** OPN expression; **c**, **g**, **k** OCN expression; **d**, **h**, **l** TRAP expression. *Arrow* OCN-positive cells; *filled triangle* chondrocyte-like cells; *filled diamond* blood vessels; *OR* ossified region, *filled star* TRAP-positive multi-nucleated giant cells; *T* tendon; magnification: ×200 (**a–h**); ×400 (**i–l**)

additionally observed in the rounded healing tendon cells and matrix throughout the tendon. Weak expression of OPN (Fig. 3f, j) was also observed in the rounded healing tendon cells, chondrocyte-like cells and matrix around the ossified regions. Weak expression of OCN was observed in the matrix and some osteoblast-like cells around the ossified regions (Fig. 3g, k, arrows). TRAP-positive multi-nucleated cells were located around the ossified deposits (Fig. 3h, l, filled star). These changes were not observed in the healthy patellar tendon sample (Figs. 2a–c, 3a–d).

Immunohistochemical staining of BMP-2, -4 and -7

There was no expression of BMP-2/-4/-7 (Fig. 4a, d, g) in the healthy patellar tendons. Weak expression of BMP-2 were observed in the rounded healing tendon cells, chondrocyte-like cells and their matrices, while moderate expression of BMP-2 was noted in the blood vessels, in the un-ossified patellar tendinopathy samples (Fig. 4b). Strong expression of BMP-4/-7 (Fig. 4e, h) was observed in the rounded healing tendon cells, chondrocyte-like cells and their matrices as well as blood vessels, especially in the blood vessels, in the ossified patellar tendinopathy samples (Fig. 4e, h). There was stronger expression of BMP-2 (Fig. 4c) and weaker expression of BMP-4/-7 (Fig. 4f, i) in the chondrocyte-like cells in the two ossified patellar tendinopathy samples compared to their expression in the chondrocyte-like cells in the three un-ossified tendinopathy samples (Fig. 4b, e, h), respectively.

A summary of the immunohistochemical staining is shown in Table 1.

Discussion

The most important finding of this study was the ectopic expression of BMP-2, -4 and -7 in all the patellar tendinopathy samples without or with ossification. This was

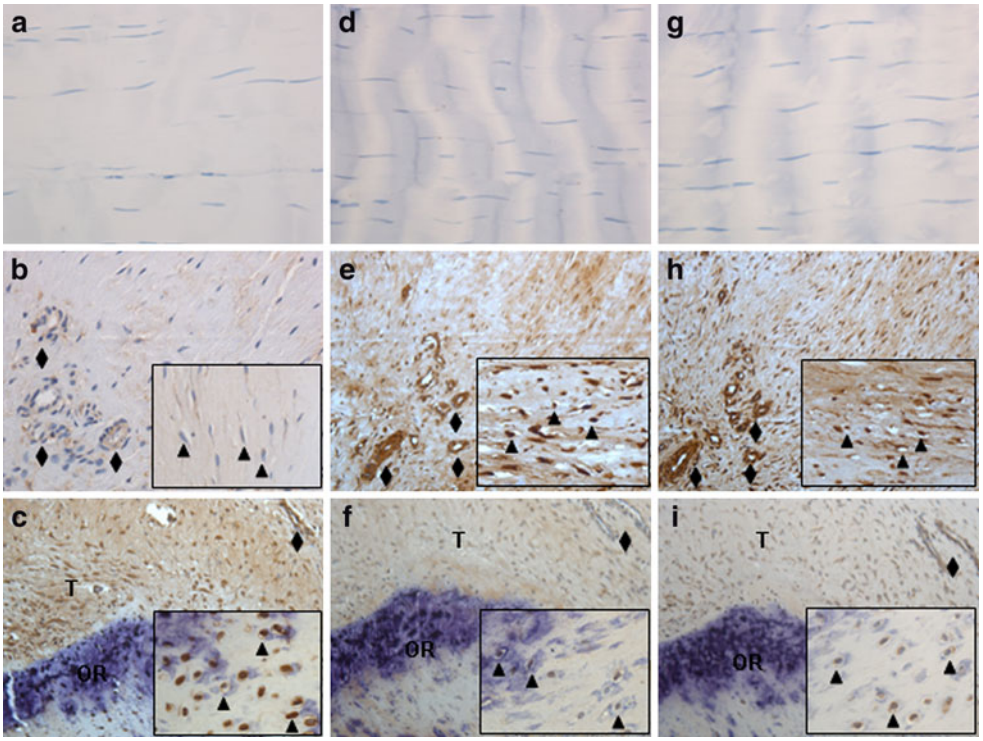

Fig. 4 Photomicrographs showing the immunohistochemical staining of BMP-2 (**a**–**c**), BMP-4 (**d**–**f**) and BMP-7 (**g**–**i**) in the healthy patellar tendon (**a**, **d**, **g**) and patellar tendinopathy samples without (**b**, **e**, **h**) and with (**c**, **f**, **i**) ossified deposits. *Filled triangle* chondrocyte-like cells; *filled diamond* blood vessels; *OR* ossified region, *T* tendon; magnification: ×400 (**a**, **d**, **g**, all *inserts*); ×200 (**b**, **c**, **e**, **f**, **h**, **i**)

not evident in the healthy controls. The results were consistent with the findings in the animal model of patellar tendinopathy [30]. Differential expression of BMP-2, -4 and -7 mRNA and protein has also been reported in the subacromial bursa tissue of patients with chronic degeneration of rotator cuff [21]. BMP-2, -4, -7 were expressed in the ossified matrix, chondrocytes, and fibroblasts near the ossified areas and have been suggested to contribute to ectopic ossification of spinal ligaments [15]. The expression of chondro-osteogenic BMPs in the rounded healing tendon cells and matrix, besides the chondrocyte-like cells and ossified deposits, suggested that they might induce the transformation of rounded healing tendon cells to chondrocytes/osteoblasts, stimulated erroneous cartilage/bone matrix deposition and promoted structural degeneration in patellar tendinopathy. An early in vitro study showed that BMP could induce transdifferentiation of tenocytes into chondrocytes [24]. BMP-2 could promote both osteogenic and chondrogenic differentiation of TDSCs in vitro [23, unpublished data]. When mouse tendon stem/progenitor cells (TSPCs) were treated with BMP-2 and then transplanted subcutaneously into immunocompromised mice, structures similar to osteotendinous junctions (termed entheses) were formed [3], which were similar to the ectopic chondro-ossified structures observed both in the tendinopathy animal model [19] and in the human samples reported in this study.

Of the sixteen patellar tendinopathy samples, ossification was observed in two samples. The results showed that the ossified deposits were formed by endochondral ossification. The histopathology of these ossified patellar tendinopathy samples was consistent with the previous findings in the ossified failed healing animal model of patellar tendinopathy [19] and ossified clinical samples of rotator cuff tendinopathy, Achilles tendinopathy and patellar tendinopathy [7, 27].

While ossification was only observed in two patellar tendinopathy samples, the healing tendon cells in all the patellar tendinopathy samples were round and typical chondrocyte-like cells were additionally observed in 3 samples. Sox9 and OPN were expressed in rounded healing tendon cells and matrix other than chondrocyte-like cells around the ossified deposits. These observations added further support to the hypothesis that erroneous differentiation of healing tendon cells to chondrocytes and/or osteoblasts might account for ectopic chondro-ossification and failed healing in tendinopathy [18, 22]. This hypothesis was also supported by other studies [2, 5, 21].

Ossified patellar tendinopathy is rare, and most patellar tendinopathies are presented without ossification [6, 9–11]. Four out of 82 spontaneously ruptured quadricep tendons

Table 1 Summary of immunohistochemical staining in human samples from the healthy patellar tendons, un-ossified and ossified patellar tendinopathy

Stain/markers/ BMPs	Healthy patellar tendons	Un-ossified patellar tendinopathy	Ossified patellar tendinopathy
Alizarin red S staining	−	NA	+ Ossified deposits
Alcin blue staining	−	NA	+ Ossified deposits
Sox9	−	NA	+ Chondrocyte-like cells around ossified deposits, rounded healing tendon cells
OPN	−	NA	Chondrocyte-like cells around ossified deposits, rounded healing tendon cells
OCN	−	NA	+ Osteoblast-like cells around ossified deposits
TRAP staining	−	NA	+ Multi-nucleated cells around ossified deposits
BMP-2	−	+ Rounded healing tendon cells, chondrocyte-like cells, blood vessels	+ Rounded healing tendon cells, chondrocyte-like cells, blood vessels
BMP-4	−	+ Rounded healing tendon cells, chondrocyte-like cells, blood vessels	+ Rounded healing tendon cells, chondrocyte-like cells, blood vessels
BMP-7	−	+ Rounded healing tendon cells, chondrocyte-like cells, blood vessels	+ Rounded healing tendon cells, chondrocyte-like cells, blood vessels

−, Null expression; +, positive expression; *NA* not tested

and patellar tendons showed calcified deposits while none of the age- and sex-matched controls ($n = 40$) showed calcification in histopathological analyses in a previous study [10]. Hyperechoic regions within the tendon, considered to be calcification, were seen in ultrasound imaging in 8 out of 28 tendons with patellar tendinopathy scheduled to undergo open tenotomy, and dystrophic ossification was present at histopathological examination in all eight cases [11]. In a retrospective study evaluating the outcome of open versus arthroscopic patellar tenotomy for the treatment of patellar tendinopathy, 9 out of 19 tendons in the open patellar tenotomy group and 9 out of 22 tendons in the arthroscopic patellar tenotomy group showed calcification in ultrasound imaging at the mean follow-up of 3.8 and 4.3 years, respectively, after surgery [6]. However, no calcification was observed in 24 knees with patellar tendonitis resistant to conservative therapy in plain radiography [9].

In this study, chondrocyte-like cells were observed in three clinical samples of un-ossified patellar tendinopathy. The ectopic expression of chondro-osteogenic BMPs in the un-ossified patellar tendinopathy samples suggested that they might promote chondrogenesis in patellar tendinopathy. The osteogenic or chondrogenic effects of BMP-2, -4 and -7 might depend on the combination of different types of BMP, local concentration and duration [13].

Besides ectopic chondro-osteogenesis, BMPs might have additional function in mediating neurogenic pain in the disease process. Sprouting of nerve fibers and expression of substance P (SP) and calcitonin gene-related peptide (CGRP) were suggested to be the mediators of neurogenic inflammation and pain in tendinopathy [16, 20]. BMPs and activin, both belong to the TGF-beta family of proteins, increased the expression of SP and CGRP in dorsal root ganglia (DRG) neurons in vitro [1, 4].

Both mechanical and biological factors might contribute to the ectopic expression of BMPs. It was known that the expression of BMP-2 and -7 were sensitive to mechanical load [26]. Changes in the extracellular matrix (ECM) composition might also modulate the effects of BMPs [3, 25].

This study is not without limitation. First, the sample size was small. Although stronger expression of BMP-2 and weaker expression of BMP-4/-7 were observed in the chondrocyte-like cells in two ossified patellar tendinopathy samples compared to the chondrocyte-like cells in three un-ossified tendinopathy samples, no statistical analysis could be done to confirm the observation due to small sample

size. More clinical samples are needed to confirm the observation. No causal relationship of the expression of BMP-2/-4/-7 and tendinopathy could be drawn. This study was mainly descriptive. However, no BMP-2/-4/-7 was observed in the healthy patellar tendon samples and hence the conclusion was confirmed. All the healthy subjects and patients with patellar tendinopathy were recruited from one hospital and hence the results might not be representative of all patients with patellar tendinopathy. In addition, only tendinopathic cases at the patella tendon were included in this study, hence the results could not be generalized to other types of tendinopathy. Whether there is also increased expression of BMPs in other types of tendinopathy needs further study. No standardized knee score was not performed for the patients with patellar tendinopathy in this study, and this might affect the generalization of the research findings. However, all the subjects with patellar tendinopathy had more than 6 months of ineffective non-operative treatment that might give some indications of the severity of the problem. The documentation of knee score would provide more information about the relationship of severity of the disorder and the expression of BMPs.

As the expression of BMP-2, BMP-4 and BMP-7 was increased which might contribute to the pathogenesis of patellar tendinopathy, strategies that inhibit the expression of BMPs might inhibit ectopic chondro-osteogenesis and promote tendon healing of patellar tendinopathy. Future study is required to understand the effectiveness of this strategy.

Conclusion

In conclusion, clinical samples of patellar tendinopathy showed ectopic expression of BMP-2, BMP-4 and BMP-7. This was not evident in control samples from healthy patellar tendons.

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