Identification of age-related genes in rotator cuff tendon

a preliminary transcriptome study on tendon ageing

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Aims

Rotator cuff tear (RCT) is the leading cause of shoulder pain, primarily associated with age-related tendon degeneration. This study aimed to elucidate the potential differential gene expressions in tendons across different age groups, and to investigate their roles in tendon degeneration.

Methods

Linear regression and differential expression (DE) analyses were performed on two transcriptome profiling datasets of torn supraspinatus tendons to identify age-related genes. Subsequent functional analyses were conducted on these candidate genes to explore their potential roles in tendon ageing. Additionally, a secondary DE analysis was performed on candidate genes by comparing their expressions between lesioned and normal tendons to explore their correlations with RCTs.

Results

We identified 49 genes in torn supraspinatus tendons associated with advancing age. Among them, five age-related genes showed DE in lesioned tendons compared to normal tendons. Functional analyses and previous studies have highlighted their specific enrichments in biological functions, such as muscle development (e.g. myosin heavy chain 3 (*MYH3*)), transcription regulation (e.g. CCAAT enhancer binding brotein delta (*CEBPD*)), and metal ion homeostasis (e.g. metallothionein 1X (*MT1X*)).

Conclusion

This study uncovered molecular aspects of tendon ageing and their potential links to RCT development, offering insights for targeted interventions. These findings enhance our understanding of the mechanisms of tendon degeneration, allowing potential strategies to be made for reducing the incidence of RCT.

Article focus

- This study focuses on identifying potential differential gene expressions in rotator cuff tendons across different age groups, which may be related to tendon ageing.
- Further investigations into these age-related genes aim to elucidate their role in tendon degeneration and tear.

Key messages

- This study included two transcriptome datasets of supraspinatus tendon from patients with rotator cuff tears.

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- Bioinformatics analysis identified 49 gene expressions differentially expressed with advancing age. Five of the age-related genes were associated with tendon tears in further investigation.

Strengths and limitations

- This study utilized two independent datasets to identify novel age-related genes, which is an innovative and effective method for identifying lesion-related genes.
- The relatively small sample sizes and lack of validation experiments limit the interpretation of our findings.

Introduction

Rotator cuff tears (RCTs) are a leading cause of shoulder pain and disability, imposing a considerable burden on society.[1] Advancing age is a prominent and well-established risk factor for the morbidity and prognosis of RCTs. The estimated prevalence of RCTs reaches approximately 20% to 30% among individuals aged 60 years and older.[2] Each additional decade of age accounts for a 1.2-fold elevated risk of developing RCTs.[3] Moreover, as age advances, the challenge of post-surgical healing becomes more pronounced, including prolonged healing times or an increased incidence of re-tears.[4]

Age-related degenerative changes in the rotator cuff tendon play a pivotal role in the development of RCTs, from both physiological and pathological perspectives.[5] Various biochemical and structural changes of the tendon become evident as age progresses, including collagen fibre arrangement and tendon tissue density. These changes may impact the mechanical properties and tissue stability of the tendon.[6] Previous studies have indicated that the expression of genes that code collagens and actin cytoskeleton is downregulated in aged mice compared to younger subjects.[7,8] These genes are linked to the severity of RCTs and their healing outcomes across multiple clinical studies.[9,10] Additionally, modulating ageing-related genes or pathways can mitigate the ageing phenotype in tendon stem/progenitor cells (TSPCs),[11] which are essential for healing of tendon injury.[12] Therefore, identifying molecular markers for tendon ageing assumes paramount importance, as it could facilitate a comprehensive understanding of tendon degeneration mechanisms and the development of targeted interventions to decelerate or reverse cellular ageing processes. This approach may ultimately reduce the incidence of RCTs or enhance the tendon's healing capacity.

Transcriptomics is a robust approach for investigating disease mechanisms and identifying potential biomarkers. Several transcriptomics studies have documented gene expression variations associated with the chronicities,[13] severity,[14] and outcomes[15] of RCTs. However, to date, no transcriptomics study has explored gene expression variations in human tendon related to age, which could represent an effective approach for uncovering genes contributing to tendon degeneration. Primarily, this study aimed to identify differentially expressed genes (DEGs) in tendons across different age groups, and investigate their roles in tendon degeneration. We hypothesized that specific genes are differentially expressed in tendon tissues across age groups, potentially serving as biomarkers for tendon ageing. Secondarily, this study aimed to explore whether these genes also exhibit differential expression (DE) in tendons with varying degrees of degeneration. Utilizing two public datasets from the Gene Expression Omnibus (GEO) database, we identified a set of age-related genes and assessed their expression variations in lesioned tendons compared to normal tendons. Furthermore, we conducted an in-depth exploration of their functions, pathways, and potential mechanisms contributing to the development of RCTs.

Methods

Data source

Two datasets of tendon tissues were retrieved from the GEO database. GSE180836[13] comprises RNA sequencing (RNA-seq) data of torn supraspinatus tendon from 31 patients with RCTs. GSE26051[16] includes microarray expression profiling data of lesioned and normal tendon samples in pairs from 23 patients (15 patients with supraspinatus tendon tears and eight patients with tendinopathy of other joints), which consist of lesioned tendons and normal tendons from nearby muscles (including subscapularis, biceps, or teres).

Study design

To identify gene expressions correlated with advancing age, we analyzed the relationship between age and gene expressions of 31 torn tendons in the GSE180836 dataset and 15 torn tendons in the GSE26051 dataset. Genes exhibiting consistent correlation trends across datasets and surpassing the significance threshold were classified as age-related genes. Subsequently, we performed secondary analysis in GSE26051 on the age-related genes by comparing the expression difference between lesioned and normal tendons, to explore their clinical associations with the development of RCTs. Functional analyses were performed to determine the biological functions, pathways, and potential clinical relevance of these genes (Figure 1).

Baseline characteristics

In this study, we included all of the 31 subjects in the dataset GSE180836 and 15 out of 23 subjects from the dataset GSE26051, who received rotator cuff repairs on their supraspinatus tendons. Baseline characteristics are summarized in Table I.

Linear regression analysis on advancing age

RNA-seq data (GSE180836) were normalized in terms of gene specificity using DESeq2 (version 1.40.2; Michael I. Love, USA) package, by dividing the count of each gene with a calculated normalization factor. Similarly, the microarray profiling data (GSE26051) underwent normalization by global scaling, which employed the signal intensities from all probes to normalize the distributions among samples, as reported originally.[16] Additionally, both normalized data were $\log_2$ transformed to stabilize the variance, and scaled to [0,1] range to facilitate comparability of estimated effects.

Univariate linear regression (LR) analysis was conducted on both datasets to examine the correlation between age and gene expressions. Genes displaying a p-value < 0.05 in both datasets were identified as candidates. Subsequently, a meta-analysis using metafor package (v4.4.0; Wolfgang Viechtbauer, Netherlands) was performed to synthesize the effects of these candidate genes across the two datasets. A

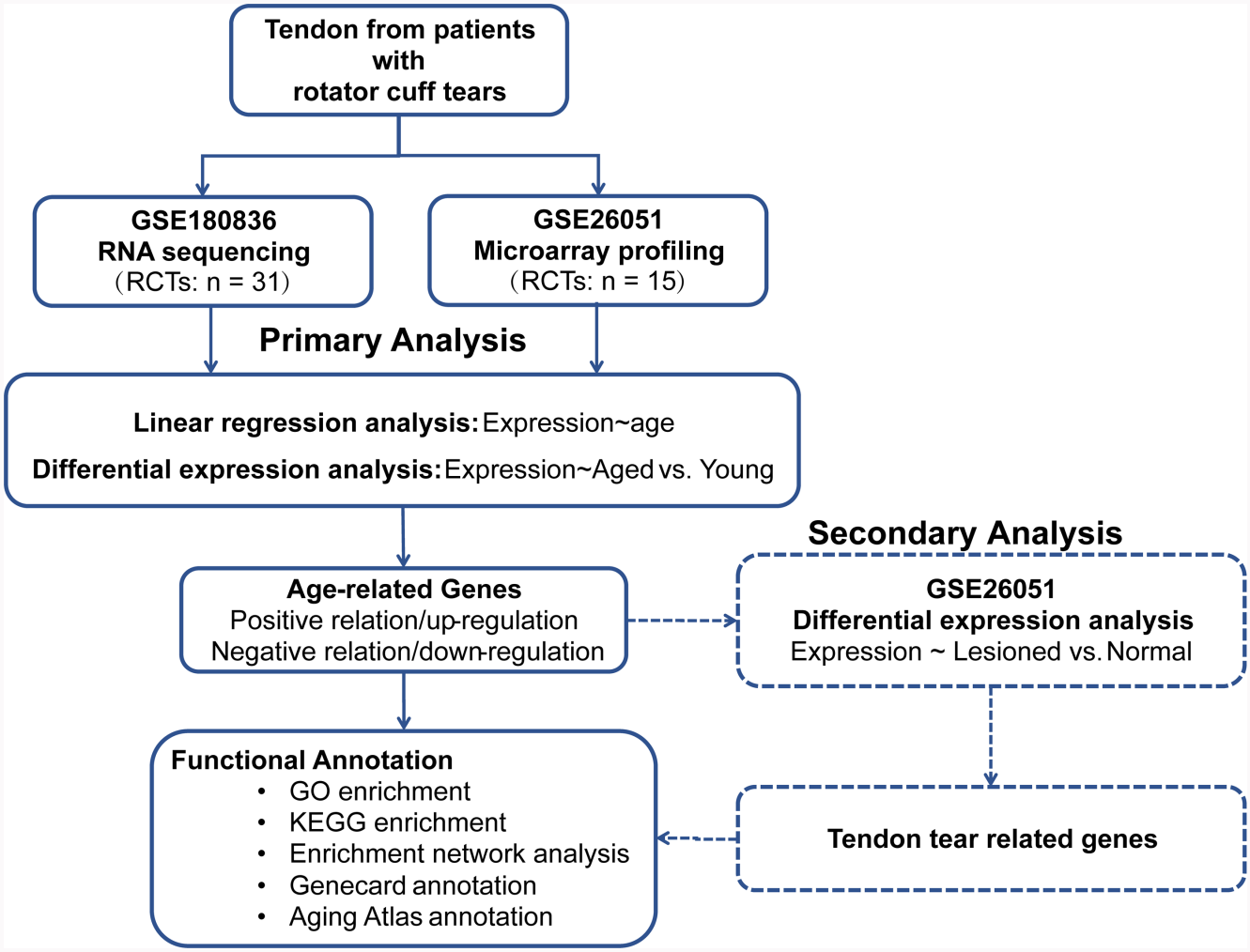

Fig. 1
Flowchart of study design. GO, Gene Ontology; KEGG; Kyoto Encyclopedia of Genes and Genomes; RCT, rotator cuff tear.

Table I. Baseline characteristics of included subjects.

Characteristic	GSE180836	GSE26051
Sample size, n	31	15
Mean age, yrs (SD; range)	58.6 (8.9; 27 to 72)	55.0 (8.7; 41 to 66)
Sex (male), n (%)	15 (48.4)	7 (46.7)
Group (male), n (%)		
Young (age ≤ 50 yrs)	4 (2)	7 (5)
Middle-aged (50 < age ≤ 60 yrs)	11 (5)	2 (1)
Aged (age > 60 yrs)	16 (8)	6 (1)

gene was considered to be significant in the meta-analysis if it had a false discovery rate (FDR) < 0.05, heterogeneity index (I^2) < 25%, and a p-value > 0.1 for quantified heterogeneity (QEp), indicating low evidence of heterogeneity. All analyses were conducted using R software (version 4.3.2; R Foundation for Statistical Computing, Austria).

DE analysis on different age groups

We further stratified the subjects in both datasets into three age groups, using cutoff at ≤ 50 and > 60 years, to assess significant disparities of gene expression among different age groups. DE analysis was conducted using the DESeq2 package for the GSE180836 dataset, and the Limma package (v3.58.1; Gordon K. Smyth, Australia) for the GSE26051 dataset. Genes with a raw p-value < 0.05 and consistent directional expression across both datasets were considered as candidates. Subsequently, a meta-analysis using MetaVolcano package (v1.14.0; Fábio R. Pires, Brazil) was performed to combine the effects of these candidate genes across the two datasets. A gene was considered to be significant in the meta-analysis if it exhibited an expression change > 1.5-fold, an FDR < 0.05, and a QEp > 0.1.

Functional analyses of age-related genes

Gene set enrichment analysis (GSEA) based on Gene Ontology (GO) gene sets was conducted on significant genes exhibiting consistent directions of expression change in DE analysis using clusterProfiler package (v4.8.3; Guangchuang Yu, China). A term with an absolute value of normalized enrichment score (|NES|) > 1 and adjusted p-value < 0.05 was considered to be significantly enriched. Additionally,

Metascape (v3.5.20240101, Yanhui Hu, USA)[17,18] was used to construct the enrichment network for the overall age-related gene set based on their similarities in biological functions found in databases, including Kyoto Encyclopedia of Genes and Genomes Pathway (KEGG), GO Biological Processes, Reactome Gene Sets, Canonical Pathways, CORUM, and Wiki Pathways. In the enrichment network, nodes represent enriched terms with $p < 0.01$, gene count > 2, and enrichment factors > 1.5. Nodes connected with lines represent the Kappa scores > 0.3, indicating the agreement between two function terms.

DE analysis on age-related genes and lesion of RCTs

To explore the potential associations between age-related genes and lesion development, we further performed DE analysis in the GSE26051 dataset, comparing gene expressions between lesioned tendons and normal tendons. DEGs were then comprehensively reviewed for correlations with tendon degeneration using the GeneCards[19] and Aging Atlas[20] databases.

Statistical analysis

In this study, all statistical analyses were performed using RStudio software (version 2023.09.1+494; Posit, USA). Linear regression analysis, DE analysis, and meta-analysis were used to assess the correlation between age and gene expression. P-values for the linear regression and DE analyses were calculated using independent-samples *t*-tests, while p-values for the meta-analysis were calculated by a z-test. The p-values of heterogeneity test in the meta-analysis (QEp) were calculated by Cochran's Q test. Identification of age-related genes was based on the significance criteria, p-values adjusted for FDR < 0.05. The p-values for GSEA analysis were calculated by permutation tests and a term with Benjamini-Hochberg adjusted p-values < 0.05 was considered to be statistically significant. GO enrichment network was conducted using Metascape, for which the p-value was calculated by permutation test.

Results

LR analysis and DE analysis on age

After data preprocessing, a total of 15,195 genes overlapped in the two datasets. LR analysis identified 59 candidate genes nominally associated with age. A subsequent meta-analysis determined 27 of these genes that had a FDR < 0.05 and passed the heterogeneity test; 13 of these genes were significantly positively correlated with age, while 14 were negatively correlated (Figure 2a and Supplementary Table i). Simultaneously, DE analysis identified 32 genes as nominally differentially expressed across both datasets. In a subsequent meta-analysis, 26 of these genes meeting the adjusted significance threshold (|Fold-change| > 1.5 and FDR < 0.05) and heterogeneity criteria were considered to be DEGs. Compared with the Young group, 12 genes were upregulated and 15 were downregulated in the Aged group (Figure 2b and Supplementary Table ii). Among these genes, five were overlapped in both the LR and DE analyses (Figure 3). In summary, we identified 49 genes as age-related genes, of which 23 genes were upregulated and 26 were downregulated as age advances.

Functional analyses on age-related genes

Based on the |NES| and adjusted p-values from the GSEA, the top three ranked GO terms enriched by age-related genes in each category are presented in Supplementary Table iii, totaling 95 functional GO terms. Of these, 20 terms were specifically related to ageing or development of the musculoskeletal system (Figure 4), covering aspects of biological processes, cellular components, molecular functions, and human phenotypes. The enrichment network was then constructed using the overall age-related gene set that revealed nine clusters of biological processes (Figure 5a and Supplementary Table iv). In conjunction with the GSEA and enrichment network analysis, overlapping biological pathways involved in muscle cell development (including myosin heavy chain 3 (*MYH3*) and *SYPL2*) and metal ion homeostasis (including *MT1E* and metallothionein 1X (*MT1X*)) are represented in Figures 5b and 5c.

DE analyses on age-related genes and lesion of RCTs

Among the 49 age-related genes identified from our primary analysis, we found five DEGs in the GSE26051 dataset (|Fold-change| > 1.5 and $p < 0.05$ (independent-samples *t*-test)). Of these, two were upregulated and the other three were downregulated in the lesioned tendons compared to normal tendons (Table II). Box plots illustrating the distribution of gene expressions across different age groups in the GSE180836 dataset are presented in Figure 6.

We focused on the five DEGs in lesioned tendons and consulted the GeneCards and Aging Atlas databases to review their functions and linkages to tendon ageing (Supplementary Tables v and vi). In Aging Atlas, these gene expressions were recorded to be associated with ageing in certain tissues or cells of specific species in transcriptome studies, but the regulation directions with ageing could be varied.

Discussion

In this study, we primarily identified 49 age-related genes in tendon from patients with RCTs, which could serve as candidate biomarkers for tendon ageing. Secondarily, we identified five of the age-related genes differentially expressed in lesioned tendons compared to normal tendons. To our knowledge, this is the first study to undertake a comprehensive transcriptome profiling to explore the effects of age on torn rotator cuff tendons.

Tendon degeneration with ageing, characterized by structural and biomechanical changes,[21] is associated with changes in gene expressions involved in extracellular matrix (ECM), transcription factors (TFs), and inflammation at the molecular level.[22] In this study, we identified genes involved in ECM development, including cartilage oligomeric matrix protein *(COMP)* and *fibromodulin* (*FMOD)*. We also identified a set of TFs with altered expression patterns associated with advancing age, including *CCAAT enhancer-binding protein delta* (*CEBPD)*, Spi-B transcription factor (*SPIB)*, and E2F transcription factor 2 (*E2F2)*. Additionally, genes such as *LDL receptor related protein 1* (*LRP1)*, *calcium dependent protein kinase I* (*CAMK1)*, and cysteine dioxygenase type 1 (*CDO1)* were noted for their involvement in the inflammatory response process.

Within the spectrum of ECM genes, a previous investigation into tendon ageing primarily focused on COMP, known for its crucial roles in collagen synthesis and tendon

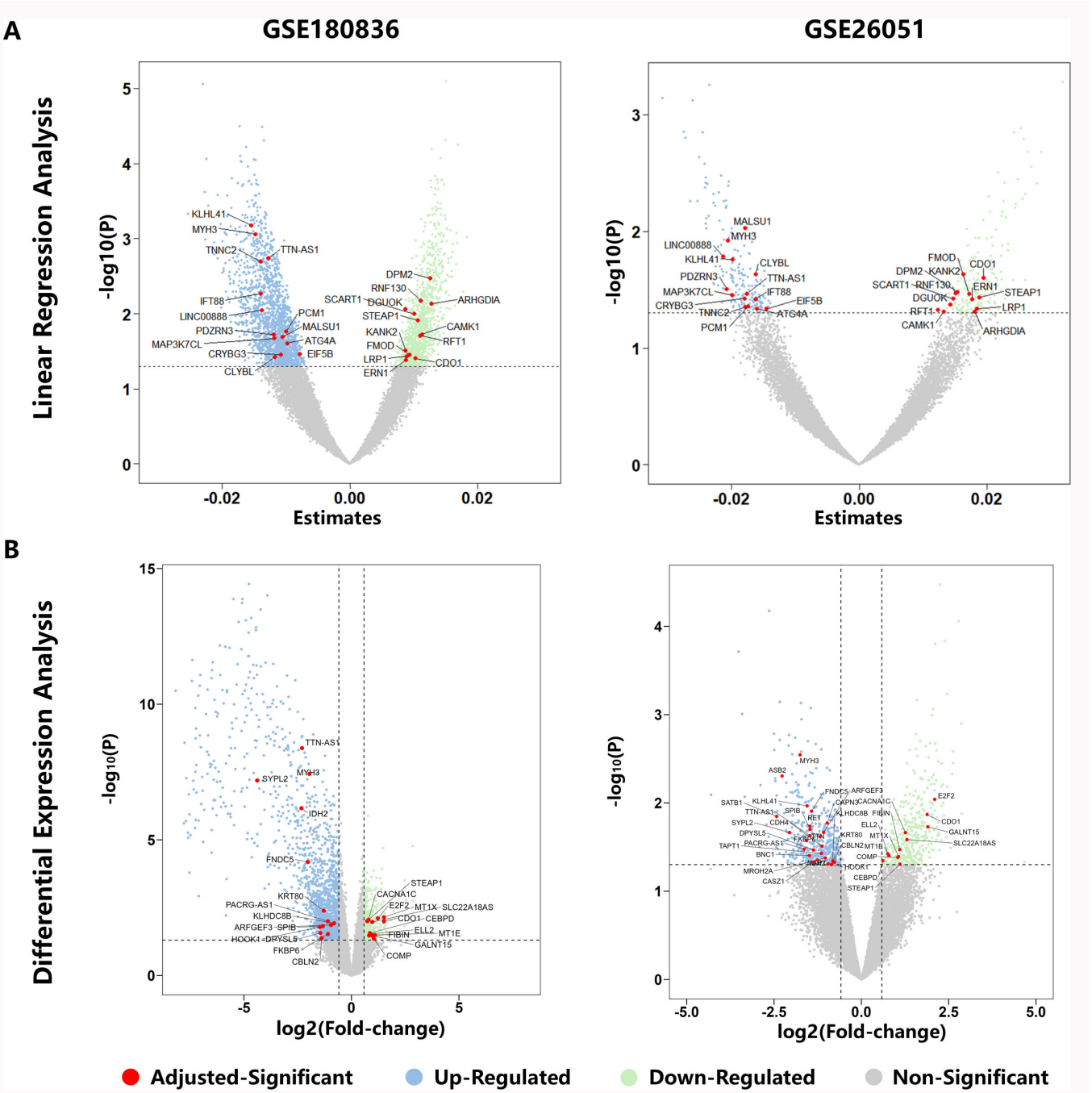

Fig. 2

Scatterplots of age-related genes involved in the present study. a) Linear regression analysis on gene expressions and age in GSE180836 (left) and GSE26051 (right). Candidate genes significantly related to age were plotted in light green for positive correlations and light blue for negative correlations ($p < 0.05$, independent-samples *t*-test). A total of 27 age-related genes determined by adjusted significance threshold (false discovery rate (FDR) < 0.05) in the meta-analysis were further filled in red and labelled with gene symbols. b) Differential expression analysis comparing the Aged group to the Young group in GSE180836 (left) and GSE26051 (right). Candidate genes significantly related to age were plotted in light green for upregulated correlations and light blue for downregulated correlations ($p < 0.05$ (independent-samples *t*-test) and |Fold-change| > 1.5). A total of 27 age-related genes determined by adjusted significance threshold (FDR < 0.05 and |Fold-change| > 1.5) in the meta-analysis were further filled in red and labelled with gene symbols.

development in early life and post-injury recovery.[23] We observed an upregulation in *COMP* with age, suggesting its potential involvement in compensatory mechanisms against tendon ageing to maintain ECM homeostasis.[24] However, a previous study showed decreased COMP levels in torn tendon of aged patients compared to normal tendon from young individuals.[25] This apparent contradiction may stem from differences of subjects in disease states and age groups. Given that *COMP* expression can be affected by factors such as mechanical force,[26] inflammation,[27] and injury,[28] further research considering confounding factors such as the dominant arm, chronicity course, and tear size is needed. Limited research has been conducted into the associations between tendon ageing and the expression of *FMOD*, which is involved in collagen fibre organization in tendons and

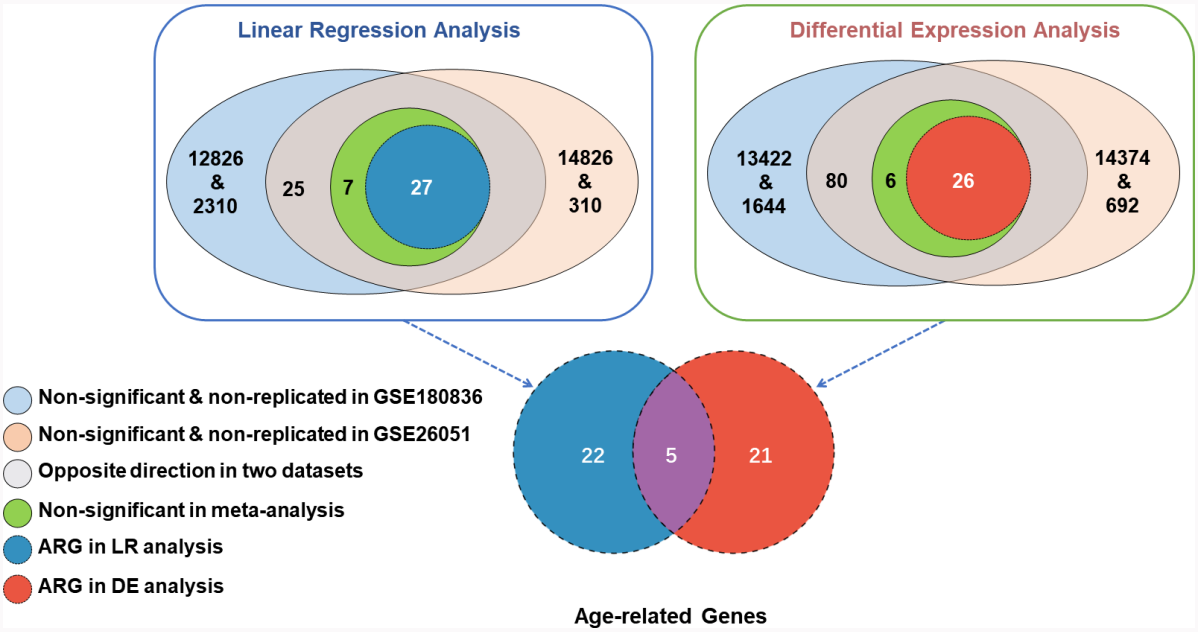

Fig. 3

Venn diagrams of age-related genes. Venn diagram inside the blue box (left) showed the association results of linear regression (LR) analysis, and the other in the green box (right) for results of differential expression (DE) analysis. Gene counts were represented within each piece of the intersections. The numbers in the outermost circle represent the quantities of non-significant and non-replicated genes. Intersections in grey represent the gene sets beyond threshold of significance in both datasets, but with opposite trends of association. Intersections in green represent candidate genes that failed to meet the adjusted significance threshold. The blue and red intersections inside each diagram represent the age-related genes (ARGs) passing the adjusted significance thresholds. The Venn diagram below indicates a total of 49 ARGs found in the present study, five of which were significant in both analysis methods.

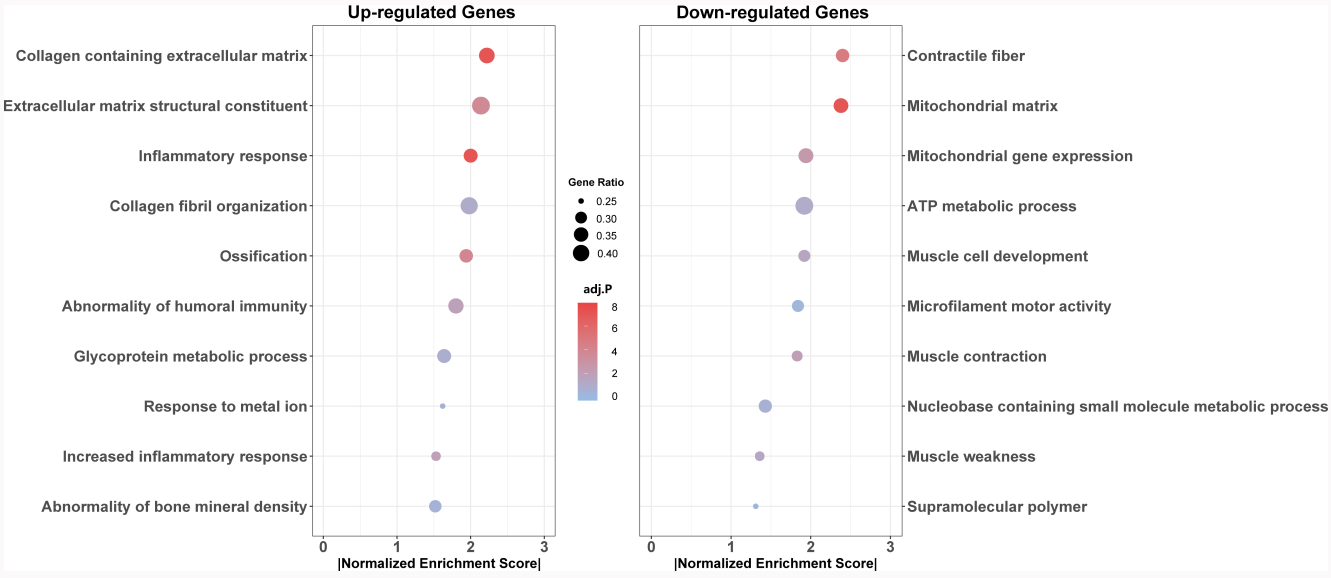

Fig. 4

Bubble plots of the gene set enrichment analysis (GSEA) enrichment terms for age-related genes. Each bubble represents an enriched term, ranked by the absolute value of the normalized enrichment score (|NES|) across categories including molecular function, cellular component, biological function, and human phenotype. The colour of each bubble indicates its adjusted p-value (permutation test), and the size reflects the gene ratio (hit gene count/total gene count) within that term. Terms associated with upregulated genes are displayed on the left, while those related to downregulated genes are shown on the right. ATP, adenosine triphosphate.

is crucial for promoting healing after tendon injury via the upregulation of matrix metalloproteinases.[29]

Alterations in the expressions of TFs (*CEBPD* and *E2F2*) in the skeletal muscle or joint cartilage of ageing mice have been documented previously.[30,31] Notably, the correlation directions between certain gene expressions and ageing may appear contradictory among different tissues or our results from human tendon. For example, the upregulation of *CEBPD* in the knee joint of aged mice aligns with our findings in aged human tendon, while it is downregulated in the limb muscle

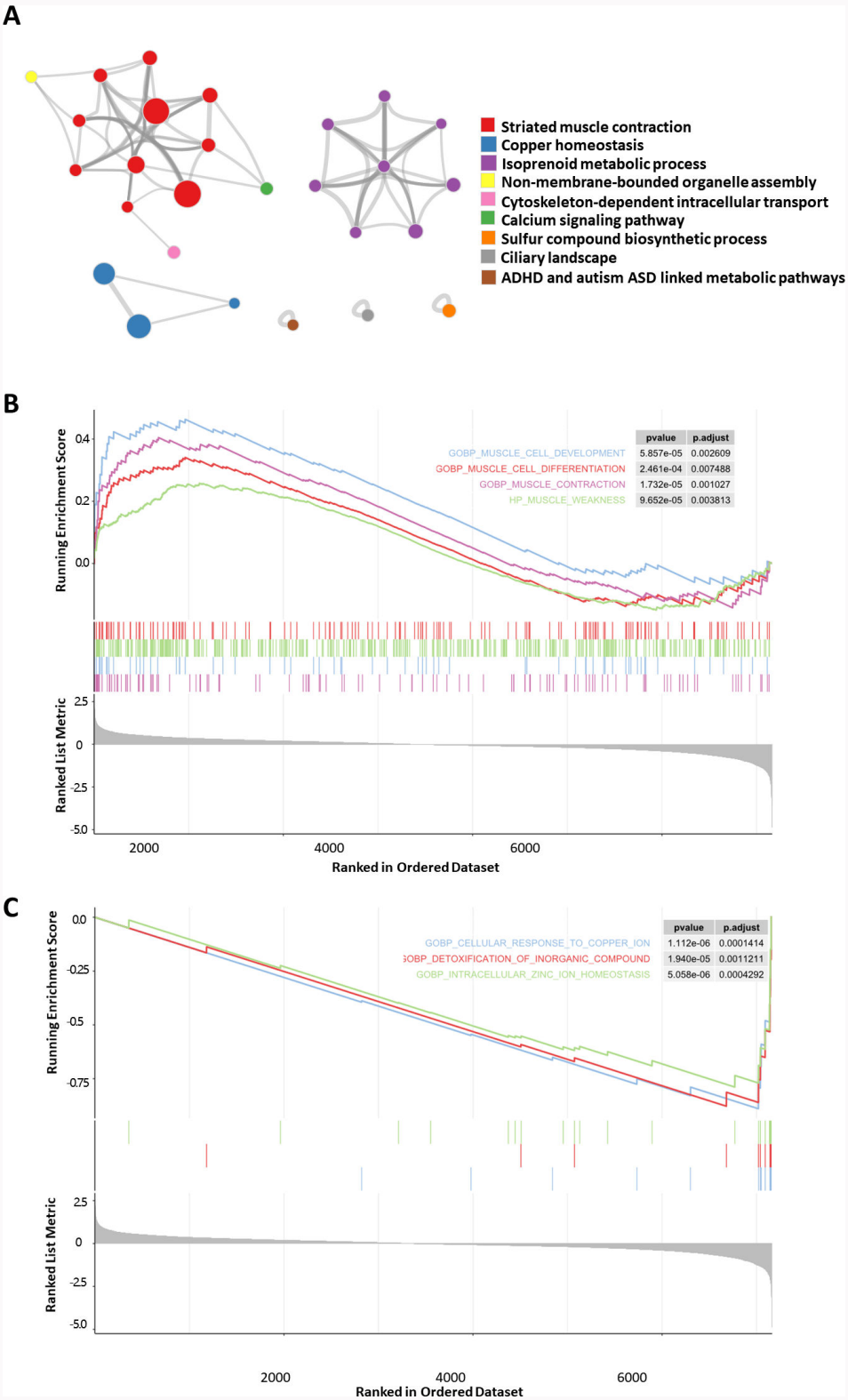

Fig. 5

Enrichment network of age-related genes. a) The enrichment network displays nine hub clusters represented in different colours, whose nodes were the enriched terms of age-related genes with $p < 0.01$ (permutation test), gene count > 2, and enrichment factors > 1.5. The size of each node indicates the hit gene count in the enriched term. The edges represent the similarity between the enriched terms measured by Kappa scores. The width of the grey line indicates the strength of the similarity. The nine clusters were annotated by the top terms of enrichment scores in each cluster. b) The enrichment terms involved in muscle cell development. In the upper part of the graph, the overall trends of the curves are upward, with the peaks of the enrichment scores located in the upper-left corner, indicating a positive normalized enrichment score (NES). This suggests that the majority of the core genes are positioned before the peak. In this study, most core genes on these pathways are downregulated, including the age-related genes myosin heavy chain 3(*MYH3*) and synaptophysin like 2 (*SYPL2*). The vertical lines in the middle represent the position of each gene in this pathway. The lower part of the graph illustrates the distribution of input genes in the background gene list. c) Enrichment terms involved in metal ion homeostasis. In the upper part of the graph, the overall trends of the curves are downward, with the peaks of the enrichment scores located in the lower-right corner, indicating a negative NES. This suggests that the majority of core genes are positioned before the peak. In this study, most core genes on this pathway are upregulated, including the age-related genes metallothionein 1X (*MT1X*) and metallothionein 1E (*MT1E*). The vertical lines in the middle represent the position of each gene in this pathway. The lower part of the graph illustrates the distribution of input genes in the background gene list. ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder.

Table II. Differential expression analysis on age-related genes and lesion situation of tendon (lesioned vs normal).

	Correlation with lesion status		Correlation with age	
Symbol	**Log_2(Fold-change)**	**p-value**	**Log_2(Fold-change)**	**FDR**
ARFGEF3	1.29	0.010	-1.06	0.025
KLHDC8B	0.68	0.047	-0.81	0.010
CEBPD	-0.79	0.010	0.76	0.010
MT1X	-1.05	0.015	1.33	0.010
DPYSL5	-1.34	0.023	-1.21	0.017

*Independent-samples *t*-test.
ARFGEF3, ARFGEF family member 3; *CEBPD*, CCAAT enhancer binding brotein delta; DPYSL5, dihydropyrimidinase like 5; FDR, false discovery rate; KLHDC8B, kelch domain containing 8B; *MT1X*, metallothionein 1X.

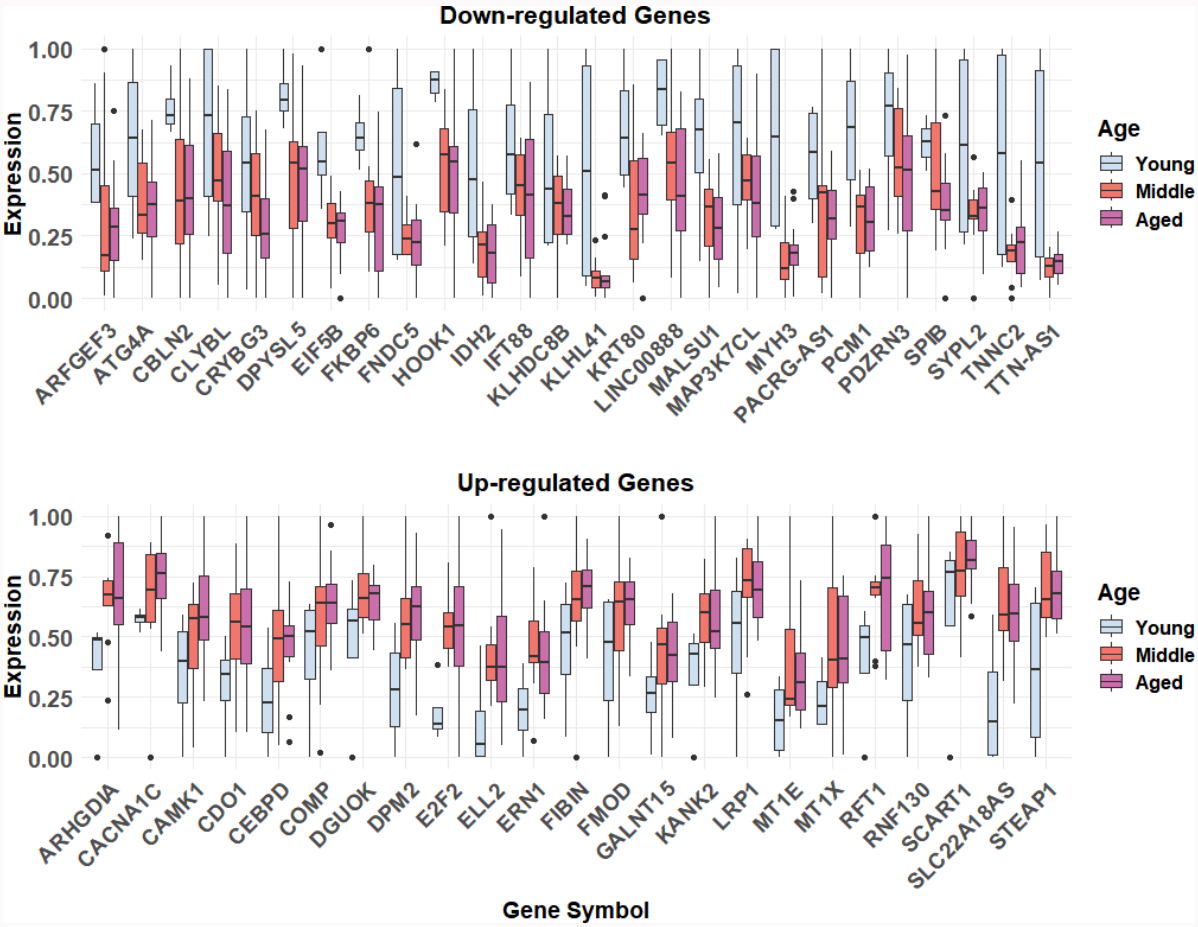

Fig. 6
Box plots of lesion-related gene expressions in different age groups. The normalized expression distributions of 49 differentially expressioned genes (DEGs) of lesion tendon are presented with their expression variations in different age groups in the GSE180836 dataset.

of aged mice.[31] Although *CEBPD* has not been associated with tendon ageing previously, its homolog, *CEBPB,* is recognized as an adipogenic gene that promotes adipogenic differentiation and inhibits osteogenic differentiation in TSPCs,[32] a feature of tendon ageing.[33] This suggests a potential role of upregulated *CEBPD* in adipogenesis with tendon ageing. Furthermore, we found *CEBPD* to be downregulated in lesioned tendons compared to normal tendons. In a recent single cell transcriptomic study,[34] *CEBPD* expression was found to be downregulated in tenocytes from patients with rotator cuff tendinopathy compared to patients without the disorder, consistent with our findings. Previous research indicates that *CEBPD* enhances *IL-6* transcription either alone or in cooperation with *CEBPB,*[35] both of which are involved in macrophage-mediated muscle fibre regeneration from micro-injury in a mouse model.[36] This suggests that downregulation of *CEBPD* could potentially serve as a biomarker for reduced repair capacity against tendon degeneration.

Existing research offers preliminary insights into the potential regulatory mechanisms of age-related genes on tendon ageing. In our study, we identified upregulated metallothionein 1X (*MT1X)* and metallothionein 1E (*MT1E)* in aged subjects, which are involved in metal ion homeostasis. Metal ions are crucial in modulating cell growth, differentiation, and apoptosis.[37] Imbalances in metal ion metabolism, associated with ageing due to their roles in cellular redox processes,[38] may exacerbate tendon ageing. Particularly, upregulated *MT1X* was considered as a beneficial factor for longevity because of its stronger control over zinc homeostasis.[39] We observed that *MT1X* was downregulated in lesioned tendon, suggesting a potential disruption in metal homeostasis or a causal factor for tendon degeneration. Additionally, genes such as myosin heavy chain 3 (*MYH3)*, troponin C2 (*TNNC2)*, kelch like family member 41 (*KLHL41)*, and synaptophysin like 2 (*SYPL2)*, which encode structural or functional proteins specific to the muscular system, were observed to be downregulated in aged subjects. These genes play critical roles in muscle cell development, differentiation, and contraction. In summary, our findings underscore the potential significance of genes involved in the regulation of inflammation, metal homeostasis, and muscle development in the context of tendon ageing and RCTs.

Furthermore, we observed that the patterns of gene expression change in tendons associated with advancing age varied among genes (Figure 6). Some genes exhibited an abrupt expression change at the age of 50 years (e.g. *CEBPD* and *E2F2* in DE analysis), while others showed a gradual change as age advanced (e.g. dentin matrix acidic phosphoprotein 2 (*DMP2)* and intraflagellar transport 88 (*IFT88)* in LR analysis). A previous study has demonstrated that steroid hormones regulate *CEBPD* expression in vitro, and since disorders of gonad function and hormone secretion generally occur around the age of 50 years, our strategy of analyzing age and gene expressions is rational.[40]

However, there were several limitations in this study. First, the datasets were acquired from published studies, which lacked comprehensive baseline and clinical characteristics for adjustment. Second, while external validation with another independent dataset and the practice of adjusted significance thresholds in our study effectively reduced the false positive rate, possible biases still existed due to the relatively small sample sizes and the use of different transcriptome profiling techniques in the original datasets. Finally, functional experiment was not available to validate the association discovered in this study. Therefore, we cautiously interpreted the results in this preliminary study by carefully reviewing published associations between genes and tendon ageing to support our findings. These associations suggest that the expressions of specific genes change with age, which provides a foundation for future studies where rigorous study designs, comprehensive analyses, and functional experiments are considered.

In conclusion, this preliminary study employed transcriptome profiling data from human rotator cuff tendon and identified 49 gene expressions associated with advancing age, which could be indicative of tendon ageing. Moreover, five of these genes were expressed differentially in torn lesioned tendons compared to normal tendons, suggesting their potential relevance to tendon degeneration and the pathophysiology of RCTs. Future studies are needed to validate the efficacy of these genes as potential biomarkers for assessing individuals' healing abilities after rotator cuff repair.

Supplementary material

Tables comprising detailed gene analysis results.

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