MicroRNAs associated with inflammation in shoulder tendinopathy and glenohumeral arthritis

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Abstract

Inflammation is associated with glenohumeral arthritis and rotator cuff tendon tears. Epigenetically, miRNAs tightly regulate various genes involved in the inflammatory response. Alterations in the expression profile of miRNAs and the elucidation of their target genes with respect to the pathophysiology could improve the understanding of their regulatory role and therapeutic potential. Here, we screened key miRNAs that mediate inflammation and linked with JAK2/STAT3 pathway with respect to the co-incidence of glenohumeral arthritis in patients suffering from rotator cuff injury (RCI). Human resected long head of the biceps tendons were examined for miRNA profile from two groups of patients: Group-1 included the patients with glenohumeral arthritis and massive rotator cuff tears and the Group-2 patients did not have arthritis or rotator cuff tears. The miRNA profiling revealed that 235 miRNAs were highly altered (fold change less than −3 and greater than +2 were considered). Data from the NetworkAnalyst program revealed the involvement and interaction between 3,430 different genes associated with inflammation out of which 284 genes were associated with JAK2/STAT3 pathway and interconnects 120 different pathways of inflammation. Around 1,500 miRNAs were found to play regulatory role associated with these genes of inflammatory responses and 77 miRNAs were found to regulate more than 10 genes. Among them 25 genes with <−10-fold change were taken to consideration which altogether constitute for the regulation of 102 genes. Targeting these miRNAs and the underlying regulatory mechanisms may advance our knowledge to develop promising therapies in the management of shoulder tendon pathology.

Keywords

Glenohumeral arthritis; Inflammation; Rotator cuff injury; Tendinopathy; miRNAs; miRNA regulators

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Introduction

Hyper-activation and persistence of inflammation has been reported in several musculoskeletal diseases, especially rotator cuff injury (RCI). Inflammation and pain are the two major symptoms of the patients with RCI and the conventional treatment approaches are aiming in the management of these symptoms [1]. RCI can be both degenerative and traumatic in nature depending on the multifactorial etiology and these causative factors may be either extrinsic or intrinsic [2]. Interestingly, irrespective of the causative factors and severity, the inflammation is a prominent hallmark in most clinical cases and the sustainability of inflammation delays the response to medications and healing [3]. This was evident by the upregulation of proinflammatory cytokines, biomarkers and receptors in the tendon tissues even after the setting of clinical symptoms. The mechanism and driving force behind the prolonged inflammatory reactions are yet to be unveiled for a mechanically robust minimally vascular tissue like tendon [4].

The co-existence of clinical conditions like osteoarthritis is reported to exasperate the inflammatory reactions of joints [5]. The rheumatoid arthritis and glenohumeral arthritis has been reported to be the aggravating factors of inflammation in patients with degenerative RCI [6]. The cumulative effects of arthritis along with preexisting RCI demonstrate osteopenia, glenohumeral/acromioclavicular erosions and proximal humeral migration which offer challenges in repair pathways and therapeutic strategies of the rotator cuff [7]. The inflammation will be prolonged at cellular and molecular level for several months even after the setting of arthritis which creates a higher chance for reoccurrence of RCI. The molecular events leading to the persistence of inflammation in RCI regarding the presence/ absence of arthritis is still unknown.

Cytokine release is considered to be the initial event in inflammation and most of the cytokine expression and action was reported to be regulated by STAT (signal transducers and activators of transcription) proteins [8]. Apart from cytokines, the association of JAK (Janus kinase) and the downstream molecules with STAT pathway activates inflammation by facilitating chemokine expression, differentiation and maturation of hematopoietic cells, stem cell activation, and production of reactive oxygen/nitrogen species [9]. The interplay between cytokine signals and STAT proteins are necessary for the execution of inflammatory responses associated with infection or injury. The extracellular interaction of ligand activates the JAK/STAT pathway by inducing conformational changes to the receptor which in turn triggers a phosphorylation cascade to downstream substrates including STATs. The phosphorylated STATs (activated) translocate to nucleus and assemble as dimeric or oligomeric complex at the specific enhancer sequences of the target inflammatory genes, thereby regulating their transcription. Seven STATs and four JAKs exist in mammalian system and each of which are recruited depending on tissue specificity and receptors and/or ligands involved [10].

The inflammatory signaling pathways and molecules are also regulated epigenetically by miRNAs. miRNAs like miR-29, miR-133a, miR-155, miR-221, miR-223, miR-652, etc. are well known for their active role in inflammatory diseases including arthritis [11]. Similarly, the specific roles of miR-9, miR-127, miR-125 were reported to trigger M1 macrophage

polarization while miR-223, let-7c, miR-124, miR-132, miR-34a, miR-146a and miR-125a-5p mediates M2 polarization [12]. Immune cell differentiation and proliferation are also under the regulation of miRNAs. To cite, miR-106a, miR-20a and miR-17-5p mediates monocyte differentiation and miR-106a, miR-106b and miR-19 are actively involved in T cell differentiation and signaling [13]. miR-223, let-72, miR-147 and miR-9 regulate inflammation by targeting the downstream signaling molecules of TLR signaling pathway [14]. The miRNA mediated regulation of cytokines, which are associates with JAK/STAT pathway, was also well established. For example, miR-27a, miR-23a and miR-24a targets IFN-γ, miR-10a regulates TGF-β, miR-9 and miR-31 are the regulators of IL-2 [15]. The transcription factor NF-κβ, which regulate a battery of pro/ant-inflammatory genes were also reported to be regulated by miR-146, miR-125, and miR-21 [16] and miR-181a, miR-19a, and miR-124 regulates TNF-α [17], [18].

Even though limited number reports are available [19], [20], the miRNA mediated regulation of inflammation in human rotator cuff tendon has not been well established. The implications of miRNA mediated inflammatory responses (especially targeting JAK/STAT signaling) coinciding with arthritis and/or non-arthritis environments of RCI are still unknown. The goal of the study is to characterize miRNAs associated with JAK2/STAT3 pathway of inflammation and to identify their target genes involved in the pathophysiology of glenohumeral arthritis and its co-incidence with rotator cuff tears. The findings from this study could unravel their regulatory role and therapeutic potential. The present study compares the alterations in miRNA profile among the patients with and without glenohumeral arthritis and rotator cuff tears in combination with the integrative expression analysis of inflammatory genes associated with JAK/STAT pathway and their cross talk with other inflammatory pathways using the meta-analysis program NetworkAnalyst.

Materials and methods

Tissue collection and processing

The Institutional Review Board (IRB) of Creighton University approved the research protocol. All patient volunteers signed the consent form and the HIPPA form. The RCI patients were explained in layman terms about the details of the procedures and written informed consent was signed before the surgery. Eight RCI patients were recruited for the study over a 6-month period. Four patients were undergoing reverse shoulder arthroplasty to treat glenohumeral arthritis with massive rotator cuff tears (Group-1). The second group was undergoing arthroscopic biceps tenodesis surgery without rotator cuff tear of glenohumeral arthritis (Group-2). The grouping was based on severity of inflammation and presence of arthritis based on preoperative imaging. The biceps were tenotomized in all patients, resected, and sent for analysis. The tissue specimens were collected in UW (University of Wisconsin) solution at 4°C for transportation and temporary storage. One part of the tissues was fixed in 10% formalin for histology and another part was stored in RNA-later for RNA isolation.

Histology

The formalin fixed tissues were embedded in paraffin wax, sectioned along the horizontal axis (5μm thickness were cut using microtome, Leica, Germany) and heat-fixed on microscopic slides [21]. After deparaffinizing the sections in xylene and dehydrated with graded concentrations of ethanol Hematoxylin and Eosin (H&E) staining was carried out [22]. The H and E slides were mounted using xylene-based mounting media and imaged using an inverted microscope attached with CCD camera (Olympus BX51; Olympus America, Center Valley, PA) under bright field mode [23]. The histological evaluation of slides was performed qualitatively and independently by two blinded investigators which were confirmed by the third one.

Immunofluorescence

The antigen retrieval of deparaffinized sections was done in HIER (Heat Induced Epitope Retrieval) buffer at 95°C for 20 min. After washing in PBS, the sections were subjected to blocking using 0.25% Triton X-100 and 5% horse serum in PBS at room temperature for 2h. Primary antibody solution (1:50 diluted in blocking solution) was added and kept overnight at 4°C. After washing in PBS fluorochrome-conjugated corresponding secondary antibody (1:100 dilution) was added and incubated for 2h at room temperature followed by washing and mounting with 4′,6- diamidino-2-phenylindole (DAPI)-containing mounting media. The sections were viewed using the fluorescent microscope (Olympus BX51; Olympus America, Center Valley, PA) and the images were taken and merged using Olympus DP71 camera and associated software. All the antibodies were procured from SantaCruz Biotech and a negative control with secondary antibody alone was also maintained to fix the exposure [24]. The primary antibodies used were: mouse anti-human CD-68 and mouse anti-human CD-16. The donkey anti-mouse-FITC was used as the corresponding secondary antibodies [25].

RNA isolation from tendon

Around 200mg tissue from the proximal portion of the biceps tendons were minced to small pieces and Trizol reagent (1ml) was added followed by homogenization. After 10 min at room temperature Chloroform-isoamyl alcohol reagent (200μl) was added, centrifuged at 12,000 rpm for 15 min. 500μl isopropanol was added to the aqueous layer to precipitate RNA and again centrifuged at 12,000 rpm for 10 min to pellet down the RNA. The pellet was washed with 75% ethanol, dissolved in sterile RNase free water, quantified and stored at −80°C [26].

miRNA microarray

RNA integrity number (RIN) score was obtained by bioanalyzer before hybridizing the samples onto the miRNA microarray (miRNA 4.0 array). The RIN score of the isolated RNA was between (2.1 to 4.8) due to the tissue characteristic. MiRNA microarray analysis was conducted at Kansas University Medical Center and the raw data was analyzed using Expression Console software (Alegent). The analysis was performed in two separate batches (two specimens from each group) and compared for the consistency of results [27].

Gene identification using NetworkAnalyst

The interrelationship among the genes associated with inflammatory pathways was identified by using NetworkAnalyst program from published data base [27] – [28]. The major genes and associated regulators of JAK/STAT pathway of inflammation [29] were used as input in NetworkAnalyst to assess the cross talk of these genes with other signaling pathways of inflammation (Table 1; Fig. 1). The list of genes generated was assessed individually for their target miRNA from the microarray data.

Results

Histology

Group-1 consists of 4 patients with glenohumeral arthritis and a massive rotator cuff tear and Group-2 was included with 4 patients without arthritis. Altered physiological architecture of tendon tissue was observed on the patients of both the groups and disorganization of extracellular matrix was prominent as evident from histomorphological changes which was prominent in Group-1. The inflammation was prominent in Group-1 as confirmed by the presence of immune cells while the mild/negligible in Group-2. The MRI analysis of the patients was also provided a similar trend (data not shown in the article). Apart from ECM disorganization, the Group-2 patients displayed normal tendon cells as well as normal tissues towards the median side and the normal tenocytes were characterized by their less dense distribution of nuclei surrounding an intact ECM. But, the tenocytes were found to be clustered at the vicinity of ECM disorganization. Two patients of Group-1 showed fatty infiltration. Neoangiogenesis was also predominant in Group-1 while it was completely absent in Group-2 tendons which is a hallmark of inflammation and associated repair. The results are shown in Fig. 1.

Immunofluorescence

Immunofluorescence assessment of the tendon tissue specimens revealed the presence of CD68^{+} macrophages (Fig. 2) and CD16^{+} neutrophils (Fig. 3) predominantly in the Group-1 patients. This indicates inflammation associated with arthritis and the existence of macrophages and neutrophils was greater with respect to the severity of arthritis as evident from MRI analysis (data not shown in this article). All the Group-2 tendons showed absence of macrophages and neutrophils.

miRNA alterations Group-1 vs Group-2

The micro array profiling of whole miRNA showed several fold alterations in the expression status of miRNAs which has regulatory roles in inflammatory pathways and associated genes. 196 miRNAs were found to be down regulated with a fold change less than -3. Several miRNAs like hsa-miR-191-5p, hsa-miR-361-5p, hsa-miR-1273g-3p, hsa-miR-99b-5p, hsa-miR-145-5p, hsa-miR-99a-5p, and hsa-miR-100-5p were exhibited to have a fold change less than −50. Thirteen miRNAs were downregulated with a fold change between −50 and −20, thirty-one miRNAs possessed fold change between −20 and −10 while the rest of 144 miRNAs showed fold change in between −10 and −3 (Table 2). Thirty-nine miRNAs were upregulated and out of which twenty-six were in the fold change

between 2- and 3-fold and nine were in between 3-fold and 4-fold change (Table 3). The hsa-miR-4467 (4.09), hsa-miR-6723-5p (4.57), hsa-miR-8071 (5.14), and hsa-miR-5001-5p (5.57) were the highly-upregulated ones.

The key genes associated with JAK/STAT pathway of inflammation as reported elsewhere were evaluated [29] and were used as input in NetworkAnalyst to assess the cross talk of these genes with other signaling pathways of inflammation. The data revealed the involvement and interaction between 3430 different genes (Supplementary Table 1). From this gene repository, the interactions among JAK2/STAT3 signaling pathway and corresponding cross talk were chosen and the gene numbers narrow down to 284 (Table 4). These 284 genes are active members of 120 different pathways connected with inflammation. Each of the pathways and number of genes (hits) are displayed in (Supplementary Table 2). The 113 genes associated with the immune system were displayed to be closely linked to inflammation via JAK2/STAT3 signaling and around 1500 miRNAs were found to play regulatory roles associated with these genes (Supplementary Table 3).

Hundreds of miRNAs obtained were exhibited multiple targets, where 77 miRNAs were found to regulate more than 10 genes (which altogether regulates 966 genes) where hsa-miR-21-5p was prominent with 22 target genes (Table 5). Among these hsa-miR-498 and hsa-miR-297 were upregulated ones. Neglecting the downregulated genes above −10FC the miRNA list was again narrowed down to 25 which altogether regulate 330 genes associated with inflammation (Table 6). On considering the fold change hsa-miR-145-5p (−55.42), hsa-miR-100-5p (−51.6), hsa-miR-23b-3p (-35.32), hsa-let-7d-5p (−31.63), hsa-miR-146a-5p (−29.52), hsa-miR-150-5p (−23.41), hsa-miR-181a-5p (−21.34) and hsa-miR-193b-3p (−20.35) were downregulated more than 20-fold suggesting their potential role in regulating inflammation. These miRNAs participate in the regulation of 102 genes.

Discussion

Tendon disorders are mostly associated with pain and inflammation and a high proportion of patients are susceptible to other inflammatory diseases like glenohumeral arthritis and/or osteoarthritis. Coincidence of such clinical conditions aggravates clinical symptoms and hurdles the repair and regeneration of injured tendon. The histology of patients with tendinopathy (without arthritis) presents degenerative lesions even without classical symptoms of inflammation [30]. However, in molecular level the upregulation of inflammatory receptors and immunoglobulins and an increased infiltration of immune cells were found to be coupled with tendinopathies [31]. The molecular mechanism leading to inflammation and enhanced cell infiltration in an otherwise avascular tissue like rotator cuff tendon is poorly revealed. Also, how the tendinopathies deal with the coincidence of arthritis is yet to be explored. The role of cytokines like IL-6, TNF-α, IL-1β and IFN-γ and inflammatory mediators like prostaglandins were established in tendon tissue as well but their regulatory mechanisms are largely unknown [4], [32]. Moreover, the epigenetic regulation of inflammatory responses by miRNAs in shoulder tendinopathies is rare in the literature. Our previous article evaluates and describes the regulatory roles of several potential miRNAs associated with the ECM integrity of long head of the biceps with respect to glenohumeral arthritis. The miRNAs common to ECM regulation and inflammation

signifies the interrelation of these two events in the pathogenesis of RCI [27]. The focus of this study was to elucidate and screen the miRNAs associated with inflammatory events regarding JAK2/STAT3 pathway using the data from same set of patients.

JAK2/STAT3 pathway is one of the predominantly operated signaling which ends up in the activation of a battery of pro-inflammatory genes. Members of JAK2/STAT3 form potential targets for anti-inflammatory therapies [33] and the pathway has been aggravated in inflammatory diseases like arthritis [34]. Limited reports are available on the execution of JAK2/STAT3 pathway in tendon tissue and the actual trigger for this pathway is largely unknown. JAK2/STAT3 pathway has also been reported to be regulated epigenetically by miRNAs as well as DNA and histone modifications. To cite, let-7 miRNA suppress SOCS3 expression and blocks STAT3 phosphorylation by JAK2 and subsequent downstream signaling in PDAC (pancreatic ductal adeno carcinoma) cell lines while the inhibition of let-7 resulted in IL-6 coupled activation of STAT3 [35]. But, to the best of our knowledge the reports regarding miRNA mediated regulation of inflammation in shoulder tendon and the correlation with arthritis are unavailable in the literature. Our attempt was to screen and elucidate the roles of miRNAs associated with inflammation regarding JAK2/STAT3 pathway based on the coincidence of glenohumeral arthritis. In the current study, 10 miRNAs were found to be crucial for the JAK2/STAT3 pathway in shoulder tendon which has implications on inflammation associated with arthritis.

hsa-miR-145-5p was one of the top ten downregulated miRNAs from our array data which has been reported to be key to innate immune response [36]. miR-145-5p was reported to be involved in phagocyte differentiation, migration and action, migration and proliferation of endothelial cells, smooth muscle cells and lymphocytes, TNF family of ligand mediated apoptosis by activating NF-κβ and MAPKs [37]. Our results also revealed the association of hsa-miR-145-5p with STATs and IFN-γ and corresponding receptors which are related to JAK2/STAT3 pathway of inflammation. Also, the presence of CD68+ macrophages, CD16+ neutrophils, and angiogenesis, which were completely absent in Group 2, in our Group 1 patients substantiates the role of hsa-miR-145-5p in aggravating inflammation of shoulder tendon tissue which can be correlated with arthritis.

hsa-miR-100-5p is another highly regulated miRNA whose targets were found to be NF-κβ, MAPK (MAPK8 and MAPK1), IL-6 receptor, PTK2 and so on. Another potent target of hsa-miR-100-5p is mTOR which signifies its role in mTOR pathway resulting in cell growth and proliferation. The downregulation of hsa-miR-100-5p in Group 1 patients can be a reason for their delayed repair responses and persistence of tissue damage. The direct involvement of hsa-miR-100-5p in inflammatory pathways is unknown and our results suggest that it can be linked to inflammation via NF-κβ and JAK2/STAT3 pathway through IL-6R.

hsa-miR-23b-3p has been established to be a regulator of inflammatory cytokines including NF-κβ and TNF-α and inhibits inflammation. hsa-miR-23b-3p is multifunctional and regulates pathways mediating cell proliferation, adhesion, differentiation and apoptosis [38], [39]. Contrastingly, hsa-miR-23b-3p exhibited a 35.2-fold decrease in Group 1 tendons, where the inflammation and ECM damage was severe, suggesting the existence of alternate

routes of regulation. From our data, it was evident that IL-6R, STAT5 and EP300 are regulated by hsa-miR-23b-3p and its downregulation results in the upregulation of these genes. Being a histone modifier (due to histone acetyl transferase activity), EP300 can facilitate the transcription of a battery of pro-inflammatory genes.

let-7 (human lethal-7) miRNAs are highly conserved and common of all miRNAs which is a family of 13 miRNAs. hsa-let-7d-5p is involved in the regulation of cell cycle genes and its downregulation is linked with carcinogenesis [40]. The active role of hsa-let-7d-5p in inflammation is yet to be unveiled. IL5 and STAT5 form the target of hsa-let-7d-5p, as evident from our data, suggesting its role in inflammatory pathways. As with other highly regulated miRNAs the downregulation of hsa-let-7d-5p can be correlated with the severity of inflammation in Group 1 tendons.

hsa-miR-146a-5p is also a potent regulator of innate inflammatory responses and are found to be upregulated in cells challenged with TNF-α and lipopolysaccharide. hsa-miR-146a-5p inhibits inflammation by targeting interleukin-1-receptor-associated kinase-1 (IRAK1) and TNF receptor associated factor 6 (TRAF6), the modulators of NF-κβ [41], [42]. hsa-miR-146a-5p was found to mediate IRAK, TRAF6, NF-κβ, EGFR and ICAM1 in our data which substantiate these findings. Also, the hsa-miR-146a-5p mediated regulation of IL-1R and STATs in our results signifies the involvement of JAK/STAT pathway in RC tendon inflammation. Moreover, the downregulation of hsa-miR-146a-5p can be a potent reason for the severity of inflammation of Group 1 patients.

The main function of hsa-miR-150-5p was reported to be the regulation of angiogenesis [43]. It is also expressed in NK cells, and B and T cells of immune system and the high levels of hsa-miR-150-5p is associated with defect in immune system and sustenance of inflammation [44]. Our data shows several other targets of hsa-miR-150-5p of which NF-κβ is typical for inflammation. The action of hsa-miR-150-5p causes the inhibition of NF-κβ signaling and there by inflammation [45]. This may be the reason why the downregulation of hsa-miR-150-5p correlates with the severity of inflammation and enhanced angiogenesis in Group 1 tendons.

hsa-miR-181a-5p is involved in inflammation associated with cancer and its main target was found out to be IL-1a. The anti-inflammatory effects of hsa-miR-181a-5p has been established in lipopolysaccharide challenged macrophage and monocyte cell lines [46], [47]. Apart from IL-1a, TNF-α and IL-6 were also established to be the targets for hsa-miR-181a-5p suggesting its anti-inflammatory roles. The feedback regulation elicited by hsa-miR-181a-5p against TNF-α induced inflammation on targeting p300/CBP on hepatic epithelial cells reveals its mechanism of regulation at the molecular level. TLR4 and NF-κβ form other potential targets for hsa-miR-181a-5p and it has significant role in relieving oxidative stress associated with inflammation as well [46]. Moreover, hsa-miR-181a-5p is one of the prominent miRNAs reported to be regulated during exercise [48] and so has significant implications on tendinopathies. But, their regulatory roles in tendons, especially in shoulder tendons, are largely unknown. Our data revealed the IFN-γ, MAPK1, IL-5, and IRAK1 to be the targets for hsa-miR-181a-5p which can be linked to JAK2/STAT3 pathway through IFN-γ and MAPK1. The inflammation in Group 1 tendons can be a result of

downregulation of hsa-miR-181a-5p and subsequent upregulation of these pro-inflammatory mediators.

hsa-miR-193b-3p was demonstrated to be associated with tumor suppression by targeting D1 cyclins in prostate cancer cells [49]. hsa-miR-193b-3p also targets collagen type-2, aggrecan and SOX5 and regulates chondrocyte metabolism [50]. To the best of our knowledge, the reports of hsa-miR-193b-3p mediated regulation of inflammatory responses are rare. Our data show genes associated with inflammatory signaling especially, MAPK1, MAPK8, IRF1, IRAK1 and NF-κβ are being targeted by hsa-miR-193b-3p as the miRNA is downregulated in Group 1 patients.

hsa-miR-498 was reported to be associated with rheumatoid cancer and arthritis as well as allergy [51], [52]. hsa-miR-498 has tumor suppressor function as it targets FOXO3 gene to inhibit cell proliferation [53]. Also, this miRNA activates smooth muscle cell proliferation mediated through VEGFR [54]. Even though, limited reports are available regarding the regulation of hsa-miR-498 in inflammatory signaling, it can be linked to inflammation because EP300 and IL1R1 form potential targets [54, 55]. The results from our analysis showed hsa-miR-498 was upregulated in Group 1 tendons and acts by JAK3, NF-κβ and MAPK1 showing their role in inflammation. Still, the upregulation of NF-κβ and MAPK1 by hsa-miR-498 can be less effective due to lesser fold degree of fold change (more than 10 times) when compared with miRNAs targeting the same. hsa-miR-297, another potentially upregulated miRNA in Group 1 tendons, is involved in multidrug resistance (MDR) in cancer cells by modulating MRP-2 (MDR associated protein −2) [56]. IFN-γ, IFNGR1, IRAK1, SOSC1, and Bcl2 are the target genes of inflammation mediated by hsa-miR-297 as derived from our data.

We are the first to report whole miRNA profile from shoulder tendon based on the coincidence of glenohumeral arthritis and 10 miRNAs (8 downregulated and 2 upregulated) were chosen from the pool of highly regulated miRNAs based on their potent target inflammatory genes about JAK2/STAT3 pathway. Inflammatory pathways other than JAK2/STAT3 (TREM-1 signaling, TLR signaling etc.) are also prevalent in shoulder tendons [25]. The compilation of all the inflammatory pathways, interconnecting genes and associated miRNAs may pool up bulk of information which is beyond the scope of this article. Interestingly, we have reported similar approach to screen the miRNAs associated with ECM disorganization and several miRNAs were found to be common for ECM disorganization and JAK2/STAT3 pathway [27]. However, integration of miRNAs associated with the pathological events and pathways could be appreciated for screening and identification of miRNAs with therapeutic potential for the management of RCI.

These miRNAs could be valuable as signature miRNAs in the pathophysiology of RCI and might help in the treatment strategies for the repair of rotator cuff. Obviously, additional studies are warranted to elucidate their therapeutic potential. *In vitro* and *in vivo* evaluations of these miRNAs using appropriate mimics and inhibitors need to be validated before extending these to therapeutic arena. The supplementation of the downregulated miRNAs either individually or in combination can benefit millions of RCI sufferers throughout the globe. Moreover, the lack of normal control specimen, variations in clinical history patients,

and lesser RNA yield (being collagenous and lesser cellularity of tendons particularly of Group 2 makes RNA isolation and purification challenging from the available biceps tendon) form major hurdles to the study. Still, the study has thrown new insights into the key miRNA players in shoulder tendon inflammation by effectively correlating with coincidence and severity of glenohumeral arthritis.

Conclusion

The miRNAs were screened with respect to their targets of inflammation mediated by JAK2/ STAT3 pathway on patients with RCI and glenohumeral arthritis and patients without glenohumeral arthritis or rotator cuff tears. The levels of hsa-miR-145-5p, hsa-miR-100-5p, hsa-miR-23b-3p, hsa-let-7d-5p, hsa-miR-146a-5p, hsa-miR-150-5p, hsa-miR-181a-5p and hsa-miR-193b-3p were predominantly downregulated in glenohumeral arthritis tendon where the severity of inflammation was greater. This suggests their regulatory roles in eliciting inflammatory responses by targeting key inflammatory genes JAK2/STAT3 and interconnecting pathways. Targeting these miRNAs and the knowledge of their regulatory mechanisms would be critical to develop promising therapies in the management of shoulder pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

List of abbreviations

DAPI	4′,6- diamidino-2-phenylindole
ECM	extracellular matrix
JAK	janus activated kinase
miRNA	microRNA
PBS	phosphate buffered saline
RCI	rotator cuff injury
RIN	RNA integrity number
STAT	signal transducers and activators of transcription

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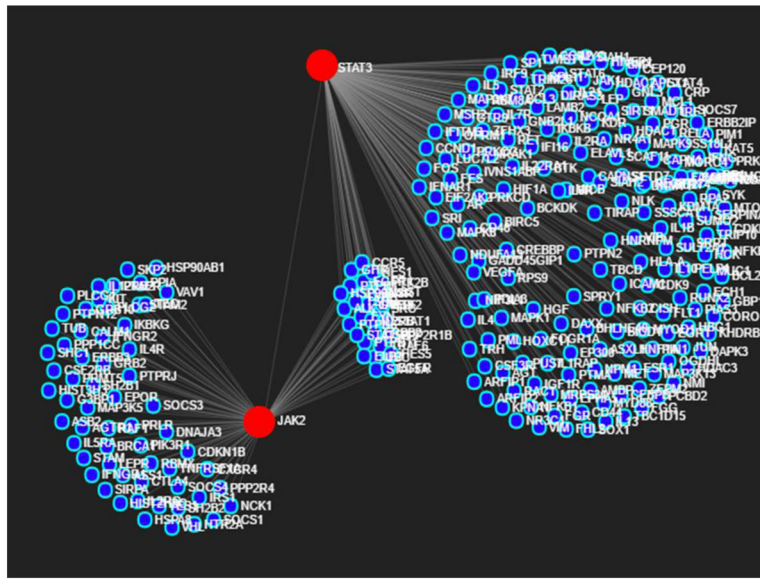
Fig. 1.
Determination of genes associated with JAK2 and STAT3 by NetworkAnalyst program.
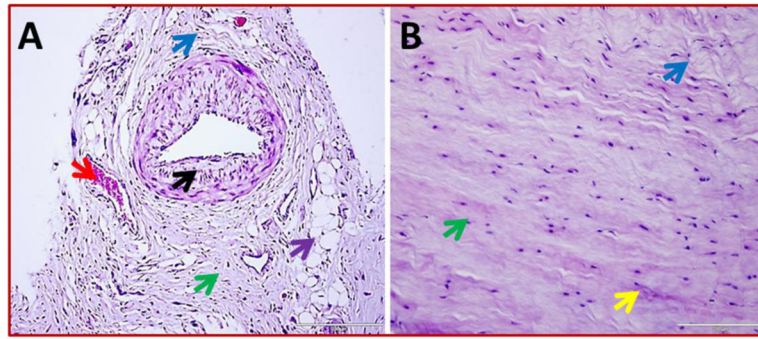
Fig. 2.
Representative images for H&E staining of biceps tendons – (A) Group-1 (represents four patient) and (B) Group-2 (represents four) showing difference in tissue organization. ECM disorganization and inflammation are more prominent in Group-1. The key features are indicated by colored arrows: green arrows - tendon cells, black arrows – inflammation, red arrows – angiogenesis, blue arrows - ECM disorganization, violet - fatty infiltration, and yellow arrows - normal ECM with dense collagen deposition. The Figs are shown in 400× magnification.
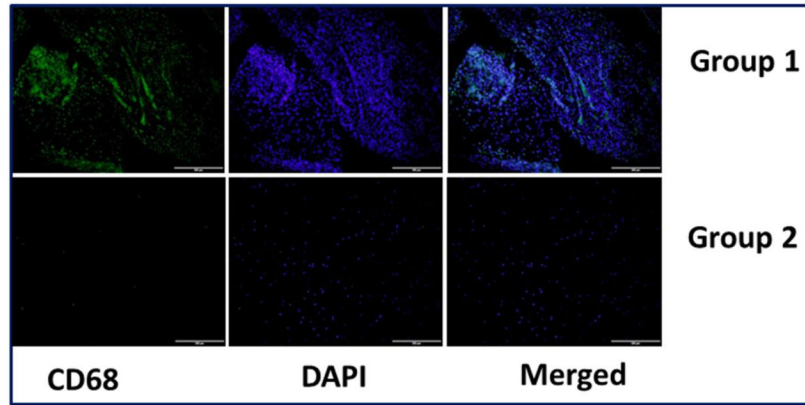
Fig. 3.
Representative images for CD68$^+$expression in the tendon tissues of Group-1 and Group-2 patients by immunofluorescence. (A) Group-1 (represents four patient) and (B) Group-2 (represents four patient) patients. Group-1 tendons displayed higher density of macrophages than Group-2.
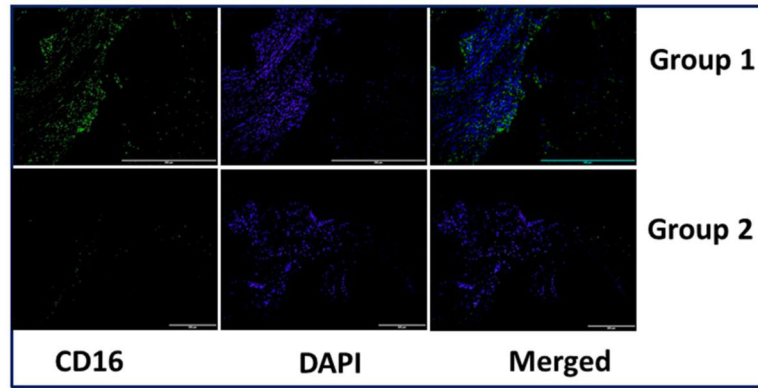
Fig. 4.
Representative images for CD16^{+}expression in the tendon tissues of Group-1 and Group-2 patients by immunofluorescence. (A) Group-1 (four patient) and (B) Group-2 (represents four patient) patients. Group-1 tendons displayed higher density of neutrophils than Group-2.

Table 1

The genes associated with JAK/STAT pathway of inflammation used as input to NetworkAnalyst.

Activity	Genes
Janus Kinase Activity	JAK1, JAK2, JAK3, TYK2
STAT Family	STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6
Receptors	CSF1R, CSF2RB, EGFR, EPOR, F2R, GHR, IFNAR1, IFNGR1, IL10RA, IL2RA, IL2RG, IL4R, IL6ST, INSR, MPL, PDGFRA, SH2B1
Phosphorylation	F2, F2R, IL20, PPP2R1A, PRLR, STAT1
Regulators	HMGA1, SMAD3, SLA2, SPI1, STAT3, JUNB, SP1, USF1, PIAS1, YY1, SMAD1, SMAD5, CEBPB, CRK, GATA3, IRF1, IRF9, JUN, MYC, NFKB1, NR3C1, PPP2R1A, SMAD2, SMAD4
STAT induced genes	CXCL9, IRF1, NOS2, A2M, BCL2L1, CDKN1A, CRP, FAS, MMP3, MYC, SOCS1, FCGR1A, IFNG, MYC, CCND1, CDKN1A, IL2RA, OSM, GATA3, GBP1, OAS1
Negative Regulators	PIAS1, PIAS2, PTPN1, PTPRC, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5

Table 2

Downregulated miRNAs – Group-1 vs Group-2 comparison

miRNAs	FC
hsa-miR-191-5p	−71.26
hsa-miR-361-5p	−69.26
hsa-miR-1273g-3p	−64.17
hsa-miR-99b-5p	−59.57
hsa-miR-145-5p	−55.42
hsa-miR-99a-5p	−51.68
hsa-miR-100-5p	−51.6
hsa-miR-23b-3p	−35.32
hsa-miR-425-5p	−33.79
hsa-miR-151a-3p	−32.1
hsa-let-7a-5p	−31.63
hsa-miR-22-3p	−30.84
hsa-let-7d-5p	−29.9
hsa-miR-146a-5p	−29.52
hsa-miR-409-3p	−28.06
hsa-miR-127-3p	−26.62
hsa-miR-150-5p	−23.41
hsa-miR-181a-5p	−21.34
hsa-miR-4269	−21.09
hsa-miR-193b-3p	−20.35
hsa-miR-30d-5p	−19.32
hsa-miR-378c	−19.16
hsa-miR-342-3p	−18.02
hsa-miR-199a-3p	−17.88
hsa-miR-199b-3p	−17.88
hsa-miR-378i	−15.69
hsa-miR-486-5p	−15.04
hsa-miR-103a-3p	−14.87
hsa-miR-134-5p	−14.82
hsa-miR-31-5p	−14.44
hsa-miR-23a-3p	−14.18
hsa-miR-422a	−14.1
hsa-miR-195-5p	−14.04
hsa-miR-1246	−14.01
hsa-miR-26a-5p	−13.97
hsa-miR-382-5p	−13.14
hsa-let-7c-5p	−12.58

miRNAs	FC
hsa-miR-378f	−12.46
hsa-miR-497-5p	−12.3
hsa-miR-10b-5p	−12.27
hsa-mir-361	−12.13
hsa-miR-199a-5p	−11.9
miRNAs	**FC**
hsa-let-7e-5p	−11.7
hsa-miR-30a-5p	−11.48
hsa-miR-193a-5p	−10.93
hsa-miR-3178	−10.86
hsa-miR-15a-5p	−10.66
hsa-miR-574-3p	−10.54
hsa-miR-451a	−10.45
hsa-miR-500a-3p	−10.25
hsa-miR-151a-5p	−10.22
hsa-miR-21-5p	−10.11
hsa-miR-125a-5p	−9.69
hsa-miR-4532	−8.88
hsa-miR-29a-3p	−8.8
hsa-miR-125b-2-3p	−8.66
hsa-let-7i-5p	−8.38
hsa-miR-370-3p	−8.38
hsa-miR-28-5p	−8.31
hsa-miR-130a-3p	−8.11
hsa-miR-214-5p	−8.1
hsa-miR-106b-5p	−8.08
hsa-miR-3651	−8.03
hsa-miR-16-5p	−7.98
hsa-let-7b-5p	−7.73
hsa-miR-30c-5p	−7.67
hsa-miR-132-3p	−7.55
hsa-miR-663a	−7.43
hsa-miR-25-3p	−7.23
hsa-miR-339-5p	−7.22
hsa-miR-146b-5p	−7.14
hsa-miR-193a-3p	−7.13
hsa-miR-532-3p	−7.12
hsa-miR-378d	−7.01
hsa-miR-744-5p	−6.94

miRNAs	FC
hsa-miR-193b-5p	−6.85
hsa-mir-711	−6.77
hsa-miR-27b-5p	−6.64
hsa-miR-3195	−6.59
hsa-miR-708-5p	−6.41
hsa-miR-4674	−6.29
hsa-miR-491-5p	−6.22
hsa-miR-130b-3p	−6.11
hsa-miR-24-2-5p	−5.92
miRNAs	**FC**
hsa-miR-652-3p	−5.88
hsa-miR-339-3p	−5.8
hsa-miR-221-3p	−5.72
hsa-miR-381-3p	−5.67
hsa-miR-139-5p	−5.65
hsa-miR-4741	−5.58
hsa-miR-4443	−5.45
hsa-miR-874-3p	−5.31
hsa-miR-21-3p	−5.23
hsa-miR-324-3p	−5.2
hsa-miR-4417	−5.2
hsa-miR-6722-3p	−5.15
hsa-miR-154-5p	−5.1
hsa-miR-362-5p	−5.1
hsa-miR-665	−5.1
hsa-miR-1233-5p	−5.09
hsa-miR-487b-3p	−5.06
hsa-miR-99b-3p	−5.02
hsa-miR-30a-3p	−5.01
hsa-miR-28-3p	−4.94
hsa-miR-27a-3p	−4.89
hsa-miR-337-5p	−4.85
hsa-miR-664b-5p	−4.82
hsa-miR-6779-5p	−4.78
hsa-miR-122-5p	−4.69
hsa-miR-20a-5p	−4.67
hsa-miR-7977	−4.67
hsa-miR-1271-5p	−4.61
hsa-miR-17-3p	−4.6

miRNAs	FC
hsa-miR-6771-5p	−4.59
hsa-miR-3687	−4.52
hsa-miR-378g	−4.51
hsa-miR-342-5p	−4.46
hsa-miR-671-5p	−4.45
hsa-miR-4507	−4.42
hsa-miR-4651	−4.41
hsa-miR-324-5p	−4.31
hsa-miR-4707-5p	−4.31
hsa-miR-2110	−4.28
hsa-miR-151b	−4.2
hsa-miR-664b-3p	−4.19
hsa-miR-143-3p	−4.17
hsa-miR-93-5p	−4.15
hsa-miR-378a-3p	−4.14
hsa-miR-6816-5p	−4.13
hsa-miR-494-3p	−4.06
hsa-miR-197-3p	−4.03
hsa-miR-10a-5p	−4.02
hsa-miR-432-5p	−4.02
hsa-miR-532-5p	−4.02
hsa-miR-7975	−3.99
hsa-miR-15b-5p	−3.98
hsa-miR-345-5p	−3.98
hsa-miR-409-5p	−3.93
hsa-miR-1343-5p	−3.92
hsa-miR-143-5p	−3.9
hsa-miR-1587	−3.9
hsa-miR-433-3p	−3.89
hsa-miR-664a-5p	−3.89
hsa-miR-3197	−3.89
hsa-miR-379-5p	−3.88
hsa-miR-3621	−3.86
hsa-miR-4433-3p	−3.83
hsa-miR-140-3p	−3.81
hsa-miR-20b-5p	−3.72
hsa-mir-28	−3.71
hsa-miR-10b-3p	−3.7
hsa-miR-6789-5p	−3.66

miRNAs	FC
hsa-miR-6798-5p	−3.63
hsa-miR-1909-3p	−3.61
hsa-miR-377-5p	−3.56
hsa-mir-4281	−3.54
hsa-miR-17-5p	−3.53
hsa-miR-24-3p	−3.53
hsa-miR-155-5p	−3.49
hsa-miR-4299	−3.49
hsa-miR-4505	−3.46
hsa-miR-455-3p	−3.45
hsa-miR-92b-3p	−3.43
hsa-miR-1268b	−3.41
hsa-miR-185-5p	−3.39
hsa-miR-224-3p	−3.38
hsa-miR-4758-5p	−3.35
hsa-miR-106b-3p	−3.33
hsa-miR-127-5p	−3.33
hsa-miR-4632-5p	−3.33
hsa-miR-4649-5p	−3.32
hsa-miR-4688	−3.31
hsa-miR-1225-5p	−3.3
hsa-miR-6765-5p	−3.29
hsa-miR-493-3p	−3.27
hsa-miR-92b-5p	−3.26
hsa-miR-3175	−3.25
hsa-miR-4646-5p	−3.25
hsa-miR-654-3p	−3.23
hsa-miR-1307-3p	−3.2
hsa-miR-106a-5p	−3.18
hsa-miR-503-5p	−3.17
hsa-miR-6132	−3.17
hsa-miR-619-5p	−3.16
hsa-miR-181a-3p	−3.14
hsa-miR-629-5p	−3.12
hsa-miR-378e	−3.11
hsa-miR-139-3p	−3.09
hsa-miR-181b-5p	−3.09
hsa-miR-452-5p	−3.06
hsa-miR-23b-5p	−3.04

miRNAs	FC
hsa-miR-937-5p	−3.03
hsa-miR-378a-5p	−3
hsa-miR-6126	−3

Table 3

Upregulated miRNAs – Group-1 vs Group-2 comparison

miRNAs	FC
hsa-miR-4327	2.02
hsa-miR-8072	2.04
hsa-miR-498	2.05
hsa-miR-1281	2.05
hsa-miR-2861	2.05
hsa-miR-7110-5p	2.13
hsa-miR-6775-5p	2.16
hsa-mir-6722	2.18
hsa-miR-6831-5p	2.27
hsa-mir-320e	2.29
hsa-miR-6769b-5p	2.29
hsa-miR-4487	2.3
miRNAs	**FC**
hsa-miR-6749-5p	2.52
hsa-miR-483-5p	2.56
hsa-miR-1229-5p	2.56
hsa-mir-6836	2.59
hsa-miR-6127	2.73
hsa-mir-550a-1	2.84
hsa-mir-550a-2	2.84
hsa-mir-550a-3	2.84
hsa-miR-4745-5p	2.86
hsa-miR-7107-5p	2.87
hsa-miR-4481	3.01
hsa-miR-6732-5p	3.06
miRNAs	**FC**
hsa-miR-8075	3.16
hsa-miR-4668-5p	3.73
hsa-miR-297	3.81
hsa-miR-6124	3.85
hsa-miR-7150	3.86
hsa-miR-6870-5p	3.95
hsa-miR-4467	4.09
hsa-miR-6723-5p	4.57
hsa-miR-8071	5.14
hsa-miR-5001-5p	5.57

Table 4

Specific cross talk and interactions between genes of JAK2/STAT3 pathway of inflammation as revealed by NetworkAnalyst.

Id	Label
P40763	STAT3
O60674	JAK2
P42224	STAT1
P18031	PTPN1
P12931	SRC
P30154	PPP2R1B
Q06124	PTPN11
P42229	STAT5A
P51692	STAT5B
P10912	GHR
P0CG48	UBC
P06241	FYN
Q6IA86	ELP2
P07948	LYN
Q9Y4K3	TRAF6
P00533	EGFR
P04626	ERBB2
P29350	PTPN6
P52333	JAK3
Q14289	PTK2B
P06213	INSR
P78347	GTF2I
P40189	IL6ST
P51681	CCR5
Q9UM73	ALK
Q05397	PTK2
P16473	TSHR
P14784	IL2RB
P07900	HSP90AA1
P11362	FGFR1
Q14469	HES1
Q5TA89	HES5
Q9UER7	DAXX
O43318	MAP3K7
Q9UBE8	NLK
Q13287	NMI
Q13283	G3BP1
P51617	IRAK1

Id	Label
Q06187	BTK
P05129	PRKCG
P45983	MAPK8
P23458	JAK1
P31785	IL2RG
P06454	PTMA
P08670	VIM
P63244	GNB2L1
P01019	AGT
Q9Y6X2	PIAS3

Id	Label
P26358	DNMT1
Q13547	HDAC1
Q00653	NFKB2
P42345	MTOR
Q14765	STAT4
Q9Y5S9	RBM8A
Q14192	FHL2
P16333	NCK1
P62993	GRB2
P19174	PLCG1
Q99683	MAP3K5
P01584	IL1B
Q13263	TRIM28
P38936	CDKN1A
P22301	IL10
P20396	TRH
P35372	OPRM1
P01282	VIP
P69891	HBG1
P02741	CRP
P15941	MUC1
O15392	BIRC5
P12314	FCGR1A
P32455	GBP1
P05451	REG1A
P15529	CD46
Q16666	IFI16
Q00978	IRF9
P15692	VEGFA
P24385	CCND1
P50750	CDK9

Id	Label
Q8IZL8	PELP1
P17676	CEBPB
P20749	BCL3
O15379	HDAC3
Q92769	HDAC2
Q9Y6K9	IKBKG
O75177	SS18L1
O14543	SOCS3
Q99062	CSF3R
P07949	RET
P52294	KPNA1
Q04206	RELA
O00570	SOX1
P61073	CXCR4
Q29980	MICB
P05412	JUN
P15260	IFNGR1
Id	**Label**
P35568	IRS1
P15927	RPA2
P29353	SHC1
P16471	PRLR
P04150	NR3C1
P10275	AR
P20823	HNF1A
Q92783	STAM
O75886	STAM2
P08887	IL6R
P08238	HSP90AB1
P21802	FGFR2
P16410	CTLA4
P11142	HSPA8
Q99665	IL12RB2
Q9NPH3	IL1RAP
Q92993	KAT5
P38398	BRCA1
Q96Q27	ASB2
Q15257	PPP2R4
Q07666	KHDRBS1
P03372	ESR1
Q05513	PRKCZ
P04632	CAPNS1

Id	Label
Q8TE76	MORC4
Q9ULD0	OGDHL
Q06520	SULT2A1
P46781	RPS9
Q9GZT8	NIF3L1
P62913	RPL11
P09769	FGR
O43255	SIAH2
P07384	CAPN1
Q8N6P7	IL22RA1
P15822	HIVEP1
P04439	HLA-A
P02760	AMBP
O60232	SSSCA1
Q8IXJ9	ASXL1
P45984	MAPK9
P06748	NPM1
P38159	RBMX
O43283	MAP3K13
P00966	ASS1
Q07820	MCL1
Q13950	RUNX2
Q16695	HIST3H3
P46527	CDKN1B
Id	**Label**
P06401	PGR
P08631	HCK
Q9Y383	LUC7L2
P62158	CALM1
Q9BT73	PSMG3
O95661	DIRAS3
P52630	STAT2
P11309	PIM1
P21860	ERBB3
P10914	IRF1
P16885	PLCG2
P28482	MAPK1
O43293	DAPK3
P17948	FLT1
P35968	KDR
P05362	ICAM1
P17181	IFNAR1

Id	Label
P24394	IL4R
P08069	IGF1R
Q09472	EP300
P02679	FGG
Q8WXH5	SOCS4
O14512	SOCS7
Q13526	PIN1
P19438	TNFRSF1A
P01579	IFNG
Q8TAE8	GADD45GIP1
P32927	CSF2RB
P27986	PIK3R1
O15524	SOCS1
P01100	FOS
P08047	SP1
P01589	IL2RA
Q9NSE2	CISH
P01106	MYC
P14210	HGF
Q15672	TWIST1
Q9HBE4	IL21
Q99836	MYD88
P01011	SERPINA3
Q9NZQ7	CD274
Q12913	PTPRJ
P21246	PTN
Q01628	IFITM3
P41597	CCR2
Q96EY1	DNAJA3
Q16665	HIF1A
P27695	APEX1
P35228	NOS2
P16871	IL7R
Q9BYH8	NFKBIZ
P06400	RB1
Q92793	CREBBP
P51532	SMARCA4
Q9H0N5	PCBD2
Q6PD62	CTR9
O43248	HOXC11
P42226	STAT6
Q9NRF2	SH2B1

Id	Label
O14492	SH2B2
O14920	IKBKB
P18146	EGR1
P58753	TIRAP
Q13322	GRB10
P38484	IFNGR2
P50607	TUB
Q01344	IL5RA
P29590	PML
P15172	MYOD1
Q15642	TRIP10
O14744	PRMT5
Q9P0J0	NDUFA13
P41159	LEP
P54756	EPHA5
P51813	BMX
P30101	PDIA3
P19838	NFKB1
P63000	RAC1
P19235	EPOR
P19525	EIF2AK2
P42680	TEC
Q15788	NCOA1
P08581	MET
P62937	PPIA
P15498	VAV1
P78324	SIRPA
Q15797	SMAD1
Q05209	PTPN12
P07332	FES
Q13309	SKP2
Q05655	PRKCD
P04049	RAF1
P07947	YES1
P17706	PTPN2
P10721	KIT
P28223	HTR2A
O14503	BHLHE40
P30626	SRI
Q92665	MRPS31
Q99590	SCAF11
O14874	BCKDK

Id	Label
P55268	LAMB2
Q8IUQ4	SIAH1
Q96RT1	ERBB2IP
P31146	CORO1A
Q13011	ECH1
P53365	ARFIP2
P22736	NR4A1
P52272	HNRNPM
Q8WW38	ZFPM2
Q9BVP2	GNL3
Q96G01	BICD1
Q8N960	CEP120
O43609	SPRY1
Q9BXP5	SRRT
Q8NEM7	FAM48A
P05112	IL4
P05113	IL5
P35225	IL13
P10415	BCL2
P40337	VHL
Q96EB6	SIRT1
O60341	KDM1A
Q71DI3	HIST2H3C
P16070	CD44
Q8WTS6	SETD7
Q15717	ELAVL1
P61956	SUMO2
P36873	PPP1CC
Q8TC07	TBC1D15
Q9BTW9	TBCD
Q96PZ0	PUS7
P53367	ARFIP1
P30556	AGTR1
P43246	MSH2
Q9Y6Y0	IVNS1ABP
P25105	PTAFR
Q15911	ZFHX3
P43405	SYK
P48357	LEPR

Table 5

The 77 miRNAs of Group 2 vs Group 1 tendons which were found to be regulating more than 10 target genes.

miRNAs	FC	No: of Hits
hsa-miR-21-5p	−10.11	22
hsa-miR-30a-5p	−11.48	21
hsa-miR-23a-3p	−14.18	19
hsa-miR-125a-5p	−9.69	18
hsa-miR-155-5p	−3.49	18
hsa-miR-16-5p	−7.98	17
hsa-miR-744-5p	−6.94	17
hsa-miR-181a-5p	−21.34	16
hsa-miR-15a-5p	−10.66	16
hsa-miR-93-5p	−4.15	16
hsa-miR-15b-5p	−3.98	16
hsa-miR-181b-5p	−3.09	16
hsa-miR-145-5p	−55.42	15
hsa-miR-132-3p	−7.55	15
hsa-miR-339-3p	−5.8	15
hsa-miR-665	−5.1	15
hsa-miR-23b-3p	−35.32	14
hsa-miR-30d-5p	−19.32	14
hsa-miR-195-5p	−14.04	14
hsa-miR-130a-3p	−8.11	14
hsa-miR-652-3p	−5.88	14
hsa-miR-221-3p	−5.72	14
hsa-miR-17-5p	−3.53	14
hsa-miR-185-5p	−3.39	14
hsa-miR-150-5p	−23.41	13
hsa-miR-103a-3p	−14.87	13
hsa-miR-382-5p	−13.14	13
hsa-miR-497-5p	−12.3	13
hsa-miR-25-3p	−7.23	13
hsa-miR-140-3p	−3.81	13
hsa-miR-92b-3p	−3.43	13
hsa-let-7a-5p	−31.63	12
hsa-let-7b-5p	−7.73	12
hsa-miR-30c-5p	−7.67	12
hsa-miR-130b-3p	−6.11	12
hsa-miR-20a-5p	−4.67	12
hsa-miR-342-5p	−4.46	12
hsa-miR-143-3p	−4.17	12
hsa-miR-10a-5p	−4.02	12

miRNAs	FC	No: of Hits
miRNAs	FC	No: of Hits
hsa-miR-20b-5p	−3.72	12
hsa-miR-377-5p	−3.56	12
hsa-miR-654-3p	−3.23	12
hsa-miR-503-5p	−3.17	12
hsa-miR-100-5p	−51.6	11
hsa-miR-146a-5p	−29.52	11
hsa-miR-134-5p	−14.82	11
hsa-miR-574-3p	−10.54	11
hsa-miR-151a-5p	−10.22	11
hsa-let-7i-5p	−8.38	11
hsa-miR-106b-5p	−8.08	11
hsa-miR-532-3p	−7.12	11
hsa-miR-381-3p	−5.67	11
hsa-miR-139-5p	−5.65	11
hsa-miR-324-3p	−5.2	11
hsa-miR-122-5p	−4.69	11
hsa-miR-433-3p	−3.89	11
hsa-miR-24-3p	−3.53	11
hsa-miR-17-5p	−3.53	11
hsa-miR-455-3p	−3.45	11
hsa-miR-23b-5p	−3.04	11
hsa-miR-193b-3p	−20.35	10
hsa-miR-199a-3p	−17.88	10
hsa-let-7c-5p	−12.58	10
hsa-miR-199a-5p	−11.9	10
hsa-miR-370-3p	−8.38	10
hsa-miR-28-5p	−8.31	10
hsa-miR-339-5p	−7.22	10
hsa-miR-193b-5p	−6.85	10
hsa-miR-708-5p	−6.41	10
hsa-miR-491-5p	−6.22	10
hsa-miR-874-3p	−5.31	10
hsa-miR-337-5p	−4.85	10
hsa-miR-24-3p	−3.53	10
hsa-miR-92b-5p	−3.26	10
hsa-miR-498	2.05	10
hsa-miR-297	3.81	10
Total		**966**

Table 6

Multiple target miRNAs of genes associated with JAK2/STAT3 pathway genes where ten or more target genes were considered.

miRNAs	FC	No: of Hits
hsa-miR-145-5p	−55.42	15
hsa-miR-100-5p	−51.6	11
hsa-miR-23b-3p	−35.32	14
hsa-let-7a-5p	−31.63	12
hsa-miR-146a-5p	−29.52	11
hsa-miR-150-5p	−23.41	13
hsa-miR-181a-5p	−21.34	16
hsa-miR-193b-3p	−20.35	10
hsa-miR-30d-5p	−19.32	14
hsa-miR-199a-3p	−17.88	10
hsa-miR-103a-3p	−14.87	13
hsa-miR-134-5p	−14.82	11
hsa-miR-23a-3p	−14.18	19
hsa-miR-195-5p	−14.04	14
hsa-miR-382-5p	−13.14	13
hsa-let-7c-5p	−12.58	10
hsa-miR-497-5p	−12.3	13
hsa-miR-199a-5p	−11.9	10
hsa-miR-30a-5p	−11.48	21
hsa-miR-15a-5p	−10.66	16
hsa-miR-574-3p	−10.54	11
hsa-miR-151a-5p	−10.22	11
hsa-miR-21-5p	−10.11	22
hsa-miR-498	2.05	10
hsa-miR-297	3.81	10
	Total	**330**