Antibody to peptidoglycan recognition protein (PGLYRP)-2 as a novel biomarker in rheumatoid arthritis

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Abstract

Objective

To identify novel autoantigens from circulating immune complexes (CICs) in rheumatoid arthritis (RA) patients and further explore their clinical significance.

Methods

From serum samples of 10 early RA (ERA) patients and 10 healthy donors, CICs were isolated and subjected to orbitrap mass spectrometry for autoantigen identification. Antibodies against the peptidoglycan recognition protein-2 (PGLYRP-2) derived from CICs were further detected by indirect enzyme-linked immunosorbent assay (ELISA) in 178 patients with RA, compared with 59 osteoarthritis (OA), 59 systemic lupus erythematosus (SLE), 55 ankylosing spondylitis (AS), 95 primary Sjögren's syndrome (pSS) and 50 healthy controls (HC).

Results

Thirty-three potential antigens out of 323 proteins were identified from CICs of RA patients. The autoantibodies to PGLYRP-2 were significantly increased in RA patients with 42.70% sensitivity and 85.20% specificity in comparison to other rheumatic diseases and healthy controls. The prevalence of anti-PGLYRP-2 was also elevated in subgroups of RA, with 34.72% in ERA, 35.29% in RF negative and 42.86% in anti-CCP negative patients. Further analysis suggested that anti-PGLYRP-2 was potentially accompanied with production of other autoantibodies in RA. In addition, we found by homology analysis that an epitope of PGLYRP-$2_{442-447}$ mimics amino acid residues 431-436 of N-acetylmuramoyl-L-alanine amidase (NAMLAA) in actinomyces naeslundii.

Conclusion

Autoantibody against PGLYRP-2 was identified as a promising biomarker in RA, especially in early and seronegative patients.

Key words

rheumatoid arthritis, biomarker, autoantibody, peptidoglycan recognition protein-2

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Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease featured by inflammatory synovitis and progressive joint destruction (1), seriously impairing physical function and reducing life quality. The prevalence of RA was approximately 0.5–1.0% worldwide and 0.28% in China (2, 3). Rheumatoid factors (RF) and anti-cyclic citrullinated peptide (CCP) antibodies have been the prevalent diagnostic markers for RA (4). Notwithstanding, owing to the inadequate sensitivity and specificity of current biomarkers, some ERA patients especially those with negative anti-CCP are still in a state of delayed diagnosis and treatment (5). We attempted to study whether there are novel biomarkers associated with RA and can be valuable clinically.

In the last decades, proteomics study focusing on biomass spectrometry technology has been utilised in the exploration of pathogenic mechanism of human diseases and the discovery of new biomarkers. With the development of methodology including mass spectrometry, a number of disease-relevant molecules have been found in RA serum and synovial fluid, including heat shock protein 60, fibrinogen, vimentin, retinol-binding protein 4, zinc finger protein 658, plasminogen, serum amyloid A4 protein, gelsolins, vitamin D-binding protein (6-10). As circulating immune complexes (CICs) contain a variety of antigens that directly combined to underlying autoantibodies, autoantigens from CICs are promising candidates for screening novel biomarkers and studying molecular mechanisms. Therefore, it is obligatory to further study the CICs in peripheral blood of RA patients (11). Autoantigenic components in CICs may be related with RA and biomarkers clinically.

In this study, we identified potential RA autoantigens in CICs using high-resolution mass spectrometry with nano-LC joined with Orbitrap Q Exactive mass spectrometer. As a result, 33 differentially expressed autoantigens were identified from RA CICs. The autoantibody against one of these autoantigens, PGLYRP-2, was clinically significant in RA.

Materials and methods

Patients

Serum samples were collected from 446 patients admitted to the Rheumatology and Immunology Department of Peking University People's Hospital from April 2017 to August 2019. 178 patients with RA (mean age 58.11±14.13 years, 147 females and 31 males), including 72 ERA (disease time ≤2 years), 106 advanced RA (disease time >2 years), 21 anti-CCP-negative RA, 34 RF-negative RA and 19 anti-CCP & RF-negative RA, 59 patients with OA (mean age 62.66±8.97 years, 51 females and 8 males), 59 patients with SLE (mean age 42.62±16.08 years, 52 females and 7 males), 55 patients with AS (mean age 42.64±12.28 years, 23 females and 32 males) and 95 patients with pSS (mean age 55.13±14.38 years, 81 females and 14 males) were enrolled in the indirect ELISA verification array. Fifty blood donors' serum samples were served as healthy controls. RA was diagnosed fulfilling 2010 American College of Rheumatology (ACR) criteria (12), OA fulfilling 1995 ACR criteria (13), SLE fulfilling 1997 ACR criteria (14), AS fulfilling 1984 New York criteria (15), and pSS fulfilling 2012 ACR criteria (16).

Patients had been informed and gave their signed consent to participate. This study was approved by the ethics committee of Peking University People's Hospital.

Immunoprecipitation

CICs derived from 10 ERA patients' and 10 healthy controls' mixed serum were collected and extracted by protein G Plus-Agarose beads. In brief, 30 μl of mixed serum sample were incubated in 50 μl of beads at 4°C overnight. The beads attached ICs were gathered via centrifugation (4°C, 1000 g, 30 min). Discard the supernatants. The beads were washed three times with 1 ml PBS and blended with 40 μl of electrophoresis sample buffer. Boil the samples for 2–3 min for subsequent SDS-page and gel staining.

In-gel trypsin digestion

Every lane was divided into 10 slices and then degraded with 25 mM of dith-

iothreitol (DTT) and alkylated with 55 mM iodoacetamide. Sequenced-modified trypsin (Promega, Fitchburg, WI) was used in gel digestion overnight at 37˚C. Peptide digests of CICs in the supernatants were purified twice with trifluoroacetic (1%) acid in acetonitrile aqueous solution (50%) for 30 min. Final extractions were centrifuged to adjust the volume to 80 ul approximately.

LC-MS/MS analysis

These peptides (10 ul) were presented to LC-MS/MS with nano-LC joined with Orbitrap Q Exactive mass spectrometer. The original data was matched with ipi. HUMAN. v. 3.87 database using Protein Discovere 1.3.0. The matched parameters were set as follows: peptide ms tolerance is 20 ppm; ms/ms tolerance is 20 mmu.

Recombinant human PGLYRP-2 protein expression

RT-PCR was applied to amplify the cDNA encoding human PGLYRP-2 with forward primer (ATGCGAATTCCGGACTTCTCTGCCGCTGCTGATGG), and reverse primer (ATGCCTCGAGTGCGGCCTTTACTGCAGGTCGGTAGCCGGCAGGG). The amplified PGLYRP-2 cDNA was subcloned into the PET28a expression vector. The 76 kDa recombinant human PGLYRP-2 was expressed in Rosetta (DE3), purified by the immobilised metal ion affinity chromatography (IMAC), and stored in buffer containing 10 mM Tris-HCL, 1 mM EDTA, PH 8.0, 50% glycerol.

ELISA experiments

Serum anti-PGLYRP-2 levels were tested by indirect ELISA. In detail, recombinant PGLYRP-2 protein (0.4 μg/ml in 0.05 M carbonate buffer) coated in the 96 well polysorp plates (NUNC, Denmark) at 4˚C overnight. Then wash wells with PBS plus 0.05% Tween-20 (PBST) three times and block with 5% BSA-PBST at 37˚C for 3 hours. All serum samples were diluted at 1:30 with 1% BSA-PBST. Each 96-well plate was filled with 100 ul diluted samples. Wells filled only by 1% BSA-PBST were used to control nonspecific background. Wells were washed for four times with 0.05% PBST after 1 hour of incubation at 37˚C, Then, 100 ul of goat anti-human IgG conjugated to peroxidase (1:10000 in 1% BSA-PBST) was added to wells and incubated at 37˚C for 40 minutes. The bound antibodies were tested with tetramethylbenzidine (TMB) as substrate after washing four times. This reaction was terminated by adding 50 ul of 2 M sulfuric acid to wells. The absorbance density (OD) was read by a Bio-Rad plate reader at 450 nm and 630 nm. Like the following formula, the OD values of anti-PGLYRP-2 were converted into arbitary units (AU) values.

$$AU = \frac{\text{[OD peptide – OD nonspecific background]}_{\text{test serum}}}{\text{[OD peptide - OD nonspecific background]}_{\text{positive control serum}}} \times 100$$

The AU value was considered positive if it was greater than the mean + 3 × standard deviation of the healthy group.

Laboratory and clinical information of RA patients

The following laboratory and clinical information was searched from the database of digital medical records: rheumatoid arthritis-associated interstitial lung disease (RA-ILD), secondary Sjögren's syndrome (sSS), xerophthalmia, rheumatoid nodules, bone erosion, morning stiffness, disease activity score 28 (DAS28), tender joint counts (TJCs), swollen joint counts (SJCs), rheumatoid 8 items [RF, immunoglobulins (IgG, IgM, IgA), anti-streptolysin O (ASO), complement (C3, C4)], rheumatoid 5 items [anti-CCP, antiperinuclear factor (APF), hidden rheumatoid factor immunoglobulin (HRF-IgA, HRF-IgG), glucose-6-phosphate isomerase (GPI)], antinuclear antibodies (ANA), antineutrophil cytoplasmic autoantibodies (ANCA-PR3), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), blood routine and liver function.

Data analysis

Acquired experimental data was analysed by SPSS software 17.0 and GraphPad Prism v. 8.0. Normally distributed data was expressed as mean ± standard deviation (M ± SD); t-test was used to analyse the differences between groups. Data not distributed normally expressed as median (range), differences were analysed with the Mann-Whitney U-test. Categorical data was presented as n (%) and analysed by chi-square (^{2}) test. Spearman correlation was adopted for correlation analysis. Differences were considered to be significant at $p<0.05$.

Results

Identification of autoantigens derived from CICs in RA

The proteins identified by orbitrap mass spectrometry from CICs in 10 ERA patients were showed in Table I. A potential antigen profile consisted of 323 proteins, in which 33 proteins were enriched in RA patients but not in 10 of the healthy controls. In the identified 33 potential autoantigens, most of them have been studied previously including heat shock protein 90, mannan binding lectin serine peptidase, immunoglobulin lambda constant, and vimentin variant 3, etc. (9, 17-25). PGLYRP-2 (IPI00163207.1) was the potential autoantigen with high binding score and its structure was similar to bacterial NAMLAA. We hypothesised that it might be a novel auto-antigen. Therefore, it was selected for further investigation.

The prevalence of anti-PGLYRP-2 in RA and its subgroups

Serum autoantibodies against PGLYRP-2 were detected by indirect ELISA in RA, disease and healthy groups. As expected, anti-PGLYRP-2 was significantly elevated in RA with a 42.70% of sensitivity and 85.20% of specificity ($p<0.001$, Table II). To examine the clinical value of anti-PGLYRP-2 in ERA and sero-negative RA (SNRA) subgroups, 72 ERA and 55 SNRA serum samples were detected. We found that the prevalence of anti-PGLYRP-2 was 34.72% (25/72) in ERA patients with disease time no more than 2 years. The prevalence of anti-PGLYRP-2 in SNRA patients without RF or anti-CCP was 35.29% (12/34) or 42.86 (9/21), respectively. Moreover, 36.84% patients with anti-PGLYRP-2 were found in 7 of 19 patients with anti-CCP and RF double negative (Table II). Thus,

Table I. Potential antigens identified from circulating immune complexes (CICs) in RA patients.

IPI number	Antigens	Gene name	Ms score
IPI00878720.1	kringle containing transmembrane protein 1	KREMEN1	17
IPI00386765.4	Isoform N3 of cAMP-specific 3',5'-cyclic phosphodiesterase 4D	PDE4D	17
IPI00027107.5	Elongation factor Tu, mitochondrial precursor	TUFM	17
IPI00102281.3	Retroviral-like aspartic protease 1	ASPRV1	17
IPI00045600.6	Isoform 3 of Disabled homolog 2-interacting protein	DAB2IP	18
IPI00830107.1	V4-2 protein	V4-2	18
IPI00646774.1	cDNA FLJ78714, highly similar to Homo sapiens corneodesmosin, mRNA	CDSN	18
IPI00979294.1	cDNA FLJ34521 fis, clone HLUNG2007041	NA	19
IPI01014108.1	Carbonic anhydrase 2	CA2	19
IPI00477357.3	Isoform 3 of Inactive phospholipase D5	PLD5	19
IPI00181283.2	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase beta-3 isoform 2	PLCB3	19
IPI00297550.8	Coagulation factor XIII A chain	F13A1	20
IPI00844156.2	SERPINC1 protein	SERPINC1	20
IPI00791534.2	Solute carrier family 4, anion exchanger, member 1	SLC4A1	21
IPI00003351.2	Isoform 1 of Extracellular matrix protein 1	ECM1	21
IPI00984754.1	Glutathione peroxidase 3	GPX3	21
IPI00982057.1	Scribble planar cell polarity protein	SCRIB	22
IPI00163207.1	Isoform 1 of N-acetylmuramoyl-L-alanine amidase	PGLYRP-2	34
IPI00794082.2	cDNA FLJ57650, highly similar to Bleomycin hydrolase	BLMH	34
IPI00940494.2	13 kDa protein	NA	35
IPI00640129.2	Heat shock protein 90 alpha family class B member 1	HSP90AB1	35
IPI00925177.1	Mannan binding lectin serine peptidase 1	MASP1	37
IPI00796316.4	cDNA FLJ53327, highly similar to Gelsolin	GSN	43
IPI00025753.2	Desmoglein-1	DSG1	56
IPI01018306.1	Fibrinogen gamma chain	FGG	62
IPI00029717.1	Isoform 2 of Fibrinogen alpha chain	FGA	86
IPI00296099.6	Thrombospondin-1	THBS1	88
IPI00018534.4	Histone H2B type 1-L	H2BC13	122
IPI00827485.1	BRE (Fragment)	NA	497
IPI00647704.1	cDNA FLJ41552 fis, clone COLON2004478, highly similar to Protein Tro alpha1 H,myeloma	NA	152
IPI00852577.3	Immunoglobulin lambda constant 1	IGLC1	452
IPI00556287.1	Putative uncharacterized protein	NA	759
IPI00975690.1	Vimentin variant 3	VIM	170

NA: not applicable.

Table II. The prevalence of anti-PGLYRP-2 in RA patients and controls.

Groups	n. of patients	anti-PGLYRP-2 antibody		
		no. of positives	Sensitivity (%)	Specificity (%)
RA	178	76	42.70	85.20 [a]
ERA	72	25	34.72	-
RF (-) RA	34	12	35.29	-
Anti-CCP (-) RA	21	9	42.86	-
Anti-CCP (-)/RF (-) RA	19	7	36.84	-
OA	59	11	18.64	-
SLE	59	10	16.95	-
AS	55	8	14.55	-
SS	95	17	17.89	-
HC	50	1	2	-

Anti-PGLYRP-2: anti-peptidoglycan recognition protein 2; Anti-CCP: anti-cyclic citrullinated peptide; RF: rheumatoid factor; RA: rheumatoid arthritis; ERA: early rheumatoid arthritis; OA: osteoarthritis; SLE: systemic lupus erythematosus; AS: ankylosing spondylitis; SS: primary Sjögren's syndrome; HC: healthy controls; n.: number. [a] Compared with the positivity for anti-PGLYRP-2 in other rheumatic diseases and healthy controls, $p<0.001$.

these results support anti-PGLYRP-2 as a valuable biomarker in early and serological negative RA patients.

The value of anti-PGLYRP-2 combination with other antibodies in RA

We further assessed the potential value of the combined detection of anti-PGLYRP-2 and other antibodies in 150 RA patients and 78 disease controls (Table III). The results suggested the sensitivity can reach 92.00% with a specificity of 76.92% when either anti-PGLYRP-2 or anti-CCP was positive. In addition, the group of "Anti-PGLYRP-2 and anti-CCP" has the highest specificity (98.72%) but the sensitivity was only 34.67%. The data indicated that anti-PGLYRP-2 might provide additional diagnostic value besides anti-CCP.

Laboratory and clinical relevance of serum anti-PGLYRP-2 in RA patients

To clarify the clinical significance of anti-PGLYRP-2, the correlation between clinical manifestations and anti-PGLYRP-2 levels was analysed. As shown in Figure 1, anti-PGLYRP-2 was positively correlated with HRF-IgG, RF, IgA, IgM and IgG ($p<0.05$). In addition, higher levels of RF, IgG, IgM and ANCA-PR3 were associated with the titre of anti-PGLYRP-2 ($p<0.05$, Table IV). However, no other clinical significance was found in regard of serum concentration of anti-PGLYRP-2 between the anti-PGLYRP-2-elevated and anti-PGLYRP-2-normal groups.

Discussion

In our study, we used high resolution mass spectrometry with nano-LC combined with Orbitrap Q Exactive mass spectrometer to identify an autoantigen profile from ERA CICs. At last, there were 33 potential autoantigens successfully identified. It is noticeable that these antigens were related to immune response, coagulation cascade, cell differentiation and so on, indicating that these antigens' corresponding autoantibodies might disequilibrate the above physiological environments and result in pathogenic abnormalities. PGLYRP-2 has been referred to connecting innate and adaptive immunity since it may play a role of scavenger by digesting bioactive peptidoglycan (PGN) into biologically inactive fragments and regulate NOD1/2 mediated inflammation (26, 27). Additionally, PGLYRP-2 is also reported to be associated with RA (9, 10). Subsequently, we also found that PGLYRP-2 was a specific autoan-

Table III. The distinguished properties of anti-PGLYRP-2, anti-CCP and RF in RA.

	Sensitivity (%)	Specificity (%)	PPV. (%)	NPV. (%)
Anti-PGLYRP-2	39.33	85.90	84.29	42.41
Anti-CCP	87.33	89.74	88.41	68.89
RF	81.33	79.49	94.24	78.65
Anti-PGLYRP-2 or anti-CCP	92.00	76.92	88.46	83.33
Anti-PGLYRP-2 and anti-CCP	34.67	98.72	98.11	44.00
Anti-PGLYRP-2 or RF	87.33	67.95	83.97	73.61
Anti-PGLYRP-2 and RF	33.33	97.44	96.15	43.18
RF or Anti-CCP	87.33	75.64	87.33	75.64
RF and Anti-CCP	81.33	93.59	96.06	72.28

PPV/NPV: positive/negative predictive value; anti-PGLYRP-2: anti-peptidoglycan recognition protein 2; Anti-CCP: anti-cyclic citrullinated peptide; RF: rheumatoid factor.

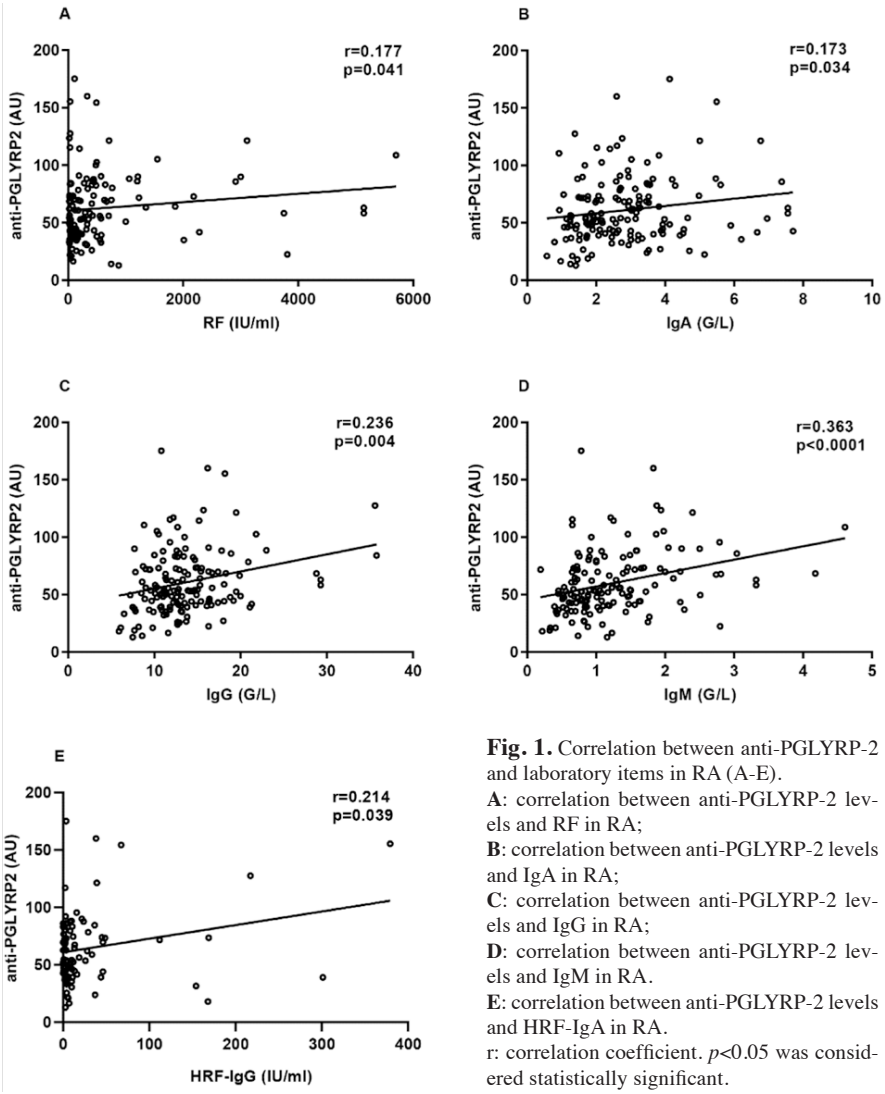

Fig. 1. Correlation between anti-PGLYRP-2 and laboratory items in RA (A-E).
A: correlation between anti-PGLYRP-2 levels and RF in RA;
B: correlation between anti-PGLYRP-2 levels and IgA in RA;
C: correlation between anti-PGLYRP-2 levels and IgG in RA;
D: correlation between anti-PGLYRP-2 levels and IgM in RA.
E: correlation between anti-PGLYRP-2 levels and HRF-IgA in RA.
r: correlation coefficient. $p<0.05$ was considered statistically significant.

tigen of RA by testing anti-PGLYRP-2 levels, which were strikingly higher in RA group than that in controls.

Mammals have four peptidoglycan recognition proteins (PGRPs): PGLYRP 1, 2, 3, 4, of which, PGLYRP-2 is the only NAMLAA that hydrolyses bacterial peptidoglycan and lessens its pro-inflammatory properties (28). Qing *et al*. has reported the serum levels of PGLYRP1 were increased in RA patients (29), while our study is among the first studies to suggest the contribution of anti-PGLYRP-2 in RA. There may be some connections between these two different subtypes of PGRPs. In this research, anti-PGLYRP-2 levels were obviously increased in RA patients than that in control groups with 42.70% sensitivity and 85.20% specificity. In the patients at early stage, the prevalence of anti-PGLYRP-2 was 34.72% (25/72). As well, for RF (-), anti-CCP (-) and anti-CCP (-)/RF (-) patients, about 30–40% detection rate may be accessible for the recognition of serologically negative patients. This indicated that anti-PGLYRP-2 could be a promising biomarker for RA diagnosis, especially in the diagnosis of RA with negative RF and/or anti-CCP antibodies. We also recommend parallel and serial testing for RF, anti-CCP, and anti-PGLYRP-2 because of the complementary effect.

Previous studies have demonstrated that anti-peptidylarginine deiminase 4 (anti-PAD4) and anti-carbamylated protein (anti-Carp) were novel diagnostic markers for RA. These antibodies were correlated with bone erosion and disease activity. The sensitivity and specificity of anti-PAD4 and anti-Carp were 20–50% and 85–98% respectively (30-35), which were similar with anti-PGLYRP-2 in our RA cohort. Anti-mutated citrullinated vimentin (anti-MCV) was one of the most valuable diagnostic marker in ERA, its sensitivity and specificity (60–80%, 80–98%) (34-37) were higher than anti-PGLYRP-2, anti-PAD4 and anti-CARP. Nevertheless, anti-PGLYRP-2 yet contributed to RA diagnosis, especially in SNRA patients. There were about 40% seronegative RA patients who was anti-PGLYRP-2 positivity. Therefore, we suggested that anti-PGLYRP-2 might be a useful diagnostic biomarker.

Furthermore, we found that the anti-PGLYRP-2 was positively correlated with the levels of HRF-IgG, RF, total IgA, IgG and IgM, which indicated anti-PGLYRP-2 might be involved in B cell activation and antibody production in patients of RA. However, it was noted that there was no association between anti-PGLYRP-2 level and disease activity (DAS28, ESR, CRP). It's likely that anti-PGLYRP-2 in RA might be play roles in the triggering stage of RA pathogenesis instead of the exacerbation stage, but a definite conclusion is too early to be drawn. We need more de-

Table IV. Measurements of laboratory and clinical indicators in RA patients with positive and negative serum anti-PGLYRP-2 levels.

Measures	Serum anti-PGLYRP-2		t /z/χ^2	*p*
	Positive (n=76)	Negative (n=102)		
Age (years)	56.97±14.75	58.96±13.66	-0.928	0.335
Disease duration (months)	60 (1-600)	42 (1-720)	-0.78	0.435
Female	63 (82.9)	84 (82.4)	0.009	0.925
SJCs	2 (0-24)	2 (0-28)	-0.143	0.886
TJCs	1 (0-28)	4 (0-28)	-1.609	0.108
DAS28 (ESR)	4,02±1.88	4.41±1.55	-1.374	0.172
DAS28 (CRP)	3.62±1.75	3.95±1.53	-1.258	0.210
ESR (mm/h)	29 (3-98)	27 (3-112)	-0.274	0.784
CRP (mg/L)	10.06 (0.26-122.65)	12.51 (0.31-164.13)	-0.867	0.386
Anti-CCP (U/Ml)	193.18 (2.51-283.6)	186.2 (2.35-261.88)	-0.620	0.535
RF (IU/ML)	326 (20-5700)	103 (20-5140)	-2.984	0.003**
lgA (G/L)	3.05±1.33	2.75±1.60	1.198	0.233
lgG (G/L)	14.84±5.65	12.92±4.22	2.374	0.019*
lgM (G/L)	1.53±0.87	1.05±0.60	3.973	<0.001***
ANCA-PR3 (RU/ml)	4.29 (0-9.82)	0.65 (0-22.15)	-2.648	0.008**
APF +	26 (60.5)	43 (59.7)	0.006	0.937
ANA +	27 (46.6)	48 (59.3)	2..197	0.138
Bone erosion	35 (46.1)	60 (58.8)	2.854	0.091
ILD	15 (19.7)	30 (29.4)	2.158	0.142
sSS	16 (21.1)	14 (13.7)	1.668	0.196
Xerophthalmia	33 (43.4)	44 (43.1)	0.001	0.97
Morning stiffness	42 (55.3)	63 (61.8)	0.761	0.383
Rheumatoid nodules	5 (6.6)	2 (2.0)	2.52	0.112

For normally distributed data, results were expressed as mean ±SD; differences between groups were analysed with the t-test. Data not distributed normally expressed as median (range), differences were analysed with the Mann-Whitney U-test. Classified data were presented as n (%) and analysed by chi-square (χ^2) test.

$p<0.05$ were considered statistically significant. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

anti-PGLYRP-2: anti-peptidoglycan recognition protein; RA: rheumatoid arthritis; SJCs: swollen joint counts; TJCs: tender joint counts; DAS28: 28-joint Disease Activity Score; ESR: erythrocyte sedimentation rate; CRP: C-reaction protein; Anti-CCP: anti-cyclic citrullinated peptide; RF: rheumatoid factors; IgA, IgG, IgM: immunoglobulins A, G, M; ANCA-PR3: anti-neutrophil cytoplasmic autoantibodies; APF: anti-perinuclear factor; ANA: anti-nuclear antibody; ILD: interstitial lung disease; sSS: secondary Sjögren's syndrome.

tailed studies to understand its explicit mechanism in RA pathogenesis. In addition, we discovered that RF, IgG, IgM and ANCA-PR3 were also increased in anti-PGLYRP-2 positive group. However, we failed to find other relevant significant differences between anti-PGLYRP-2 positive and negative groups.

Previous studies have reported that PGLYRP-2-/- mice were resistant to peptidoglycan-induced arthritis (38) and bacterial keratitis symptoms in these mice were efficiently resolved (39), but others found oppositely that PGLYRP-2-/- mice had a significantly increased cecal inflammation (40) and a protective effect on psoriasis skin (41). Moreover, inflammatory properties of peptidoglycan were reduced after PGLYRP-2 degradation (42). Recently, PGLYRP-2 has been described as a candidate biomarker for the diagnosis of critically ill sepsis (43) and the adequate immune response against hepatocellular carcinoma (HCC) (44). PGLYRP-2 also played an important part in the early defense against streptococcus pneumoniae infection (45). Subsequently, serum PGLYRP-2, identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS), was differentially expressed in RA by more than 1.26-fold to spondyloarthropathies (SpAs) (10). A similar study then reported that PGLYRP-2 was >2.0-fold upregulated in RA compared with healthy controls (9). Unfortunately, these studies did not further verify the expression and function of PGLYRP-2 in RA, and the role of PGLYRP-2 in RA still remained unknown. In summary, PGLYRP-2 was a meaningful regulatory molecule in the process of innate immunity, and may have some implications in RA as infection contributes to its pathogenesis.

However, it is worth noting that most of the study's findings derived from cross-sectional data, not longitudinal data, and whether the taking of immunosuppressive drugs or corticosteroids impacts the expression of serum anti-PGLYRP-2 remains uncertain. Future studies recruiting consecutive patients are necessary to further evaluate the clinical significance of anti-PGLYRP-2 in RA disease progression.

Besides, what interests us is whether bacteria also have antigenic epitopes aligned by anti-PGLYRP-2 antibody, since NAMLAA were found in bacterial genomes such as escherichia coli (E. coli) and their structures were similar to eukaryotic PGRPs (46, 47). This amidase in bacteria has the functions of regulating cell wall growth, overturning peptidoglycan during growth, separating daughter cells during cell division and autolysis (46). Furthermore, we found that the "WGDIGY" sequence of PGLYRP-2 was identical to that of $NAMLAA_{431–436}$ in actinomyces naeslundii using the BLAST network service of the NCBI. Therefore, the study of antigenic determinants perhaps also needs further validation in the future.

In conclusion, our study has successfully established a CICs antigen profile for RA patients and healthy controls via high-resolution orbitrap mass spectrometry and first identified the prevalence and importance of antibodies against the CIC-associated PGLYRP-2 in RA.

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