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ORIGINAL ARTICLE

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The prevalence of autoantibodies against cartilage intermediate layer protein, YKL-39, osteopontin, and cyclic citrullinated peptide in patients with early-stage knee osteoarthritis: evidence of a variety of autoimmune processes

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Abstract We studied arthritis-related autoantibodies in 136 patients with knee osteoarthritis (OA) and 67 age- and sex-matched healthy individuals in the Shanghai District of China. Serum antibody titers for recombinant fusion proteins of cartilage intermediate layer protein (CILP) and YKL-39 were analyzed using enzyme-linked immunosorbent assay (ELISA) and Western blot. Serum antibody titers against recombinant osteopontin (OPN) and cyclic citrullinated peptide (CCP) antibodies were measured also using ELISA. Anti-CILP antibodies were detected in 25/136 OA patients but only 1/67 controls. Anti-YKL-39, anti-OPN, and anti-CCP antibodies were detected in 9/136, 11/136, and 7/136 of the OA patients, respectively, and 0/67 controls. There was rarely overlap of these antibodies in a single patient, suggesting distinct antigen specificity in each case. The antibodies were detected in patients with OA of grades II and III but not grade IV. The prevalence of autoantibodies to various arthritis-related proteins in early-stage knee OA supports the involvement of a specific immune response in initial cartilage degeneration in OA.

Keywords Autoantibody · Cartilage intermediate layer protein · Cyclic citrullinated peptide · Osteoarthritis · Osteopontin · YKL-39

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Introduction

Osteoarthritis (OA) is a chronic joint disease that is common among aged populations. Histologically, it is characterized by the presence of cartilage surface irregularities such as fissures and fibrillation, loss of cartilage matrix, osteophyte formation, and patchy, chronic synovitis. In general, it is a degenerative disease caused mainly by the aging process and/or joint overload.

A growing body of evidence supports the role of immunologic involvement in the pathogenesis of OA. First, infiltration of immunocompetent cells such as T lymphocytes, B lymphocytes, and monocytes/macrophages into the synovial tissue in OA patients has been reported [1, 2, 3]. Second, immunoglobulins and immune complexes are occasionally detected in OA patient cartilage [4]. Third, a subset of OA patients produce autoantibodies in association with human leukocyte antigen (HLA)-A and -B haplotypes and, moreover, some OA patients have other, concurrent autoimmune diseases [4, 5, 6]. Finally, oligoclonal T-cell expansion occurs in synovial membranes in OA patients [7, 8], suggesting activation and proliferation of a limited number of T-cell clones with distinct antigen specificity. Taken together, these data indicate the involvement of immunologic mechanisms in the pathogenesis of OA [9, 10].

Although the role of antigen recognition by T cells in OA has been studied, only a small number of the autoantigens they recognize has been identified. Target antigens include cartilage matrix molecules such as collagens and proteoglycans; however, it is likely that T cells in OA also recognize specific peptides that are quantitatively minor among the joint components and not known to be potentially antigenic.

We previously demonstrated that a subset of Japanese OA patients possess autoantibodies against several cartilage-derived proteins. Specifically, they produced au-

toantibodies against two chondrocyte-derived proteins, cartilage intermediate layer protein (CILP) and YKL-39. The former is a 91.5-kD glycoprotein secreted from chondrocytes, of which mRNA encodes a proform of two domains with a region homologous to the porcine nucleotide pyrophosphohydrolase (NTPPHase) at its carboxyl terminal. The CILP is distributed in the middle layer of cartilage, and its expression is enhanced in elderly people and also in patients with early-stage OA [9, 10].

The other chondrocyte-derived protein, YKL-39, belongs to the chitinase protein family, sharing amino acid homology with human cartilage glycoprotein-39, a candidate potent autoantigen in rheumatoid arthritis (RA) [11]. As for the immune response to these proteins, anti-CILP and anti-YKL-39 autoantibodies were detected in 10.5% and 11.1%, respectively, of OA patients in a Japanese population. In addition, immunization with recombinant proteins of these antigens in mice induced mild but chronic arthritis, suggesting the potent arthritogenicity of these antigens [12, 13].

In addition to CILP and YKL-39, osteopontin (OPN) is a target of autoantibody response in OA and RA patients [14]. Osteopontin is an extracellular matrix protein that contains an Arg-Gly-Asp sequence. It is expressed in a variety of cells, including chondrocytes, and has diverse bioactivity, such as cell adhesion. The anti-OPN antibody in RA patients seems to be linked with clinical activity, such as high C-reactive protein level and high erythrocyte sedimentation rate, suggesting a role of anti-OPN-antibody in the pathogenesis or pathophysiology of arthritis [14]. The mechanisms by which the production of these antibodies is involved in OA pathophysiology are, however, not yet fully understood.

Recent reports have focused on the importance of anticyclic citrullinated peptide (CCP) antibodies in the pathogenesis of RA. The target, CCP, was produced from filaggrins (filament-aggregating proteins) by citrullination by peptidylarginine deiminase. Filaggrins are an RA-specific antigen recognized by antikeratin antibodies or antiperinuclear factor antibodies, and RA sera react specifically with the citrulline-containing peptides [15]. The anti-CCP antibody is exclusive in RA patients; that is, the frequency of the antibody is much lower in patients with other autoimmune diseases, such as systemic lupus erythematosus [16]. Nevertheless, the anti-CCP antibody is suggested to be detectable in a subset of OA patients at an even higher frequency than in SLE (according to preliminary data by the manufacturer of the anti-CCP ELISA kit) (see Materials and methods). This suggests that the immune response to CCP might be involved in the pathogenesis of OA as well as that of RA. There are no detailed reports, however, on the presence of the anti-CCP antibody among OA patients.

These data taken together suggest that a variety of autoimmune responses involved in OA are also involved in joint degeneration. Nevertheless, it is not known whether autoimmunity against various antigens is a general phenomenon in human OA patients. Further, it is also unknown whether the autoimmune responses to different antigens exist in a single patient or if these responses are completely exclusive. Here, we explored the presence of these recently identified autoantibodies in OA patients, using a panel of serum samples of freshly diagnosed OA patients in the Shanghai District in China.

Materials and methods

Patients

One hundred thirty-six patients (29 men and 107 women) aged 40–88 years (mean 67) were interviewed in the Shanghai District, China, and diagnosed with knee OA according to the American College of Rheumatology criteria [17]. All patients were radiographically graded using the Kellgren and Lawrence scale and treated with nonsteroidal anti-inflammatory drugs. In addition, 67 age- and sex-matched control individuals were enrolled in the study. No arthritic diseases were detected in the control group, whose grading was confirmed to be 0 in all individuals. All samples were obtained with signed, informed consent, and the study was approved by the local institutional ethics committee.

Preparation of recombinant proteins

Recombinant CILP proteins were prepared as previously described [12] (the constructs of each CILP fusion protein are shown in Fig. 1A). Briefly, cDNA fragments encoding the first half ($cDNA^{C1}$) and second half ($cDNA^{C2}$) of the non-NTPPHase-homologous region of CILP were obtained from mRNA of human articular chondrocytes by reverse transcription-polymerase chain reaction. The cDNA fragments were subcloned into a plasmid expression vector of pTEX-2-eHis. Using these constructs, recombinant CILP proteins of C1 and C2 were produced in *Escherichia coli* as a fusion protein with beta-galactosidase (β-gal). β-Gal alone was similarly produced as a negative control. The proteins were purified using a histidine tag as described previously [12].

The recombinant YKL-39 protein was prepared as described previously [13]. Briefly, the DNA fragment encoding YKL-39, amplified by reverse transcription-polymerase chain reaction, was cloned into a plasmid vector of pMAL-eHis, a derivative of pMAL-c2 (New England Biolabs, Beverly, Mass., USA), by which YKL-39 was produced in *E. coli* as a fusion protein with maltose binding protein (MBP). The MBP alone was similarly produced as a negative control. The proteins were purified as above.

Enzyme-linked immunosorbent assay

To detect the anti-CILP and anti-YKL39 antibodies, ELISA was performed as previously described [12, 14]. Antibody binding reactivity with a fusion protein was

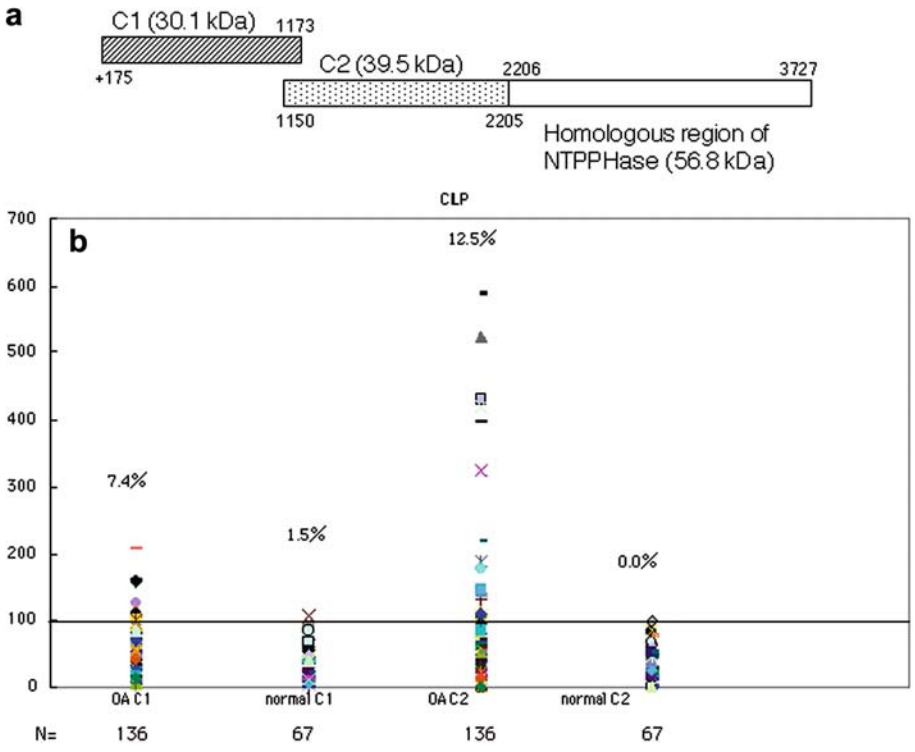

Fig. 1a, b Cartilage intermediate layer protein autoantibodies in OA patients. **a** Constructs of the CILP fusion proteins C1 and C2 are shown. *Numbers* denote the nucleotide numbering of the human CILP cDNA fragments that encode C1 (999 bp, molecular weight 30.1 kDa) and C2 (1056 bp, molecular weight 39.5 kDa). Both proteins were expressed as β-gal fusion proteins in *E. Coli*. **b** Autoantibody titers against recombinant CILP-C1 and CILP-C2 proteins in sera from 136 Chinese patients with knee OA and 67 age- and sex-matched healthy individuals, diluted to 1:500 and tested by ELISA. Results are shown in binding units. *Horizontal solid line* represents the positive cutoff limit

expressed as "binding units" according to a formula using optical density (OD) values: sample (binding units) = [OD sample of normal serum/(mean OD sample + 3 SD of normal serum)] × 100. In each sample, the OD value of MBP or β-gal was subtracted from the OD value of the fusion protein to obtain the OD of the sample. According to this formula, 100 binding units were used as the cutoff point.

The anti-OPN antibody titer was assayed by ELISA as described previously [14] with minor modifications, using purified recombinant human OPN (Chemicon International, Temecula, Calif., USA). Anti-CCP antibody was analyzed semiquantitatively using a commercially available ELISA kit (DIASTAT, Axis-Shield Diagnostics, UK) according to the manufacturer's instructions. In this assay, concentrations of the anti-CCP antibody in sera could be calculated, and samples were marked positive if the concentration was greater than 5 U/ml. Each assay was performed in triplicate.

Western blotting

The Western blotting procedure was described previously [14]. Briefly, 1 μg of the purified fusion protein, MBP or β-gal, separated by 8% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was transferred onto nitrocellulose membranes. After blocking with phosphate-buffered saline (PBS) containing 3% bovine serum albumin and 0.1% Tween-20 for 1 h and washing in PBS with 0.1% Tween-20 three times, each membrane sample was incubated with serum samples diluted adequately in PBS containing 3% bovine serum albumin and 0.1% Tween-20 for 1 h. The serum samples, preincubated with 20 μg/ml of bacterial lysate containing nonrecombinant pTEX-2-eHis product for 2 h at room temperature, were reacted before the membranes. After similar washing, the bound antibodies were reacted with horseradish peroxidase-conjugated goat antihuman IgG antibodies (Zymed, San Francisco, Calif., USA) for 30 min. Finally, the bound antibodies were visualized by enzymatic reaction of horseradish peroxidase with diaminobenzidine.

Results

Detection of CILP autoantibodies

Autoantibodies to the C1 protein were detected in ten (7.4%) of 136 OA serum samples and in one (1.5%) of 67 serum samples from healthy controls (Fig. 1b). Similarly, autoantibodies to the C2 fusion protein were detected in 17 (12.5%) of the same 136 OA serum samples but none of the controls. Only two samples reacted to both the C1 and C2 regions; 25 of 136 (18.4%) of the OA patients had the anti-CILP autoantibodies. All of the ELISA-positive samples were confirmed by Western blotting.

Detection of YKL-39 autoantibodies

We next detected autoantibodies to YKL-39 by ELISA in the same panel of serum samples using the MBP-fused recombinant protein of YKL-39. Autoantibodies to the

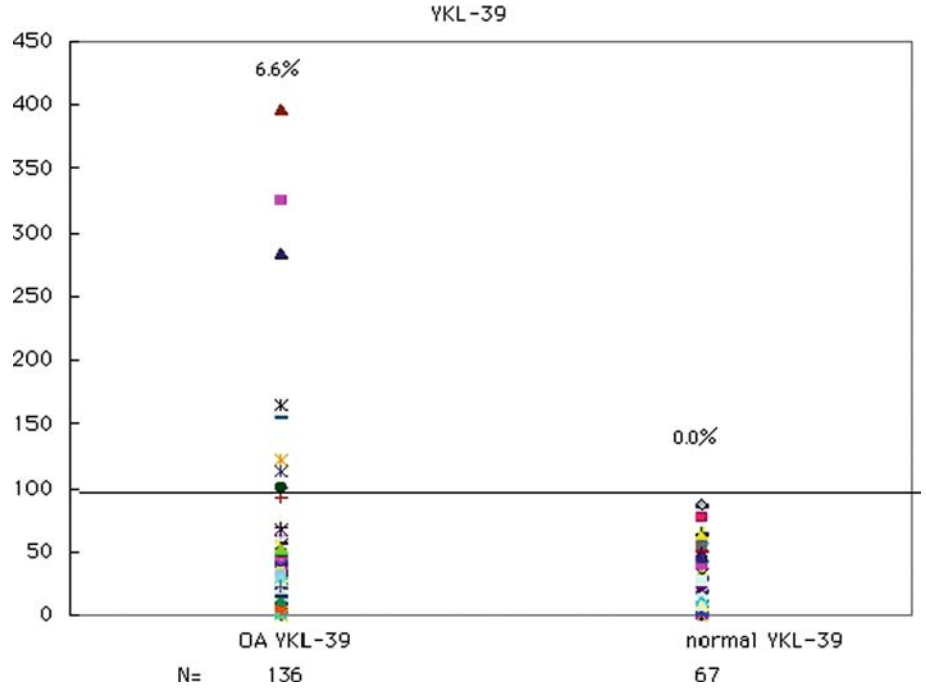

Fig. 2 Autoantibody titers to the YKL-39 fusion protein tested by ELISA in serum samples, diluted to 1:300, from 136 Chinese patients with knee OA and 67 age- and sex-matched healthy individuals. Results are shown in binding units. *Horizontal solid line* represents the positive cutoff limit

YKL-39 fusion protein were detected in nine of 136 OA serum samples (6.6%) and none of the control samples (Fig. 2). All of the ELISA-positive serum samples were confirmed to be positive by Western blotting (representative cases are shown in Fig. 3).

Detection of OPN autoantibodies

Of 136 serum samples, 11 (8.1%) from OA patients reacted with OPN (Fig. 4), whereas there was no such antibody reaction in the control group.

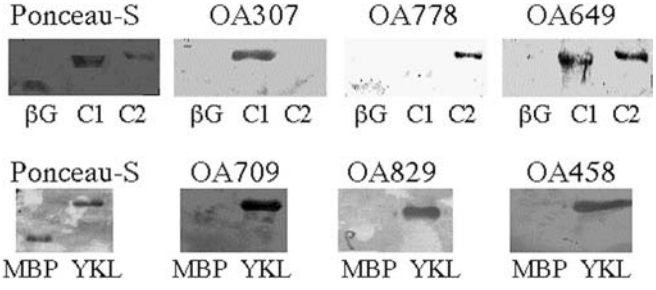

Fig. 3 Western blotting of the anti-CILP and anti-YKL-39 antibodies. Serum samples with positive antibody titers by ELISA were further tested by Western blotting. *Upper row* C1 and C2 fusion protein and the fusion partner β-gal as a negative control were separated by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Then the membranes were reacted with serum samples. Representative results from three patients are shown. βG β-galactosidase. *Lower row* The YKL-39 fusion protein and its fusion partner, MBP, were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Then the membranes were reacted with the serum samples. Representative results from three patients are shown

Detection of anti-CCP antibodies in osteoarthritis patients

Anti-CCP antibodies are present in RA sera with high specificity. Thus, they are considered to be a specific marker for the diagnosis of RA. It is not clear, however, whether sera from OA patients react to CCP. Of the 136 patients with knee OA, seven (5.2%) were positive for anti-CCP antibody (Fig. 5). Only one of 67 control patients (1.5%) was anti-CCP antibody-positive.

Prevalence of multiple autoantibodies in osteoarthritis patients of different clinical grade

We detected various autoantibodies in a subset of knee OA patients as described above. We then examined whether the presence of the various autoantibodies overlapped in the tested OA patients.

As summarized in Table 1, there was infrequent overlap between two distinct antibodies. For example, none of the patients had both anti-CILP and anti-YKL39 antibodies or anti-CCP and anti-YKL-39 antibodies simultaneously. Among these antibodies, dual positivity of anti-CILP and anti-CCP was more frequent than any of the other combinations. Specifically, four of 25 anti-CILP-positive patients (16%) were also positive for the anti-CCP antibody, and four of seven (57.1%) with anti-CCP also had the anti-CILP antibody. Table 2 shows a list of eight patients with positive titers for two or more of the autoantibodies tested. There was no correlation between age, sex, or positive titer of a particular autoantibody (Table 2 and data not shown).

Finally, we investigated the association between the presence of the autoantibodies and radiographic grading

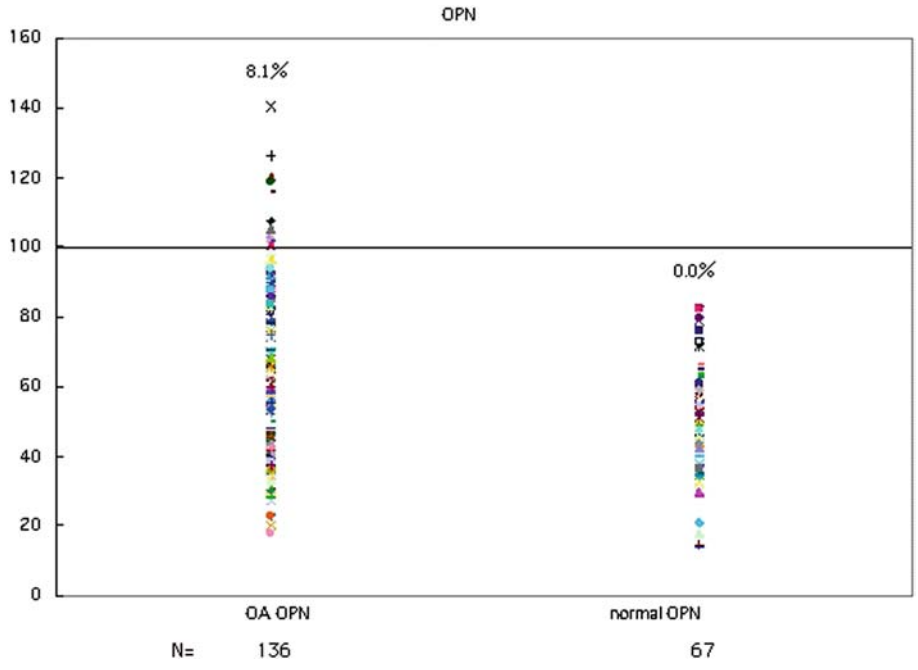

Fig. 4 Anti-OPN antibody in sera from 136 knee OA patients and from 67 healthy individuals, diluted to 1:200, and tested by ELISA. Results are shown in binding units. *Horizontal solid line* represents the positive cutoff limit

of OA. Table 3 shows the percentage of patients with positive antibody titers among each OA grade. As demonstrated, the autoantibodies tested in this study were detected most frequently in the early to middle stages of OA and never in advanced knee OA. More specifically, anti-CILP and anti-CCP autoantibodies were detected in patients with grade II OA more frequently (22.0% and 7.0%, respectively) than in those with grade III (10.3% and 0%). The anti-YKL-39 antibody was detected in patients with grades II and III OA with similar frequency. Only the anti-OPN antibody was detected more frequently in patients with grade III OA than in those with grade II. None of the antibodies were detected in patients with grade IV OA.

In total, 43 of 136 knee OA patients (31.6%) tested positively for at least one of the autoantibodies among anti-CILP, anti-YKL39, anti-OPN, and anti-CCP antibodies.

Discussion

The present study demonstrates the prevalence of autoantibodies in knee OA patients in Shanghai, China. The autoantibodies examined are relatively ''new'' and have not been fully recognized. The results indicate that the frequency of these antibody titers is similar between Japanese and Chinese populations and that autoanti-

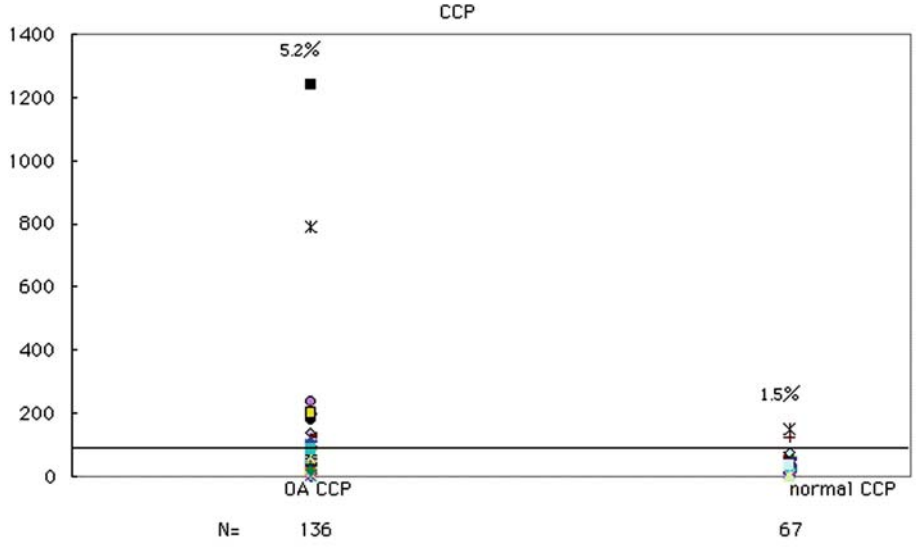

Fig. 5 Anti-CCP antibody in serum samples from 136 knee OA patients and 67 healthy individuals, tested by ELISA. Results are shown in U/ml of the antibody. *Horizontal solid line* represents the positive cutoff limit

Table 1 Comparison of the presence of anti-CILP and anti-YKL-39 in 136 knee OA patients. + Positive,− negative

CILP vs YKL-39	Anti-CILP + ($n=25$)	Anti-CILP − ($n=111$)
Anti-YKL-39 + ($n=9$)	0	9
Anti-YKL-39 − ($n=127$)	25	102
CILP vs OPN	Anti-CILP + ($n=25$)	Anti-CILP − ($n=111$)
Anti-OPN + ($n=11$)	3	8
Anti-OPN − ($n=125$)	22	103
CILP vs CCP	Anti-CILP + ($n=25$)	Anti-CILP − ($n=111$)
Anti-CCP + ($n=7$)	4	3
Anti-CCP − ($n=129$)	21	108
OPN vs YKL-39	Anti-OPN + ($n=11$)	Anti-OPN − ($n=125$)
Anti-YKL-39 + ($n=9$)	2	7
Anti-YKL-39 − ($n=127$)	9	118
CCP vs YKL-39	Anti-CCP + ($n=7$)	Anti-CCP − ($n=129$)
Anti-YKL-39 + ($n=9$)	0	9
Anti-YKL-39 − ($n=127$)	7	120
CCP vs OPN	Anti-CCP + ($n=7$)	Anti-CCP − ($n=129$)
Anti-OPN + ($n=11$)	1	10
Anti-OPN − ($n=125$)	6	119

Table 2 List of eight patients with positive titers of two or more autoantibodies

Patient no.	Age	Sex	Grade	CILP	YKL-39	OPN	CCP
35	43	F	2	+		+	+
81	88	M	2	+			+
461	70	F	2	+			+
694	58	M	2	+			+
353	67	F	2	+		+	
650	79	M	2	+		+	
403	48	F	2		+	+	
592	80	M	2		+	+	

body production is a relatively common phenomenon in early-stage OA patients. The antibodies, however, are not present in the sera of patients with advanced knee OA.

In our previous study, 10.5% of Japanese OA patients possessed the anti-CILP antibody [12] and 11.1% were positive for the anti-YKL-39 antibody [13]. Antibodies against the recombinant fusion protein of human OPN were detected in 0.95% of OA patients, whereas more serum samples (9.5%) reacted with native human OPN [14]. Those frequencies are slightly different from those of Chinese patients in the current study, in which 25 of 136 (18.3%) had antibodies against CILP (Fig. 1). The sera of 6.6% of the Chinese knee OA patients in the present study were anti-YKL-39 antibody-positive (Fig. 2).

In our experiments using recombinant purified human OPN, antibodies against rhOPN were present in 8.1% of the patients (Fig. 4), confirming the presence of anti-OPN antibodies in a subset of OA patients in both Japan and China, even when different types of OPN are used. Differences in the frequency of positive antibody titers might depend on differences in genetic background, e.g., the distribution of HLA haplotypes between Chinese and Japanese populations. Otherwise, it might relate to differences in the therapies between the two countries. Further investigation of the genetic background, clinical information, and prognosis of tested patients will clarify this issue.

Table 3 Clinical grade of knee OA and correlation to prevalence of the autoantibodies

Grade	II ($n=100$)	III ($n=29$)	IV ($n=7$)
Anti-CILP (n)	22.0% (22)	10.3% (3)	0% (0)
Anti-YKL-39 (n)	7.0% (7)	6.9% (2)	0% (0)
Anti-OPN (n)	8.0% (8)	10.3% (3)	0% (0)
Anti-CCP (n)	7.0% (7)	0% (0)	0% (0)
Percent of patients with one or more antibodies (n)	35.0% (34)	27.6% (8)	0% (0)

The anti-CCP antibody is a specific marker of RA, and the target antigen, CCP, is also thought to be involved in the pathogenesis of RA. To our knowledge, this is the first study to report the presence of anti-CCP antibody in a panel of patients with various degrees of knee OA as graded by the Kellgren and Lawrence scale. The anti-CCP antibody was present in a subset of patients with grade II knee OA but not other grades. These findings suggest the presence of the specific immune response to CCP only in the early stage of cartilage degradation, and the response seems to disappear at the late stage. The reason for this phenomenon is not clear; however, release of the CCP antigen might occur only during early-stage OA. In addition, anti-CCP-positive RA patients might develop significantly more severe radiographic damage than those who are anti-CCP antibody-negative [18]. It is therefore important to observe the prognosis of cartilage degradation in OA patients with the anti-CCP antibody, as in RA patients.

Overlapping of antibodies in a single patient was infrequent. Thus, each individual might have a distinct autoreactivity to different antigens, probably depending on the HLA haplotype, which determines the specificity of antigen presentation. In particular, none of the patients possessed the anti-CILP and anti-YKL-39 antibodies simultaneously, indicating that a distinct mechanism underlies the immune responses to the respective autoantigens. The coexistence of anti-CILP and anti-CCP antibodies was relatively frequent. In this regard, it has been suggested that CILP expression is upregulated in early OA and, as described above, CCP might be involved in triggering the arthritis. In fact, in our study, all of the patients positive for the antibodies were in early stages of OA. It is, therefore, necessary to investigate further the release of these antigens in each stage of OA to explore the mechanism of dual antibody production.

In addition to the anti-CILP and anti-CCP antibodies, anti-YKL39 and anti-OPN antibodies were detected only in patients with OA of grades II or III but not grade IV. This finding suggests that these antibodies or the immune responses to these (relatively minor) antigenic proteins are specific for the early cartilage degradation in OA, and it is possible that patients with advanced OA have antibodies with different specificities. Thus, the antigen specificity of T and B cells in different stages of OA should be analyzed.

The present study reveals a variety of autoimmune processes in a subset of patients with knee OA in a Chinese population. In all, 43 of 136 OA patients (31.6%) had at least one of the autoantibodies analyzed here (i.e., anti-CILP, anti-YKL39, anti-OPN, and anti-CCP). Because there are probably many other autoantibodies to known or yet unidentified antigens, it is possible that a majority of OA patients have autoantibodies. Identification of the target autoantigens in OA patients, especially those specific to OA, will aid in understanding the immune-mediated pathogenesis of OA.

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