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Coexistence of fibrotic and chondrogenic process in the capsule of idiopathic frozen shoulders

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SUMMARY

Objective: To analyze changes in the capsule from idiopathic frozen shoulders and clarify their etiology.
Materials and methods: Samples (the rotator interval capsule, middle glenohumeral ligament (MGHL), and inferior glenohumeral ligament (IGHL)) were collected from 12 idiopathic frozen shoulders with severe stiffness and 18 shoulders with rotator cuff tears as a control. The number of cells was counted and the tissue elasticity of the samples was calculated by scanning acoustic microscopy (SAM). The amount of glycosaminoglycan content was assessed by alcian blue staining. Gene and protein expressions related to fibrosis, inflammation, and chondrogenesis were analyzed by quantitative polymerase chain reaction (qPCR) and immunohistochemistry (IHC). Furthermore, the total genes of the two groups were compared by DNA microarray analysis.
Results: The number of cells was significantly higher and the capsular tissue was significantly stiffer in idiopathic frozen shoulders compared with shoulders with rotator cuff tears. Staining intensity of alcian blue was significantly stronger in idiopathic frozen shoulders. Gene expressions related to fibrosis, inflammation, and chondrogenesis were significantly higher in idiopathic frozen shoulders compared with shoulders with rotator cuff tears assessed by both qPCR and DNA microarray analysis.
Conclusion: In addition to fibrosis and inflammation, which used to be considered the main pathology of frozen shoulders, chondrogenesis is likely to have a critical role in pathogenesis of idiopathic frozen shoulders.

Introduction

Shoulder stiffness is a major cause of shoulder disability and pain. It disturbs the quality of life in various aspects; however, its etiology has remained unclarified. The term '*frozen shoulder*' was first described by Codman in 1934[1] and defined in 1992 by the American Academy of Orthopaedic Surgeons[2]. Though neither entity nor classification of frozen shoulders has come to consensus, it is generally divided into two categories: idiopathic and acquired. In the former, there is no known intrinsic shoulder disorder. In the latter, there is some sort of predisposing condition affecting the shoulder (e.g., surgery or trauma)[3]. Clinical features of idiopathic frozen shoulders are classically characterized by three stages: freezing, frozen, and thawing[4].

The estimated prevalence of frozen shoulders is 2–5% in the general population[5]. Patients with frozen shoulders are predominantly women in their 40s–70s[6]. The natural history of frozen shoulders is considered to be a self-limited disease[1]. However, some patients show little or no improvement with residual limited range of motion. Non-operative treatment is the initial approach. Mobilization and stretching are commonly prescribed as conservative treatment for frozen shoulders[7]. However, when patients could not regain satisfactory range of motion after prolonged non-operative treatment, operative treatment (such as arthroscopic capsular release) would be the treatment of choice[8]. This indicates the joint capsule is one of the main pathologies of frozen shoulders.

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In our previous reports, elasticity of the joint capsule decreased (the capsule became stiffer)[9] and the range of motion was almost back to normal after the capsular release in an immobilized knee model in rats[10].

In 1945, Neviaser reported perivascular infiltration with inflammatory cells and capsular fibrosis in stiff shoulders[11]. Lundberg described increased collagen density and glycosaminoglycan distribution in the capsule with no significant number of inflammatory cells in the synovial membrane of frozen shoulders[12]. Bunker analyzed biopsies of the coracohumeral ligament and the rotator interval capsule from frozen shoulders, and reported active fibroblast proliferation and transformation to myofibroblasts[13]. Hand collected biopsies of the rotator interval capsule, revealed proliferating fibroblasts and chronic inflammatory cells[14]. Rodeo reported that the following growth factors such as transforming growth factor-beta (TGF-β), platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF) were involved in frozen shoulders[15]. Though some growth factors, cytokines, and matrix metalloproteinases (MMPs) may play an important role in the pathogenesis of frozen shoulders[16], its mechanism is still unclear. The aim of this study was to elucidate the fibrotic, inflammatory, and chondrogenic differentiation process in the pathogenesis of idiopathic frozen shoulders.

Patients and methods

The protocols of this study were approved by both institutional review boards of Funabashi Orthopaedic Clinic and Tohoku University. From July 2007 to June 2009, we performed arthroscopic capsular release in 12 patients with idiopathic frozen shoulders, whose condition had failed to improve or had deteriorated after 6-months of conservative treatment. Two patients had mild diabetes mellitus, which was well controlled. As a control group, 16 patients with rotator cuff tears without limited range of motion were selected. Biopsy materials from the rotator interval capsule, middle glenohumeral ligament (MGHL), and inferior glenohumeral ligament (IGHL) were obtained during arthroscopic surgery. Thirty-six samples from idiopathic frozen shoulders and 48 samples from shoulders with rotator cuff tears were collected. There was no case including bilateral shoulders. The average age was 50.5 years (41–61, three males and nine females) in the group of idiopathic frozen shoulders and 56.6 years (43–71, ten males and six females) in the control group of shoulders with rotator cuff tears. The difference of age distribution between these two groups was marginal but not statistically significant (mean difference: 6.1 years, 95% CI: −0.3 years to 12.4 years, P=0.065). One sample of MGHL from an idiopathic frozen shoulder was eliminated from evaluation because of a technical problem.

Tissue preparation

For histological and mechanical property evaluation for scanning acoustic microscopy (SAM), the tissues were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS), pH 7.4. The paraffin embedded tissue was cut into 5-μm sagittal sections. The hematoxylin and eosin-stained sections were used for cell counts and morphologic evaluation. At least four areas of each section were captured at high magnification (×200, DMLB 100 HC light microscope; LEICA, Wetzlar, Germany) and the number of cells were counted. The amount of proteoglycans was assessed by alcian blue staining.

SAM

In general, it is known that the sound speed is in proportion to the square route of Young's elastic modulus[17]. The sound speed of a tissue on a slide glass can be measured *in situ* using SAM. Details of this system used in this study have been reported elsewhere[9,17,18]. The optical and acoustic images were compared to ensure morphological congruence in the analyses.

Image comparisons of SAM

To analyze influence factors for tissue elasticity in idiopathic frozen shoulders, SAM images of IGHL were compared with immunohistochemistry (IHC) of collagen type I and alcian blue staining. Appropriate mask patterns were made for the images and Pearson's product–moment correlation coefficient was calculated with gray scale images. To clarify the influence of each the component on the sound speed, the color images were separated into light's three primary colors or divided into three components for principal component analyses. The correlation coefficient of each factor was independently calculated.

IHC

The sections were deparaffinized and immersed in 3.0% hydrogen peroxide for 10 min. For Ki-67, the slides were incubated at 87°C with Target Retrieval Solution (S2367, DAKO, dilution at 1:10) for 30 min. For collagen types I, II, and III, and α-smooth muscle actin (α-SMA), the slides were incubated with 0.1% Trypsin 0.1% $CaCl_2$/Tris Buffer at room temperature. Endogenous immunoglobulins were blocked by incubation with 10% normal goat serum (Nichirei) in PBS. The slides were incubated with antibodies (Supplementary Table 1). The final detection step was carried out using 3, 3′-diaminobenzidine tetrahydrochloride (3, 3′-diaminobenzidine (DAB), Sigma–Aldrich), 0.1 M imidazole, and 0.03% hydrogen peroxidase. These slides were counterstained with hematoxylin. For negative controls, normal mouse IgG was used as a primary antibody.

RNA extraction and purification

All the samples were immediately placed in a vessel containing 1-ml QIAzol (Qiagen, Hilden, Germany) and homogenized with a Polytron® (Kinematica AD, Switzerland). The total RNA of the homogenate was purified using RNeasy Lipid Tissue Mini Kit (Qiagen). All the IGHL samples from idiopathic frozen shoulders and three IGHL samples from shoulders with rotator cuff tears were used for DNA microarray analysis.

Quantitative polymerase chain reaction (qPCR)

Complementary DNA was synthesized using Cloned avian myeloblastis virus (AMV) first-strand cDNA synthesis kit (Invitrogen). Gene expression was evaluated quantitatively by real-time polymerase chain reaction (PCR) on a LightCycler (Roche Diagnostics, Basel, Switzerland). PCR efficiencies and relative expression levels of fibrotic, inflammatory, and chondrogenic differentiation factors as a function of EF1α1 expression were calculated as previously described[19]. The primer sequences for expression analyses are in Supplementary Tables 2 and 3.

In situ hybridization (ISH)

Three IGHL samples from idiopathic frozen shoulders and two IGHL samples from shoulders with rotator cuff tears were assessed. Paraffin embedded blocks and sections of the tissue for ISH were obtained from Genostaff Co., Ltd. The tissues were dissected, fixed with Tissue Fixative (Genostaff), and then embedded in paraffin by their proprietary procedures[20]. Fragments encoding human

aggrecan (2392–3007 bp: GenBank accession number NM_013227), human collagen type II (4647–4904 bp: GenBank accession number NM_001844) and human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (460–895 bp: GenBank accession number NM_002046.3) were used as RNA probes.

DNA microarray analysis

After treatment with DNase I (Invitrogen, Carlsbad, CA), RNAs were amplified using Amino Ally MessageAmp II aRNA Amplification Kit (Ambion) and then labeled with Cy3 and Cy5. Whole Human Genome Microarray Kit 4 × 44 K (Agilent) was applied to the Cy3- and Cy5-labeled amplified RNAs, which were then competitively hybridized at 65°C for 17 h[21].

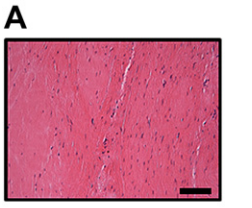
Statistical analyses

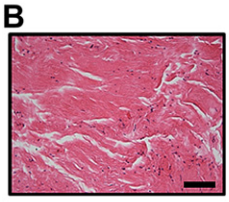
Differences between idiopathic frozen shoulders and shoulders with rotator cuff tears for number of cells and sound speed were compared by Student's *t* test, because their distribution was expected to be Gaussian by Shapiro–Wilk normality test. Because distribution of qPCR data was so skewed, we used Mann–Whitney's *U* test. Of all variables, 95% confidence interval was estimated by bootstrap percentile method ($B = 1000$). All statistical tests were two-sided, and a value of $P < 0.05$ was accepted as statistically significant. All data were analyzed by using R2.13.0.

Results

Fibrotic and inflammatory process in idiopathic frozen shoulders

The collagen bundles were dense with less space in idiopathic frozen shoulders [Fig. 1(A), Supplementary Fig. 1(A) and (B)]. On the other hand, the bundles were sparse and well-organized in shoulders with rotator cuff tears [Fig. 1(B), Supplementary Fig. 1(C) and (D)]. There were spindle shaped cells with sharp or ellipsoidal nuclei, mainly fibroblast-like cells, in both groups. The number of cells was significantly higher in idiopathic frozen shoulders [Fig. 1(C)]. Gradation color images of SAM in idiopathic frozen shoulders were composed of high sound speed area (yellow to red) [Fig. 1(D), Supplementary Fig. 1(E) and (F)], whereas those in shoulders with rotator cuff tears were composed of low sound speed area (blue) [Fig. 1(E), Supplementary Fig. 1(G) and (H)]. The average sound speed was significantly higher in idiopathic frozen shoulders

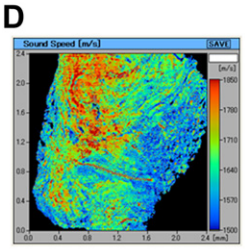


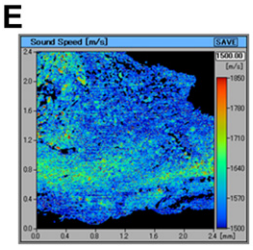


C

Number of cells

		N	Mean (95% CI)	P
RI	IFS	12	1489 (1310 - 1673)	<0.001
	RCT	16	991 (850 - 1270)	
MGHL	IFS	11	1620 (1517 - 1730)	<0.001
	RCT	16	915 (801 - 1024)	
IGHL	IFS	12	1097 (969 - 1201)	0.013
	RCT	16	811 (647 - 994)	





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Sound speed (m/s)

		N	Mean (95% CI)	P
RI	IFS	12	1623 (1613 - 1634)	<0.001
	RCT	16	1575 (1562 - 1589)	
MGHL	IFS	11	1639 (1629 - 1650)	<0.001
	RCT	16	1593 (1577 - 1609)	
IGHL	IFS	12	1674 (1660 - 1687)	<0.001
	RCT	16	1579 (1563 - 1594)	

Fig. 1. Histological features and elastic changes of capsules. A: H–E staining of IGHL in idiopathic frozen shoulders (IFS), B: H–E staining of IGHL in shoulders with rotator cuff tear (RCT), C: the number of cells in capsules, D: SAM gradation color image of IGHL in IFS, E: SAM gradation color image of IGHL in RCT, and F: average sound speed of capsules. Collagen bundles were dense in all the samples of IFS (A), however, there were interspaces between the bundles in all the samples of RCT (B). The number of cells was significantly higher in all the samples in IFS compared with those in RCT (C). High sound speed area (yellow to red) was observed in IFS (D), however, low sound speed area was in RCT (E). Average sound speed was significantly higher in all the samples in IFS compared with those in RCT (F). Scale bars: 100 μm (A and B). RI: the rotator interval capsule, MGHL: middle glenohumeral ligament, IGHL: inferior glenohumeral ligament.

Table I
Pearson's product-moment correlation coefficient of image comparison analyses

	Gray scale	Red	Green	Blue	The first component	The second component	The third component
Collagen type I	0.075	0.039	0.077	0.138	−0.104	−0.137	0.175
Alcian blue	−0.304	−0.311	−0.285	−0.160	−0.279	−0.174	0.224

than that in shoulders with rotator cuff tears [Fig. 1(F)]. Comparing gray scale images of SAM, high sound speed area (white) or low sound speed area (black) did not correspond with any images in IHC of collagen type I [Supplementary Fig. 2(A)–(H)]. The Pearson's product–moment correlation coefficient was low in all the images (Table I). Though the number of cells was significantly higher in idiopathic frozen shoulders, there were few cells expressing immunoreactivity of Ki-67 in both groups [Fig. 2(A) and (E), Supplementary Fig. 3(A)–(D)]. Immunoreactivity of collagen type I was stronger in idiopathic frozen shoulders compared with that in shoulders with rotator cuff tears [Fig. 2(B) and (F), Supplementary Fig. 3(E)–(H)]. There was no significant difference of immunoreactivity in collagen type III between the two groups [Fig. 2(C) and (G), Supplementary Fig. 3(I)–(L)]. Immunoreactivity of α-SMA was not detected in fibroblast-like cells, but was detected in blood vessels [Fig. 2(D) and (H), Supplementary Fig. 3(M)–(P)]. The number of blood vessels in the capsule was significantly higher in idiopathic frozen shoulders. The gene expressions of COL1A1 and PDGF B were significantly higher in all the samples of idiopathic frozen shoulders. However, the gene expressions of COL3A1, interleukin (IL)1-β, α-SMA, and Substance P were significantly higher in two-third of samples in idiopathic frozen shoulders. The gene expression of calcitonin gene-related peptide (CGRP) was significantly lower in MGHL, but significantly higher in IGHL in idiopathic frozen shoulders. The gene expression of tissue inhibitor of metalloproteinase (TIMP)-1 was significantly lower in the rotator interval capsule and IGHL in idiopathic frozen shoulders (Table II). There was no statistical difference of connective tissue growth factor (CTGF), HGF, and tumor necrosis factor (TNF)-α in all the samples between the two groups. However, the gene expressions of TGF-β1 and PDGF A were significantly lower in one-third of samples in idiopathic frozen shoulders (Supplementary Table 4). Though the gene expression of MMP-1 in MGHL was significantly lower and that of MMP-14 in IGHL was significantly higher in idiopathic frozen shoulders, there was no statistical difference in all the other samples of MMP-2, MMP-3, MMP-9, TIMP-2, and TIMP-3 between the two groups (Supplementary Table 5). Higher gene expressions of COL1A1, COL3A1, PDGF B, Substance P, and CGRP, and protein expression of collagen type I indicated fibrotic process in idiopathic frozen shoulders. Higher gene expression of IL-1β indicated inflammatory process in idiopathic frozen shoulders.

Chondrogenic differentiation process in idiopathic frozen shoulders

The intensity of alcian blue staining was significantly stronger in idiopathic frozen shoulders than that in shoulders with rotator cuff tears [Fig. 3(A) and (B), Supplementary Fig. 1(I)–(L)]. There was no immunoreactivity of collagen type II in both groups (data not shown). Comparing gray scale images of SAM, high sound speed area (white) or low sound speed area (black) did not correspond with any image of alcian blue staining [Supplementary Fig. 2(A), and (I)–(O)]. The Pearson's product–moment correlation coefficient was low in all the images (Table I). The gene expressions of aggrecan (ACAN) and COL2A1 were significantly higher in all the samples of idiopathic frozen shoulders. The gene expression of COL10A1 in MGHL and IGHL was also significantly higher in idiopathic frozen shoulders [Fig. 3(C)]. There was no statistical difference in SOX9, A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)4, and ADAMTS5 between the two groups (Supplementary Table 6). Fibroblast-like cells expressed ACAN signals in idiopathic frozen shoulders [Fig. 3(D)], but no signal in shoulders with rotator cuff tears [Fig. 3(E)]. Though strong signals of GAPDH were detected in both groups (data not shown), there was no signal of COL2A1 in both groups [Fig. 3(F)]. Higher intensity of alcian blue staining indicated chondrogenic differentiation in idiopathic frozen shoulders. Furthermore, higher gene expressions of ACAN, COL2A1, and COL10A1 supported chondrogenic differentiation in idiopathic frozen shoulders.

Gene expression profiles

To characterize the microarray expression profiles, probes whose expressions changed by more than twofold up-regulation or less than a half down-regulation in all the idiopathic frozen

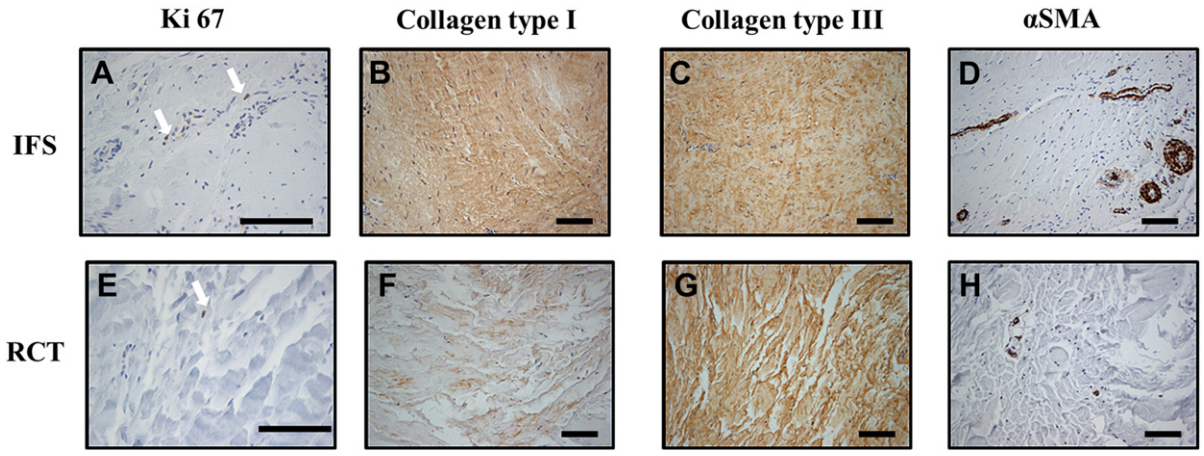


Fig. 2. Immunoreactivity of fibrotic factors in IGHL. A–D: IFS, E–H: RCT. Few immunoreactivity of Ki-67 was detected both in IFS and RCT (A and E). Stronger immunoreactivity of collagen type I was observed in IFS compared with those of RCT (B and F). There was no significant difference in immunoreactivity of collagen type III between IFS and RCT (C and G). Strong immunoreactivity of α-SMA was detected in blood vessels both in IFS and RCT. No immunoreactivity of α-SMA was observed in fibroblasts-like cells in the two groups. The number of blood vessels in the substance of the capsule increased in IFS (D and H). Scale bars: 100 μm. Arrow heads: immunoreactivity of Ki-67 (A and B).

Table II
Inflammatory and fibrotic gene expressions of capsules

			N	Mean (95% CI)	P
COLA1	RI	IFS	12	3.27 (1.6–5.0)	*0.006*
		RCT	16	1.51 (0.5–2.5)	
	MGHL	IFS	11	4.16 (1.9–6.4)	*0.006*
		RCT	16	1.52 (0.6–2.5)	
	IGHL	IFS	12	3.88 (1.4–6.3)	*<0.001*
		RCT	16	0.816 (0.1–1.5)	
COL3A1	RI	IFS	12	2.28 (1.3–3.5)	0.14
		RCT	16	1.48 (0.9–2.2)	
	MGHL	IFS	11	3.26 (2.0–5.1)	*0.047*
		RCT	16	1.73 (1.0–2.5)	
	IGHL	IFS	12	2.78 (1.2–5.0)	*0.011*
		RCT	16	1.05 (0.4–2.1)	
PDGFB	RI	IFS	12	4.60 (3.1–6.6)	*0.003*
		RCT	16	1.86 (1.2–2.6)	
	MGHL	IFS	11	1.80 (1.3–2.3)	*0.001*
		RCT	16	0.737 (0.5–1.0)	
	IGHL	IFS	12	1.25 (0.9–1.6)	*0.016*
		RCT	16	0.711 (0.5–0.9)	
IL-1β	RI	IFS	12	4.19 (3.0–5.8)	*0.023*
		RCT	16	2.50 (1.4–3.8)	
	MGHL	IFS	11	4.49 (2.4–7.1)	*0.005*
		RCT	16	1.16 (0.6–2.0)	
	IGHL	IFS	12	5.28 (2.6–8.5)	0.063
		RCT	16	2.81 (1.4–4.6)	
TIMP-1	RI	IFS	12	0.831 (0.6–1.1)	*0.011*
		RCT	16	1.34 (1.1–1.6)	
	MGHL	IFS	11	1.30 (0.8–1.8)	0.35
		RCT	16	1.75 (1.1–2.6)	
	IGHL	IFS	12	0.58 (0.5–0.7)	*<0.001*
		RCT	16	1.67 (1.0–2.4)	
α-SMA	RI	IFS	12	1.32 (1.0–1.8)	0.54
		RCT	16	1.65 (1.1–2.3)	
	MGHL	IFS	11	1.35 (1.1–1.6)	*0.002*
		RCT	16	0.714 (0.5–1.0)	
	IGHL	IFS	12	2.00 (1.4–2.8)	*<0.001*
		RCT	16	0.725 (0.5–1.0)	
Substance P	RI	IFS	12	2.99 (1.7–4.5)	0.063
		RCT	16	1.27 (0.9–1.6)	
	MGHL	IFS	11	5.68 (1.3–11.7)	*0.024*
		RCT	16	1.01 (0.7–1.4)	
	IGHL	IFS	12	4.60 (2.0–7.5)	*0.005*
		RCT	16	0.854 (0.5–1.4)	
CGRP	RI	IFS	12	3.77 (1.6–6.6)	0.36
		RCT	16	4.70 (1.5–8.6)	
	MGHL	IFS	11	3.10 (1.0–5.4)	*0.034*
		RCT	16	7.63 (3.8–12.0)	
	IGHL	IFS	12	39.5 (23.1–58.8)	*0.001*
		RCT	16	14.2 (4.6–28.3)	

Italic values significant the statistically of $P < 0.05$.

shoulders compared with those in shoulders with rotator cuff tears were selected. Gene expression of 33 genes was significantly higher (Table III) and that of 11 genes was significantly lower (Table IV) among approximately 20,000 genes. Notably, fibrotic factors such as COL1A1, chondrogenic factors such as FBJ murine osteosarcoma viral oncogene homolog (FOS), FBJ murine osteosarcoma viral oncogene homolog B (FOSB), ACAN, and COL10A1, and angiogenic factors such as CYR61 were significantly higher in idiopathic frozen shoulders. The gene expressions of FOS and FOSB in MGHL and IGHL were significantly higher in idiopathic frozen shoulders. The gene expressions of CYR61 and SPARC were significantly higher in all the samples of idiopathic frozen shoulders (Table V, Primer sequences: see to Supplementary Table 2).

Discussion

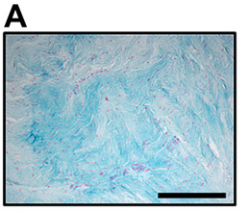
Though the term '*frozen shoulder*' was first reported more than 100 years ago[22], its etiology is still unknown. Accordingly, the prevention and treatment methods have not been established. The rotator interval capsule containing the coracohumeral ligament is considered as the predominant area of frozen shoulders[23]. However, it is difficult to gain full range of motion after the release of only the rotator interval capsule in our clinical experience. Wide arthroscopic capsular release is necessary in regaining the full range of motion in frozen shoulders[8], which indicates that the capsule including the glenohumeral ligaments is one of the main causes of restricted range of motion. Therefore, biopsy materials were taken from three regions (the rotator interval capsule, MGHL, IGHL) in this study.

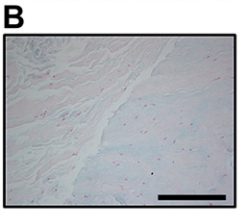
Many studies reported the microscopic pathology of frozen shoulders. Based on these reports, three schools of thought have been proposed as follows[14]: (1) an inflammatory process[11]; (2) a fibrotic process[11,13,23]; and (3) an inflammatory process with subsequent reactive capsular fibrosis[15]. In addition, Lundberg reported increased density of collagens fibrils and altered glycosaminoglycan contents in frozen shoulders[24]. Though some growth factors, cytokines, and MMPs may play an important role in the pathogenesis of frozen shoulders[16], there has been no studies measuring elasticity of the capsule and performing systemic gene expression profiling for frozen shoulders.

Collagen fibril density of the capsule in idiopathic frozen shoulders was apparently higher in this study, which was similar to a previous report[12]. Excisional biopsies of the coracohumeral ligaments including the rotator interval capsule from frozen shoulders in a previous study indicated moderate to hyper cellularity with nodular and/or laminar patterns[13]. However, there were no prominent nodular or laminar patterns in idiopathic frozen shoulders in this study. This might be caused by the patients' condition. Ten out of 12 patients had arthroscopic surgery before the biopsy[13]. In our cases, none of them had prior shoulder surgeries before the biopsy and we collected only the capsule itself. The higher cell density in idiopathic frozen shoulders might be explained either by (1) increased cell proliferation, or (2) contraction of the capsule. Ki-67 is known as a cellular marker for proliferation[25]. In this study, little immunostaining of Ki-67 was detected in both groups, which might be caused by collecting samples at the latest stage of frozen shoulders or other factors suppressing Ki-67 expressions. At least in the samples collected in this study, contraction of the capsule is more likely to explain the higher cell density in idiopathic frozen shoulders.

Determination of structural collagens is important to understand the elastic changes and pathology of the capsule in idiopathic frozen shoulders. The capsule in frozen shoulders was contracted like Dupuytren's contracture[12,13] and the sound speed of the capsule measured on the slide glass using SAM was significantly higher in this study, which means the capsule of frozen shoulders is stiffer than the control. In our study, expressions of collagen type I (IHC, qPCR) and type III (qPCR) were significantly higher in idiopathic frozen shoulders. However, image comparisons of SAM with IHC of collagen type I indicated low correlations in idiopathic frozen shoulders. In this series, almost all the patients had received steroid injections for pain controls in both groups. Though, the steroids might have affected biologic changes and biologic processes in the shoulder capsule, we cannot assess their effects and degrees precisely. There remain other factors such as abnormal cross-linking of collagens by advanced glycation endproducts.

Rodeo reported higher expression of cytokines such as TGF-β, PDGF, IL1-β, TNF-α, and HGF in frozen shoulders by IHC[15], whereas Bunker reported no up-regulation of MMP-1, MMP-2, MMP-3, MMP-9, MMP-14, and TIMP-1by RT-PCR[16]. In our previous report in the immobilized knee in rats, expressions of TGF-β1 and CTGF were significantly higher in the capsule[26]. These data did not correspond well with this study. Disease stages, sampling portions may have affected the results. Moreover, higher gene expressions of COL3A1, PDGF B, Substance P, and CGRP indicated a fibrotic process, whereas higher gene expression of IL-1β indicated an inflammatory

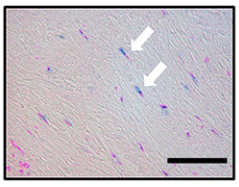


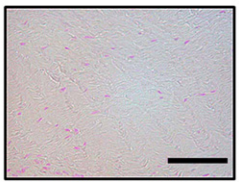


C

Gene expressions

			N	Mean (95%CI)	P
ACAN	RI	IFS	12	152 (14.7 - 389.5)	*0.005*
		RCT	16	10.6 (2.2 - 25.2)	
	MGHL	IFS	11	46.0 (11.1 - 95.7)	*0.005*
		RCT	16	5.71 (2.1 - 10.3)	
	IGHL	IFS	12	46.5 (15.0 - 94.9)	*0.005*
		RCT	16	9.20 (0.7 - 22.8)	
COL2A1	RI	IFS	12	3.03 (1.9 - 4.2)	*0.003*
		RCT	16	0.981 (0.6 - 1.6)	
	MGHL	IFS	11	2.98 (1.5 - 4.8)	*0.008*
		RCT	16	1.07 (0.6 - 1.6)	
	IGHL	IFS	12	4.53 (2.2 - 7.5)	*0.002*
		RCT	16	1.03 (0.5 - 1.7)	
COL10A1	RI	IFS	12	2.23 (1.4 - 3.1)	0.063
		RCT	16	1.27 (0.7 - 1.9)	
	MGHL	IFS	11	3.33 (1.3 - 6.8)	*0.005*
		RCT	16	0.734 (0.4 - 1.1)	
	IGHL	IFS	12	3.31 (2.1 - 5.0)	*<0.001*
		RCT	16	0.948 (0.4 - 1.7)	





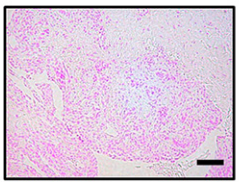


Fig. 3. Chondrogenic differentiation of capsules. A: alcian blue staining of IGHL in IFS, B: alcian blue staining of IGHL in RCT, C: gene expressions, D and E: ISH of ACAN, F: ISH of collagen type II. The gene expressions are shown as a ratio of expression of EF1a1. Strong alcian blue staining was observed in IFS compared with that in RCT (A and B). The gene expressions of ACAN and COL2A1 were significantly higher in all the samples of IFS compared with those of RCT. The gene expression of COL10A1 was significantly higher in MGHL and IGHL of IFS compared with those of RCT (C). Stronger signals of ACAN were detected in IFS (D), however, there was no signal in RCT (E). There was no signal of collagen type II in IFS (F). Scale bars: 200 μm (A and B), 100 μm (D–F). Arrow heads: signals of ACAN (D).

process in idiopathic frozen shoulders. This data supports that the inflammatory and fibrotic processes are one of the main pathologies in idiopathic frozen shoulders.

Transformation from fibroblasts to myofibroblasts is a key to understanding the pathology of frozen shoulders. Bunker reported α-SMA positive cells by IHC in frozen shoulders[13]. Myofibroblasts were significantly higher in post-traumatic contractures in human elbow joints[27]. However, there was little evidence of myofibroblasts in this study, which supported a report by Hand[14]. Though the gene expression of α-SMA was significantly higher in idiopathic frozen shoulders, fibroblast-like cells had less immunoreactivity of α-SMA and blood vessels had strong immunoreactivity in both groups. Higher gene expression of α-SMA in idiopathic frozen shoulders by qPCR may be explained by proliferation of angiogenesis, which is supported by the higher gene expression of CYR61, which is known as an angiogenic factor. Besides angiogenic factors, CYR61 is a component of the extra-cellular matrix that activates pro-inflammatory genes in macrophages[28]. In addition, CYR61 drives fibroblasts into senescence and up-regulates the expression of antifibrotic genes to restrict fibrosis during tissue repair[29]. Though the number of cells was significantly higher, there was less immunoreactivity of Ki67 and α-SMA of fibroblast-like cells in idiopathic frozen shoulders. CYR61 may have increased the number of blood vessels as an angiogenic factor and may have driven myofibroblasts into senescence in idiopathic frozen shoulders. The differences in immunoreactivity of α-SMA in the capsule of frozen shoulders may also be influenced by joint hemorrhage.

Mast cells seem to play an important role in the pathology of frozen shoulder as they are believed to induce a fibrotic response *via* involvement in the connective tissue of fibroblasts and myofibroblasts[30]. A higher number of mast cells were reported in frozen shoulders[14] and in joint contractures after fractures[31]. Neuropeptides Substance P and CGRP can cause mast cell degranulation[32]. Though myofibroblasts did not increase, Substance P was significantly higher in idiopathic frozen shoulders in this study.

Alcian blue staining intensity was stronger in idiopathic frozen shoulders in this study. This data supported a previous report by Lundberg[24]. However, there were no chondrocyte-like cells in idiopathic frozen shoulders, which indicate that the fibrocartilage might be produced in idiopathic frozen shoulders. The rotator cuff tendons contain a large number of aggrecan, but the histological structures do not resemble those of mature fibrocartilage[33]. From these results, we selected chondrogenic factors, such as SOX9, ACAN, COL2A1, and COL10A1 to analyze the differentiation process in chondrocytes by qPCR. SOX9 was characterized as the master gene of chondrogenesis[34] and regulates ACAN, COL2A1, and COL11A1 along with SOX5 and 6[35]. Aggrecan and collagen type II were cartilage specific extra-cellular matrix molecules[36]. Collagen type X is a well-established marker for late chondrocyte differentiation[34]. Though gene expression of SOX9 was not significantly

Table III
Increased gene profiles in microarray analysis

GenBank	Gene name	Description
NM_005252	FOS	Homo sapiens FBJ murine osteosarcoma viral oncogene homolog
NM_058197	CDKN2A	Homo sapiens cyclin-dependent kinase inhibitor 2A (melanoma, pi6. inhibits CDK4), transcript variant 3
NM_013227	ACAN	Homo sapiens aggrecan. Transcript variant 2
NM_006732	FOSB	Homo sapiens FBJ murine osteosarcoma viral oncogene homolog B, transcript variant 1
NM_001964	EGR1	Homo sapiens early growth response 1
NM_002922	RGS1	Homo sapiens regulator of G-protein signaling 1
NM_002928	RGS16	Homo sapiens regulator of G-protein signaling 16
NM_001554	CYR61	Homo sapiens cysteine-rich, angiogenic inducer, 61
NM_000399	EGR2	Homo sapiens early growth response 2 (EGR2), transcript variant 1
NM_002135	NR4A1	Homo sapiens nuclear receptor subfamily 4, group A, member 1, (NR4A1), transcript variant 1
NM_005203	COL13A1	Homo sapiens collagen, type XIII, alpha 1 (COL 13A1), transcript variant 1
NM_178172	GPIHBP1	Homo sapiens glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1
THC2724906		Q96HL9_HUMAN, CHP protein
NM_174858	AK5	Homo sapiens adenylate kinase 5, transcript variant 1
Z74615	COL1A1	Homo sapiens mRNA for prepro-alphal(I) collagen
NM_001050	SSTR2	Homo sapiens somatostatin receptor 2
NM_001321	CSRP2	Homo sapiens cysteine and glycine-rich protein 2
NM_032181	FAM176A	Homo sapiens family with sequence similarity 176, member A (FAM176A), transcript variant 2
XR 015254	LOC728295	Homo sapience similar to FL00310 protein (LOC728295)
NM_001040619	ATF3	Homo sapiens activating transcription factor 3 (ATF3), transcript variant 4
NM_004417	DUSP1	Homo sapiens dual specificity phosphatase 1
NM_003118	SPARC	Homo sapiens secreted protein, acidic, cysteine-rich (osteonectin)
NM_001114734	PABPC4L	Homo sapiens poly (A) binding protein, cytoplasmic 4-like
AK055302		Homo sapiens cDNA FLJ30740 fis, clone FEBRA2000319
NM_052970	HSPA12B	Homo sapiens heat shock 70kD protein 12B, transcript variant 1
NM_001004343	MAP1LC3C	Homo sapiens microtubule-associated protein 1 light chain 3 gamma
NM_144586	LYPD1	Homo sapiens LY6/PLAUR domain containing 1, transcript variant 1
NM_000493	COL10A1	Homo sapiens collagen, type X. alpha 1
NM_032510	PARD6G	Homo sapiens par-6 partitioning defective 6 homolog gamma (C. elegans)
NM_006033	LIPG	Homo sapiens lipase, endothelial
NM_031910	C1QTNF6	Homo sapiens Clq and tumor necrosis factor related protein 6
NM_020130	C8orf4	Homo sapiens chromosome 8 open reading frame 4
NM_033438	SLAMF9	Homo sapiens SLAM family member 9, transcript variant 1

higher by qPCR, those of ACAN, COL2A1 and COL10A1 were significantly higher in idiopathic frozen shoulders, which indicated the chondrogenic gene expression patterns in idiopathic frozen shoulders were different from those in the articular cartilage. Fibroblast-like cells expressed strong gene expression of ACAN, but COL2A1 was not detected by ISH. These discrepancies may be explained by the fact that PCR can detect a small amount of mRNA.

On the basis of explant studies, it appears that a higher proportion of the aggrecan in the tendon, relative to the cartilage, is degraded by constitutive ADAMTS-aggrecanases[37]. Aggrecan is present within the compressed fibrocartilagenous regions and also the non-compressed tensional regions of the tendons[38]. This may explain the existence of aggrecan in the capsule although the capsule, in general, is under less tension than the tendons. Uncontrolled expression of aggrecan may be one of the main etiologies of idiopathic frozen shoulders.

Table IV
Decreased gene profiles in microarray analysis

GenBank	Gene name	Description
AB172662	MYOC	Macaca fascicularis brain cDNA clone: QflA-19129, similar to human myocilin. Trabecular meshwork inducible glucocorticoid response
NM_001890	CSN1S1	Homo sapiens casein alpha si, transcript variant 1
NM_002084	GPX3	Homo sapiens glutathione peroxidase 3 (plasma)
NM_021146	ANGPTL7	Homo sapiens angiopoietin-like 7
NM_003012	SFRP1	Homo sapiens secreted frizzled-related protein 1
NM_020415	RETN	Homo sapiens resistin, transcript variant 1
NM_031950	FGFBP2	Homo sapiens fibroblast growth factor binding protein 2
NM_000065	C6	Homo sapiens complement component 6, transcript variant 1
NM_015101	GLT25D2	Homo sapiens glycosyltransferase 25 domain containing 2
NM_012431	SEMA3E	Homo sapiens sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3E
NM_145791	MGST1	Homo sapiens microsomal glutathione S-transferase 1 (MGST1), transcript variant lc

Table V
Confirmation of increased gene expressions by microarray analysis in capsules

			N	Mean (95% CI)	*P*
FOS	RI	IFS	12	2.26 (1.0–4.0)	0.20
		RCT	16	1.64 (0.6–3.2)	
	MGHL	IFS	11	2.88 (1.5–5.0)	*0.022*
		RCT	16	1.17 (0.6–1.8)	
	IGHL	IFS	12	3.39 (2.1–4.8)	*0.043*
		RCT	16	2.29 (1.0–3.9)	
FOSB	RI	IFS	12	2.69 (1.2–4.3)	0.15
		RCT	16	1.45 (0.6–2.5)	
	MGHL	IFS	11	4.80 (1.6–10.3)	*0.012*
		RCT	16	0.917 (0.6–1.3)	
	IGHL	IFS	12	7.47 (4.1–11.6)	*0.015*
		RCT	16	3.09 (1.3–5.6)	
CYR61	RI	IFS	12	5.03 (2.5–8.8)	*0.043*
		RCT	16	2.05 (1.2–3.0)	
	MGHL	IFS	11	5.46 (2.2–10.8)	*<0.001*
		RCT	16	1.10 (0.7–1.5)	
	IGHL	IFS	12	3.13 (1.9–4.5)	*0.043*
		RCT	16	1.82 (0.9–3.1)	
SPARC	RI	IFS	12	4.92 (3.2–6.8)	*<0.001*
		RCT	16	1.51 (0.9–2.2)	
	MGHL	IFS	11	4.74 (2.5–7.2)	*0.01*
		RCT	16	1.34 (0.8–2.4)	
	IGHL	IFS	12	4.07 (2.3–6.5)	*<0.001*
		RCT	16	0.883 (0.4–1.6)	

Italic values significant the statistically of $P < 0.05$.

Production, deposition, and turnover of aggrecan in fibrous tissues represent a critical process in the reparative response[39]. Aggrecan accumulates in the tendon specifically due to a lack of ADAMTS5 activity, and a resultant switch in TGFb1-signaling from fibrogenic to chondrogenic[40]. For fibrogenic differentiation, aggrecan must be eliminated via an ADAMTS5-dependent process[41]. Though expression of ACAN was significantly higher, expressions of ADAMTS4 and ADAMTS5 were not significantly higher in idiopathic frozen shoulders by qPCR. This data might indicate a reparative process against a fibrotic process in idiopathic frozen shoulders. On the other hand, almost no increase of MMPs in idiopathic frozen shoulders may contribute to the fibrotic process in idiopathic frozen shoulders. Further studies are needed to elucidate a signaling pathway in idiopathic frozen shoulders.

From DNA microarray analysis, gene expressions related to fibrosis such as COL1A1 and COL13 A1, chondrogenesis such as FOS, ACAN, FOSB, and COL10A1, angiogenesis such as CYR61 were significantly higher in idiopathic frozen shoulders. Considering other genes in microarray analysis, there are some possibilities in the pathogenesis of idiopathic frozen shoulders. FOS, known as immediate early gene family of transcription factors, has a critical role in chondrogenesis[42]. However, over expression of FOS inhibits collagen type II expression in vitro[42]. EGR1 is rapidly induced by mitogens, hypoxia, shear stress, or mechanical injury in fibroblasts, endothelial cells, and other cell types via mitogen- and stress-activated protein kinases[43]. COL2A1 promoter activity is inhibited by interleukin-1β induced by EGR1 binding[44]. These results may indicate suppression of COL2A1 in idiopathic frozen shoulders, which might influence the result of ISH. ATF3 reduces the activity of a SOX9 dependent promoter[45]. Gene expression of SOX9 was not significantly higher in idiopathic frozen shoulders in our study. There seemed to be a condition that chondrogenesis was suppressed in idiopathic frozen shoulders.

EGR1 up-regulates COL1A2 promoter activity and further enhances the stimulation induced by TGF-β in fibroblasts[46]. Furthermore, EGR1 plays a key role in orchestrating tissue response to acute injury by activating the transcription of many proliferation-associated genes, such as PDGF A and B, VEGF, TNF-α, ICAM-1, FGF-2, IGF-2, IL-2, and EGR-1 itself[43,47]. Though the gene expressions of TGF-β1, PDGF A, and TNF-α were not significantly higher in idiopathic frozen shoulders, the gene expression of PDGF B was significantly higher in this study. Other pathways to control gene expression of COL1A1 may exist in idiopathic frozen shoulders. Together with c-fos, EGR1 also up-regulates collagenase expression in periodontal gingival fibroblasts and rheumatoid synovial fibroblasts[48,49]. In this study, only expression of MMP-14 was significantly higher in idiopathic frozen shoulders. SPARC is an extra-cellular matrix protein and was over expressed in cirrhotic livers and its down-regulation ameliorated liver fibrosis[50]. SPARC may accelerate the higher expression of COL1A1 in idiopathic frozen shoulders.

In conclusion, fibrosis, inflammation, and chondrogenesis are the main pathologies of idiopathic frozen shoulders.

Contributions

- Conception and design: YH.
- Analysis and interpretation of the data: YH, TW, EI.
- Drafting of the article: YH, AA, TM, EI.
- Critical revision of the article for important intellectual content: YH, EI.
- Final approval of the article: YH, EI.
- Provision of study materials or patients: YH, AA, NT, HS.
- Statistical expertise: TW.
- Administrative, technical, or logistic support: YO, MT, EC, HS, YS.
- Collection and assembly of data: YH, AA, YO, TT, TM, NH.

Role of the funding source

All of the authors had no funding source concerning this study.

Conflict of interests

The authors declare that they have no conflict of interests.

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Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.joca.2011.12.008.

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