Reverse expression of α2,6-sialic acid ratios on IgG, IgM, and IgG/IgM autoantibodies correlates with mouse arthritis and rheumatoid arthritis disease activity

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Abstract

Background: Sialic acids (SIAs), for example, α2,6-SIAs, can link to conserved *N*-glycans of immunoglobulin G (IgG). In this study, we investigated the correlation between α2,6-SIA on IgG and IgM and the disease activity of arthritis and rheumatoid arthritis (RA) in mice.

Methods: We measured α2,6-SIA levels in IgGs and IgMs in collagen-induced arthritis (CIA). Additionally, α2,6-SIA levels in rheumatoid factors (RFs) and anti–cyclic citrullinated peptide (anti-CCP) antibodies in RA patients were measured. Correlations between α2,6-SIA on Igs and CIA were analyzed and also in RA patients by utilizing the disease activity score 28 (DAS28). The ability to differentiate RA progression by Ig and autoantibody α2,6-SIA levels was examined.

Results: In CIA mice, plasma IgG-α2,6-SIA/IgG ratios decreased, whereas plasma IgM-α2,6-SIA/IgM ratios increased. Moreover, arthritis was not observed in collagen-injected mice with decreased IgG-α2,6-SIA/IgG ratios and without increased IgM-α2,6-SIA/IgM ratios. Isolated IgG-α2,6-SIA/IgG ratios displayed a significant inverse correlation with DAS28 scores (r = –0.383, p = 0.037). In contrast, isolated IgM-α2,6-SIA/IgM ratios correlated positively with DAS28 (r = 0.351, p = 0.009). Isolated IgG-anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios were differentiated into either the remission (higher ratios) or the nonremission (lower ratios) category (p = 0.061), which is similar to the pattern for C-reactive protein (CRP) (p = 0.041) but different from that for the erythrocyte sedimentation rate (ESR) (p = 0.421). Using multiple linear regression analysis, plasma IgMRF-α2,6-SIA/IgMRF ratios displayed a correlation with DAS28 (p = 0.006), which was also observed in the ESR (p = 0.005), but was different from that for CRP (p = 0.222).

Conclusion: Concurrent reverse expression of α2,6-SIA ratios on IgM and IgG correlated with the occurrence of CIA and RA disease activity. Thus, α2,6-SIA ratios on IgG-anti-CCP antibodies and IgMRF are potential markers for evaluating RA disease activities.

Keywords: Anti-citrullinated protein antibodies; Arthritis, Experimental; Immunoglobulin G; Rheumatoid factor; Sialic acids

1. INTRODUCTION

Autoantibodies (antibodies produced by the immune system) directed against immunoglobulin G (IgG) Fc fragments are a primary characteristic of rheumatoid arthritis (RA). However, whether IgM, IgG, or IgA rheumatoid factors (RFs) correlate with RA disease activity remains controversial,[1–4] especially after IgG anti–cyclic citrullinated peptide (anti-CCP) antibodies were found as an excellent indicator of erosive joint disease.[5,6]

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A previous study reported lower levels of IgG-linked galactose in RA patients.[7,8] Although one study supported this finding,[9] another study produced contradictory results.[10] Recently, sialic acid (SIA) was ascertained to be covalently bound to lipids and proteins through α2-3, α2-6, or α2-8 linkages.[11] One earlier study stated that serum asialylated IgG correlated with RFs and C-reactive protein (CRP) levels.[12] Additionally, another report suggested a reduction in galactosylation and sialylation of IgG RFs by an increase in RF activity.[13] Further, higher sialylation levels of human IgGs were associated with decreased RF activity in antibody-dependent cellular cytotoxicity (ADCC) assays.[14] Nevertheless, these reports did not specifically measure α2,6-SIA levels, which play an essential role in anti- and proinflammatory IgG activities (see below).

The carbohydrate moiety of IgG is vital for certain antibody effector functions.[15,16] Recently, it was noted that IgG contains anti-inflammatory properties following sialylation of the Fc fragment (ie, covalent attachment of SIA through α2-6-linkage), which is reduced in an antigen-specific immune reaction.[17] Moreover, sialylated anti-collagen II antibodies and anti–citrullinated protein antibodies suppress the development

of collagen-induced arthritis (CIA) as well as arthritic activity in mice.[18] Similarly, sialylated anti-collagen IgG autoantibodies decrease autoimmune-induced inflammation and disease development of CIA.[19] Nevertheless, the association of α2,6-SIA found on IgG, IgM, and autoantibodies with arthritis activity in RA patients remains unknown.

Hence, we hypothesized that α2,6-SIA contents on IgG, IgM, and autoantibodies might correlate with the occurrence of arthritis and RA disease activity. These perspectives will add to the currently used laboratory parameters for determining inflammation, such as CRP level and erythrocyte sedimentation rate (ESR), to improve the measures of disease activity in RA. Accordingly, we analyzed the α2,6-SIA contents on IgG, IgM, and autoantibodies (including anti-CCP and RF antibodies), as well as their correlations with the occurrence of mouse arthritis and clinical activity in RA patients.

2. METHODS

2.1. CIA in mice

Six- to 8-year-old C57B6 mice were obtained from the National Animal Center (Taipei, Taiwan) and housed in a temperature-controlled, specific pathogen-free room with a 12-hour day/night cycle at the Animal Center of Chang Gung Memorial Hospital at Linkou (Taoyuan, Taiwan). We followed the same method as described previously to elicit CIA.[20,21] Briefly, mice were separated into three groups of five each: one control group (injected with phosphate-buffered saline [PBS] on day 0) and two experimental groups (injected on day 0 with collagen and Freud's adjuvant [Chondrex, Inc., Redmond, WA]). At indicated time points, 0.1 mL of blood was obtained from the mouse tail vein. Incomplete Freud's adjuvant (IFA; Chondrex, Inc., Redmond, WA) mixed with collagen was injected at the beginning of the third week. Blood was obtained from five mice in each group at the beginning of day 0, week 2, week 4, and week 6. At the sixth week, CIA was seen in 40% of C57B6 mice injected with type II collagen. Hence, we designated the mice in the experimental group with the occurrence of arthritis (the ankle/hind foot swelling only [Fig. 1B] or with the forelimb swelling together) as CIA mice. Moreover, the mice in the experimental group with no occurrence of gross arthritis were designated as non-CIA mice, and the mice injected with PBS were designated as control mice (Table 1).

2.2. Patients and clinical and laboratory parameters

In total, 108 volunteer RA patients (female to male ratio, 85:23) provided their informed consent before their inclusion in this

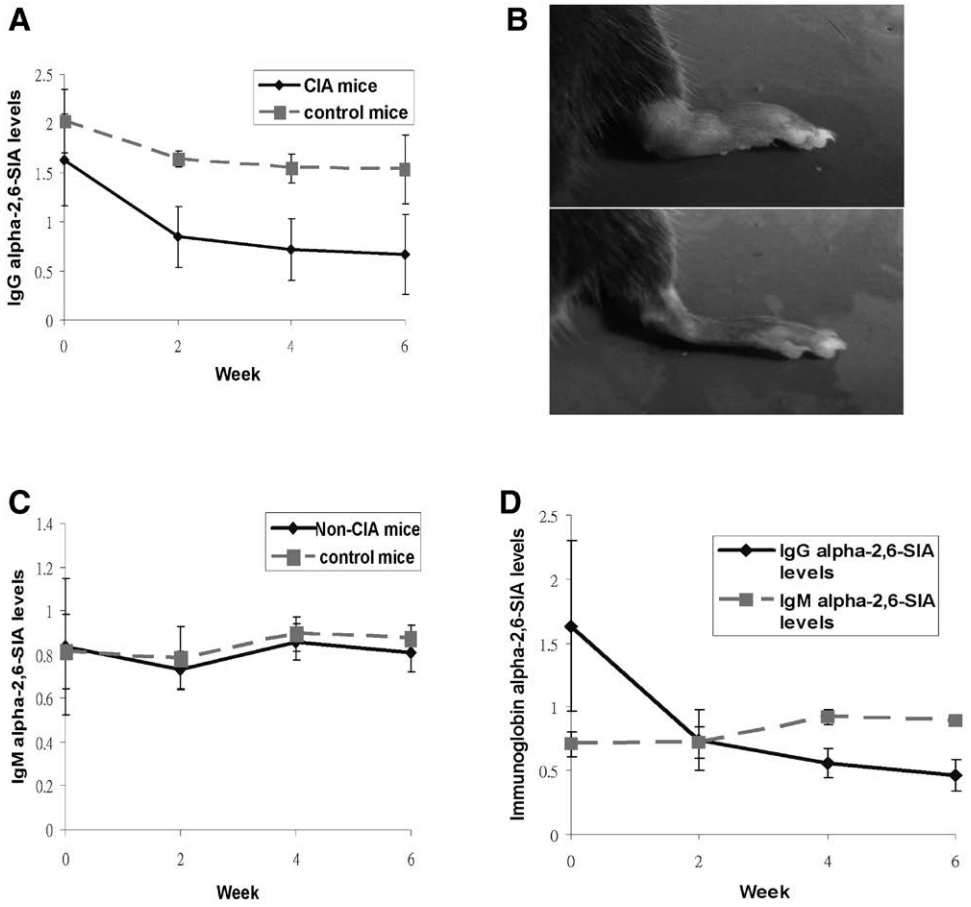

Fig. 1 The whole course comparison of IgG and IgM alpha-2,6-sialic acid (SIA) ratios between collagen-induced arthritis (CIA) mice and control mice. A, The IgG alpha-2,6-SIA/IgG ratios expressed as mean ± SD over a 6-wk period. Comparison between week 0 and week 6 in CIA mice gave $p < 0.05$ (95% confidence interval (CI): 0.123 to 2.213); that between week 0 and week 6 in control mice gave $p > 0.05$ (95% CI: −0.957 to 1.067); that between day 0 and week 2 in control mice were not different ($p = 0.095$, 95% CI:−0.114-0.877). (B) Hind leg arthritis in CIA mice vs no arthritis in control mice. (C) IgM-α2,6-SIA/IgM ratios are expressed as mean ± SD during the 6-wk period. Comparison between week 0 and week 6 in non-CIA mice yielded $p > 0.05$ (95% CI: −0.194 to 0.493); in control mice $p > 0.05$ (95% CI: −0.151 to 0.277). D, IgG and IgM α2,6-SIA ratio changes (expressed as mean ± SD) in CIA mice during a 6-wk period. The comparison of IgG α2,6-SIA ratios between week 0 and week 6 in CIA mice yielded $p < 0.05$ (95% CI: 0.123 to 2.213) and that of IgM α2,6-SIA ratios surrendered $p < 0.05$ (95% CI: −0.336 to −0.025). This is representative of two experiments.

Table 1

Contrasting Ig-α2,6-SIA/Ig ratios in different mouse groups

Groups	Day 0	Week 2	Week 4	Week 6
SIA/IgG ratios				
CIA mice	1.631 ± 0.670	0.741 ± 0.237	0.559 ± 0.118	0.463 ± 0.125* (4)
Non-CIA mice	1.629 ± 0.376**	0.906 ± 0.351	0.830 ± 0.365	0.811 ± 0.476**,*** (6)
Control mice	2.022 ± 0.322	1.640 ± 0.084	1.545 ± 0.144	1.536 ± 0.349*,*** (4)
SIA/IgM ratios				
CIA mice	0.706 ± 0.103****	0.720 ± 0.121	0.916 ± 0.056	0.887 ± 0.036**** (4)
Non-CIA mice	0.910 ± 0.372*****	0.737 ± 0.064	0.822 ± 0.076	0.761 ± 0.083*****,****** (6)
Control mice	0.923 ± 0.155	0.847 ± 0.153	0.875 ± 0.103	0.860 ± 0.082****** (4)

Blood samples were obtained on day 0 (the same day when collagen was injected), and in weeks 2, 4, and 6 as described in Methods. Parentheses indicate the number of mice used in individual treatment group. Upper half: blood samples were directly tested for mouse IgG α2,6-SIA contents and the results were divided by IgG amounts to give final ratios. No difference in day 0 data of the IgG α2,6-SIA level among three groups was observed (compared using the *t* test: control mice day 0 vs CIA mice day 0: $p = 0.348$ [95% confidence interval, –0.612 to 1.393]; control mice day 0 vs non-CIA mice day 0: $p = 0.108$ [–0.111 to 0.897]); control mice between day 0 and week 2 were not different ($p = 0.095$ [–0.114 to 0.877]), between day 0 and week 4 were comparable ($p = 0.052$ [–0.006 to 0.959]) and between day 0 and week 6 were similar ($p = 0.087$ [–0.096 to 1.067]). Lower half: mouse IgM α2,6-SIA contents tested in blood were divided by IgM amounts to give final ratios. Day 0 IgM SIA levels among three groups were not different (CIA mice day 0 vs non-CIA mice day 0 and vs control mice day 0 provided $p = 0.213$ [–0.554 to 0.146] and 0.065 [–0.452 to 0.019], respectively). This is representative of two experiments. CIA mice = mice with collagen-induced arthritis; control mice = mice injected with phosphate buffered saline without arthritis; Ig = immunoglobulin; non-CIA mice = mice injected with collagen and did not develop arthritis; SIA = sialic acid.

*$p = 0.005$ [0.545 to 1.600].

**$p = 0.007$ [0.278 to 1.358].

***$p = 0.025$ [0.121 to 1.329].

****$p = 0.016$ [–0.313 to –0.047].

*****$p = 0.338$ [–0.196 to 0.495].

******$p = 0.107$ [–0.028 to 0.226].

study. All patients fulfilled the 1987 ACR criteria for classification of RA. Blood samples were collected from 30 newly diagnosed/untreated and 78 regularly treated RA patients. None of the recruited patients had secondary Sjogren's syndrome with systemic lupus, tuberculosis, Crohn's disease,[22,23] recent infection, or recent vaccination. The tender joint count (TJC), swollen joint count (SJC), visual analogue scale for general health, ESR, and CRP were collected to calculate the disease activity score 28 (DAS28) for each patient.

2.3. Study approval

The Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital at Linkou (Taoyuan, Taiwan) approved the protocol for mouse experiments. The "Principles of Laboratory Animal Care" (NIH Publication Vol 25, No. 28 revised 1996), as well as specific national laws where applicable, were followed. The Chang Gung Institutional Review Board (Taoyuan, Taiwan) approved the human study, and the subjects' written consent was obtained in accordance with the ethical standards of the 1975 Declaration of Helsinki (as revised in Brazil, 2013).

2.4. Isolation of immunoglobulins

Plasma was obtained from blood collected in ethylenediaminetetraacetic acid tubes undergoing centrifugation and separation as previously described.[24] Then the plasma was passed through a protein G column (Sigma-Aldrich, St. Louis, MO). Next, IgGs in the protein G column were eluted and collected (designated as isolated IgG, contrary to plasma IgG examined directly in plasma). Human IgM was isolated by passing a blood sample once through a protein A column (Sigma-Aldrich) and passed through a protein G column for an additional three times (as isolated IgM, contrary to plasma IgM directly analyzed in plasma).

2.5. Preparation of F9.4 mouse IgM and AF1.9-L2 mouse IgG

Mouse IgM from the F9-4 cell line [25] (F9-4 IgM) and a subclone mouse IgG from the AF1.9-L2 cell line (AF1.9-L2 IgG) were obtained from Professor David W. Scott at the Uniformed Services University of Health Sciences (Bethesda, MA). F9-4 was used as the standard for assaying mouse IgM α2,6-SIA. Furthermore, AF1.9-L2 IgG was used as the standard for estimating the α2,6-SIA levels of mouse IgG and human IgG. This was due to that there was no available human IgG carrying a high α2,6-SIA content that could be obtained at that time.

2.6. ELISA for quantification of human or mouse IgG and IgM

The ELISA assays were performed as described previously, with modifications.[26] Briefly, ELISA microtiter plates were coated with the $F(ab')_2$ fragment of a goat anti–human IgG, Fc fragment–specific antibody (Jackson ImmunoResearch Inc., West Grove, PA). Diluted isolated IgGs or plasma samples were then added and followed by the horseradish peroxidase (HRP)–conjugated $F(ab')_2$ fragment of a goat anti–human IgG to capture the target antibodies. Human IgG (range, 100-0.048 ng/well, 2-fold serial dilutions; contains little or no α2,6-SIA content; Sigma-Aldrich) served as a standard.

Similarly, human IgM quantification was measured with human IgM (range, 200-0.195 ng/well, 2-fold serial dilutions; Sigma-Aldrich) as a standard. Analogous procedures were followed for quantifying mouse IgG and mouse IgM. AF1.9-L2 IgG (range, 200-0.195 ng/well, 2-fold serial dilutions) and F9-4 IgM (range, 200-0.195 ng/well, 2-fold serial dilutions) were used as standards for mouse IgG and IgM, respectively.

2.7. ELISA for quantification of α2,6-SIA contents of human IgG and IgM

ELISA microtiter plates were coated with *Sambucus nigra* lectin (SNA-1, which binds α2,6-SIA; EY Laboratories, Inc., San Mateo, CA). After washing, isolated IgGs (or plasma) were added at appropriate dilutions. Incubation with secondary antibodies, with either a biotin-conjugated anti–human IgG antibody or a biotin-conjugated anti–mouse IgG antibody (Jackson ImmunoResearch Inc.), followed by incubation with an HRP–streptavidin detector. This was used to quantify the α2,6-SIA contents in either human IgG or AF1.9-L2 mouse IgG (range, 400-25 ng/well, 2-fold serial dilutions, used as a standard for detecting human IgG α2,6-SIA), respectively. Thereafter, ELISA

results for α2,6-SIA were correlated with results assayed by the lectin blot method.[27]

Human IgM α2,6-SIA was quantified with human IgM (range, 200-0.195 ng/well, 2-fold serial dilutions; carrying a high α2,6-SIA content; Sigma-Aldrich) as a standard.

2.8. Lectin blotting

As described previously,[28] once sodium dodecyl sulfate-polyacrylamide gel electrophoresis was completed, 20 μL of each isolated IgG sample was transferred and incubated with an SNA–alkaline phosphatase solution and then visualized with a 1 mL of NBT/BCIP solution (Amresco Inc., Cochran Road Solon, OH).

2.9. ELISA for quantification of α2,6-SIA contents of mouse IgG and IgM

The same procedure as above was used for quantifying mouse IgG and IgM α2,6-SIA contents. AF1.9-L2 (range, 200-0.195 ng/well, 2-fold serial dilutions) was used as the standard for α2,6-SIA of IgG. Mouse IgM SIAs were measured with F9-4 IgM (range, 200-0.195 ng/well, 2-fold serial dilutions) as the standard.

2.10. ELISA for plasma IgMRF and for IgMRF α2,6-SIA contents

ELISA plates were coated with human IgG Fc fragment (Jackson ImmunoResearch Inc.) at 10 μg/mL for IgMRF detection.[29,30] Plasma (1:1000 dilution) was added and followed by an HRP-conjugated goat anti–human IgM antibody (Jackson ImmunoResearch Inc.) for IgMRF measurement (designated as plasma IgMRF). Alternatively, an SNA (*Sambucus nigra*)–HRP solution (EY Laboratories, Inc.) was coated on the plates for measurement of the α2,6-SIA content of IgM (designated as the IgMRF-α2,6-SIA).[30] Human IgGRF and its α2,6-SIA content were detected with rabbit IgG Fc fragment (Jackson ImmunoResearch Inc.) by a similar method.[29,30] An RA patient with a serum IgMRF level of 943 IU/mL (determined by kits from Inova Diagnostics, Inc., San Diego, CA) was used as the standard for RF amounts and for the α2,6-SIA content of RF.

2.11. Quantification of plasma anti–CCP antibodies and of their SIA contents

Plasma was analyzed for IgG anti-CCP antibodies (Quantalite CCP3 IgG ELISA; Inova Diagnostics, Inc.). For α2,6-SIA quantification, plates were incubated with 1:100 dilutions of isolated human IgG samples for 1 hour. Next, an SNA–HRP solution (EY Laboratories Inc.) was added. Furthermore, isolated IgG-anti-CCP-α2,6-SIA was assayed with the standard using the serum containing high IgG-anti-CCP antibody levels (at 250 units, measured as above) with a relatively high α2,6-SIA content.

2.12. Definition of immunoglobulin SIA contents and ratios

Plasma IgG-α2,6-SIA/IgG ratios were defined as the α2,6-SIA contents of plasma IgG divided by the plasma IgG levels (ng/mL:ng/mL). Plasma IgM-α2,6-SIA/IgM ratios were delineated as the α2,6-SIA contents of plasma IgM divided by the plasma IgM levels.

Isolated IgG-α2,6-SIA/IgG ratios were defined as the α2,6-SIA contents of isolated IgG (from a protein G column) divided by the plasma IgG levels. Isolated IgM-α2,6-SIA/IgM ratios were delineated as the α2,6-SIA contents of isolated IgM (from a protein A column together with a protein G column) divided by the plasma IgM levels.

Similarly, isolated IgG anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios were defined as the α2,6-SIA contents of isolated IgG-anti-CCP divided by the plasma IgG-anti-CCP antibody levels. Plasma IgMRF-α2,6-SIA/IgMRF ratios were delineated as the α2,6-SIA contents of plasma IgMRF divided by the plasma IgMRF levels. Plasma IgGRF-α2,6-SIA/IgGRF ratios were similarly defined.

2.13. Statistical analyses

The correlation between groups was analyzed by means of Pearson's correlation coefficient (r) and linear regression when outcome variables (DAS28) were normally distributed. Otherwise, the correlation was assessed using Spearman's correlation coefficient (rho, ρ). The comparison in mouse experiments was conducted by two-sample t or paired t test. All analyses were performed using SPSS 16.0 software (SPSS Inc, Chicago, IL, USA). A value of $p < 0.05$ was considered statistically significant. A trend was considered for $0.05 \leq p < 0.10$ for the correlation of variables in RA patients because patient characteristics were diversely distributed[31] unlike those in mice. Receiver-operating characteristic (ROC) curves for different variables against DAS28 were plotted to calculate areas under the curve (AUC).

3. RESULTS

3.1. Changes in blood IgG or IgM α2,6-SIA contents in CIA

No difference was observed in the plasma IgG α2,6-SIA/IgG ratios among three groups (CIA mice, non-CIA mice, and control mice as described in Section 2.1.) on day 0 (Table 1). Plasma IgG-α2,6-SIA/IgG ratios of CIA mice were significantly lower than the control mice at week 6 (Table 1; Fig. 1A). Plasma IgG-α2,6-SIA/IgG ratios of non-CIA mice were also lower compared with control mice at week 6 (Table 1). In addition, plasma IgG-α2,6-SIA/IgG ratios of CIA mice at week 6 were significantly lower than those at week 0, but no difference was observed in control mice between week 6 and week 0 (Fig. 1A). Similarly, no difference was observed in plasma IgG-α2,6-SIA/IgG ratios of control mice between day 0 and week 2 ($p = 0.095$, 95% confidence interval:–0.114 to 0.877) (Fig. 1A; Table 1). Thus, both CIA and non-CIA mice had decreased plasma IgG-α2,6-SIA/IgG ratios in week 6, which is indicative of inflammation. Note, such an observation was not seen in control mice (Table 1). This phenomenon raises the question regarding what other factors might be associated with arthritis in CIA mice but not in non-CIA mice.

Furthermore, we examined plasma IgM-α2,6-SIA/IgM ratios in mice. We found that there was no change in the plasma IgM-α2,6-SIA/IgM ratio in non-CIA and control mice between week 0 and week 6 (Table 1; Fig. 1C). In contrast, the IgM-α2,6-SIA/IgM ratio in week 6 was significantly higher in CIA mice than that in week 0 (Table 1; Fig. 1D). Moreover, there was a notable difference between the decreasing IgG-α2,6-SIA/IgG ratios over time and the increasing IgM-α2,6-SIA/IgM ratios over time in CIA mice (Fig. 1D). Hence, α2,6-SIA ratio changes occurred in an inverse proportionality for IgG and IgM in CIA mice. In contrast, α2,6-SIA ratio changes affected only IgG in non-CIA mice (Table 1). We then tested if similar phenomena existed in human patients with RA.

3.2. Correlation between α2,6-SIA ratios of isolated IgG and DAS28 in RA patients

The female to male ratio of the 108 RA patients was 85:23, and the average age was 55.5 ± 11.7 years (mean ± SD). Mean CRP levels were 14.90 ± 27.66 mg/L (range, 0.21-196.45); mean ESR levels were 31.64 ± 28.81 mm/h (range, 3-134); mean RF titers were 80.73 ± 198.42 IU (range, 0-943); mean SJC was 2.31 ± 4.13 (range, 0-26); mean TJC was 6.56 ± 6.90 (range,

0-28); and mean DAS28 were 3.88 ± 1.40 (range, 1.46-9.18). In newly diagnosed/untreated RA patients, isolated IgG-α2,6-SIA/IgG ratios significantly correlated inversely with the DAS28 (Fig. 2A).[32] This result was also supported by the univariable linear regression result: isolated IgG-α2,6-SIA/IgG ratios against DAS28 gave t =–2.120 and p = 0.043. Additionally, as determined by ELISA, the α2,6-SIA contents of isolated IgG correlated modestly with those measured by the lectin blot (n = 30, ρ = 0.331, and p = 0.074).

3.3. Correlation between α2,6-SIA ratios of isolated IgM and DAS28 in RA patients

The isolated IgM-α2,6-SIA/IgM ratio from RA patients exhibited a positive correlation with the DAS28 (Fig. 2B). This result was also supported by the univariable linear regression result: isolated IgM-α2,6-SIA/IgM ratios against DAS28 gave t = 2.391 and p = 0.021. These data suggest that high IgM-α2,6-SIA/IgM ratios correlate with the high arthritic activity scores in RA patients, similar to the occurrence of arthritis in CIA mice at week 6, when compared with week 0 (Table 1; Fig. 1D).

3.4. The ability of SIA ratios of anti-CCP antibodies to differentiate different RA disease activities

Isolated IgG-anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios differed, with a trend between remission (DAS28 < 2.6) and nonremission (DAS28 ≥ 2.6), as was observed for CRP but not for ESR (Table 2). The ratios differed significantly between moderate and high disease activity (but not CRP and ESR, Table 2). In other words, lower isolated IgG-anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios were seen in RA patients with higher disease activity (Table 2). Consequently, isolated IgG-anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios may be useful in differentiating between various disease activities in RA patients.

3.5. The ability of SIA ratios of plasma IgM RF to differentiate diverse RA disease activities

By using multivariable linear regression, plasma IgMRF-α2,6-SIA/IgMRF ratios significantly correlated with the DAS28 (as an outcome variable, p = 0.006), similar to ESR (p = 0.005), but different from CRP (p = 0.222). That is, CRP was not as useful as IgMRF-α2,6-SIA/IgMRF ratios and ESR in predicting DAS28 in multivariable analysis.

Moreover, plasma IgGRF-α2,6-SIA/IgGRF ratios (r =–0.012, p = 0.906) did not correlate with DAS28. Nevertheless, plasma IgGRF-α2,6-SIA/IgGRF ratios were (not significantly) inversely associated with DAS28, which is contrary to the positive correlation of plasma IgMRF-α2,6-SIA/IgMRF ratios with DAS28.

3.6. AUCs of various laboratory parameters against different DAS28 disease activities with sex distinction

The ROC curves of ESR, CRP, and anti-CCP against DAS28 ≥ 2.60 (nonremission) yielded meaningful AUCs (Table 3). It was demonstrated that ESR and CRP had significantly higher AUCs than isolated IgG-anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios (Table 3). In contrast, isolated IgG-anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios were inclined to predict a DAS28 < 2.60 better than ESR and CRP (Table 3). Intriguingly, isolated IgG-anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios also predicted a DAS28 ≦ 3.2 significantly better than ESR and CRP (Table 3).

Moreover, when we classified RA patients into male and female subgroups, ESR and CRP of male patients predicted DAS28 ≦ 5.1 much better than DAS28 > 5.1 (Table 4). In contrast, ESR and CRP of female patients predicted DAS28 > 5.1 better than DAS28 ≦ 5.1 (Table 4). However, isolated IgG-anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios did not distinguish between different DAS28; especially between gender.

4. DISCUSSION

CIA (Table 1; Fig. 1) demonstrated the following: (1) collagen immunization induced significantly lower plasma IgG-α2,6-SIA/IgG ratios compared with the PBS control; (2) collagen immunization significantly increased plasma IgM-α2,6-SIA/IgM ratios in arthritic mice, but not in collagen-injected mice without gross arthritis; (3) plasma IgG-α2,6-SIA/IgG ratios and plasma IgM-α2,6-SIA/IgM ratios displayed an inverse relationship in CIA mice. The third trend was also observed in RA patients (Fig. 2A, 2B). A similar tendency for IgG SIA was noted in a previous report.[17]

However, whether high-α2,6-SIA-carrying IgMs in CIA mice and RA patients are anti-inflammatory or proinflammatory is an interesting question that has not yet been answered (Figs. 1D and 2B and Section 3). Nevertheless, mouse and human data tentatively suggest that high-α2,6-SIA-carrying IgMs were associated with the occurrence of arthritis and higher arthritis activity in addition to the anti-inflammatory role of α2,6-SIA–rich IgGs in serum-induced arthritis and ADCC.[14,17] Therefore, the use of IgMSIA as a novel indicator of RA disease activity needs to be further studied.

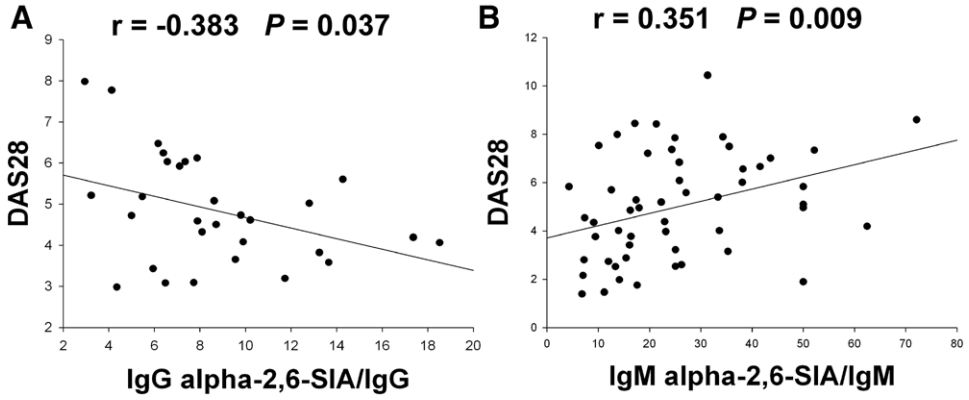

Fig. 2 Correlation between the IgG or IgM α2,6-sialic acid (SIA) ratios and disease activity score 28 (DAS28). A, IgG α2,6-SIA/IgG ratios in 30 newly diagnosed/untreated rheumatoid arthritis (RA) patients. Isolated IgG-α2,6-SIA/IgG ratios yielded a significant and inverse correlation with the DAS28: r =–0.383 and p = 0.037. B, Correlation of isolated IgM-α2,6-SIA/IgM ratios with DAS28 in 54 RA patients: r = 0.351, p = 0.009.

Table 2

Laboratory data to differentiate disease activity score 28 disease activities in rheumatoid arthritis patients

Differentiation of disease activities	ESR	CRP	SIA/anti-CCP ratios
Remission vs non-remission	0.421	0.041	0.061
Low/moderate disease activity vs high disease activity	0.071	0.090	0.057
Moderate disease activity vs high disease activity	0.132	0.164	0.045

Data shown are *p* values. SIA/anti-CCP ratios indicate the SIA contents of isolated IgG anti-CCP antibodies divided by plasma IgG anti-CCP antibody levels. Remission means rheumatoid arthritis patients with disease activity score 28 (DAS28) < 2.6; nonremission means DAS28 ≥ 2.6; low disease activity, 2.6 ≤ DAS28 ≤ 3.2; moderate disease activity, 3.2 < DAS28 ≤ 5.1; high disease activity, DAS28 > 5.1. Comparisons were done using *t*-test or Mann-Whitney *U* test where appropriate.

CCP = cyclic citrullinated peptide; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; SIA = sialic acid.

Table 3

AUC for inflammatory markers and SIA ratios of autoantibodies against DAS28 in patients with rheumatoid arthritis

Laboratory data	DAS28 cut-off with < 2.6	DAS28 cut-off with ≧ 2.6	DAS28 cut-off with ≦ 3.2	DAS28 cut-off with > 3.2
ESR	0.321*[1] (0.177-0.465)	0.679*[1] (0.535-0.823)	0.241*[2] (0.137-0.346)	0.759*[2] (0.654-0.863)
CRP	0.266**[1] (0.120-0.441)	0.734**[1] (0.589-0.880)	0.349**[2] (0.217-0.482)	0.651**[2] (0.518-0.783)
Anti-CCP	0.335 (0.167-0.503)	0.665 (0.497-0.833)	0.452 (0.311-0.592)	0.548 (0.408-0.689)
SIA/anti-CCP ratios	0.666***[1] (0.503-0.828)	0.334***[1] (0.172-0.497)	0.649 (0.495-0.802)	0.351 (0.198-0.505)
IgM	0.465 (0.305-0.625)	0.535 (0.375-0.695)	0.482 (0.354-0.613)	0.517 (0.387-0.646)
IgMRFSIA/IgMRF ratios	0.505 (0.334-0.676)	0.495 (0.324-0.666)	0.492 (0.361-0.622)	0.508 (0.378-0.639)

Data shown are AUC values with (95% confidence interval [CI]). DAS28 with inclusion of ESR. DAS28 ≥ 2.6 indicates rheumatoid arthritis (RA) patients with nonremission; DAS28 < 2.6 designates RA patients with remission. SIA/anti-CCP ratios indicates the SIA content of isolated IgG anti-CCP divided by the amount of plasma IgG anti-CCP. IgMRF SIA/IgMRF ratios indicates the SIA content of IgMRFs divided by the amount of IgMRFs in plasma.

CCP = cyclic citrullinated peptide; CRP = C-reactive protein; DAS28 = disease activity score 28; ESR = erythrocyte sedimentation rate; SIA = sialic acid.

,*Significant difference of AUCs was impressed when 95% CI were compared between AUCs of two continuous categories (*1 vs *1; *2 vs *2; and so on) were nonoverlapped. In contrast, nonsignificant difference of AUCs was impressed when 95% CI between compared AUCs of two continuous categories were overlapped. ESR against DAS28 > 5.1 provided AUC (0.688, 95% CI: 0.515-0.822), not different from that AUC (0.373, 0.197-0.548) of isolated IgG-anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios against DAS28 > 5.1.

Table 4

Different tendencies of area under the curve represented by key laboratory markers in male and female rheumatoid arthritis patients

Laboratory markers	DAS28 ≦ 5.1	DAS28 > 5.1
Male RA (n = 23)		
ESR	0.872* (0.711 to 1.032)	0.032* (–0.102 to 0.289)
CRP	0.846** (0.591 to 1.101)	0.154** (–0.101 to 0.409)
SIA/anti-CCP ratios	0.667 (0.400 to 0.933)	0.333 (0.067 to 0.600)
Female RA (n = 85)		
ESR	0.281*** (0.128 to 0.434)	0.719*** (0.566 to 0.872)
CRP	0.390 (0.234 to 0.547)	0.610 (0.453 to 0.766)
SIA/anti-CCP ratios	0.514 (0.345 to 0.684)	0.486 (0.316 to 0.655)

DAS28 with inclusion of ESR; SIA/anti-CCP ratios = ratios of sialic acid contents of isolated IgG anti-CCP divided by plasma IgG anti-CCP levels. Data shown are area under the curve values with (95% confidence interval [CI]).

CCP = cyclic citrullinated peptide; CRP = C-reactive protein; DAS28 = disease activity score 28; ESR = erythrocyte sedimentation rate; RA = rheumatoid arthritis; SIA = sialic acid.

*,**,***Significant difference of AUCs was impressed when 95% CI between compared area under the curves of two continuous categories were nonoverlapped.

No change was found in the α2,6-SIA content of whole IgG in pregnant RA patients,[33] which differed from the data showing an elevation of the α2,6-SIA content in IgG1/IgG2 subclasses in pregnant RA patients.[34] Similarly, high isolated IgG-α2,6-SIA/IgG ratios reflected low RA disease activity in this study (Fig. 2A). This viewpoint was supported by studies in which T-cell–dependent and T-cell–independent antigens both induced tolerant immunosuppressive sialylated IgG antibodies.[35,36] In particular, successful RA treatment with glucocorticoids brought an increased IgG-α2,6-SIA/N-acetylgalactosamine ratio.[37] Similar to the present study, the aforementioned study used the IgG capture lectin ELISA immobilizing assay[37] to detect α2,6-SIA of native IgG. This approach was unlike other studies that reported α2,6-SIA contents on denatured and proteolytically sliced IgG fragments.[35,36] We used a protein G column to obtain native IgG to be assayed for SIA (see Sections 2.7 and 2.9), which methods and protocols were previously described by a prior study.[37,38]

Therefore, the strength of our study was observed through the inverse correlation between the α2,6-SIA ratios in IgG and IgM in CIA mice and human RA, regardless of the dispute on whether an SNA-column preferably selects pathogenic autoantibodies.[17,39] Second, our study is the first to analyze and assess the usefulness of isolated IgG-anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios as a potential autoantibody differentiator of RA disease activity (DAS28) (Table 2) and a better predictor for RA remission than ESR (Table 3). Third, ESR and CRP in male RA patients predicted a DAS28 ≦ 5.1 (ESR of female patients predicted a DAS28 > 5.1) (Table 4), which was not reported in the past.[40] Conclusively, we pioneered the idea that higher plasma IgMRF-α2,6-SIA/IgMRF ratios indicate an increasing RA disease activity. To verify whether sialylated IgMRF plays a protective or destructive role in RA, larger well-designed studies in mice and humans are needed. Nevertheless, IgM anti-dsDNA antibody has been purported to be protective in systemic lupus erythematosus.[41] Hence, the SIA moiety of RFs has to be taken into account when rheumatologists decide whether different types of RFs are deleterious or advantageous to RA patients beyond the type and amount of RF.[42,43]

Moreover, whether ESR and CRP play different roles in shaping disease activity between male and female RA patients (Table 4) is an ideal topic to investigate for future studies. In terms of why only IgMRFSIA/IgMRF ratios, but not IgGRF-α2,6-SIA/IgGRF ratios, correlated with DAS28 scores is currently unknown. However, it may be related to the nature of IgGRF to self-associate, hence, making measurements of IgGRF-α2,6-SIA and IgGRF less accurate.[44,45]

The primary limitation of the current study is that the correlation of laboratory measurements with DAS28 was mostly modest to reasonable (Fig. 2).[32] Nevertheless, isolated IgG-anti-CCP-α2,6-SIA/plasma IgG-anti-CCP ratios differentiated various DAS28 activities better than ESR (Table 2). Second, no anti-collagen IgG and IgM α2,6-SIA were measured, due to the unavailability of blood for testing (Fig. 1). Yet, our utmost important goal for clinical human application has been accomplished by the conclusions drawn from Fig. 2 and Tables 2 and 3. Third, only isolated IgG and IgM α2,6-SIA ratios, but not human blood IgG and IgM α2,6-SIA ratios, correlated with DAS28 (Fig. 2). Thus, these limitations could constrain the convenient use of this method for clinical use. Nevertheless, these findings open up a new direction for potential biomarkers to evaluate RA disease activity. Therefore, further investigation is warranted by enrolling a much larger population for study to possibly circumvent these limitations.

New biomarkers for RA are still desired for clinical practices,[31] since patients with active RA might have normal inflammatory indicators (ESR/CRP) and vice versa.[46,47] Furthermore, ESR and CRP have different underlying pathophysiologies that also differ from SIA contents of IgG and IgM.

In conclusion, isolated IgG-α2,6-SIA/IgG and IgM-α2,6-SIA/IgM ratios correlated negatively and positively with DAS28 scores in RA patients, respectively, similar to those in CIA. The α2,6-SIA ratio of IgG-anti-CCP antibodies and IgMRF are potential biomarkers for evaluating RA disease activities. Altogether, we described a new potential way to explore concurrent SIA changes in blood IgG and IgM, especially in autoantibodies, to differentiate RA disease activities.

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