Spatial Transcriptomic Revealed Potential Role of Exogenous Immune Cells in Extracellular Matrix Degradation and PI3K-AKT Pathway in Angiogenesis in Rotator Cuff Tendinopathy

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Disclosures: Nil

INTRODUCTION: Degenerative tendinopathy is an unmet clinical challenge in sports medicine due to its unclear etio-pathogenesis. Spatial transcriptomic adds spatial information to the RNA sequencing data and allows in-situ cell-to-cell interactions to be identified in the tissue context. This approach has been applied to study freshly isolated biopsies of healthy and diseased tendons. However, its application in formalin-fixed and paraffin-embedded (FFPE) tendon samples, which represent a rich resource for molecular profiling has not been reported. This pilot study aimed to test the feasibility of performing spatial transcriptomic on FFPE samples for an unparalleled and unbiased discovery of key players in the tissue context in degenerative tendinopathy. Specifically, we aimed to explore novel biomarkers associated with chondrogenesis and ectopic bone formation in rotator cuff tendinopathy.

METHODS: The clinical research ethics committee approved the study. Tendinopathy samples were collected from patients during surgery after getting their consent. Rotator cuff tendinopathy sample from one patient (age 55, male) in paraffin block was used for 10x Visium to obtain spatial transcriptomic data according to the established protocol. After quality control filtering, the data were normalized and scaled using Space Ranger. Using Loupe Browser 6.0, the cell clusters were defined by the software and mapped to calcification area, chondrocyte-like area, small round cell area, normal tendon area hypocellularity area and hypervascular area on the H&E image **(Figure 1)**. After normalizing the count data, LogFC (log2 fold change) of gene expression was calculated. Differentially expressed genes (DEGs) were determined by applying threshold false discovery rate (FDR) of less than 0.05 to adjusted p values, which were generated by the Benjamini and Hochberg approach. The association of DEGs with different tissue areas was examined. The DEGs were also submitted to the DAVID database (https://david.ncifcrf.gov/home.jsp)to identify the key biological processes and signaling pathways associated with each cluster.

RESULTS: The DV200 of the sample was 59%. The sequencing depth was 35,636 reads per spot and 81 M reads per sample. Immune cell markers including the T-cell marker (*CD4*), and the monocyte marker (*CD14*) were detected in cluster 1 (small cell area) and cluster 4 (hypervascular area) **(Figure 2)**. *S100A4* is an alarmin which plays a substantial role in chronic inflammation. Previous studies also reported the roles of *S100A4* in the promotion of tissue fibrosis, cell migration and chemotaxis. There was upregulation of *S100A4* in cluster 4 (hypervascular area) compared to cluster 2 (normal tendon area) **(Figure 3)**. The expression of *CD74*, which plays important roles in many inflammatory diseases, was also upregulated in cluster 1 (small cell area) and cluster 4 (hypervascular area) compared to cluster 2 (normal tendon area) **(Figure 3)**. Activation of metalloproteinases (MMPs) and collagen degradation, including *CTSK* and *MMP2*, were key biological processes associated with cluster 1 (small round cell area). Focal adhesion and activation of PI3K-AKT pathway were key biological processes and singling pathway, respectively, identified in cluster 4 (hypervascular area), with significant activation of *COL4A1*, *COL4A2*, *ITGA6*, ITGA7, *LAMA5*, *PGF*, *VWF*.

DISCUSSION: In summary, we built a spatial transcriptomic atlas of diseased tendon using a FFPE tendinopathy sample. We identified the presence of immune cells including macrophages and T-cells as well as high expression of CD74 and alarmin S100A4 in the small cell area and hypervascular area, suggesting that inflammation is involved in the disease pathogenesis. The immune cells were likely exogenous and might enter tissue via circulation. Besides inducing inflammation, the immune cells produced MMPs and induced matrix degeneration in tendinopathy. The PI3K-AKT pathway was likely involved in angiogenesis in tendinopathy. We will verify our findings with more tendinopathy and healthy tendon samples. The expression and co-localization of key genes or cell types at the gene and protein level will also be validated by fluorescent in-situ hybridization (FISH) and immunofluorescent staining (IF), respectively.

CLINICAL RELEVANCE: The study opens new opportunities of using FFPE samples for spatial transcriptomic research in tendon to improve tendon health.
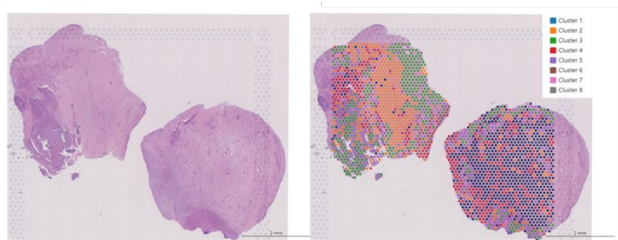
Specific region	Cluster
Calcification area	2, 3, 4, 5
Chondrocyte-like area	1, 4, 5
Small round cell area	1
Normal tendon area	2
Hypocellularity area	3
Hypervascular area	4

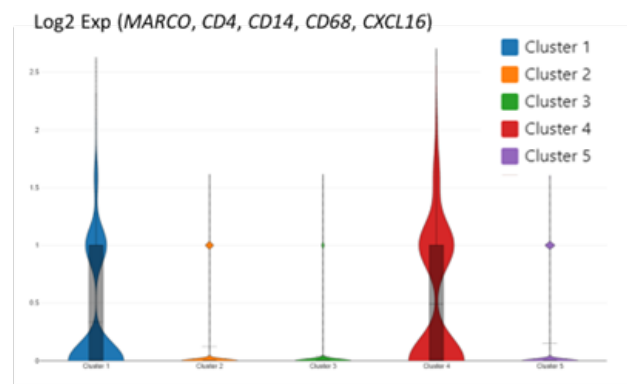Figure 1. Assignment of gene clusters to different histopathological areas in tendinopathy.

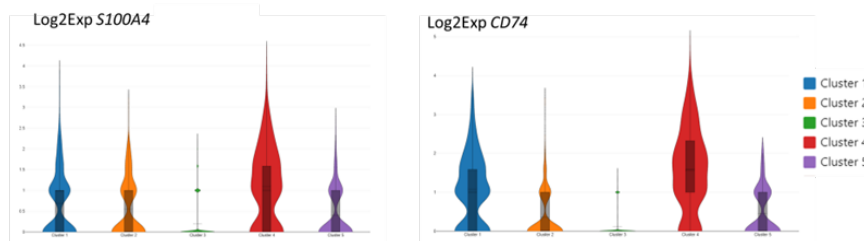Figure 2. Expression of immune cell markers in different cell clusters.

Figure 3. Expression of *S100A4* and *CD74* in different cell clusters.

ORS 2024 Annual Meeting Paper No. 1277