Expression of glucocorticoid receptor isoforms and associations with serine/arginine-rich protein 30c and 40 in patients with systemic lupus erythematosus

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Abstract

Objective

To investigate the expression of glucocorticoid receptor (GR) isoforms in patients with systemic lupus erythematosus (SLE), comfirm the main GR isoforms involving in glucocorticoids (GC) resistance, and explore the associations of GR isoforms with serine/arginine-rich protein (SRp) 30c and SRp40.

Methods

Seventy patients with SLE and thirty-eight age- and sex-matched controls were recruited. All patients received prednisone (0.5–1 mg/kg/d) as their routine therapy. According to the therapeutic effect, patients were divided into glucocorticoid-resistant (GCR) and glucocorticoid-sensitive (GCS) groups. Transcript levels of GRα, GRβ, GRγ, GR-P, SRp30c and SRp40 in peripheral blood mononuclear cells (PBMCs) were determined by real-time PCR. GRα and GRβ proteins were detected by western blotting. Trial registration number is ChiCTR-RCH-12002808.

Results

Four GR transcripts in SLE patients showed the following trend: GRα (51.85%) > GR-P (23.78%) > GRγ (13.08%) >GRβ (0.03%). GR-P transcript and ratio of GRα/GR-P in SLE patients were significantly higher than that in controls ($p<0.05$). GRα transcript and protein as well as SRp40 transcript in GCS group were significantly higher than that in the GCR group before GC treatment ($p<0.05$). In the GCS group, GRα transcript and SRp40 transcript were significantly higher after GC treatment than that before GC treatment ($p<0.05$). In the GCR group, GR-P transcript was significantly higher after GC treatment than that before GC treatment ($p<0.05$). Positive correlation between SRp40 and GRα transcript was found ($p<0.05$). Additionally, SLE Disease Activity Index scores were significantly negatively correlated with GRα transcript and protein expression ($p<0.05$).

Conclusion

Our data demonstrated that the decreased expression of GRα might be the evidence of high disease activity and help to predict GC resistance. GR-P isoform might be implicated in the development of resistance. Additionally, the preliminary finding suggested that SRp40 might be associated with GRα transcripts in SLE patients.

Key words

systemic lupus erythematosus, glucocorticoid receptor, resistance, alternative splicing, serine/arginine-rich proteins

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by a variety of auto-antibodies. Glucocorticoids (GC) as inflammation inhibitors are commonly used to treat patients with SLE. However, responses to GC vary widely from patients to patients. Up to a third patients fail to respond to GC, these individuals are known as glucocorticoid-resistant (GCR) patients (1-3). Up to now, the pathogenesis of GC resistance has not been fully understood.

The biological action of GC is mediated through the activation of GC receptor (GR). Human GR gene is located on chromosome 5q31-32 and composed of nine exons. Alternative splicing of the primary GR transcript results in five isoforms: GRα, GRβ, GRγ, GR-P and GR-A (4-8). The GRα and GRβ isoforms differ in exon 9 at their extreme C-termini. When the splicing event that joining exon 7 with exon 8 fails, the GR-P isoform that ends at intron G is produced. GRγ isoform is created when three additional bases are kept in the splicing junction between exons 3 and 4. The elimination of exons 5 to 7 by alternative splicing generates the GR-A isoform (9).

GRα is the classical active physiological and pharmacological receptor isoforms through which GC functions. GRα is a modular protein composed of an N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), and a C-terminal ligand binding domain (LBD). The LBD of GRβ and GR-P is defective (9). GRγ contains an insertion of a single arginine residue between the two zinc fingers of the DBD (8). These structural changes may confer several distinct properties on the different GR isoforms and their alternative expression may be associated with GC resistance.

It has been reported that disease activity related to the expression of GRα and GRβ in patients with SLE. Piotrowski, *et al*. reported that SLE patients with high disease activity exhibited significantly elevated GRβ transcript and protein levels in PBMCs (10). Li *et al*. found GRα expression in patients with SLE was lower than that in controls and GRα expression is negatively correlated with disease activity (11). Additionally, the expression and binding of both $CD3^+$/GRα and $CD14^+$/GRα in GCR patients with SLE, as detected by flow cytometry, were lower than those in glucocorticoid-sensitive (GCS) patients with SLE (12). To date, the expression of GRγ and GR-P isoforms has not been reported in patients with SLE.

Alternative splicing is one of many mechanisms that regulate gene expression in eukaryotes. This mRNA splicing process is mediated by spliceosomes, specialised assemblies of proteins, which include serine/arginine-rich protein (SRp) that control the level of exon inclusion/exclusion in the mRNA transcript. SRp such as SRp30 (13, 14) and SRp40 (15) could regulate the relative levels of GRα and GRβ, through alternative splicing of GR pre-mRNA. The potential role of these splicing factors in differential expression of GR isoforms (GRα, GRβ, GRγ, and GR-P) has not been investigated in peripheral blood mononuclear cells (PBMCs) of patients with SLE.

The aim of the present study was to investigate the expression of GRα, GRβ, GRγ, and GR-P in patients with SLE and evaluate their differential expression between GCS and GCR patients. Additionally, we analysed the association of GR transcripts with SRp30c and SRp40.

Materials and methods

Patients and controls

The American Rheumatism Association diagnostic criteria in 1997 for SLE and SLE Disease Activity Index (SLEDAI) scores were adopted for the diagnosis and disease activity assessment of SLE patients, respectively (16, 17). A total of 70 patients (male/female, 5/65; median age, 42 years) with SLE and 38 age- and gender-matched healthy controls (male/female 3/35; median age, 40 years) were included. Among the patients, 49 patients with disease relapse took no other drugs during remission, excluding physiological doses of GC (prednisone 5 mg/day orally).

All patients took prednisone (0.5–1 mg/kg/day orally) as part of their routine therapy for four weeks in combina-

Table I. Primers used to discriminate the different GR transcripts (GRα, GRβ, GRγ, and GR-P) and SRp30c, SRp40 expression, normalised to β-actin expression.

Gene Name	Left Primer Sequence (5' to 3')	Right Primer Sequence (5' to 3')
NR3C1(GRα)	GCAGCGGTTTTATCAACTGACA	AATGTTTGGAAGCAATAGTTAAGGAGAT
NR3C1(GRβ)	GAAGGAAACTCCAGCCAGAA	CCACATAACATTTTCATGCATAGA
NR3C1(GRγ)	TTCAAAAGAGCAGTGGAAGGTA	GGTAGGGGTGAGTTGTGGTAACG
NR3C1(GR-P)	GGAGAAAAAGGCGCATCCTA	TGCTATGTTAACCAATCCCCAAT
SFRS9(SRp30c)	CATGGAATATGCCCTGCGTAAAC	ACCGTGAGTAGCCATAGCTGGTG
SFRS5(SRp40)	TCGCAGACCTCGAAATGATAGAC	GCATCCGCAAACGTTACTTCC
ACTA1(β-actin)	CATTGCCGACAGGATGCA	CATCTGCTGGAAGGTGGACAG

tion with oral hydroxychloroquine or other immunosuppressants to achieve a smooth reduction in GC dosage after four weeks. The patients with renal involvement (24-hour urine protein>3.5g) were given 800mg cyclophosphamide monthly at least for 6 times.

After the four-week GC treatment, the patients were classified into two groups according to their response to prednisone (18, 19): the GCS group or GCR group. The criteria for classification in the GCS group were remission or partial remission of clinical symptoms with a decrease in the SLEDAI score of at least 4 points after prednisone therapy. The criteria for the GCR group were a lack of response to GC with a decrease in the SLEDAI score of less than 4 points, or even an increase.

These studies were conducted in accordance with the Declaration of Helsinki and were approved by our local Research Ethics Committee. Informed consent was obtained from all patients and controls before participation in the study. The trial registration number is ChiCTR-RCH-12002808.

Preparation of PBMCs

Peripheral blood (6 ml) was collected into EDTA-K_2 tubes each patient before the start of four-week GC administration from 6:30 to 7:00 am. Peripheral blood from 28 cases was also collected after four-week GC treatment. After the centrifugation for 10 min at 250×g, the upper plasma was transferred to a 1.5-ml Eppendorf tube. The lower blood cells were diluted by addition of an equal volume of 0.9% NaCl. Diluted samples were carefully layered over 4 ml of lymphprep (Chuan Ye Biologicalmanufacture.co., LTD, Tianjing, China) and centrifuged at 800×g for 20 min at room temperature. After centrifugation the mononuclear cells formed a distinct band at the sample interface. The harvested fraction was washed twice with 0.9% NaCl. The cells were pelleted by centrifugation for 10 min at 250×g . The pellets were used as mononuclear cells and stored in liquid nitrogen.

RNA isolation and cDNA synthesis

Total cellular RNA was extracted from PBMCs of SLE patients and controls using TRIzol reagent (Takara Bio Inc., Otsu, Japan) according to manufacturer's instructions. RNA quality was examined based on the optical density (OD) ratio at 260/280 nm and quantified by the OD at 260 nm using NanoDrop 2000c software (Thermo, USA). The mRNA with an A260/A280 ratio between 1.7 and 2.1 was reverse transcribed into first-strand cDNA synthesis. The DRR036 kit (Takara Bio Inc., Otsu, Japan) was used for first-strand cDNA synthesis. RNA samples (1.0 μg) were blended with 4μl 5×PrimeScript® RT master mix and RNase free ddH_2O in a final volume of 20 μl. This mixture was incubated for 15 min at 37°C and then heated at 85°C for 5 sec.

Real-time PCR analysis

PCR reactions were performed according to the manufacturer's instructions (SYBR® Premix Ex Taq™ , Takara Bio Inc., Otsu, Japan). Cycle conditions were as follows: initial denaturation at 95°C for 30 sec; 40 cycles of 95°C for 5 sec, and 60°C for 20 sec, and a denaturation cycle for creation of dissociation curves. Reactions for each sample and gene of interest were run in triplicate. The cycle threshold (CT) value represents the cycle in which the fluorescence intensity of each sample reached the threshold fluorescence intensity which was normalised to β-actin expression as a housekeeping gene. Comparative quantisation was performed using the lightcycle480 (Hoffmann-La Roche, Ltd, Switzerland). Only individual PCR samples with single-peak dissociation curves were selected for data analysis. The average CT value was used to calculate mRNA expression levels of the PCR targets relative to that of the reference gene using the following equation: relative expression $=2^{-[CT(target) - CT(reference\ gene)]} \times 10^3$ (20). Primers specific for GRα, GRβ, GRγ, and GR-P were localised on exons 8 to 9α, 8 to 9β, 3 to 4, and exon 7-intron G, respectively. Primers were designed by Takara Bio Inc. (Table I).

Western blotting analysis

We were not able to purchase the antibody of GRγ and GR-P because no company produced and sold them, GRα and GRβ proteins were detected only by western blotting. PBMCs (approximately 5×10^6) were lysed in 200 μl RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 14,000× *g* at 4°C for 20 min, the supernatant contained the total cell protein was removed. A sample of the supernatant was taken for protein concentration estimation using BCA protein Assay kit (KeyGEN Biotechnology, Nanjing, China) and the remainder used for testing GRα and GRβ proteins. For electrophoresis, extracted protein (50 ug) was denatured and separated in 10% SDS-PAGE and transferred onto nitrocellulose membranes. These were blocked with PBS containing 3% bovine serum albumin, 0.1% Tween 20, and then incubated with different antibodies: GRα antibody (ab3580, dilution 1:250, Abcam, Cambridge, UK) and GRβ antibody (ab130227, dilution 1:1000, Abcam). Each antibody was diluted in 5% non-fat milk and incubated overnight at 4°C. Active bands were detected using conjugated horseradish peroxidase-goat anti-mouse or horseradish peroxidase-goat anti-rabbit antibody (dilution 1:5000 ZSGB-BIO, China). Detection was performed by

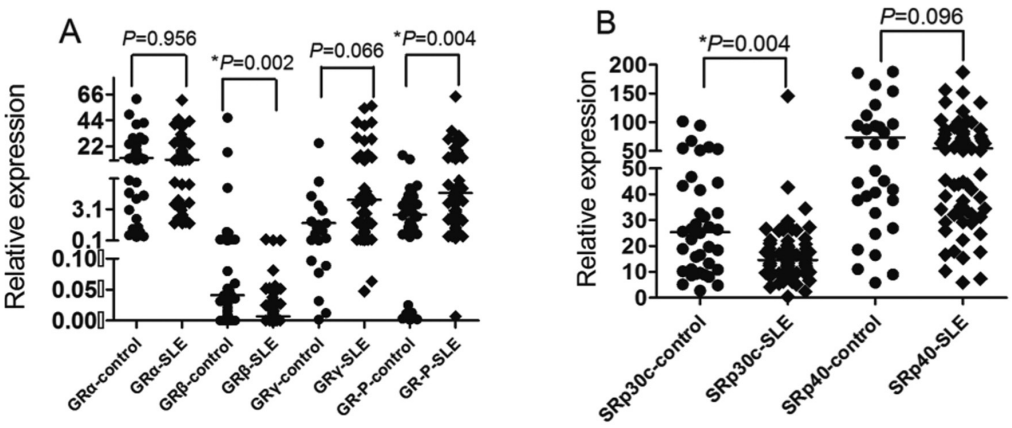

Fig. 1. The expression of GR transcripts (GRα, GRβ, GRγ, and GR-P) and SRp30c, SRp40 in peripheral blood mononuclear cells of 38 healthy controls and 70 SLE patients was measured by real-time RT-PCR. (**A**) The GR transcripts (GRα, GRβ, GRγ, and GR-P) relative expression. (**B**) SRp30c and SRp40 relative expression. The value for each individual is represented as a single point. Horizontal lines represent the median values for each group. Mann-Whitney U test used to analyse any two groups. *p<0.05 was considered significant.

enhanced chemiluminescence. Bands of the GRα protein appeared at approximately 86 kDa, bands of the GRβ protein at approximately 85 kDa. The relative intensity of each band was calculated against β-actin.

Statistical analysis

The mRNA data did not follow a normal distribution and were expressed as median and interquartile range (P25–P75). Comparisons between groups were performed using the Mann-Whitney U-test; Spearman's rank correlation test was used to identify correlations between two groups. The data of protein and SLEDAI score were expressed as the mean ± SD. Comparison between sample groups was done using ANOVA. Pearson's correlation test was used to identify correlations between two groups. SPSS.version.17.0 (SPSS, Chicago, USA) was used for all calculations. A value of p<0.05 was considered to indicate statistical significance (two-tailed test).

Results

GRα, GRβ, GRγ and GR-P mRNA expression

The expression of four GR isoforms exhibited the following trend in SLE patients and controls: GRα>GR-P>GRγ>GRβ. The percentages of GRα, GR-P, GRγ, GRβ expression of the total GR in SLE patients were 51.85%, 23.78%, 13.08% and 0.03%, controls: 68.85%, 12.27%, 11.63% and 0.20% (Supplementary Table I). GRα was the predominant isoform in SLE patients and controls, while the expression of GRβ was minimal. As shown in Figure 1, GRα and GRβ expression in patients were lower than that in controls. GRβ expression in patients were significantly lower than that in controls (0.68×10^{-2} *vs*. 4.10×10^{-2}, p=0.002), and GRα/GRβ ratio was significantly higher in patients compared with controls (1201.01 *vs*. 294.07, p=0.021). GRγ and GR-P expression in patients was higher than that in controls, significantly in GR-P expression (4.65 *vs*. 2.56, p=0.004), and GRα/GR-P ratio in patients was significantly lower than that in controls (2.07 *vs*. 5.65, p=0.038).

As suggested in Table II, the sex, age and dosage of prednisolone did not

Table II. Characteristics and the mRNA expression of objective genes in the GCS and GCR patients.

Variable	GCS (n=56)	GCR (n=14)	p-value
Sex (male/female)	4/52 1/13	0.994	
Age (years)#	46 (25-56)	40 (25.5-64)	0.579
SLEDAI before treatment@	10.77 ± 2.86	12.69 ± 2.66	0.089
SLEDAI after treatment@	5.23 ± 1.48	10.77 ± 2.09	0.004*
Prednisolone(mg/kg/d)@	0.83 ± 0.12	0.96 ± 0.28	0.442
GRα#	17.34 (11.09-27.12)	8.44 (7.08-19.35)	0.031*
GRβ#^	1.00 (0.05-2.75)	0.73 (0.22-2.55)	0.548
GRγ#	3.99 (0.49-26.64)	3.20 (1.01-14.67)	0.979
GR-P#	4.52 (1.26-9.48)	3.02 (1.71-9.01)	0.864
GRα/GRβ#	1009.34 (396.18-3921.22)	1577.93 (471.27-4472.93)	0.542
GRα/GRγ#	2.91 (0.44-19.43)	1.89 (0.74-19.75)	0.830
GRα/GR-P#	2.20 (0.82-5.72)	3.72 (1.51-7.16)	0.363
SRp30c#	16.35 (7.64-19.71)	12.83 (10.11-19.27)	0.818
SRp40#	71.08 (57.77-104.87)	47.61 (21.08-69.00)	0.024*

GCR: glucocorticoid resistant; GCS: glucocorticoid sensitive; SLEDAI: systemic lupus erythematosus disease activity index; SRp: serine/arginine-rich protein; ^GRβ mRAN expression $\times10^2$. #the data were shown as median (P25-P75);@the data were shown as mean ± SD; *p<0.05 was considered significant.

Table III. GR transcripts (GRα, GRβ, GRγ, and GR-P) and SRp30c, SRp40 mRNA expression in GCS patients before and after GC treatment.

Variable	BGT	AGT	p-value
GRα	13.38 (11.73-18.04)	21.70 (16.73-33.11)	0.008*
GRβ	1.47 (0.44-4.91)	2.54 (0.19-6.59)	0.841
GRγ	2.72 (1.19-4.26)	3.07 (2.03-5.54)	0.411
GR-P	5.14 (2.19-18.07)	5.37 (2.19-8.58)	0.248
SRp30c	13.63 (9.72-18.52)	16.18 (11.94-20.91)	0.871
SRp40	48.00 (33.23-64.41)	78.86 (60.63-142.99)	0.007*

GCS: glucocorticoid-sensitive; BGT: before GC treatment; AGT: after GC treatment. *p<0.05 was considered significant.

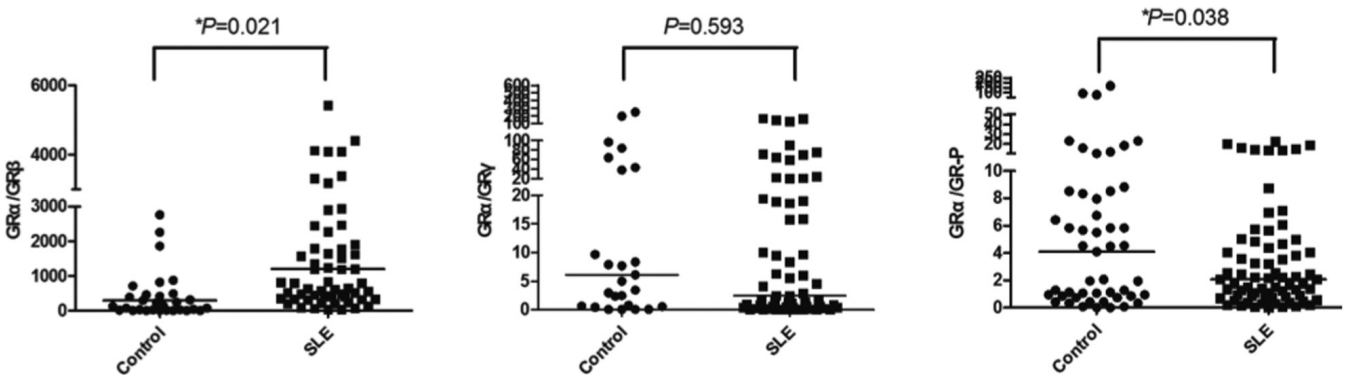

Fig. 2. The ratio of GRα/GRβ, GRα/GRγ, and GRα/GR-P in controls and patients with SLE. The value for each individual is represented as a single point. Horizontal lines represent the median values for each group. *p<0.05 was considered significant.

differ significantly between the GCS and GCR groups (p>0.05). In GCS group, SLEDAI score after four-week GC treatment was significantly lower than that before GC treatment, while no difference was found in GCR group before and after GC treatment. GRα, GRβ, GRγ and GR-P expression in GCR group were all lower than that in GCS group. GRα in GCR group were significantly lower than that in GCS group (8.44 *vs*. 17.34, p=0.031). No difference was found in GRα/GRβ ratio, GRα/GRγ ratio, and GRα/GRp ratio between GCS and GCR groups (p>0.05).

Four GR isoforms were increased after GC treatment, especially GRα (Supplementary Table II). It was distinct that the auto-upregulation role of GC on different GR isoforms in GCS and GCR patients. In GCS patients, the GRα was significantly increased after GC treatment. However, the GR-P was significantly increased in the GCR group after GC treatment (Table III and IV). Additionally, GRα in GCR group was lower than that in GCS group, while GR-P was significantly higher than that in GCS group after GC treatment (Supplementary Table III).

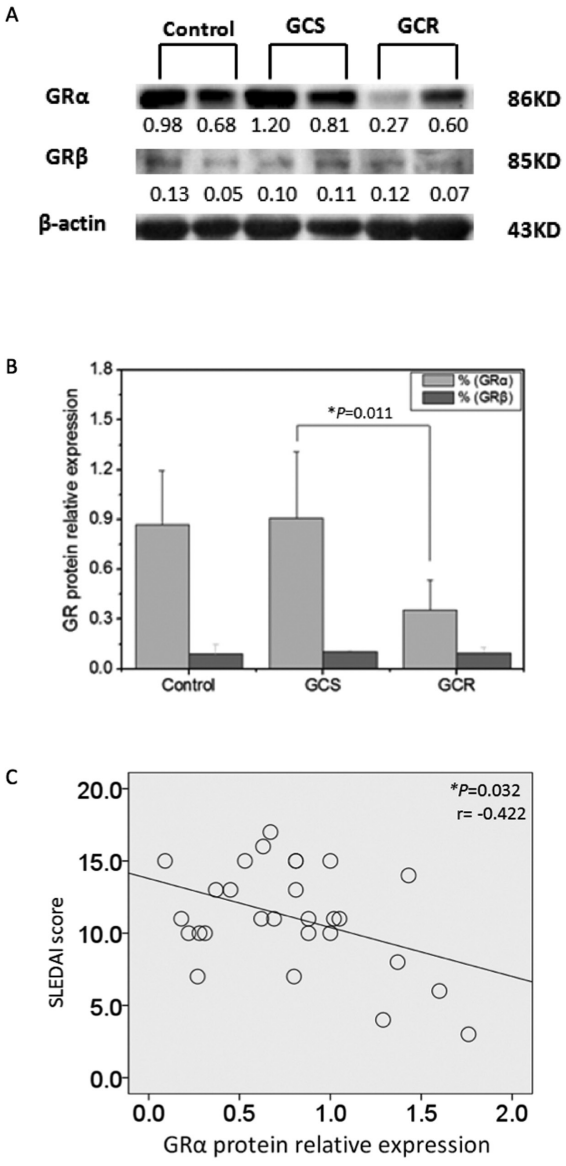

Fig. 3. (**A**) The protein expression of GRα and GRβ by western blotting analysis in controls, glucocorticoid-resistant (GCR) and glucocorticoid-sensitive (GCS) patients with SLE, each lane represents one sample of each group. (**B**) Mean densitometry of GRα and GRβ proteins in controls, glucocorticoid-resistant (GCR) and glucocorticoid-sensitive (GCS) patients with SLE. GRα protein was significantly lower in GCR than that in GCS patents (p=0.011). No difference was found in GRβ protein among three groups. (**C**) The correlation between GRα protein and corresponding SLEDAI score. *p<0.05 was considered significant.

GRα and GRβ protein expression

The relative expressions of GRα and GRβ proteins were calculated against β-actin in 32 SLE patients (24 GCS patients, 8 GCR patients), and 22 controls. Consistent with their mRNA expression, GRα protein expression was significantly higher in GCS patients than that in GCR patients (0.91±0.28 *vs*. 0.35±0.12, p=0.011). No difference in GRβ protein was found among three groups (Fig. 3).

SRp30c and SRp40 mRNA expression

SRp40 expression in GCR group were significantly lower than that in GCS group before GC treatment (p<0.05). Additionally, SRp40 expres-

Table IV. GR transcripts (GRα, GRβ, GRγ, and GR-P) and SRp30c, SRp40 mRNA expression in GCR patients before and after GC treatment.

Variable	BGT	AGT	*p*-value
GRα	9.02 (7.49-16.45)	18.72 (9.58-21.63)	0.184
GRβ	0.68 (0.34-3.64)	3.46 (0.88-4.90)	0.123
GRγ	3.71 (2.46-6.56)	5.03 (4.28-9.42)	0.327
GR-P	2.67 (0.73-8.06)	14.33 (6.16-26.00)	0.012*
SRp30c	14.65 (10.61-24.49)	18.52 (12.97-31.31)	0.401
SRp40	52.18 (9.58-71.63)	133.69 (32.32-236.66)	0.263

GCR: glucocorticoid-resistance; BGT: before GC treatment; AGT: after GC treatment. *$p<0.05$ was considered significant.

sion showed significant positive correlation with GRα transcript (r=0.444, $p<0.001$) and no significant correlation with GRβ, GR-P (r=0.091, $p=0.442$; r=0.207, $p=0.084$, respectively). On the contrary, there were significant correlations between the expression of SRp30c and GRβ, GR-P (r=0.238, $p=0.047$; r=0.382, $p=0.001$, respectively), and no significant correlation was found between SRp30c and GRα (r=0.169, $p=0.153$). In addition, the expression of SRp40 and SRp30c was not associated with GRγ expression shown in Figure 2 (r=0.034, $p=0.791$; r=-0.111, $p=0.426$, respectively).

Correlation between GRα expression and SLEDAI scores

GRα expression in low activity (SLEDAI score<10) group was significantly higher than that in high activity (SLEDAI score ≥10; 12.13 *vs*. 8.44, $p=0.011$; Table V). The negative correlation between GRα transcript and SLEDAI score was found (r=-0.289, $p=0.021$). GRα protein expression was also significantly negatively correlated with SLEDAI score. (r=-0.422, $p=0.032$). GRβ, GRγ and GR-P showed no significant correlation with SLEDAI score in SLE patients (Fig. 4).

GR mRNA expression in subgroups of SLE patients

According to age, proteinuria, ds-DNA qualitation, ANA titer, complement level and PLT quantitation, 70 patients were further subdivided into six groups. The expressions of four GR isoforms in subdivided groups were listed in Table V.

For equal cases in two groups, 35 patients were more than 42 year-old at diagnosis as older group, the others as younger group. GR-P expression was significantly higher in older group than that in younger group (6.75 *vs*. 4.22, $p=0.022$). In addition, GR-P expression of patients with proteinuria was significantly lower than that of patients without proteinuria (3.30 *vs*. 6.13, $p=0.018$). GRα expression of patients with positive anti-dsDNA was significantly lower than that of patients with negative

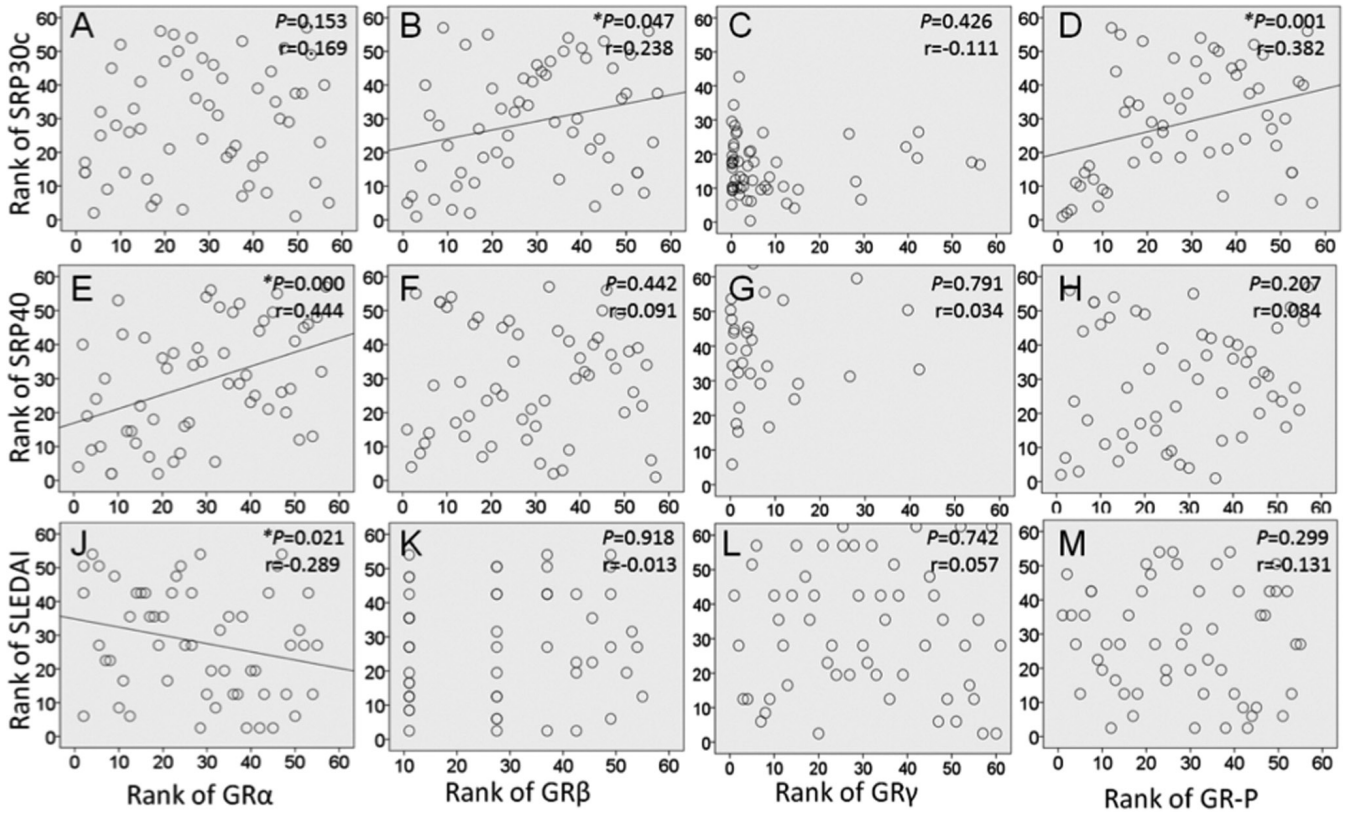

Fig. 4. Correlation analyses. (**A-D**) Rank correlation between GR transcripts (GRα, GRβ, GRγ, and GR-P) and SRp30c mRNA expression, respectively. (**E-H**) Rank correlation between GR transcripts (GRα, GRβ, GRγ, and GR-P) and SRp40 mRNA expression, respectively. (J-M) Rank correlation between GR transcripts (GRα, GRβ, GRγ, and GR-P) and SLEDAI score, respectively. *$p<0.05$ was considered significant.

Table V. The different GR transcripts (GRα, GRβ, GRγ, and GR-P) in subgroups of in SLE patients.

	NO.	GRα mRNA		GRβ mRNA		GRγ mRNA		GR-P mRNA	
		Expression	*p*-value	Expression[#]	*p*-value	Expression	*p*-value	Expression	*p*-value
Age									
<42	35	10.67(8.09-15.20)	0.672	0.67(0.29-2.81)	0.747	2.67(0.49-14.28)	0.330	4.22(1.27-6.57)	0.022*
>42	35	9.82(3.70-19.00)		0.89(0.22-2.08)		4.57(1.09-40.26)		6.75(2.12-14.04)	
SLEDAI scores									
≤10	32	12.13(8.14-23.39)	0.011*	0.63(0.31-2.98)	0.596	3.91(0.57-11.36)	0.783	5.62(2.12-10.73)	0.402
>10	38	8.44(4.13-11.63)		0.73(0.14-2.29)		3.99(0.45-25.73)		4.39(1.67-9.23)	
Proteinuria									
without proteinuria	46	10.64(3.44-17.35)	0.422	1.07(0.27-2.76)	0.325	2.74(0.49-14.28)	0.940	6.13(2.49-13.05)	0.018*
with proteinuria	24	10.25(6.36-25.78)		0.53(0.13-2.13)		4.39(0.57-23.76)		3.30(1.07-6.35)	
Anti-dsDNA									
(-)	30	12.32(6.68-25.00)	0.037*	1.41(0.39-4.13)	0.072	4.25(1.71-42.10)	0.563	3.85(1.47-12.70)	0.597
(+)	40	9.82(3.20-11.45)		0.52(0.18-1.34)		3.83(0.51-14.48)		4.39(2.05-6.50)	
ANA titer									
<1000	12	17.51(9.23-47.77)	0.030*	0.78(0.05-2.78)	0.763	3.76(0.97-22.29)	0.780	4.20(2.91-13.39)	0.779
>1000	58	10.67(5.59-18.27)		0.68(0.27-2.56)		3.99(0.51-20.87)		4.65(1.87-9.04)	
Complement level									
Normal	35	10.97(6.12-21.85)	0.429	1.45(0.46-3.39)	0.002*	5.45(0.42-23.27)	0.743	4.79(1.78-11.29)	0.522
Low C3/C4	35	10.49(6.64-14.36)		0.32(0.06-1.32)		3.57(0.51-10.17)		3.85(1.73-8.90)	
PLT quantification									
Normal	47	10.67(6.10-16.80)	0.694	1.32(0.30-2.97)	0.038*	2.74(0.51-18.36)	0.933	4.60(1.29-8.67)	0.591
Low PLT	23	9.29(4.42-19.37)		0.38(0.05-1.11)		4.30(1.59-26.64)		6.13(2.23-13.07)	

SLEDAI: systemic lupus erythematosus disease activity index; Anti-dsDNA: anti-double-stranded DNA; ANA: anti-nuclear antibodies; PLT: platelet; [#]GRβ mRAN expression $\times 10^2$. *$p<0.05$ was considered significant.

anti-dsDNA (9.82 *vs*. 12.32, $p=0.037$). GRα expression of patients with high ANA titer (>1:1000) was significantly lower than that of patients with low ANA titer (≤1:1000) (10.67 *vs*. 17.51, $p=0.030$). No significant difference in the mRNA expression of the GRγ isoforms was observed in any subgroups.

Discussion

The expression of GR isoforms has been linked to GC resistance in various diseases treated with GC, such as in acute lymphocytic leukemia (ALL), multiple myeloma and immune thrombocytopenia (9, 20-24). However, existing data were conflicting in these diseases, and little information regarding SLE is available. This is the first investigation of GRα, GRβ, GRγ, and GR-P expression patterns in 70 patients with SLE and the association of GR isoforms with the response to GC and disease activity. Additionally, we demonstrated the positive correlation between GRα and SRp40.

GRα was the predominant isoform, accounting for up to 51.85% of total GR, lower than that in controls (68.85%). GRα expression in GCR group was even lower than that in GCS group before GC treatment. Previous studies have demonstrated that decreased expression of GRα may be associated with GC resistance in ALL (21), adult immune thrombocytopenia (22) and atopic dermatitis (23). We found GRα expression in high-activity group was significantly lower than that in low-activity group. In addition, GRα expression negatively correlated to anti-dsDNA and ANA titer. These findings indicated that GRα was the predominant isoform regulating the response to GC and lower GRα expression might be related to high disease activity and GC resistance in patients with SLE. In other words, the level of GRα isoform could predict the state of illness and the patient's sensitivity to GC. This is supported by a previous study (12). For patients with decreased expression of GRα, high dose of GC may not provide good therapeutic effect, and other immunosuppressive agents should be added to suppress inflammation.

In addition, we found the marked positive correlation between SRp40 and GRα expression in SLE patients. SRp40 expression in GCR group was significant lower than GCS group. This is the first *in vivo* evidence SRp40 may regulate alternative splicing of GR, which has been reported previously only in HeLa cells (15). Based on these results, SRp40 may contribute to the pathogenesis of GCR by regulating GRα expression in SLE patients. SRp30 has been reported to direct alternative splicing of GR pre-mRNA to GRβ in neutrophils (14), and bombesin attenuates pre-mRNA splicing of GR by regulating SRp30c expression in prostate cancer cells (13). In addition, SRp20, SRp30c, and SRp40 regulate GR splicing and the response to GC in the trabecular meshwork (24). Because the pathogenetic mechanisms of malignancies and inflammatory diseases differ, the expression of splicing factors may be heterogeneous, even within a single disease. In this study, SRp40 was shown to be associated with GRα transcript in PBMCs of SLE patients. The expression of SRp40 was also significantly increasing after GC treatment in GCS patients, as much as GRα, which implied the alternative splicing role of SRp40 might be involved in the mechanism of GR autoupregulation, especially GRα. This will

likely facilitate development of novel therapies that aim to improve the response to GC treatment and further *in vitro* studies are needed.

Our results implied GC treatment could cause an auto-upregulation of GR, consistent with a previous study in T-cell and pre-B-cell acute lymphoblastic leukemia (25). It was distinct that the auto-upregulation role of GC on different GR isoforms in GCS and GCR patients. In GCS patients, the GRα was significantly increased after GC treatment. However, the GR-P was significantly increased in GCR group after GC treatment. Our data demonstrated that GR-P isoform might be implicated in the development of resistance.

The GR-P isoform was firstly described in tumor cells from a GCR multiple myeloma patient (26). This truncated isoform lacks a large part of the ligand-binding domain, including the domains for silencing of GR in the absence of hormone and transcriptional activation. Beatriz Sánchez-Vega *et al*. reported sequentially GR-P was the predominant receptor variant in a late resistant multiple myeloma cell line, while GRα was virtually undetectable (9). In our study, GR-P expression was higher in SLE patients than that in controls. Moreover, the GRα/GR-P ratio in patients was significantly lower than that in controls. Although no difference of GR-P expression was found between GCS group and GCR group before GC treatment, the GR-P was significant higher in GCR group after four-week GC treatment. These results suggest that high expression of GR-P isoforms might interfere with GRα expression and thereby the response to GC.

In this present study, the expression of GRβ in SLE patients was minimal and even lower than that in controls. It is questionable whether this isoform is of biological relevance in the response to GC. Elevated GRβ was reported to contribute to GC resistance (27-30), such as ALL, asthma, inflammatory bowel disease. The molecular basis might be GRβ interfering with GRα through formation of non-functional dimers with GRα or by competing with GRα for binding to NF-κB and AP-1. However, GRβ was expressed 1000- and 1500-fold less than GRα in GCS and GCR patients. The expression of GRβ did not increase in GCR patients with SLE. These findings suggested an indirect or minor role of GRβ in GC resistance of SLE patients. This is supported by several studies which demonstrated that GRβ may not participate in GC resistance (20, 32, 33).

As shown in Table V, GR-P expression in patients with proteinuria was significantly lower than in those without proteinuria. GRβ expression of patients with low complement levels (C3 or/and C4) was significantly lower than that of those with normal complement levels. Also, GRβ expression of patients with thrombocytopenia was significantly lower than that of those with normal thrombocyte levels. The imbalance of GRβ and GR-P expression might provide insight into the pathogenesis and progression of SLE.

Recently, existing data of GRγ isoform are scarce. High GRγ expressionis has been reported to be associated with GC resistance in small cell lung carcinoma cells, corticotroph adenomas, and childhood ALL (33, 34). However, Sánchez-Vega reported no correlation between GRγ and GC resistance in multiple myeloma cell lines (9). In our study, GRγ expression showed no difference between GCS and GCR groups or between high activity and low activity groups. Anamino acid insertion occurs in all C-terminal splice variations (GRα, GRβ and GR-P), thus GRγ is unlikely to influence GC sensitivity. However, it is possible that GRγ binds to GC and DNA with a capacity similar to GRα, but they regulate the transcription of a different subset of target genes (35). Therefore, further studies of the function of GRγ isoform are required.

Taken together, our findings suggest that the expression pattern in SLE patients was different from controls, which might provide insight into the pathogenesis of SLE. GRα was the predominant isoform and could help to predict disease activity and the sensitivity to GC in patients with SLE. In addition, SRp40 may be associated with the alternative splicing of GR and upregulate GRα transcript; this warrants further investigation.

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