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Identification of autoantibodies associated with systemic lupus erythematosus☆

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of antinuclear antibodies. We performed serological analysis of cDNA expression library (SEREX) to identify autoantibodies associated with SLE. The screening of three different cDNA expression libraries with pooled sera of patients with SLE yielded 11 independent clones that reacted with pooled sera of patients with SLE. In this screening, autoantibodies to poly(ADP-ribose) polymerase (PARP), U1snRNP, and galectin-3 were prevalent in the sera of patients with SLE (26/68, 25/68, 12/63, respectively). The frequency of autoantibody to PARP was significantly higher in SLE than that of healthy donors (0/76) (38.2% vs 0%, $p < 0.00001$). The autoantibody to PARP was infrequently detected in the serum of patients with RA (1/50). However, autoantibody to PARP was not found in the sera of patients with other rheumatic diseases including Sjogren's syndrome (0/19), systemic sclerosis (0/18), and polymyositis/myositis (0/37). The frequency of autoantibody to human galectin-3 (12/63) was significantly higher in SLE than that of healthy donors (0/56) (19% vs 0%, $p = 0.0006$). Autoantibody to galectin-3 was not found in the sera of patients with rheumatoid arthritis (0/50), Sjogren's syndrome (0/18), and systemic sclerosis (0/19). Interestingly, autoantibody to galectin-3 was also prevalent in the sera of patients with polymyositis/dermatomyositis (16/37, 43.2%). Further functional characterization of these autoantibodies would be necessary to determine their value as diagnostic markers or to define clinical subsets of patients with SLE. Statistical analysis revealed that the presence of autoantibody to PARP was inversely related with pleurisy, and the presence of autoantibody to galectin-3 related with renal disease.

Keywords: cDNA expression library; PARP; SEREX; Systemic lupus erythematosus

Systemic lupus erythematous (SLE) is an autoimmune disease that involves multiple organ systems, and associated with autoantibodies directed against cell nuclei, membrane, and cytoplasm [1]. Autoantibodies to U1-RNP and Sm protein are known to be associated with SLE [2–7]. Studies of populations, segregation of disease in families, and twin concordance rates suggest that genetic factors influence on predisposition to SLE. The genetic basis of SLE is rather complex, with an unknown mode of non-mendelian inheritance. It was reported that systemic lupus erythematosus was associated with reduction of DNase 1 activities in the sera of patients with SLE compared with healthy controls [8]. It is believed that the disturbed removal of DNA from nuclear antigen is responsible for SLE. This suggests that lack of DNase 1 activity is closely associated with the pathogenesis of SLE. Systemic lupus erythematosus is controlled by multigenes, including FcγRIIa, FcγRIIIa, IL-6, IL-10,

☆ *Abbreviations:* SEREX, serological analysis of cDNA expression library; SLE, systemic lupus erythematosus; PARP, poly(ADP-ribose) polymerase; RA, rheumatoid arthritis; SSc, systemic sclerosis.

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Bcl-2, T-cell receptor alpha, and beta chain. Polymorphisms of these genes reportedly are associated with SLE [9–13]. The lack of complement results in SLE [14]. The SLE is associated with abnormalities of cells undergoing apoptosis [15,16]. The overexpression of anti-apoptotic protein, bcl-2, was associated with SLE [17–19]. Molecular alterations of cellular molecules, during apoptosis, such as oxidation and phosphorylation resulted in autoantibody production [20]. Autoimmune diseases are characterized by the presence of antinuclear autoantibodies. There has not been systematic study of identifying autoantibodies associated with specific autoimmune diseases. The pathogenic role or the relationship to underlying etiological events has not been well studied for most of these autoantibodies. The early and accurate diagnosis of SLE is critical for its treatment. For identification of autoantibodies specifically associated with SLE, serological analysis of cDNA expression library (SEREX) was employed. SEREX combines both molecular and immunological approach to identify molecules associated with various diseases. Since its inception in 1990, SEREX has been extensively used to identify molecules associated with cancers. The advantage of using SEREX is that it enables us to identify molecules, on a genomic scale, that are specifically associated with disease of choice. Here, we report the identification of autoantibodies associated with SLE and analyzed clinical manifestations according to the autoantibodies.

Materials and methods

Patients. Sixty-eight patients with SLE were enrolled at the Rheumatology Clinic, Department of Medicine at Seoul National University. The patients' age was 34.7 ± 11.7 years old (means $\pm$ SD) with 96% being female. The disease duration was 64.4 ± 45.9 months. The relationship between the presence of autoantibodies and clinical subsets of SLE was described in Table 3.

Samples. Poly(A)$^+$ RNA from human kidney tissue was obtained from Clontech (Palo Alto, CA, USA). Poly(A)$^+$ RNA from human testis was obtained from Bioneer (Chungwon, Korea). Lung cancer cell line used in this study was obtained from Korea Cell Line Bank (Seoul, Korea). Sera of patients with various rheumatism-related diseases including RA (50), Sjogren's syndrome (19), systemic sclerosis (41), myositis (37) were stored at −80 °C until use. Seventy-six healthy individuals were enrolled as control. All primers used in this study were obtained from Bioneer (Chungwon, Korea). NBT and BCIP were obtained from Sigma Chemical. Anti-human IgG Ab conjugated with alkaline phosphatase was obtained from KPL (Gaithersburg, MD).

Construction of cDNA expression libraries. A total of 5 μg of human testis poly(A)$^+$ RNA (Bioneer, Chungwon, Korea) or NCI H1703 lung cancer cell line or human kidney was used for the construction of cDNA expression library. Construction of cDNA expression library was carried out according to the instruction manual provided by the manufacturer (Stratagene, La Jolla, CA). Briefly, 5 μg of poly(A)$^+$ RNA was used to synthesize double stranded cDNA. To this, *Eco*RI adapter was added, and thus obtained DNA was digested with *Xho*I to yield unidirectional cDNA library. This cDNA library was ligated to λ-ZAP express vector. Each library consisted of approximately 2×10^6 primary recombinants on average and 5×10^5 of them were used for immunoscreening.

Immunoscreening of the cDNA expression library. cDNA expression libraries were screened with pooled sera of 10 patients with SLE. Pooled sera of 10 patients with SLE were preadsorbed to reduce the background sera activity. Briefly, *Escherischia coli*/phage lysate was diluted 1:10 in 1× wash solution (KPL). Nitrocellulose membrane was added to this diluted lysate and incubation continued for 30 min. Membrane was incubated with BSA blocking solution for 1 h. After blocking, membrane was washed with 1× wash solution. Membrane was incubated with diluted (1:5) sera of patients for 1 h. After incubation, membrane was removed. Subsequently obtained preadsorbed pooled sera of patients were used for immunoscreening. Immunoscreening procedure was done according to the instruction manual provided by the manufacturer (Stratagene). Immunoreactive clones were tested for reactivity against diluted sera (1:250) from individual patient with SLE and normal healthy individuals by using same screening strategy.

Sequencing of reactive clones. Sera-reactive clones were in vivo excised to plasmid form. Plasmid DNA was purified by commercial kit (Qiagen, Westburg, Leusden, The Netherlands). Sequences were obtained from phage clones. Sequencing was done by ABI PRISM 310 Genetic Analyzer Automated Sequencer (Perkin–Elmer, Foster City, CA).

Screening of allogeneic sera. Seroreactivity of patient for PARP was determined according to the standard procedures [21]. First, λ-ZAP phages without insert were mixed with test clone and served as negative control. Serum of patient was used at a dilution of 1:250. Assay was scored positive when test clones are clearly distinguishable from control clones.

Results

Identification of immunoreactive clones

To identify autoantibodies present in the sera of patients with SLE, cDNA expression libraries were constructed. These cDNA expression libraries were made from testis, kidney, and human lung cancer cell line NCI H1703. The titer of each of these cDNA expression libraries was on average 2×10^6 CFU/ml. Each of these cDNA expression libraries was screened with pooled sera (1:250 dilutions) of 10 patients with SLE. A total of 23 clones were found to react with pooled sera of patients with SLE. After in vivo excision, identities of these clones were determined by sequencing. There were 11 independent clones that reacted with pooled sera of 10 patients with SLE. Table 1 shows list of such genes. All these clones in Table 1 have significant homologies with known genes. Among these nucleolar autoantigen (55 kDa), and PARP are represented by seven, and five overlapping clones, respectively. PARP is activated by DNA strand breaks [22]. Human PARP (113 kDa) is highly conserved and abundant protein. PARP is a DNA-dependent enzyme that catalyzes ADP-ribosylation of nuclear acceptor proteins by using NAD+ as a substrate. Many data suggest that PARP is important for maintaining genomic integrity [23]. U1snRNP (small nuclear ribonucleoprotein) identified in this screen is known to be associated with SLE. The clones identified in this study are involved in diverse cellular functions

Table 1
Genes identified by SEREX of cDNA expression libraries

Designation	No. clone	Accession No.	Length (kb)	Gene	Source
S1	1	XM012219	1.7	Gene similar to phosphoglycerate mutase	H1703
S2	2	XM028790	2.9	Homolgous to Zfp161 in mouse	H1703
S3	5	NM001618	3.9	Poly(ADP-ribose) polymerase	H1703
S4	7	BC001047	1.9	Nucleolar autoantigen (55 kDa)	H1703
S5	1	NM004703	3	Rabaptin-5	H1703
S6	1	XM047728	1.9	H2A histone family, member Y	H1703
S7	1	M95724	3.1	Centromere autoantigen C	H1703
S9	2	M22636	1.7	U1 small nuclear ribonucleoprotein 70 kDa protein	H1703
S11	3	NM002520	0.88	Nucleophosmin	Testis
S14	1	XM005953	5.7	Transcription factor-like 1	Kidney
S15	1	XM051637	0.9	Galectin-3, galactose binding	Kidney

signaling, DNA repair, and transcription. Galectin-3 is a galactose- and IgE-binding protein secreted by inflammatory macrophages. It plays a potential role in macrophage extracellular matrix interaction. Deregulated expression of galectin-3 is associated with hepatocellular carcinoma [24]. Rabaptin-5 binds to GTP-ase and is involved in membrane docking or fusion [25]. Nucleophosmin is a nucleolar phosphoprotein that is more abundant in tumor cells than in normal resting cells. It was shown to be a substrate of cyclinE/CDK2 [26]. We have not detected autoantibody to dsDNA or Sm protein, well-known markers of SLE. This might be due to the method we used in this study. Detection of autoantibodies by SEREX gives unbiased selection unlike Western blot or immunofluorescence staining.

Frequencies of autoantibodies in the sera of patients with various autoimmune diseases

Next, we determined frequencies of IgG autoantibodies against the autoantigens detected in this study. We limited our analysis to IgG antibodies by using 1:250 diluted sera of patients with SLE for the screening of cDNA expression library and by using anti-human IgG antibody conjugated with alkaline phosphatase. To determine frequency of each autoantibody, λ-ZAP vector without insert was mixed with test clone. These coplated plaques were reacted with sera of patients with various rheumatic diseases including SLE and the detection of immunoreactivity was carried out by color reaction using NBT and BCIP. Fig. 1 shows reactivity of patient with SLE for PARP. Table 2 shows result of screening of allogenic sera for reactivity with autoantigens detected by pooled sera of patients with SLE. We found that PARP (26/68) and U1snRNP (25/68) showed the highest frequencies in the sera of patients with SLE. U1snRNP was also present in the sera of patients with polymyositis/dermatomyositis (2/37) and systemic sclerosis (3/18). U1snRNP was shown to interact with phosphorylated serine/arginine (SR) splicing factor in cells undergoing apoptosis [27]. This suggests that SLE is related to regulation of alternative splicing of apoptotic effector molecules. Many of the autoantibodies in Table 2 showed specificity for sera of patients with SLE. For example, autoantibody to PARP did not react with sera of patients with Sjogren's syndrome or SSc or myositis while it showed low frequency in the sera of patients with RA (2%). This suggests that PARP is a valuable serologic marker for diagnosis of SLE. Most of the clones in Table 2 did not react with sera of healthy donors. This suggests that these autoantibodies are specific for autoimmune diseases. Autoantibody to human galectin-3 was present in 12/63 sera of patients with SLE. Interestingly, it also was present in 16/37 sera of patients with polymyositis/dermatomyositis. In this screen, we did not detect autoantibody against Sm protein, which is a well-known marker of SLE. This suggests racial variation in frequency of autoantibodies associated with SLE. We checked whether there was a

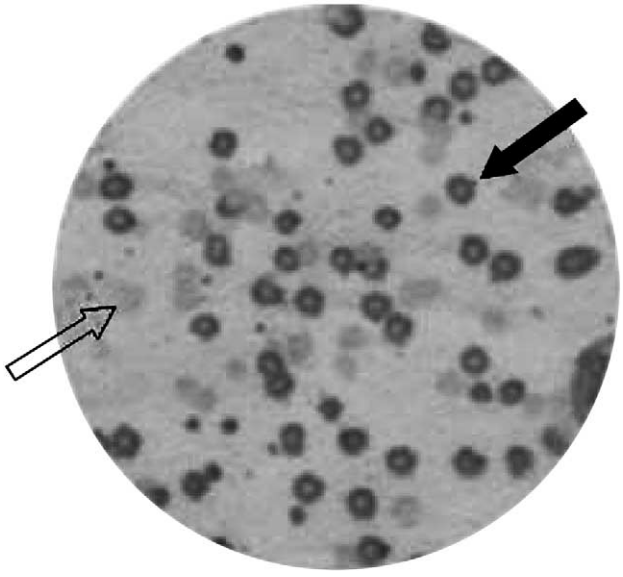

Fig. 1. Seroreactivity of patient with SLE for PARP. λ-ZAP phages without insert were mixed with test clones and served as negative control. Assays were scored positive only when test clones were clearly distinguishable from control phages. Bold arrow indicates test clone and blank arrow indicates control clone.

Table 2
Reactivity of autoantigens with IgG autoantibodies in the sera of patients with rheumatic diseases or healthy donors

Designation	SLE	RA	Myositis	SSc	Sjogren	Normal
S1	3/68	0/50	0/37	1/18	0/19	0/76
S2	5/68	0/50	0/37	0/18	0/19	0/76
S3	26/68	1/50	0/37	0/18	0/19	0/76
S4	2/68	3/50	1/37	0/18	0/19	1/76
S5	1/68	0/50	0/37	0/18	0/19	0/76
S6	4/68	0/50	0/37	0/15	0/19	0/76
S7	5/68	0/50	0/37	12/18	0/19	0/76
S9	25/68	0/50	2/37	3/18	0/19	0/76
S11	4/57	0/50	0/37	1/18	0/19	1/56
S14	14/68	1/50	0/37	0/18	0/19	0/56
S15	12/63	0/50	16/37	0/18	0/19	0/56

Table 3
Clinical characteristics of the patients with SLE

	Reactivity with PARP			Reactivity with galectin-3		
	Positive ($n = 26$)	Negative ($n = 42$)	P-value	Positive ($n = 12$)	Negative ($n = 53$)	P-value
Age (years, means ± SD)	30.7 ± 9.8	37.3 ± 12.2	0.02	39.1 ± 13.7	33.5 ± 11.3	0.14
Disease duration (months)	58.2 ± 48.7	68.3 ± 44.3	0.38	59.3 ± 38.8	67.5 ± 48.9	0.59
Malar rash	15/26	21/42	0.54	4/12	30/51	0.17
Discoid lesion	1/26	1/42	1.0	0/12	2/51	1.0
Oral ulcer	10/26	16/42	0.98	3/12	21/51	0.35
Photosensitivity	9/26	8/42	0.15	2/12	14/51	0.71
Arthritis	18/26	29/42	0.99	6/12	38/51	0.1
Pleurisy	3/26	17/42	0.01	4/12	13/51	0.72
Percarditis	1/26	8/42	0.13	1/12	6/45	1.0
Hemolytic anemia	2/26	11/42	0.11	1/12	12/51	0.43
Leukopenia	15/26	25/42	0.88	9/12	28/51	0.33
Thrombocytopenia	3/26	13/42	0.06	3/12	11/51	1.0
Renal disease	14/26	18/42	0.38	9/12	19/51	0.02
CNS disorder	1/25	7/42	0.14	0/12	6/45	0.58
Anti-ds DNA Ab	24/26	36/40	0.75	12/12	45/49	0.58
Anti-Sm Ab	4/10	4/15	0.48	0/7	8/18	0.06

Chi-square test was used in statistical test.

relationship between the presence of autoantibodies to PARP and clinical subsets of SLE. As shown in Table 3, the presence of autoantibodies to PARP was not related with the presence of autoantibodies to dsDNA or Sm protein. However, the presence of autoantibodies to PARP was inversely related with pleurisy ($p = 0.01$). We found that autoantibody to galectin-3 was related with renal disease. Throughout this study, we identified autoantibodies associated with SLE and studied the relationship between autoantibodies and clinical manifestations.

Discussion

Systemic lupus erythematosus is an autoimmune disease resulting from the interactions of multiple genes and environmental factors. SLE is associated with impaired humoral and cellular immune responses. It is characterized by autoantibodies that recognize autoantigens displayed on the surface of cells undergoing apoptosis. Lack of information on autoantiodies associated with SLE makes it difficult to understand the mechanism of pathogenesis leading to SLE, let alone design of therapy. The SEREX approach has been used to identify autoantibodies associated with various cancers [28,29]. The theoretical advantage of SEREX is unbiased selection of target autoantigens (autoantibodies). Recently, the SEREX approach was used to identify autoantibodies associated with autoimmune diseases such as polymyalgia rheumatica [21]. In this study, we carried out SEREX analysis to identify autoantibodies associated with SLE. By screening three different cDNA expression libraries with pooled sera of patients with SLE, we identified clones that reacted with sera of patients with SLE but not with those of healthy donors. In this study, we constructed cDNA expression library from testis. Testis is characterized by genome-wide hypomethylation [28]. This ensures the expression of a wide spectrum of the human genes. In this screening,

clones encoding for PARP (26/68) and U1snRNP (25/68) protein showed highest frequencies in the sera of patients with SLE. It was interesting to note that clone encoding for human galectin-3 reacted with sera of patients with myositis (16/37) and SLE (12/63). This suggests that clinically distinct autoimmune diseases might be controlled by the same genes. Many of the autoantigens identified in this screen represent nuclear antigens (ZFP161, nucleolar autoantigen 55 kDa, centromere autoantigen C, and U1 small nuclear ribonucleoprotein 70 kDa protein). We carried out screening of allogenic sera three times, and the frequency of each autoantibody shown in Table 2 was consistent. By linkage analysis, PARP was shown to confer susceptibility to SLE [30]. The altered metabolism of PARP was found in patients with SLE [31]. PARP did not react with sera of patients with various rheumatic diseases including systemic sclerosis, Sjogren's syndrome, and polymyositis/dermatomyositis while it showed low frequency (1/68) in the sera of patients with rheumatoid arthritis. The presence of autoantibody against PARP is interesting because this enzyme is involved in DNA repair and apoptosis. Previously, it was reported that autoantibody against PARP was present in the sera of patients with autoimmune rheumatic and bowel diseases [32]. In this report, patients' sera with various rheumatic diseases were tested by ELISA with peptides corresponding to the zinc finger domains of PARP, and more than 50% of the sera of patients with SLE or MCTD (mixed connective tissue disease) showed positive reaction. It was also reported that autoantibodies against PARP were found in rheumatic diseases including Sjogren's syndrome [33]. In other report, autoantibodies against PARP were found in the sera of patients with RA, Sjogren's syndrome, scleroderma, sarcoidosis, and cryptogenic fibrosing alveolitis [34]. However, we did not find autoantibodies against PARP in the sera of patients with Sjogren's syndrome or scleroderma. This discrepancy might be due to the methods used for screening or racial variations. The high prevalence of PARP in the sera of patients with SLE gives glimpse of the mechanism of pathogenesis leading to SLE. PARP, just like other targets for autoantibodies (U1snRNP, DNA topoisomerase, and lamins), is cleaved during the apoptosis. PARP is cleaved into 24 and 89 kDa protein fragments. It would be interesting to check whether autoantibodies react better with fragments of PARP. In this study, we identified several autoantibodies associated with systemic lupus erythematosus. Among these, three autoantibodies (PARP, U1snRNP, and galectin-3) merit further analysis. These autoantibodies are highly prevalent in the sera of patients with SLE. Serial analysis of sera taken at different stages during the course of disease is necessary for determining the value of these autoantibodies as markers for disease activity and the definition of clinically distinct subgroups of patients. Although it is highly sensitive, SEREX is not suitable for analysis of large series of sera because of extensive serum adsorptions. ELISA would be more suitable for analysis of large series of sera of patients with SLE. It would be necessary to develop monoclonal antibodies against these autoantigens identified in this study. This will make it easier for the screening of large number of sera of patients with autoimmune diseases and the determination of correlation of serum autoantibody titers with clinical subsets of the disease of interest. Also, identification of more autoantibodies would be necessary for better understanding of mechanisms leading to SLE.

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