



Diagnostic performance of anti-citrullinated protein/peptide antibodies in juvenile idiopathic arthritis

S.Y. Pang¹, H.Y. Liu¹, Y.J. Huang¹, Y.F. Liu¹, Y.M. Dai¹, P. Zeng² and H.S. Zeng²

¹Clinical Laboratory, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, China

²Department of Immunology and Rheumatology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangzhou, China

Corresponding author: H.Y. Liu
E-mail: xiangliuhaiying@aliyun.com

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ABSTRACT. The prevalence rates of anti-citrullinated protein/peptide antibodies (ACPAs) were investigated in a cohort of juvenile idiopathic arthritis (JIA) patients, and their diagnostic performances were compared. ACPAs, including anti-cyclic citrullinated peptide IgG (anti-CCP), anti-CCP IgG/IgA (anti-CCP3.1), citrullinated recombinant rat filaggrin antibodies (CPA), anti-mutated citrullinated vimentin (anti-MCV), and antibodies to citrullinated human IgG-derived peptides (RA/CP), were measured in the sera from 81 JIA patients. Serum samples from 55 children with other joint diseases or viral infections and 49 healthy donors were tested as controls. Of the 81 JIA patients, 7 (8.6%), 8 (9.9%), 17 (21.0%), 23 (28.4%), and 18 (22.2%) were found to be positive for anti-CCP, anti-CCP3.1, CPA, anti-MCV, and RA/CP, respectively, with specificities of 98.1, 95.1, 93.3, 84.6, and 86.5%. Analysis by subtype revealed that 7/7 (100%) of RF-positive polyarticular JIA patients tested positive at high serum levels for anti-

MCV or RA/CP, and 5/7 (71.4%) were positive for anti-CCP, anti-CCP3.1, or CPA ($P < 0.001$, compared with controls). Eighteen of 81 JIA patients demonstrated joint erosions on radiographs and erosive arthritis occurred more often in ACPAs positive patients ($P < 0.01$). Our findings indicate that although ACPAs are not satisfactory screening biomarkers for JIA due to low sensitivity, ACPA measurement can aid in diagnosing RF-positive polyarticular JIA and identifying JIA patients with severe bone involvement. The diagnostic performance of each ACPA in JIA is different, and the careful selection of assays is necessary.

Key words: Anti-citrullinated protein/peptide antibodies; Diagnosis; Juvenile idiopathic arthritis

INTRODUCTION

Juvenile idiopathic arthritis (JIA) is a clinically heterogeneous group of arthritis subtypes that occur in children under 16 years old with an onset characterized primarily by arthritis persisting for at least 6 weeks for which the cause is unknown (Petty et al., 2004). JIA causes persistent synovitis, pain, joint destruction, and functional disability. Irreversible joint destruction can be prevented by intervention at an early stage, and thus, the accurate diagnosis of JIA is important. The diagnosis of JIA depends primarily on clinical manifestations of the disease, with little in terms of serological support. A wide variety of autoantibodies have been described in patients with this syndrome, but none of these is specific to JIA. Only rheumatoid factor (RF) and antinuclear antibodies are routinely used for classifying, diagnosing, and evaluating the clinical status of JIA (Borchers et al., 2006).

JIA is the most common rheumatic disease in childhood and a major cause of disability; the pathological change in joints is considered to mimic that in adult rheumatoid arthritis (RA). Since a citrulline residue of a filaggrin was found to be an essential constituent of the antigenic determinants recognized by specific antibodies that were present in RA patients in 1998 (Schellekens et al., 1998), through the optimization of the antigen, an enzyme-linked immunoassay (ELISA) detecting RA-specific antibodies, namely antibodies against the citrullinated cyclic peptide (anti-CCP), has been developed. Since this assay was developed, anti-CCP has been used widely in clinical laboratories and accepted by clinical rheumatologists, and positivity for anti-CCP antibodies has been included in the 2010 revised ACR criteria for RA (Aletaha et al., 2010), with a reported sensitivity of 60~80% and specificity of up to 90%. Citrullination, a posttranslational change in which arginine is converted to citrulline by the loss of a NH_2 residue, is believed to be a common phenomenon in RA and has been proven to play a pivotal role in the pathogenesis of RA (Rubin and Sønderstrup, 2004). Subsequently, several other citrullinated proteins have been identified to be targets of the autoimmune response in RA, including vimentin, the glycolytic enzyme α -enolase, and the Epstein-Barr virus protein EBNA-1, and ELISAs detecting antibodies against these citrullinated proteins/peptides for the diagnosis of RA were developed accordingly, with comparable sensitivities and specificities (Bizzaro et al., 2007; Coenen et al., 2007). In addition to being serological markers for RA, especially for early diagnosis, these anti-citrullinated protein/peptide antibodies (ACPAs) have been found in many studies over the last decade to be predictive of the development of erosive joint lesions (Mathsson et al., 2008; Goëb et al., 2009).

Recent studies have evaluated the role of anti-CCP in JIA in diagnosis and prediction of disease outcome and found that anti-CCP can be detected mainly in patients with polyarthritic onset and who are RF positive (Avcin et al., 2002; Ferucci et al., 2005; Dewint et al., 2006; Syed et al., 2008). Although these ACPs have similar sensitivities and specificities in diagnosing RA, because they differ in terms of the antigen source, their patterns of reactivity are not completely identical in the same patients, and little data have been obtained about ACPs other than anti-CCP in JIA. Therefore, it is necessary to explore whether these ACPs will aid in the diagnosis of JIA and determine the disease severity. The aims of the present study are to determine the prevalence of ACPs in JIA using commercially available ELISA kits and to compare their diagnostic performance and clinical significance in a cohort of patients.

MATERIAL AND METHODS

Study population

Serum samples of 81 patients with JIA and 104 controls were collected at Guangzhou Women and Children's Medical Center. The JIA patients, 18 girls and 64 boys with a mean age of 8.6 (range 1.3-15.6) years, had a mean disease duration of 1.4 (range 0-7.4) years were diagnosed with JIA according to International League of Associations for Rheumatology classification (Petty et al., 2004); among the JIA patients, there were 7 with RF-positive polyarthritis, 15 with RF-negative polyarthritis, 13 with oligoarthritis, 7 with enthesitis-related arthritis (ERA), and 39 with systemic-onset arthritis. Data concerning the clinical signs of the disease (clinical arthritis was defined as swelling and/or pain with limitation of motion and radiological joint damage defined as the presence of joint space narrowing and/or bone erosion) were collected from the patients' files. The 104 controls consisted of 55 disease controls (34 patients with juvenile systemic lupus erythematosus (JSLE), 7 with hepatitis B virus infection, 4 with reactive arthritis, 3 with mixed connective tissue disease, 3 with tumor-invasive joint disease, 2 with streptococcus-infection syndrome, 1 with acute nephrotic syndrome, and 1 with infectious mononucleosis) and 49 healthy donors consisting of 29 girls and 20 boys (mean age: 9.5 years; range: 5-14 years). All patients in the control group were carefully checked (through oral questionnaire and hospital records) for the presence of signs or symptoms of JIA; none had any clinical features indicating possible undiagnosed JIA. This study was approved by the local Ethics Committee, and informed consent was obtained from all participating patients for the storage of sera and its use in an anonymous form for research purposes.

Detection of ACPs

ACPs were detected with five commercially available ELISA kits using the following reagents: anti-CCP IgG (anti-CCP, Euroimmun, Lubeck, Germany), anti-CCP IgG/IgA (anti-CCP3.1, Quanta Lite™ CCP3.1, INOVA Diagnostics, San Diego, CA, USA), citrullinated protein antibodies (CPA, Genesis Diagnostics, Cambridgeshire, UK), antibodies against mutated citrullinated vimentin (anti-MCV, Orgentec Diagnostika GmbH, Mainz, Germany), and antibodies against citrullinated human IgG-derived peptides (RA/CP, Aeskulisa RA/CP-Detect, Aesku Diagnostika, Wendelsheim, Germany). Tests were performed using serum sample aliquots that had been stored at -80°C. The key characteristics of each method are shown in Table 1. Citrullinated synthetic peptides included those derived from the filaggrin and

human IgG sequences; citrullinated proteins included recombinant rat filaggrin and mutated human vimentin. The procedures were conducted according to the manufacturer protocols. The quantitative results were read against the standard curve drawn using calibrators following the method recommended by the manufacturer, and the qualitative results were interpreted using the suggested cutoffs provided in the kits. RF was measured by nephelometry (Beckman-Coulter Immage 800). A positive result was recorded when the RF concentration was more than 20 IU/mL.

Table 1. Antigenic characteristics of the diagnostic methods used for the detection of anti-citrullinated protein/peptide antibodies (ACPAs).

Name of Ab	Manufacturer	Citrullinated antigen	Conjugate	Cut-off (suggested)	Calibrator range
anti-CCP	Euroimmun	Synthetic CCP	HRP-rabbit anti-human IgG	5.0	1-200
anti-CCP3.1	Innova	Synthetic CCP	HRP-goat anti-human IgG/IgA	20.0	15.62-250
CPA	Genesis	Recombinant rat filaggrin	HRP-rabbit anti-human IgG	6.25	0-100
anti-MCV	Orgentec	Human mutated vimentin	HRP-rabbit anti-human IgG	20	0-1000
RA/CP	Aesku	IgG-derived synthetic peptide	HRP-rabbit anti-human IgG	18.0	0-300

Statistical analysis

Statistical analyses were performed using SPSS 13.0 software for Windows. Frequencies were compared using the chi-square test of Fisher's exact test as appropriate. ACPA levels were compared using the Mann-Whitney U test. Spearman's test was used to establish correlations between ACPAs and radiological data. Two-sided P values <0.05 were considered statistically significant throughout. The scattergram was plotted using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

RESULTS

ACPAs in JIA patients and control groups

The distributions of ACPA concentrations in JIA patients and control patients for the different assays are shown in Figure 1. Using the cutoff values provided by the manufacturers, 7 (8.6%), 8 (9.9%), 17 (21.0%), 23 (28.4%), and 18 (22.2%) of 81 JIA patients were found to be positive for anti-CCP, anti-CCP3.1, CPA, anti-MCV, and RA/CP, respectively. Table 2 presents the number of ACPA-positive results for the JIA patient subtypes and control groups. Six (27.3%), 6 (27.3%), 9 (40.9%), 10 (45.5%), and 9 (40.9%) of 22 patients with polyarticular JIA were identified by anti-CCP, anti-CCP3.1, CPA, anti-MCV, and RA/CP, respectively. Out of the 7 patients with RF-positive polyarticular JIA, 7 (100%) were positive for anti-MCV or RA/CP, and 5 (71.4%) were positive for anti-CCP, anti-CCP3.1, or CPA at high serum levels.

Between 3.6 and 23.6% of the 55 disease controls were positive for ACPAs, which did not differ from the JIA group ($P > 0.05$), but the antibody levels of ACPA-positive patients in the disease controls group were much lower ($P < 0.05$). Each ACPA could be detected in patients with juvenile systemic lupus erythematosus (5.9~29.4%). Three (6.1%) and 2 (4.1%) of the 49 healthy controls showed reactivity to anti-MCV and RA/CP, while no anti-CCP-, anti-CCP3.1-, or CPA-positive results were found in this group. Forty (49.4%) of the 81 JIA patients, 24 (43.6%) of the 55 disease controls, and 4 (8.2%) of the 49 healthy controls showed a positive reaction in one or more assay. Among them, 5 JIA patients with the RF-

positive polyarticular subtype and 1 patient with JSLE in the disease controls group tested positive for all the ACPAs measured. Based on the 81 JIA patients and 104 controls in this study, the diagnostic specificities of anti-CCP, anti-CCP3.1, CPA, anti-MCV, and RA/CP were 98.1, 95.1, 93.3, 84.6, and 86.5%, respectively, with positive predictive values and negative predictive values of 4.526 and 0.931, 2.020 and 0.947, 3.13 and 0.847, 1.844 and 0.846, and 1.644 and 0.899.

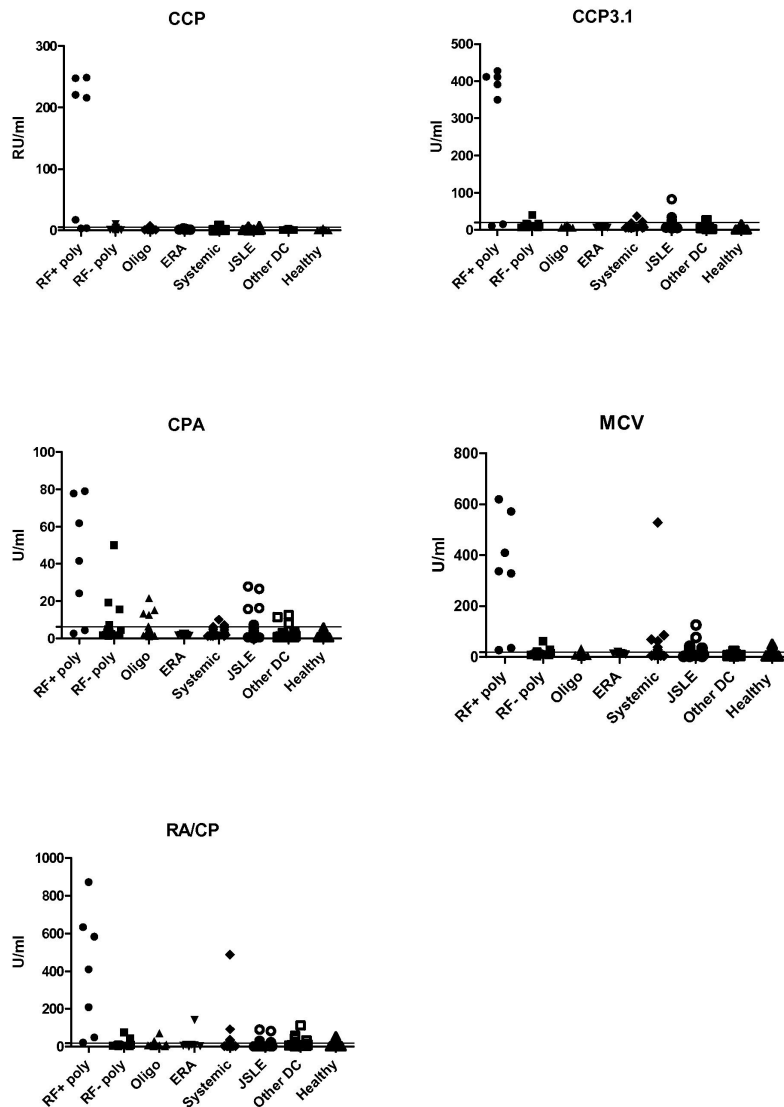


Figure 1. Scatter diagram of ACPAs in JIA patients and controls. RF-positive polyarticular JIA (RF⁺ poly), RF-negative polyarticular JIA (RF⁻ poly), oligoarticular JIA (Oligo), enthesitis-related arthritis (ERA), systemic-onset JIA (Systemic), juvenile systemic lupus erythematosus (JSLE), and disease controls (DC). The horizontal line shows the cutoff value recommended by the manufacturer.

Table 2. Positivity of ACPAs in juvenile idiopathic arthritis (JIA) patients and control groups.

Groups	N	Positive [N (%)]				
		Anti-CCP	Anti-CCP3.1	CPA	Anti-MCV	RA/CP
JIA patients	81	7 (8.6)	8 (9.9)	17 (21.0)	23 (28.4)	18 (22.2)
RF-positive polyarthritis	7	5 (71.4)	5 (71.4)	5 (71.4)	7 (100)	7 (100)
RF-negative polyarthritis	15	1 (6.7)	1 (6.7)	4 (26.7)	3 (20)	2 (13.3)
Oligoarthritis	13	1 (7.7)	-	5 (38.5)	2 (15.4)	2 (15.4)
Enthesitis-related arthritis	7	-	-	-	1 (14.3)	1 (14.3)
Systemic-onset JIA	39	-	2 (5.1)	3 (7.7)	10 (25.6)	6 (15.4)
Disease controls	55	2 (3.6)	5 (9.1)	7 (12.7)	13 (23.6)	12 (21.8)
Juvenile systemic lupus erythematosus	34	2 (5.9)	4 (11.8)	5 (14.7)	10 (29.4)	5 (14.7)
Hepatitis B virus infection	7	-	-	-	-	2 (28.6)
Reactive arthritis	4	-	-	-	-	1 (25.0)
Multiple connective tissue disease	3	-	-	-	2 (66.7)	2 (66.7)
Tumor-invasive joint disease	3	-	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)
Streptococcus infection syndrome	2	-	-	1 (50.0)	-	1 (50.0)
Acute nephrotic syndrome	1	-	-	-	-	-
Infectious mononucleosis	1	-	-	-	-	-
Healthy controls	49	-	-	-	3 (6.1)	2 (4.1)

Association of ACPAs with RF and clinical features of the disease

The relationships between the presence of ACPAs and RF and the clinical features of JIA, including age at onset, disease duration, and radiographic erosion, were evaluated. There were no differences between ACPA-positive JIA patients and the negative group regarding age at onset and disease duration at the time of serum sample collection.

RF was positive in 10 patients with JIA (12.3%), out of which 7 had polyarticular disease, and 3 had systemic-onset arthritis. Of these 10 patients, 5 (50%), 5 (50%), 5 (50%), 7 (70%), and 8 (80%) were positive for anti-CCP, anti-CCP3.1, CPA, anti-MCV, and RA/CP, respectively, whereas 2 (2.8%), 3 (4.2%), 12 (16.9%), 15 (21.1%), and 10 (14.1%) were positive among the 71 RF-negative JIA children. The number of positive results for the five ACPAs among RF-positive JIA patients was significantly higher than that among RF-negative patients ($P < 0.05$), which indicated that the presence of ACPAs correlated significantly with the presence of RF.

Radiographs were performed on each JIA patient within 1 month of sample collection. Eighteen (22.2%) of the 81 JIA patients showed joint space narrowing or bone erosion on the radiograph, including 5/7 RF-positive polyarthritis cases, 3/15 RF-negative polyarthritis cases, 3/13 oligoarthritis cases, 1/7 ERA cases, and 6/39 systemic-onset JIA cases (Table 3). The frequencies of anti-CCP, anti-CCP3.1, CPA, anti-MCV, and RA/CP among the 18 JIA patients with joint erosions were 27.8, 27.8, 50, 55.6, and 38.9%, respectively, which were higher than those of patients without joint erosions ($P < 0.05$). In addition, the presence of erosive arthritis was seen in 5/7 (71.4%), 5/8 (62.5%), 9/17 (52.9%), and 10/23 (43.5%) JIA patients who were positive for anti-CCP, anti-CCP3.1, CPA, and anti-MCV, respectively; these rates of erosive arthritis were higher than the respective rates of patients who were negative for these factors ($P < 0.05$), but there was no difference between RA/CP-positive and RA/CP-negative JIA patients (38.9 vs 17.5%, $P > 0.05$). Spearman correlation analysis showed that, except for RA/CP, the presence of anti-CCP, anti-CCP3.1, CPA, or anti-MCV was closely related to joint erosive disease in JIA patients ($P < 0.05$). Moreover, the five ACPAs were detected simultaneously in the sera of 5 RF-positive polyarticular patients with erosive joint disease, and anti-MCV was found in 4/6 (66.7%) systemic JIA patients with radiological damage.

Table 3. Distribution of ACPAs among JIA subtypes with joint erosions.

Subtypes	N	Joint erosions	Positive [N (%)]				
			Anti-CCP	Anti-CCP3.1	CPA	Anti-MCV	RA/CP
RF-positive polyarthritis	7	5	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)
RF-negative polyarthritis	15	3	-	-	1 (33.3)	1 (33.3)	-
Oligoarthritis	13	3	-	-	1 (33.3)	-	-
Enthesitis related arthritis	7	1	-	-	-	-	1 (100.0)
Systemic-onset JIA	39	6	-	-	2 (33.3)	4 (66.7)	1 (16.7)
Total	81	18	5 (27.8) ^a	5 (27.8) ^a	9 (50.0) ^a	10 (55.6) ^a	7 (38.9) ^b

Compared with the frequency of ACPAs in patients without joint erosions: ^aP < 0.05, ^bP = 0.108.

DISCUSSION

In this study, we compared for the first time the diagnostic performances of several commercial EIA methods, representing the most commonly used methods in clinical laboratories, in a single JIA population. Our findings showed that the prevalence rates of all ACPAs in the whole population of JIA patients were less than 30% and much lower than those of adult RA patients, which agrees with the results reported in previous studies on anti-CCP (Avcin et al., 2002; Bongi et al., 2004; Kasapçopur et al., 2004; Kwok et al., 2005; Gilliam et al., 2011; Tebo et al., 2012). Therefore, we confirmed that ACPAs cannot serve as screening biomarkers for the general JIA population due to their low sensitivity.

However, JIA is a heterogeneous disease consisting of seven subtypes, and each subtype exhibits its own clinical, immunologic, and genetic characteristics; these subtypes will develop into different diseases in adulthood. Although there are differences in the prevalence rates of ACPAs among all JIA types or within each subtype because different antigenic probes were used for each ACPA, among the four subsets evaluated in this study, polyarticular JIA has the higher frequencies of ACPAs (27.3~45.5%) (Ozawa et al., 2012). In particular, polyarticular JIA that is RF positive, which is considered the early phase of adult RA, has comparative ACPA positivity rates (over 71.4%) as adult RA (Bizzaro et al., 2007). The results of the present study also are supported by most previous studies using anti-CCP. The results of our study strongly suggest that the determination of ACPAs is helpful for the polyarticular subtype of JIA, especially for RF-positive polyarticular JIA because of its high specificity.

Anti-CCP has been widely accepted as a predictor of erosion in adult RA. van Rossum et al. (2003) reported radiological damage in 80% of anti-CCP-positive JIA patients. Anti-CCP was strongly associated with erosive disease at an OR of 14.3 in a large cohort of JIA children including 77 polyarticular and 153 pauciarticular or systemic-onset JIA cases (Ferucci et al., 2005). Detection of anti-CCP was helpful for identifying RF-negative JIA patients and pediatric JIA patients who will need further care as adults (Bacos et al., 2014; Sparchez et al., 2015). However, the lack of an association with erosion in some studies was explained by the short disease duration, as unlike in adults, in children, erosion often occurs late in the course of arthritis. In this study, we assessed the relation of anti-CCP, recombinant citrullinated rat filaggrin, mutated citrullinated vimentin, and citrullinated IgG-derived peptide with the presence of joint space narrowing and/or bone erosion examined by X-ray and found a significant correlation between anti-CCP and anti-MCV and the occurrence of radiologically identified pathological damage in joints. Although this study was not prospective, our findings again demonstrate that high ACPA levels could suggest a more severe disease course and are probably important for prognostic use.

Meanwhile, each ACPA had a different prevalence among the JIA subtypes, mainly because of the antigens used. Anti-CCP has been extensively studied in JIA, and the sensitivity of anti-CCP has been reported to be between 1.8 and 27.9% (Machado et al., 2005; Brunner and Sitzmann, 2006; Gilliam et al., 2008; Habib et al., 2008; Kuna et al., 2009). In the present study, anti-CCP was identified in 7 (8.9%) of 81 JIA patients, agreeing with the reported rates in most published studies, and the positivity (9.9%) was slightly improved by when anti-CCP3.1 IgG and IgA were used simultaneously to detect CCP. Our data demonstrate again that the sensitivity of anti-CCP in the whole JIA population is too low to be a screening marker for the diagnosis of JIA, despite its reported high specificity. Citrullinated vimentin has been shown to be the target for the previously described RA-specific Sa antibodies (Vossenaar et al., 2004). The prevalence of anti-MCV has been reported recently to vary between 5.4 and 10.5%, and it was also found in disease controls (Kuna et al., 2009; Morbach et al., 2010; Gilliam et al., 2013). The prevalence of anti-MCV in our JIA patient group was 28.4%, which was higher than that of any other ACPA; however, 3 of the 49 healthy controls were found to be positive for anti-MCV, and 23.6% of disease controls also showed positive reactivity, very similar to the results reported by Kuna. Interestingly, anti-MCV was detected in 25.6% of patients with systemic-onset JIA, the most common subtype of JIA (48.1% of cases) in the study area (Zeng et al., 2009); but this subtype has a worse prognosis - one-third of children develop severe polyarthritis, and the disease has a significant mortality rate as the result of carditis, macrophage activation, and sepsis. Whether any relation exists between anti-MCV and these serious complications remains to be clarified. CPA and RA/CP, which target the antigens citrullinated rat recombinant filaggrin protein and the citrullinated Fc fraction of human IgG, were investigated in JIA for the first time in this study. Of 81 JIA patients, 17 (21.0%) were positive for CPA and 18 (22.2%) were positive for RA/CP, with specificities of 93.3 and 86.5%, respectively. Detection of CPA is useful in some subtypes of JIA, such as polyarthritis and oligoarthritis, with a sensitivity of 26.7-71.4% and specificity of 93.3%.

In the ACPAs investigated in this study, anti-CCP, anti-CCP3.1, and CPA have been found to be specific for JIA and closely correlate with joint erosions, and antibodies against citrullinated recombinant filaggrin seem to have a higher sensitivity than anti-CCP. Although similar sensitivities have been found for anti-MCV and RA/CP, their use in JIA is limited because they are also found in patients with MCTD and viral infections, as has been observed in adults (Coenen et al., 2007). It is worth noting that SLE may be the main disease affecting the specificity of ACPAs in JIA. We found that 5.9-29.4% of patients with JSLE tested positive for the five ACPAs, and one of them was positive in all ACPA assays, which is similar to the prevalence (5-28.7%) reported in adult SLE (Chan et al., 2008; Damián-Abrego et al., 2008; Zhao et al., 2009) but much higher than previously reported in JSLE. It is important to differentiate JSLE from JIA because arthritis presents in almost 70% of SLE patients as the most common initial feature at the early stage. Regarding this aspect, 34 children with SLE were enrolled into the disease control group in this study. The clinical significance of the presence of ACPAs in JSLE is unknown, but the presence of anti-CCP in adult SLE is closely associated with the onset of arthritis and bone erosion. In addition, it is important to use ACPAs to differentiate JIA from JSLE.

In summary, ACPAs are not sensitive biomarkers for JIA; however, the measurement of ACPAs could be useful for the diagnosis of a certain subtype of JIA (RF-positive polyarticular arthritis), but it is not useful for the diagnosis of JIA in general or for predicting whether a JIA patient will have severe bone involvement. Nevertheless, because the diagnostic performance of each ACPA is different, careful selection of assays is needed.

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