Paediatric knee anterolateral capsule does not contain a distinct ligament: analysis of histology, immunohistochemistry and gene expression

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ABSTRACT

Objectives The presence of a discrete ligament within the knee anterolateral capsule (ALC) is controversial. Tendons and ligaments have typical collagens, ultrastructure, transcription factors and proteins. However, these characteristics have not been investigated in paediatric ALC. The purpose of this study was to characterise the paediatric ALC in terms of tissue ultrastructure and cellular expression of ligament markers scleraxis (SCX)—a basic helix-loop-helix transcription factor—and the downstream transmembrane glycoprotein tenomodulin (TNMD), as compared with the paediatric lateral collateral ligament (LCL) and paediatric quadriceps tendon (QT). We hypothesised that, in comparison to the LCL and QT, the ALC would possess poor collagen orientation and reduced SCX and TNMD expression.

Methods 15 paediatric ALCs (age 6.3±3.3 years), 5 paediatric LCLs (age 3.4±1.3 years) and 5 paediatric QTs (age 2.0±1.2 years) from fresh cadaveric knees were used in this study. Fresh-frozen samples from each region were cryosectioned and then stained with H&E immunohistochemistry and gene expression analysis.

Results The histological sections of the paediatric LCL and QT showed well-organised, dense collagenous tissue fibres with elongated fibroblasts, while the ALC showed more random collagen orientation without clear cellular directionality. The aspect ratio of cells in the ALC was significantly lower than that of the LCL and QT (p<0.0001 and p<0.0001, respectively). The normalised distribution curve of the inclination angles of the nuclei in the ALC was more broadly distributed than that of the LCL or QT, indicating random cell alignment in the ALC. The paediatric LCL also stained positive for this tendon/ligament surface molecule ACT, while the ALC was only sparsely positive for this tendon/ligament disorganised structure of the ALC was negative for SCX. Immunostaining was apparent in the paediatric LCL SCX expression. TNMD expression analysis and immunohistochemistry showed more random collagen orientation without clear tissue fibres with elongated fibroblasts, while the ALC also suggested that contradictory reports of a distinct ligament within the ALC of the knee may be due in part to the variation in dissection protocols and a lack of standardised dissection technique. Similarly, Herbst et al also suggested that contradictory reports of a distinct ligament within the ALC of the knee might be due to whether tissues were fixed prior to dissection.

Despite considerable debate and investigation, the precise anatomy and biomechanics of knee ALC, and the anterolateral complex more broadly, remain uncertain. While the ultrastructure and cellular phenotype of tendons and ligaments have been previously characterised, their presence in the ALC has heretofore been largely unexplored. In particular, tendons and ligaments are composed of aligned collagen fibrils interposed with elongated cells that express scleraxis (SCX), a basic helix-loop-helix (bHLH) transcription factor, and other proteins and molecules that contribute to their unique properties. The presence of a distinct ligament within the ALC of the knee may be due in part to the variation in dissection protocols and a lack of standardised dissection technique. This study provides new insights into the histological and immunohistochemical characteristics of the paediatric ALC, which may have implications for understanding the role of this structure in the development of rotational knee instability and the potential for using such tissues in ACL reconstruction. Further research is needed to determine the functional significance of the paediatric ALC and its role in knee stability.
and the downstream transmembrane glycoprotein TNMD.\textsuperscript{14,15}

These molecular markers distinguish developing tendons and ligament from other musculoskeletal tissues and therefore their expression would support a ligamentous phenotype. As ligaments and tendons differentiate in utero, with nearly mature ligament structure present by childhood, confirmed expression of these molecular markers of a ligament phenotype in the ALC of paediatric knees would support a discrete ALL. Therefore, the objective of the present study was to investigate the presence of the ligament phenotype in paediatric knee ALC. We hypothesised that, in comparison to the lateral collateral ligament (LCL) and quadriceps tendon (QT), the ALC would possess poor collagen orientation and reduced SCX and TNMD expression.

METHODS

Our institutional review board was consulted before the initiation of this study. Because this study included access to cadaveric specimens without any patient identifiers or contact with the family, institutional review board approval was not deemed necessary. The specimens were provided by an allograft harvesting facility, which had received family consent for use of tissue for research purposes (AlloSource, Centennial, Colorado, USA).

Dissection

Fifteen paediatric ALCs (aged 6.3±3.3 years), five paediatric LCLs (aged 3.4±1.3 years) and 5 QTs (aged 2.0±1.2 years) from unpaired, fresh cadaveric knees were used in this study. All fresh paediatric knees were dissected in a layer-by-layer fashion. After removal of the overlying skin and subcutaneous fat on the lateral side of the knee, the superficial and deep layers of the iliotibial band (ITB) and overlapping fascia of the biceps femoris muscle posteriorly were dissected from their combined insertion into Gerdy’s tubercle. The remaining structures of the anterolateral complex were then observed (figure 1A). The LCL, which was encompassed by the superficial layer of the capsule, was also identified (figure 1A). The fresh ALC, including the putative ALL, was dissected off the underlying LCL and deeper joint structures without insertion site (figure 1B–D). After harvesting, the samples were fresh-frozen and cryosectioned. The sections were then stained with H&E as well as analysed immunohistochemically for the presence of SCX and TNMD.

RNA isolation and gene expression analysis

Frozen tissue was homogenised in Trizol Reagent (Invitrogen). Total RNA was then isolated by following the manufacturer’s protocol of the RNeasy Plus Mini Kit (QIAGEN, Germantown, Maryland, USA), and subsequently quantified spectrophotometrically using a NanoDrop 2000c Spectrophotometer (ThermoFisher, Pittsburgh, Pennsylvania, USA). Reverse transcription was performed using SuperScript IV Vilo Master Mix (Invitrogen, Carlsbad, California, USA), and PCR was performed on an Applied Biosystems real-time PCR system using SYBR Green Reaction Mix (Applied Biosystems, Foster City, California, USA). Transcript levels of SCX and TNMD were analysed using primers that were previously validated (online supplementary figure 1).\textsuperscript{16}Glyceraldehyde 3-phosphate dehydrogenase served as the housekeeping gene. The relative expression of each gene in each tissue sample was normalised to the expression level in the LCL using the ΔΔCt method. The sequences of primers for each gene are listed in online supplementary table 1.

Histology and immunohistochemistry (IHC)

All samples were fresh-frozen and then embedded in Tissue-Tek Optimal Cutting Temperature Compound (Sakura Finetek USA, Torrance, California, USA), and subsequently cryosectioned at 7 μm thickness, following standard histological procedure. The sectioned samples were postfixed in acetone and stained with H&E. For IHC, rehydrated sections were incubated with primary antibodies against human SCX or TNMD (Abcam, Cambridge, Massachusetts, USA) at 4°C overnight, followed by incubation with appropriate secondary antibodies. Immunostaining was carried out using the Vectastain ABC kit and NovaRED peroxidase substrate kit (Vector Labs, Burlingame, California, USA). Digital images were acquired with an OLYMPUS CKX41 microscope.

Cell morphology analysis

The aspect ratio of the cell nuclei (cell length/width) was analysed to evaluate cell morphology and orientation within the ALC, LCL and QT. Cell length, width and inclination were measured using OsiriX MD software (OsiriX MD imaging software, Pixmeo, Geneva, Switzerland). Individual cell inclination was normalised to the average population inclination and expressed as a distribution curve (degrees). An average of 74.5±11.3 cells per specimen were analysed in each group.

Statistical analysis

All data are expressed as mean±SD. Statistical analysis was performed using either two-way independent analysis of variance or two-way independent multivariate analysis of variance,
followed by Tukey’s honestly significant difference post hoc testing. A threshold of \( p < 0.05 \) was adopted to determine statistical significance.

**RESULTS**

**Cadaver specimens**

As noted, 15 paediatric ALCs (aged 6.3±3.3 years), 5 paediatric LCLs (aged 3.4±1.3 years) and 5 paediatric QTs (aged 2.0±1.2 years) from unpaired, fresh cadaveric knees were used in this study. The mean age of QT was significantly younger than that of ALC (\( p < 0.0067 \)). The mean length of ALC, LCL and QT was 26.4±10.6 mm, 20.5±7.3 mm and 40.4±6.7 mm, respectively (online supplementary table 2).

**Gene expression for SCX and TNMD**

Paediatric ALC tissue showed a lower expression of SCX as compared with paediatric QT (\( p = 0.0005 \)) and LCL, reaching statistical significance only for the former (figure 2A). TNMD, the downstream target of SCX, was also expressed at a lower level in the ALC as compared with both the LCL and the QT (\( p = 0.0314 \) and \( p = 0.0056 \), respectively) (figure 2B). For both genes, relative expression was equal between QT and LCL (\( p = 0.285 \), \( p = 0.40 \), respectively) (figure 2B).

**Histological analysis**

H&E staining showed random fibre orientation and disorganised collagen structure in the ALC (figure 3A–D). Similar findings were also seen in the proximal and distal sides on the tissue ALC without the insertion to bone (online supplementary figure 2A–D, I–L). On the other hand, with the histological sections of the QT, the musculotendon junction was seen in the distal portion of the specimen (figure 3E, F). The tendon midsubstance showed well-organised, dense collagenous tissue with elongated nuclei running parallel to the aligned collagen fibres (figure 3G, H). Boxes indicate regions of magnification in the subsequent panel.

Cell morphology was further characterised in the ALC, as compared with the LCL and QT, to assess the magnitude of cell elongation and directionality. The aspect ratio (length/width) of cells in the ALC was significantly lower than that of the LCL and QT (\( p < 0.0001 \) and \( p < 0.0001 \), respectively) (figure 4A). The normalised distribution curve of the inclination angles of the nuclei in the ALC was more broadly distributed than that of the LCL or the QT, indicating a more random cell alignment (figure 4B).
Immunohistochemical analysis revealed low SCX and TNMD protein expression in the randomly aligned cells of the ALC compared with the elongated, aligned cells of the QT. The immunostaining pattern of the ALC was similar to that in the disorganised non-tendinous tissues around the QT (figure 5).

**DISCUSSION**

The main finding of this study was that a distinct ligament was not discernible in the ALC of paediatric knees. Neither histologically nor immunohistochemically could any structure in the ALC be considered consistent with a ligament phenotype, with immunostaining of two ligament genetic markers, SCX and TNMD, largely absent.

In terms of macroscopic anatomy of knee anterolateral structure, a distinct structure termed the ALL has been frequently reported in dissections of adult knees. Claes et al identified ALL structure in 40 of 41 embalmed cadaveric knees. Landreau et al, using 11 fresh-frozen specimens with a mean age of 82 years, and Vincent et al, using 40 knees (30 total knee arthroplasty surgical specimens and 10 fresh cadavers) with a mean age of 85 years, also concluded that the ALL was identifiable in all specimens. Compared with the present study, these studies included cadaveric specimens with an average age of more than 70 years and used different fixation methods and dissection protocols. On the other hand, Dombrowski et al reported that the anterolateral capsular morphology was variable on both macroscopic inspection and MRI assessment, concluding that a distinct lateral capsular thickening was identifiable only in 4 of 10 specimens. Similarly, in a study of 14 skeletally immature knee specimens with a median age of 8 years, Shea et al concluded that the frequency of a distinct ALL was much lower in paediatric specimens than that reported in adults. Divergent findings have also been reported in studies of fetal knees, with reports of a distinct ALL in 20 of 20 knees, as compared with 0 of 21 knees. As macroscopic determination of a putative ligament structure is most often subjective, histological analysis of microscopic ultrastructure has been used.

Daggett et al asserted that the ALL exists as a distinct structure of the anterolateral complex, consisting of four layers including the aponeurotic layer, the superficial layer including the ITB, the deep fascial layer and the ALL. In support, a single histological sample stained with H&E was photographed at low magnification. However, a ligamentous phenotype of the putative ALL was difficult to assess as the histological sample was sectioned transversely (ie, a plane orthogonal to the longitudinal axis of the proposed ligament). Helito et al showed a more organised fibrous structure with attachments to the femur and lateral meniscus, but the magnification was low and cell morphology could not be appreciated. In a subsequent study, Helito et al concluded that the ALL was composed of a deep and superficial layer, with H&E-stained sections from both layers showing modestly aligned collagen fibres (again at low magnification, preventing clear cell morphology characterisation). Vincent et al independently showed a capsular structure possessing a crimp pattern of aligned collagen fibres, possessing greater organisation than the looser connective tissue found superficially and assumed to be synovium. In the few studies in which histology of a known ligament (eg, LCL) was also included, the putative ALL/capsular thickening tended to possess a microscopic ultrastructure less organised than the bona fide ligament. Histology of paediatric specimens has not

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**Figure 5** Immunohistochemical analysis of scleraxis (SCX) (A, B; E–G) and tenomodulin (TNMD) (C, D; H, I) in the anterolateral capsule (ALC) and the quadriceps tendon (QT). (A, C, E, H) Scale bar=250 µm, (B, D, F, G, I and J) scale bar=25 µm. Boxes indicate regions of magnification in the subsequent panel. LCL, lateral collateral ligament.
been previously performed, and the two studies on the anterolateral complex of the fetal knee failed to demonstrate an aligned collagen structure in the capsule.11,20

As distinct tendons and ligaments are already formed at birth, this study sought to characterise the microscopic morphology and cell phenotype of the ALC of paediatric knees, as compared with the LCL and QT.24 Histologically, the ALC of the paediatric knees did not demonstrate a pattern of aligned collagen fibres, as seen in the LCL and QT. To more rigorously evaluate tissue organisation, quantitative measures of cell morphology were performed, revealing a nuclear aspect ratio and cell directionality suggesting random fibre alignment in the ALC, as compared with the LCL and the QT. This finding was in agreement with previous biomechanical studies.23 Namely, these past biomechanical studies demonstrated that the magnitude and direction of the strain in the ALC in response to external loads applied to the knee using a 6 df robotic testing system was much larger than typical ligament and did not demonstrate a uniform strain distribution.25 In another biomechanical study that investigated both anterior tibial load and internal tibial torque, the force in the ALC was significantly smaller than that in the other structures at 30°, 60° or 90° of knee flexion.26

Interrogation of the ligament phenotype was further achieved by immunohistochemical staining of tendon/ligament markers. Helito et al previously found the fetal ALC stained positive for collagen type 1.15 However, collagen type 1 is not a specific ligament marker but rather is expressed in many connective tissues.27 Therefore, SCX and TNMD transcription and translation were probed to clarify the presence of tendon/ligament cells in paediatric knee ALC. TNMD is predominantly expressed in mature tendon fibroblasts (tenocytes) and ligament fibroblasts (ligamentocytes) at high levels.15 SCX is a bHLH transcription factor that genetically marks tendon and ligament tissues throughout development and acts to regulate the expression of tissue-specific molecules such as TNMD.14,15 Using validated human primers (online supplementary table 1 and figure 1), SCX and TNMD were found to be expressed at significantly lower levels in the human knee ALC as compared with the LCL and QT. Likewise, SCX and TNMD synthesis, as evaluated by IHC, was confirmed in both LCL and QT, but not in their surrounding capsule nor in the ALC.

While quantitative measures of histological, IHC and molecular analyses did not support a ligament phenotype in the paediatric ALC, these methodologies have yet to be applied to adult specimens. It is possible that a ligamentous phenotype emerges with maturation, induced by mechanical loading of the ALC, but such a hypothesis has not been investigated. In addition to the exclusive use of paediatric tissues, this study is limited by a relatively small number of anatomic specimens, with a different mean specimen age between ALC and QT samples. Although a larger sample number with an equivalent mean age would be ideal, the relative scarcity of paediatric specimens, as compared with fetal or adult specimens, largely obviated such a possibility. Third, only two ligament markers were examined, SCX and TNMD, but not other ligament markers such as Mohawk and early growth response-1 (EGR1). However, the examined markers were chosen as they are more characterised to date.

CONCLUSION

A distinct ligament in the paediatric ALC could not be identified by histological, immunohistochemical or molecular analyses. SCX and TNMD were transcribed at lower levels in the ALC compared with the LCL and QT. This indicates that postnatal ALC develops in the absence of genetically defined tendon or ligament structures or their precursors. These findings confirm that ALC/ALL reconstructions may not be considered restoration of normal lateral knee capsular anatomy, but rather as non-anatomic, biomechanically based ‘augmentation’ procedures that will require long-term follow-up to assess its impact on knee function.

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REFERENCES


