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Cross-sectional study

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Mild inflammation persists in the glenohumeral joint of patients with shoulder instability: Cross-sectional study

Kyoko Muneshige, Tomonori Kenmoku, Kentaro Uchida, Lars Arendt-Nielsen, Ryo Tazawa, Mitsufumi Nakawaki, Daisuke Ishii, Masashi Satoh, Gen Inoue, Masashi Takaso

Keywords: Shoulder instability, Osteoarthritis, Tumor necrosis factor-α, Interleukin-1β, M1 macrophages

1. Introduction

The frequency of osteoarthritis (OA) is lower in the glenohumeral joint than in the hip and knee joints [1–6]. The reason for this difference may be that the shoulder joint is not a weight-bearing joint. In a prospective study, approximately two-thirds of patients with a history of shoulder dislocation were prone to developing OA in the glenohumeral joint [3]. A total of 31% of 282 patients with an anterior dislocation event in the past 40 years had confirmed glenohumeral joint osteoarthritis [5].

The OA progression mechanism has been widely investigated in weight-bearing joints [2,7–10]. OA development and progression in these joints likely involve inflammation. Elevated levels of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β produced by the OA joint synovium promote the activities of matrix metalloproteases (MMPs) and a disintegrant and metallopeptins behaviorally harboring thrombospondin motifs (ADAMTS). These proinflammatory cytokines also contribute to the
downregulation of anabolic events and upregulation of catabolic mechanisms to promote inflammatory responses. These effects of inflammation worsen the structural damage to OA joint. Nevertheless, there are few reports on the OA progression mechanism for the glenohumeral joint [1,11].

Casagrande et al. reported that cyclooxygenase 2 (Cox-2), collagen type I, TNF-α, MMP-3, and ADAMTS5 were related to OA progression since their expression was significantly increased in OA shoulders compared to controls [1]. Therefore, the mechanisms of OA in the shoulder are likely similar to those in other weight-bearing joints. A previous study showed that patients with shoulder instability (SI) exhibited higher IL1B mRNA expression in the glenohumeral synovium than in the subacromial bursa [12]. Microscopic evidence suggests that synovitis occurs in the glenohumeral joint of patients with SI [12]. Furthermore, Cox-2 expression was similar in the glenohumeral synovium of shoulders with instability and rotator cuff tears (RCTs) [13]. According to these reports, mild inflammation may be present in a shoulder with instability. Nonetheless, in patients with SI, the molecular mechanisms underlying the development of glenohumeral synovitis and OA progression remain unknown.

Macrophages are key players in the development and progression of synovitis [14,15]. They can be classified on their activation as classically activated macrophages (proinflammatory M1 phenotype) and alternatively activated macrophages (anti-inflammatory M2 phenotype). M1 macrophages have proinflammatory functions and are responsible for releasing molecules, such as IL-1β and TNF-α, which are associated with knee and hip joint inflammation [16–18]. Nonetheless, it is unclear whether increased macrophage counts and M1 polarization occur in the glenohumeral joint after shoulder dislocation, although these mechanisms may be associated with OA development during SI.

The aim of the present study was to investigate the expression of inflammatory cytokines and macrophage markers in the synovial tissue of the glenohumeral joint in patients with SI compared with patients with RCTs. We hypothesized that mild inflammation might persist in the glenohumeral joint after shoulder dislocation and that inflammation might be associated with OA progression.

2. Method

2.1. Patient selection and sampling

The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. This experimental protocol adhered to institutional guidelines and received approval from the Institutional Review Board (reference number: KMEO B20-093). Written informed consent was obtained from all participants.

From October 2017 to April 2020, synovial membrane samples acquired from the glenohumeral joint, specifically the rotator interval, were obtained from patients who underwent arthroscopic surgery. This included 30 patients who underwent Bankart repair for SI (two with single dislocation, 26 with recurrent dislocation, and two with recurrent subluxations) and 30 patients who underwent arthroscopic rotator cuff repair for degenerative RCTs. All the patients in the SI group had a traumatic episode. Patients with SI and RCT were clinically assessed prior to surgery using the Rowe score and the Constant score, respectively [19,20]. An experienced shoulder surgeon conducted all arthroscopic surgeries. The OA status was assessed radiologically using the Samilson and Prieto grade, and arthroscopically using the Outerbridge classification [21,22].

The patients were allocated to either the SI group or the RCT group based on the details of the surgery. We excluded patients with a history of rheumatoid arthritis or other collagen diseases.

All synovial membrane samples were used for quantitative PCR (qPCR). Additionally, 4 of the 30 synovial samples with sufficient sample volume in each group were analyzed using immunohistochemistry. Another 3 of the 30 samples from the SI group were used for flow cytometry.

2.2. qPCR

To extract total RNA, synovial samples were homogenized using a Polytron homogenizer (Kinematica AG, Luzern, Switzerland). After centrifugation (15,000 rpm, 4 °C, 5 min), the supernatants were mixed with an equal volume of 100% ethanol and vortexed for 30 s, before being transferred to a spin column (Direct-zol RNA Microprep kit; Zymo Research, Irvine, CA, USA). RNA was eluted as per the manufacturer’s protocol. RNA concentration was determined using a DS-11 Spectrophotometer (DeNovix, Wilmington, DE, USA). Total RNA was reverse-transcribed into complementary DNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Transcript-level expression of inflammatory cytokines (TNFA and IL1B), pan-cluster of differentiation (CD) 68, and M1 macrophage (CD80 and CD86) markers was measured by qPCR on a real-time PCR detection system (CFX-96; Bio-Rad, Hercules, CA, USA). The sequences of the PCR primer pairs used are provided in Table 1. Transcript-level expression of the cytokine and macrophage marker genes was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), before being compared between the SI and RCT groups. Additionally, we investigated the correlation between mRNA expression and preoperative features of SI patients, including age at the first dislocation, time interval between the final dislocation and surgery, and mean time interval between injury and stabilization.

2.3. Immunohistochemistry

Immunohistochemistry was performed using streptavidin–biotin peroxidase staining (Histofine SAB-PO kit; Nichirei Biosciences Inc., Tokyo, Japan) according to the manufacturer’s protocol. All the reagents used for immunohistochemistry were provided in the kit except primary antibodies or unless otherwise specified. Synovial tissue from four samples of each group was fixed with 4% paraformaldehyde, before paraffin-embedding and sectioning at 3 μm thickness. After deparaffinization, the tissue sections were incubated in a citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) using a water bath at 98 °C for 40 min, allowed to cool to 20 °C for 20 min, and then washed with PBS. Subsequently, the sections were blocked for 10 min using rabbit serum when mouse primary antibodies were used or normal goat serum when rabbit primary antibodies were used. Anti-TNF-α (dilution, 1:200; Cat. No. ab8348; Abcam, Cambridge, UK), anti-IL-1β (dilution, 1:50; Cat. No. 16806-1-AP; Proteintech Group, Inc., Rosemont, IL, USA), and anti-CD68 (dilution, 1:100; Cat. No. ab125212; Abcam, Cambridge, UK) were then added, and the sections were incubated for 1 h at room temperature (range 20–22 °C). After washing with PBS, tissue sections were incubated with anti-mouse or -rabbit IgG biotinylated antibody for 10 min before washing with PBS. The sections were subsequently incubated with avidin DH

<table>
<thead>
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<th>Direction</th>
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<th>Product size (bp)</th>
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<tr>
<td></td>
<td>R</td>
<td>GTCACTGGGGGTTGCAAGAG</td>
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</tr>
<tr>
<td>IL1B</td>
<td>F</td>
<td>GTACCTGTTCCGGGTTGTTGGA</td>
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</tr>
<tr>
<td></td>
<td>R</td>
<td>GGGAACCTGGCAAGACTCAA</td>
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</tr>
<tr>
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<td>R</td>
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</tbody>
</table>
biotinylated peroxidase for 10 min and then washed with PBS. A reaction using diaminobenzidine solution was performed for visualization. After counterstaining with Mayer’s hematoxylin nuclear counterstain, the sections were washed with tap water and covered with a coverslip.

### 2.4. Flow cytometric analysis

Synovial samples were digested with 1 mg/mL collagenase type I solution at 37 °C for 16 h and passed through a nylon mesh filter with a pore size of 100 μm to obtain single-cell suspensions. The cells were stained with anti-CD45 (conjugated to Pacific Blue, Clone: HI30; BioLegend, San Diego, CA), anti-CD68 (conjugated to PE-Cy7, Clone: IT2.2; BioLegend), and anti-CD80 (conjugated to APC, Clone: 2D10; BioLegend) for 45 min at 4 °C. Immunostaining for CD68 was performed by treating the cells with the fixation/permeabilization solution (BioLegend, San Diego, CA) before incubating with anti-CD68 (conjugated to FITC, Clone: Y1/82A; BioLegend) for 30 min at 4 °C. After washing twice in wash buffer, 100,000 total events were acquired using the BD FACSVersus system (BD Biosciences, San Jose, CA, USA). The data were analyzed using the FlowJo v10.7 software (Tree Star Inc., Ashland, OR, USA). Negative gates were determined using an isotype control.

### 2.5. Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Statistical significance was determined using the nonparametric Mann-Whitney U test or unpaired t-test for comparisons between the two groups. Correlations between mRNA expression and preoperative features in patients with SI were performed using Spearman’s correlation coefficient. We also analyzed the relationship between mRNA expression levels and sex and the correlation with age using the Mann-Whitney U test and Spearman’s correlation coefficient, respectively. P < 0.05 was considered statistically significant. The classification scheme for Spearman’s correlation is defined as follows: 0 < |p| ≤ 0.2, negligible; 0.2 < |p| ≤ 0.4, low; 0.4 < |p| ≤ 0.7, moderate; 0.7 < |p|, high. All statistical analyses and power analyses were conducted using the JMP Pro 14.1 software (SAS Institute Inc., Cary, NC, USA).

### 3. Results

#### 3.1. Preoperative features of patients in the SI and RCT groups

The clinical characteristics of the patients are summarized in Table 2. Patients in the SI group were significantly younger than those in the RCT group (P < 0.001), although there were no significant differences in the male/female ratio (P = 0.084) or body mass index (P = 0.074) between groups. The Rowe score was 33.5 ± 16.5 before surgery in the SI group, and the Constant score was 41.5 ± 17.0 before surgery in the RCT group. There was a significant difference in the OA status based on the Samilson and Prieto grading system between the SI and OA groups (P = 0.025), despite no significant difference in the Outerbridge grading system (P = 0.43).

#### 3.2. Immunohistochemical analysis of the synovial lining layer in the SI and RCT groups

Immunohistochemical analysis showed that TNF-α, IL-1β, and CD68 were predominantly expressed in the hyperplastic synovial lining layer of SI patients (Fig. 1A–1B, 1C and 1D). In contrast, no significant differences were observed in CD68 expression between the two groups (P = 0.98, Power = 0.18; Fig. 2E). Even though significant differences were observed between the two groups, there were no significant differences for any of the genes when considering the OA statuses based on the Samilson and Prieto grades (TNFA, P = 0.70, Power = 0.03; IL1B, P = 0.90, Power = 0.04; CD68, P = 0.50, Power = 0.08). The expression of TNFA was significantly correlated to that of CD68 (p < 0.001; Fig. 3A). CD80 (P = 0.60, P < 0.001; Fig. 3C) and CD86 (P = 0.46, Power = 0.21; CD68, P = 0.94, Power = 0.08).

The expression of TNFA was significantly correlated to that of CD68 (p = 0.64, P < 0.001; Fig. 3A). CD80 (P = 0.52, P = 0.0003; Fig. 3B) and CD86 (P = 0.60, P < 0.001; Fig. 3C) in the SI group. The expression of IL1B was significantly correlated to that of CD68 (p = 0.49, P = 0.06; Fig. 3D) and CD68 (p = 0.38, P = 0.06; Fig. 3E) in the SI group (Fig. 3D and E). However, there was no significant correlation between the expression of IL1B and CD80 (P = 0.33, P = 0.075; Fig. 3F).

#### 3.3. Inflammatory cytokine and macrophage marker expression in patients with SI and RCT

TNFA and IL1B mRNA levels were significantly higher in the SI group than in the RCT group (Fig. 2A: TNFA, P = 0.012, Power = 0.88; Fig. 2B: IL1B, P = 0.014, Power = 0.46; Fig. 2B: IL1B, P = 0.014, Power = 0.46; Fig. 2B: IL1B, P = 0.014, Power = 0.46). The data with two outliers removed, P = 0.027, Power = 0.74), as were CD68 and CD80 levels (Fig. 2C: CD68, P = 0.022, Power = 0.58; Fig. 2D: CD80, P = 0.003, Power = 0.67; Fig. 2D: CD80 data with two outliers removed, P = 0.008, Power = 0.57). In contrast, no significant differences were observed in CD68 expression between the two groups (P = 0.91, Power = 0.18; Fig. 2E). Even though significant differences were observed between the two groups, there were no significant differences for any of the genes when considering the OA statuses based on the Samilson and Prieto grades (TNFA, P = 0.70, Power = 0.13; IL1B, P = 0.90, Power = 0.04; CD68, P = 0.40, Power = 0.18; CD80, P = 0.66, Power = 0.13; CD86, P = 0.75, Power = 0.09) and the Outerbridge classification (TNFA, P = 0.81, Power = 0.11; IL1B, P = 0.33, Power = 0.15; CD68, P = 0.34, Power = 0.29; CD80, P = 0.46, Power = 0.21; CD68, P = 0.94, Power = 0.08).

The expression of TNFA was significantly correlated to that of CD68 (p = 0.64, P < 0.001; Fig. 3A). CD80 (p = 0.52, P = 0.0003; Fig. 3B) and CD86 (p = 0.60, P < 0.001; Fig. 3C) in the SI group. The expression of IL1B was significantly correlated to that of CD68 (p = 0.49, P = 0.06; Fig. 3D) and CD68 (p = 0.38, P = 0.037; Fig. 3E) in the SI group (Fig. 3D and E). However, there was no significant correlation between the expression of IL1B and CD80 (P = 0.33, P = 0.075; Fig. 3F).

#### 3.4. Flow cytometric analysis of CD80 and CD68 expression in macrophages isolated from SI patients

CD80 and CD68 are not only observed in macrophages, but also in B, T, and dendritic cells [23,24]. To confirm whether elevated expression of CD80 and CD68 reflects an increase in the M1 macrophage population, we used a flow cytometric analysis to investigate the presence of CD80 and CD68 in CD68+/.CD4+ macrophages harvested from the synovial membranes of patients in the SI group. Almost all CD68+/.CD4+ macrophages were positive for CD80 (96.25 ± 1.51%; Fig. 4A), whereas only 2.55 ± 1.00% CD68+/CD14+ cells were positive for CD80 (Fig. 4B).

#### 3.5. Correlation between age and gene expression and comparison between sexes

We observed significant, albeit low, negative correlations for IL1B (p = −0.28, p = 0.028; Fig. 5B) and CD80 (p = −0.39, p = 0.0022; Fig. 5D) with age. However, there were no differences between any other parameters and gene expression (Fig. 5A, C, E). In addition, there were no significant differences between sexes in the expression of any of the genes.
3.6. Relationship between clinical characteristics and gene expression in the SI group

We observed a significantly negative correlation between CD80 expression and the time interval between final dislocation and surgery in the SI group ($\rho = -0.420$, $P = 0.033$; Table 3). However, there were no differences between any other parameters (Rowe score, age at initial dislocation, and time interval between initial dislocation and surgery) and gene expression.

4. Discussion

This study showed that TNFA and IL1B expression in the synovial membrane of the glenohumeral joint were significantly elevated in patients with SI compared to that in RCT patients. These data indicate that an ongoing inflammatory process is an important part of the pathophysiology during SI. Nevertheless, there was no significant difference among OA statuses determined by the Samilson and Prieto grading system and the Outerbridge classification [21,22].

4.1. Biomarkers in SI and their importance for disease progression

Increased mRNA levels of inflammatory cytokines in the glenohumeral joint tissue have previously been reported in patients with shoulder OA and SI [1,12]. In particular, IL1B is expressed at higher levels in the synovium of the glenohumeral joint than in the subacromial bursa in patients with SI [12]. Moreover, TNF-\(\alpha\) is elevated in the cartilage from the glenoid of patients with shoulder OA [1]. IL-1\(\beta\) and TNF-\(\alpha\) mediate cartilage catabolism by inducing MMPs and ADAMTSs in the synovium of patients with OA [25]. Synovitis combined with the increased mRNA expression of inflammatory cytokines in patients with SI may explain the development and progression of OA at preoperational
stages [5,6].

Macrophages play a key role in synovial inflammation [14]. M1 macrophages in particular produce high amounts of TNF-α and IL-1β, as observed in the synovial tissue of humans and mice with knee OA [26–29]. In addition, an increase in the number of M1 macrophages is associated with knee OA severity [30]. Here, we observed higher expression levels of pan macrophage (CD68) and M1 macrophage (CD80) markers in the glenohumeral synovial tissue of patients with SI than patients with RCT. In addition, CD68 expression levels were positively correlated with TNFA and IL1B expression in the synovial tissue of patients with SI. Thus, increased levels of macrophages may play an important role in the early development of OA during SI, by increasing inflammatory cytokine production.

4.2. Risk factors for the development of OA

Risk factors for the development of OA in patients with SI have previously been identified [3–6,31–33]. Traumatic cartilage damage has been reported after shoulder dislocation [34]. Patient age at the initial dislocation is correlated with the occurrence of arthritis after surgery. Additionally, a longer time interval between the initial dislocation and surgery increases the chance of developing arthritic changes [31]. In the present study, CD80 expression was negatively correlated with the time interval between final dislocation and surgery. This observation may imply that polarization toward M1 macrophages occurs immediately after dislocation. A longer time interval between dislocation and surgery may lead to repeated inflammatory responses with recurrent dislocation, subluxation of the glenohumeral joint, or both, ultimately aggravating

Fig. 3. Correlation between the expression of inflammatory cytokines and macrophage markers in synovial tissue of the glenohumeral joint. A: Correlation between TNFA and CD68 (ρ = 0.64, P < 0.001). B: Correlation between TNFA and CD80 (ρ = 0.52, P = 0.003). C: Correlation between TNFA and CD86 (ρ = 0.60, P < 0.001). D: Correlation between IL1B and CD68 (ρ = 0.49, P = 0.006). E: Correlation between IL1B and CD80 (ρ = 0.33, P = 0.075). F: Correlation between IL1B and CD86 (ρ = 0.38, P = 0.037). Red line, regression line; red region, confidence interval; red ellipse, 0.95 confidence ellipse.

Fig. 4. Flow cytometric analysis of CD80+ and CD86-positive cellsDot plot analysis of (A) CD68+/CD14+ among CD45+ populations and (B) CD80+ and CD86+ cells among CD68+/CD14+ populations.
OA progression. Rotator cuff tears are also related with the translation of the glenohumeral joint during shoulder motion [35]. However, a capsulolabral lesion after dislocation was required to induce instability in a rotator cuff-deficient model during a cadaveric study [36]. Therefore, capsulolabral damage affects the glenohumeral joint instability more than a rotator cuff tear. In addition, capsulolabral damage can induce micro-instability during shoulder motions regardless of recurrent shoulder dislocations. Both micro-instability and cartilage damage after dislocation may have a more significant effect than RCT on the persisting inflammatory cytokines in the glenohumeral joint and on early OA progression, similar to weight bearing joints [2,7–10].

4.3. The relationship between age/sex, inflammatory cytokines and macrophages

In this study, the mean age was lower in patients with SI than in patients with RCT, and we show that age could potentially affect the expression of inflammatory cytokines and M1 macrophage proliferation. Recent studies have suggested that aging is related to chronic low-grade inflammation, referred to as inflammaging, although the precise mechanism remains largely unknown [37,38]. During inflammaging, inflammatory cytokines in the joint synovium of elderly patients can be higher than those in younger patients [37,38]. Proinflammatory cytokines produced form monocytes in the serum and intramuscular macrophage abundance in the healthy elderly are increased at or above the same...
Table 3

<table>
<thead>
<tr>
<th>Score</th>
<th>Age at initial dislocation (years)</th>
<th>Time from initial dislocation to surgery (years)</th>
<th>Time from final dislocation to surgery (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFA</td>
<td>$\rho = -0.03$</td>
<td>$p = 0.28$, $P = 0.15$, $P = 0.50$, $P = 0.49$</td>
<td>$p = 0.13$, $p = 0.15$, $p = 0.31$, $p = 0.12$</td>
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<tr>
<td>$\rho = 0.86$</td>
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<tr>
<td>IL1B</td>
<td>$\rho = -0.33$</td>
<td>$p = 0.049$, $p = 0.81$, $p = 0.43$, $p = 0.12$</td>
<td>$p = 0.15$, $p = 0.15$, $p = 0.31$, $p = 0.12$</td>
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<td>$\rho = 0.079$</td>
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</tr>
<tr>
<td>CD68</td>
<td>$\rho = -0.048$</td>
<td>$p = 0.044$, $p = 0.55$, $p = 0.33$</td>
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<td>$\rho = 0.80$</td>
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<tr>
<td>CD80</td>
<td>$\rho = -0.21$</td>
<td>$p = 0.041$, $p = 0.04$, $p = 0.16$, $p = 0.033$</td>
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<td>$\rho = 0.26$</td>
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</tr>
<tr>
<td>CD86</td>
<td>$\rho = -0.093$</td>
<td>$p = 0.23$, $p = 0.15$, $p = 0.89$</td>
<td>$p = -0.28$, $p = -0.28$, $p = 0.27$, $p = 0.27$</td>
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<td>$\rho = 0.63$</td>
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</table>

Boldface values indicate significant correlations between the two groups.

levels as in young participants regardless of sex [39–43]. Thus, the expression of macrophage markers and inflammatory cytokines in younger patients is presumed to be equal to or lower than that in elderly patients, regardless of sex. However, our findings showed significantly higher expression levels of TNFA, IL1B, CD68, and CD80 in the SI group than in the RCT group. Therefore, the instability in the glenohumeral joint may influence inflammatory cytokine expression and M1 macrophage expansion to a greater extent than that after RCT injuries or aging. However, further research is needed to confirm these hypotheses.

4.4. Limitations

The first caveat is that this study did not include a healthy population as a control group, which would enhance the validity of the findings. Second, this is a cross-sectional study. We could not confirm whether OA of the glenohumeral joint was developed or not in patients with SI. However, the OA-related inflammatory cytokines were significantly higher in the glenohumeral joints of patients with SI than in those with RCT. These findings support the increasing rate of OA in shoulders with SI. This may be one underlying molecular mechanism driving the early development and progression of OA in patients with SI.

5. Conclusion

The expression of inflammatory cytokines and M1 macrophage markers is elevated in the glenohumeral synovial tissue of patients with SI. This may be one underlying molecular mechanism driving the early development and progression of OA in patients with SI.

Author contributions

MK wrote the manuscript and performed PCR analysis and flow cytometric analysis. TK enrolled patients and organized this study. KU performed PCR and flow cytometric analysis and contributed to manuscript writing. LAN provided logistic support, interpreted PCR and flow cytometric data, and revised the manuscript. RT and MN performed PCR analysis. MS performed immunohistochemistry analysis and contributed to manuscript writing. GI is responsible for the integrity of this study, especially methodological and ethical issues. MT is responsible for the integrity of this study and contributed to revising the manuscript. All authors approved the final version of the manuscript.

Role of the funding source

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Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jocarto.2022.100241.

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