Detection of epitopes in systemic lupus erythematosus using peptide microarray

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Abstract. Systemic lupus erythematosus (SLE) is a common autoimmune disease, which features the secretion of antibodies directed against autoantigens *in vivo*. In the present study, a peptide microarray was developed to detect the epitopes recognized by autoantibodies in patients with SLE for an effective method of diagnosis. SLE-associated epitopes in 14 autoantigens were predicted using the antigenic epitope prediction software DNA star. Peptides were synthesized based on the predicted antigenic epitopes and immobilized on a slide surface and developed into a peptide microarray. Using this peptide microarray the autoantibodies in 120 patients with SLE and 110 healthy subjects were detected. A total of 73 potential antigenic epitopes in 14 autoantigens were predicted and screened. The peptide microarray based on the 73 epitopes was used to detect the autoantibodies in patients with SLE. A total of 14 epitopes with potential diagnostic values were screened out. The sensitivity and specificity of the 14 epitopes for the diagnosis of SLE were 71.6 and 85.8%, respectively. An optimal set of epitopes for SLE diagnosis was obtained. As individual patients had a specific autoantibody spectrum it was possible to detect autoantibodies in SLE and perform the diagnosis of SLE using the peptide microarray.

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Introduction

Systemic lupus erythematosus (SLE) is one of the common autoimmune diseases, mostly occurs in Asian women. The main cause of this disease is that the autoimmune system attacks its own tissues, resulting in tissue damage (1-3). The typical symptoms of SEL include specific lesions with butterfly erythema, subacute skin lupus erythematosus and discoid erythema, and non-specific lesions with light allergy, hair loss, mouth ulcers, skin vasculitis (purpura), pigmentation or depigmentation, livedo reticularis, Raynaud's phenomenon, urticaria-like rash and rare lupus lipid film Inflammation or deep lupus and bullous lupus erythematosus (4). Numerous studies have shown that the abnormalities in genetic, endocrine, infection, immune and some environmental factors are associated with the incidence of SLE (3,5). However, the pathogenesis of SLE has not yet been elucidated.

The main pathological manifestation of autoimmune diseases is that the patient produces a high level of autoantibodies to identify different autoantigens. It was demonstrated that autoantibodies associated to several autoantigens and involved in SLE including anti-double stranded antibody (anti-dsDNA antibody), anti-nuclear antibody (ANA), anti-soluble antigen antibodies (anti-ENA antibody) including anti-Jo-1, anti-U1RNP antibody (anti-nRNP antibody), anti-ribosomal P antibody (anti-rRNR antibody), anti-Scl-70, anti-Sm antibody, anti-SSA/Ro antibody and anti-SSB/La antibody, anti-nucleosome antibodies, and anti-phospholipid antibodies (6-12). The use of autoantigens to detect autoantibodies is an important technique for the diagnosis of autoimmune diseases. However, the intact autoantigen profiling is difficult to obtain and purify, and the stability of the antigens is poor. In order to avoid the high production cost and poor stability of autoantigens, some attempts have been made to detect autoantibodies by alternative. To achieve the goal of diagnosis, some studies have been attempted to detect the autoantibodies in SLE using protein chip (13-17). However, the protein chip is also limited by the difficult in expression and purification the intact antigens. Researchers try to find a more desirable alternative such as peptide microarray.

In recent years, peptide microarray has been developed rapidly (18-20). Peptide microarrays integrate many peptide active molecules on a very small surface area, so as to detect the expression and function of different biomolecules (21). Peptide microarrays has advantages in simple and fast, high-throughput and accuracy, and low-cost, compared with traditional protein chips. Using peptide microarrays, the diagnostic accuracy of lung cancer indicators was 93.1% (22). The diagnostic accuracy of plasma in lung cancer patients using peptide microarrays was also reached 92% (23,24). It was also found that peptide microarrays are useful in the detection of p53 autoantibodies, and have potential application value in head and neck cancer patients (25,26). However, thus far, the use of the peptide microarray technique in diagnosis of autoimmune diseases, particularly the early diagnosis of SLE have not been well studied yet.

In the present study, we predicted the SLE-related epitopes in 14 autoantigens using the antigenic epitope prediction software DNA star, and designed the peptide microarray for SLE detection. Then, the autoantibodies in 120 SLE patients and 110 healthy subjects were analyzed and an optimal set of epitopes were screened out. The sensitivity and specificity of the optimal set of epitopes in diagnosis of SLE were evaluated.

Materials and methods

Patients. Samples from 120 patients with SLE (including 15 males and 105 females, average age was 34.5 years) who underwent SLE treatment in the Southern China Hospital were collected. Samples from 110 healthy volunteers (including 15 males and 95 females, average age was 30.2 years) were also collected. There were no significant differences in the sex and age between the SLE patients and healthy subjects. The diagnostic criteria for SLE were acute or subacute cutaneous lupus manifestations, manifestations of chronic cutaneous lupus, arthritis, serositis, renal disorder, blood-hematologic diseases, oral or nasopharyngeal ulcers, immunological disorder, and alopecia. The diagnosis of SLE should include four of the above criteria, one clinical criterion and one immunological criterion. The immunological criteria were as follows: i) higher titers of ANA than the laboratory reference standard; ii) higher titers of anti-dsDNA than the laboratory reference standard; iii) positive anti-Sm antibody; iv) anti-phospholipid antibodies (positive anti-lupus anticoagulant/false positive serological test for syphilis/anticardiolipin antibody at twice the normal level or increased anti-B2GPI above titer); v) decreased level of complement proteins (C3, C4 and CH50); and vi) no hemolytic anemia, but Coombs test is positive. Renal disorder was confirmed as lupus nephritis (diagnosed by lupus nephritis with ANA or anti-dsDNA-positive). The exclusion criteria included patients with viral hepatitis, tuberculosis or SLE combined with other primary organ diseases. In the present study, we did not exclude the patients with other infections including influenza, EBV and HIV, because those infections in China are very low. All participants signed informed consent. The present study was approved by the Ethics Committee of Southern China Hospital.

Epitopes prediction by DNA star software. The DNA star (NIAID, USA) was used to predict the epitopes on 14 autoantigens. In the DNA STAR online analysis system, the parameters 'Epitope' and 'assay' were set as 'any epitopes' and 'all'. Followed by searching and querying, the parameters 'MHC Restriction', 'Host', and 'Disease' were set as 'any MHC Restriction', 'Humans', and 'Autoimmune Disease' for each antigen indicator. After further narrowed the search range by the peptide information and the linked literatures, we obtained the peptide sequences associated with SLE.

Peptide microarray preparation. All peptides (purity >98%) were synthesized by Sangon Biotech, Shanghai, China. The peptide indicators were prepared using a biochip spotting instrument (AD3200; BioDot, Irvine, CA, USA). The peptide microarray was prepared in a clean slide. After the peptide microarray was soaked in a 5% ammonia silane anhydrous ethanol solution for 30 min. After washed 3 times with anhydrous ethanol and deionized water for 5 min, the peptide microarray was air-dried and soaked in a phosphate-buffer solution (PBS) containing 2.5% glutaraldehyde for 30 min. After washes with anhydrous ethanol and deionized water for 5 min, three times, the peptide microarray (384-well plate) was prepared for peptide loading. The peptide was well-diluted to 0.5 mg/ml in PBS and loaded into the peptide microarray at 20 μl/well. After centrifuged 2 min at 2,000 rpm, the peptide microarrays were placed overnight, stored in a slide box, and sealed within hermetic bags at 4˚C (or -20˚C with humidity <50% for long-term storage). The bags were exposed to room temperature for 3-4 h before pick out the peptide microarray.

Diagnosis of SLE by peptide microarray. For screening, the peptide microarray was blocked with 0.1% bovine serum albumin (BSA) in PBS for 30 min, incubated with serum specimens for 4 h, followed by washes with PBS with Tween-20 5 times, and PBS 5 times. Then, the peptide microarray was incubated with a 555-Streptavidin fluorescein for 1 h at room temperature, and then washes with PBST and PBS 5 times, respectively. A biotinylated anti-human IgG was used for autoantibody detection. After air-drying, the peptide microarray was measured by Jingxin LuxScan™ 10K-B Microarray Scanners (CapitalBio Corporation, Beijing, China) with 532 nm excitation wavelength. Finally, data analysis was performed by GenePro software version 6 (GenePro, Fitchburg, WI, USA) and GraphPad Prism software version 6 (GraphPad Software, Inc., La Jolla, CA, USA).

ELISA detection. In order to verify the validity of epitopes, we selected the 2 most commonly used SLE autoantibodies in clinic including Sm, and RNP. A complete antigen of each indicator was used to detect the autoantibodies in sera of the SLE patients using the Human peripheral blood anti-Sm IgG (cat. no. EA1593-9601G) and anti-nRNP IgG (cat. no. EA1591-9601G) ELISA Kit (EUROIMMUN Medizinische Labordiagnostika AG, Lubeck, Germany) following the kit instruction. The cut-off value was 20 RU/ml. The results of ELISA were compared with those of peptide microarray. For Sm, the peptides including SMD1-2, SMD2-1, SMD2-2 and SMD3-1 were compared; for RNP, the peptides including U1-SnrnpA-2 and U1-SnRNP 68/70 kDa were compared.

Table I. Predicted epitopes on 14 antigens.

Number	Start	End	Peptide
SMD1 (Accession: CAE11897.1)			
1	83	119	VEPKVKSKKREAVAGRGRGRGR GRGRGRGRGRGGPRR
2	41	57	KAVKMTLKNREPVQLET
3	12	26	HETVTIELK
SMD2 (Accession: AAC13776.1)			
1	1	19	MSLLNKPKSEMTPEELQKR
2	112	118	NPLIAGK
3	76	90	EVPKSGKGKKKSKPV
4	93	98	DRYISK
5	22	27	EEFNTG
SMD3 (Accession: AAA57034.1)			
1	120	126	NIFQKRR
2	110	117	RGRGRGMG
3	43	61	MSNITVTYRDGRVAQLEQV
4	96	108	GRGKAAILKAQVA
5	32	39	LIEAEDNM
Proliferating cell nuclear antigen (PCNA) (Accession: NP_872590.1)			
1	253	261	PKIEDEEGS
2	57	67	FDTYRCDRNLA
3	149	157	RDLSHIGDA
4	80	87	KCAGNEDI
5	1	8	MFEARLVQ
Acidic ribosomal phosphoprotein (P1) (Accession: AAA36471.1)			
1	18	30	DDEVTVTEDKINA
Acidic ribosomal phosphoprotein (P2) (Accession: AAA36472.1)			
1	44	61	SELNGKNIEDVIAQGIGK
2	12	18	LGGNSSP
snRNP-B/B' (Accession: P14678.2)			
1	1	11	MTVGKSSKMLQ
2	48	66	FRKIKPKNSKQAEREEKRV
3	90	99	TGIARVPLAG
4	34	40	FDKHMNL
5	70	78	VLLRGENLV
6	223	231	PPPGMRGPP
7	22	45	LQDGRIFIGTFKAFDKHMNLILCD
U1-snRNP-C (Accession: NP_003084.1)			
1	82	91	SLPGPPRPGM
2	69	80	APPPAGAMIPPP
3	35	47	KDYYQKWMEEQAQ
U1-snRNP-A (Accession: NP_004587.1)			
1	1	10	MAVPETRPNH
2	60	77	KEVSSATNALRSMQGFPF
3	94	104	IAKMKGTFVER
4	80	91	KPMRIQYAKTDS
5	94	104	IAKMKGTFVER
Nucleolin (Accession: AAA59954.1)			
1	118	127	VATPGKKGA
2	214	233	TPAKGKKAAK
3	315	327	NFNKSAPELKTGI

Table I. Continued.

Number	Start	End	Peptide
4	331	337	FAKNDLA
5	347	353	RKFGYVD
6	421	430	LVSKDGKSKG
7	514	526	VPQNQNGKSKGYA
Acidic ribosomal phosphoprotein (P0) (Accession: AAA36470.1)			
1	1	13	MPREDRATWKSNY
2	21	27	LDDYPKC
3	32	41	ADNVGSKQMQ
4	45	51	MSLRGKA
5	91	100	TKEDLTEIRD
6	125	136	AQNTGLGPEKTS
7	146	152	KISRGTI
8	162	171	KTGDKVGASE
9	202	213	EVLDITEETLH
10	215	220	FLEGVR
11	243	249	NGYKRVL
12	296	312	AKVEAKEESEESDEDMG
DNA topoisomerase1 (truncated) (Accession: NP_003277.1)			
1	52	66	YDGKVMKLSPKAEEV
2	89	103	FKDWRKEMTNEEKNI
3	1	12	KKKKPKKEEEQK
4	128	136	QMSKEEKLK
5	415	422	LTAPDENI
DNA topoisomerase 1 (full length) (Accession: NP_003277.1)			
1	459	476	NQYREDWKSKEMKVRQRA
2	674	682	VMKDAKTKK
3	491	498	NEKEEGET
4	504	510	CCSLRVE
5	365	376	GNHPKMGMLKRR
U1-SnRNP 68/70 KDa (Accession: P08621.2)			
1	424	437	LAPENGYLMEAAPE
2	375	383	DREHKRGER
3	120	134	RREFEVYGPIKRIHM
4	282	294	KDKDRDRKRRSSR
5	303	315	RERKEELRGGGGD
6	1	8	MTQFLPPN
7	211	217	SGRDDTS
8	138	145	KRSGKPRG

Statistical analysis. Peptide microarray data were measured using GenePro software version 6.0 (GenePro), and analyzed by GraphPad Prism software v 6.0 (GraphPad) using Student's t-test. The ROC curves and area calculated were performed by SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). A value of $P<0.05$ was considered statistically significant.

Results

Prediction of antigens. Using the DNA star software, a total of 73 potential epitopes were obtained from 14 autoantigens were predicted. The 14 autoantigens included acidic ribosomal phosphoprotein (P0), acidic ribosomal phosphoprotein (P1), acidic ribosomal phosphoprotein (P2), DNA topoisomerase 1 (full length 0, DNA topoisomerase 1 (truncated), nucleolin, proliferating cell nuclear antigen (PCNA), SMD1, SMD2, SMD3, snRNP-B/B', U1-snRNP 68/70 kDa, U1-snRNP-A, and U1-snRNP-C. The detailed information of the epitopes are shown in Table I.

Samples detection by peptide microarray. The peptide microarray based on the 73 epitopes were used to test the serum

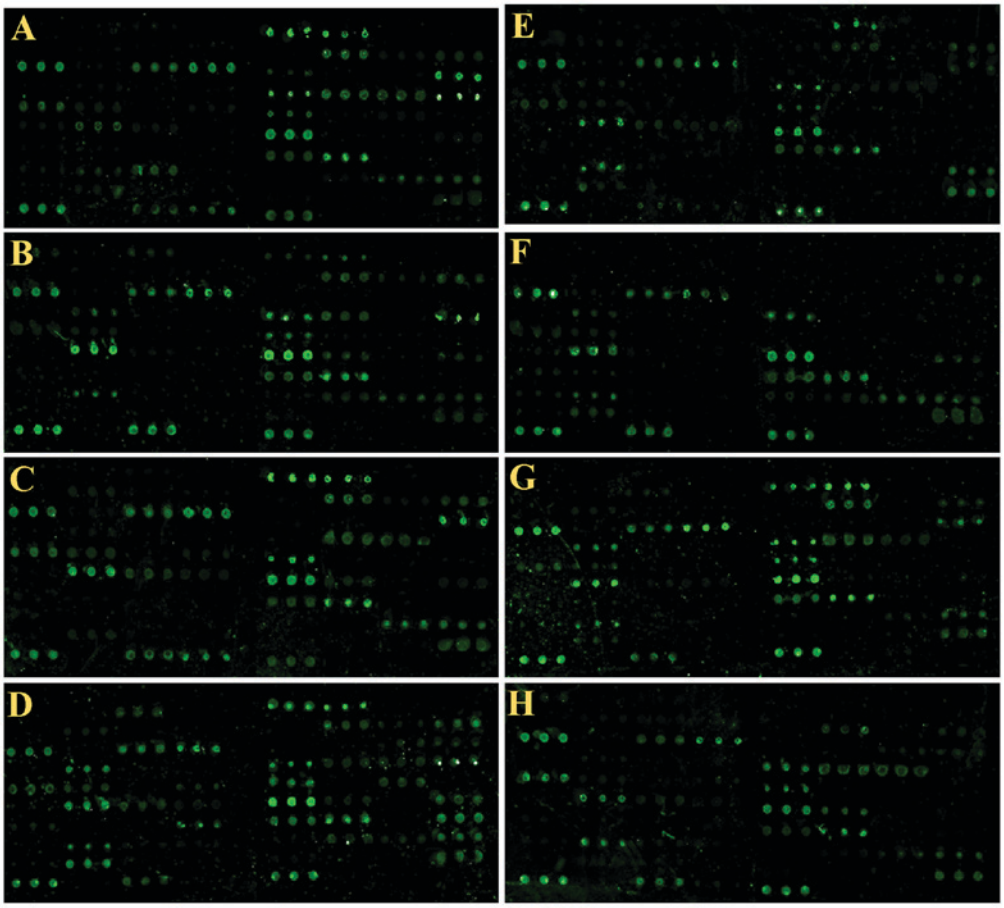


Figure 1. Representative peptide microarray detection data. (A-D) SLE patients. (E-H) healthy subjects.

autoantibodies in 120 SLE patients and 110 matched healthy subjects (Fig. 1).

The results showed that the autoantibodies that produced by different individuals recognized and bound different epitopes. If the signal information larger than the negative control, the average data plus 10 mines of SD was sued to assess the positive rate. The positive controls used in the present study was biotinylated random linear 12-peptide. The negative control was a random linear 12-peptide. We analyzed the positive rate and false positive rate of each peptide (Fig. 2). It can be seen there were 18 peptides had high positive rate with low false positive rate, including P0-2, P0-4, P2-1, SMD1-2, SMD2-1, SMD2-2, SMD3-1, PCNA-2, U1-SnrnpA-2, DNA (full length)-5, U1-68/70kDa-3, U1-68/70kDa-7, U1-68/70kDa-8, DNA (truncated)-1, DNA (truncated)-2, Nucleolin-1, Nucleolin-5, and Nucleolin-7. All the positive rate and false positive rate of the peptides were no less and no >10%, respectively. The ratio of positive rate to false positive rate was >5, indicating all the 18 peptides are valuable epitopes.

ROC curve plotting and area analysis. The specificity and 1-specificity of all cut-off points in data from the 18 screened peptides were calculated and the ROC curve was plotted (Fig. 3A). The areas under the ROC curves of peptides P0-2, P0-4, P2-1, SMD1-2, SMD2-1, SMD2-2, SMD3-1, PCNA-2, U1-SnrnpA-2, DNA (full length)-5, U1-68/20Da-3, U1-68/20kDa-7, U1-68/20kDa-8, DNA (truncated)-1, DNA (truncated)-2, Nucleolin-1, Nucleolin-5 and Nucleolin-7 was 0.662±0.051, 0.622±0.053, 0.498±0.054, 0.701±0.050, 0.760±0.045, 0.756±0.050, 0.713±0.049, 0.704±0.049, 0.550±0.054, 0.691±0.050, 0.685±0.050, 0.689±0.050, 0.795±0.042, 0.707±0.050, 0.639±0.052, 0.684±0.051, 0.651±0.053, and 0.672±0.051 (Fig. 3B). And the area under the curves of 14 peptides was >0.65, including P0-2, SMD1-2, SMD2-1, SMD2-2, SMD3-1, PCNA-2, DNA (full length)-5, U1-68/20kDa-3, U1-68/20kDa-7, U1-68/20kDa-8, DNA (truncated)-1, Nucleolin-1, Nucleolin-5 and Nucleolin-7, suggesting they are of significant diagnostic value in SLE.

Verification of the validity of epitopes. In order to verify the validity of the 14 epitopes, we selected the intact antigens of 2 most commonly used SLE autoantibodies in clinic, including Sm and RNP to detect the sera of the SLE patients using direct ELSIA kit. The results of ELISA were compared with those of peptide microarray (for Sm, the peptides including SMD1-2, SMD2-1, SMD2-2 and SMD3-1 were compared; for RNP, the peptides including U1-SnrnpA-2 and U1-SnRNP 68/70 kDa were compared) (Table II). When one peptide was positive, the results of peptide microarray was set as positive. The peptide microarray was higher in sensitivity and lower in specificity than the direct ELISA. In addition, when detected an expanded sample set including 120 SLE patients and 110 healthy subjects (Table III). When two peptides in one sample were positive, the sample was positive. The sensitivity

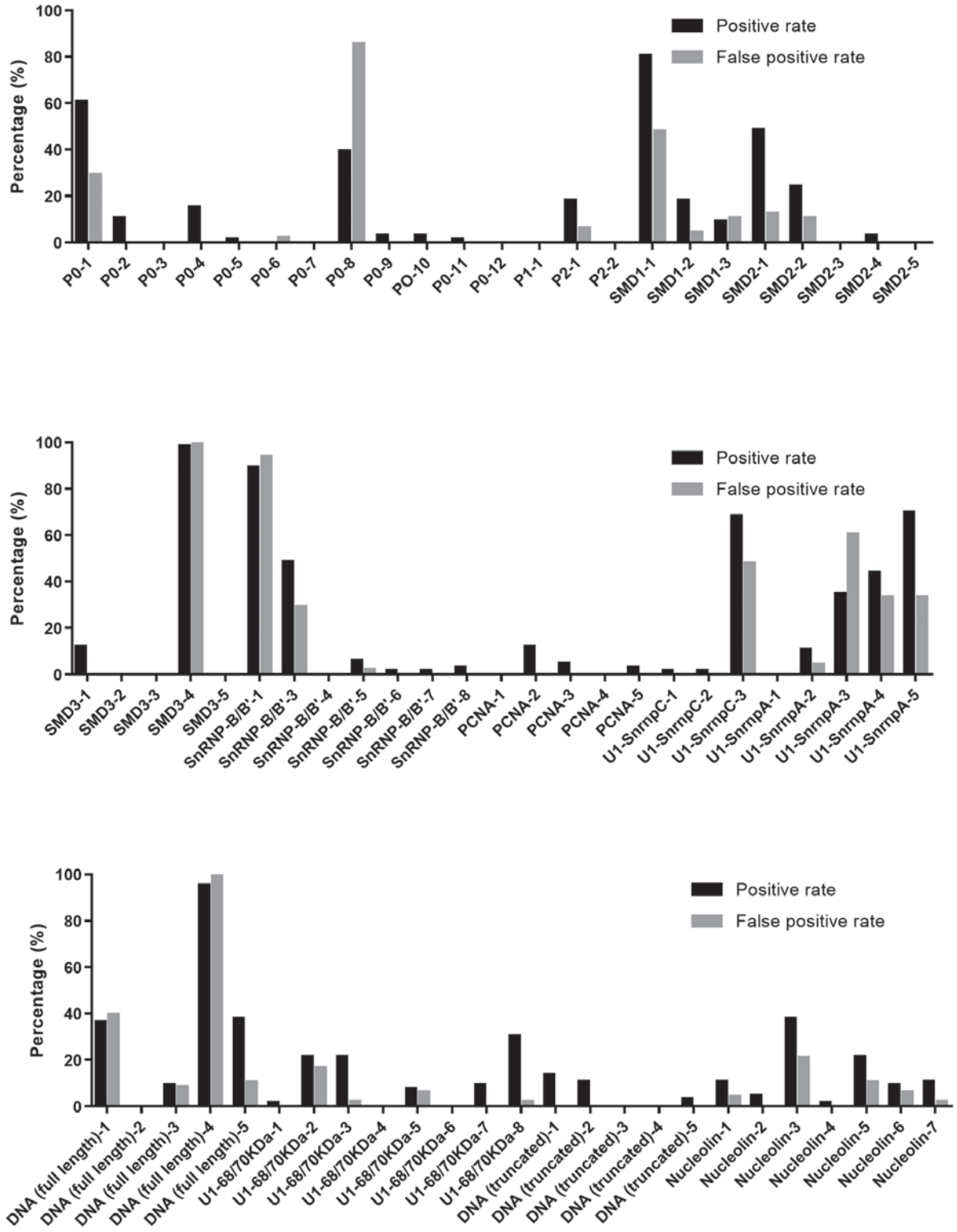
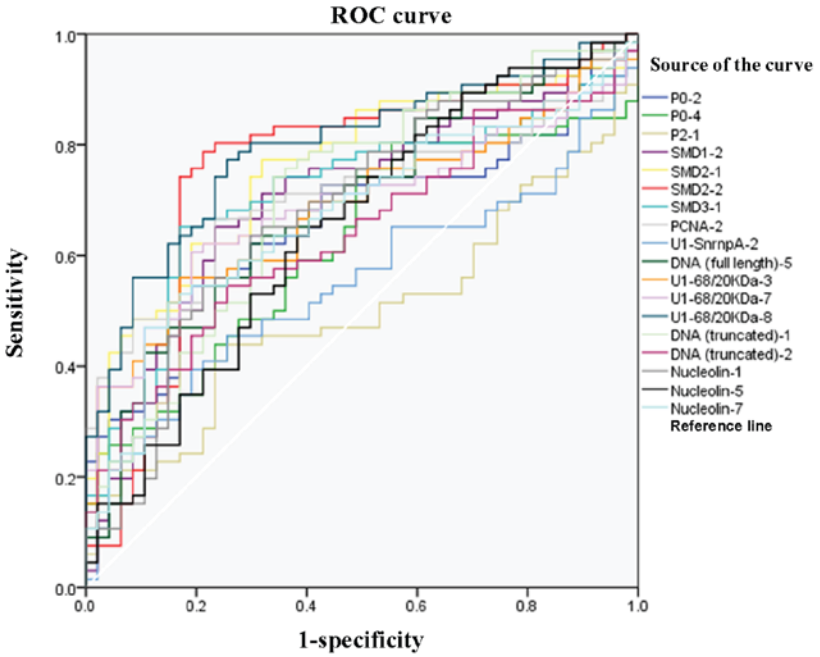


Figure 2. The positive rate and false positive rate of 73 peptides.

Table II. Comparison between ELISA and peptide microarray.

	Intact antigens		Peptide microarray		
Antigens	Sensitivity	Specificity	Sensitivity	Specificity	Related
Sm	20.1	100	57.6	79.1	0.85
RNP	41.8	100	51.5	91.6	0.88

A



B

Area under the curve

Test result variable(s)	Area	Std. error[a]	Asymptotic sig.[b]	Asymptotic 95% confidence interval	
				Lower bound	Upper bound
P0-2	0.662	0.051	0.003	0.563	0.762
P0-4	0.622	0.053	0.028	0.518	0.725
P2-1	0.498	0.054	0.977	0.392	0.605
SMD1-2	0.701	0.050	0.000	0.603	0.799
SMD2-1	0.760	0.045	0.000	0.671	0.848
SMD2-2	0.756	0.050	0.000	0.658	0.853
SMD3-1	0.713	0.049	0.000	0.617	0.810
PCNA-2	0.704	0.049	0.000	0.608	0.800
U1-SnrnpA-2	0.550	0.054	0.370	0.444	0.656
DNA (full length)-5	0.691	0.050	0.001	0.593	0.788
U1-68/20 kDa-3	0.685	0.050	0.001	0.587	0.782
U1-68/20 kDa-7	0.689	0.050	0.001	0.592	0.787
U1-68/20 kDa-8	0.795	0.042	0.000	0.713	0.877
DNA (truncated)-1	0.707	0.050	0.000	0.610	0.804
DNA (truncated)-2	0.639	0.052	0.012	0.538	0.740
Nucleolin-1	0.684	0.051	0.001	0.584	0.784
Nucleolin-5	0.651	0.053	0.006	0.548	0.754
Nucleolin-7	0.672	0.051	0.002	0.573	0.772

a Under the nonparametric assumption

b Null hypothesis: true area = 0.5

Figure 3. The ROC curve and statistical analysis of detection data by 18 peptides. (A) ROC curve. Sensitivity and 1-specificity represent the true positive rate and false positive rate, respectively. (B) The area under the curves.

Table III. Diagnostic results of SLE using 18 peptides.

Detection	SLE	Healthy	Total
Positive detection	86	16	102
Negative detection	34	94	128
Total	120	110	

of peptide microarray in the diagnosis of SLE was 71.6%, and the specificity was 85.5%.

Discussion

Autoantibodies are an important feature of autoimmune diseases and are important indicators of disease diagnosis and

progression monitoring. Autoantibodies can be detected by intact antigens, but the target epitopes by each autoantibody cannot be accurately identified. In clinic, the precise identification of autoantibodies and the detection of combining epitopes is of great significance in accurate diagnosis and treatment. The present study uses peptide microarray technology to detect the autoantibodies in peripheral blood of SLE patients. The findings suggested epitopes recognized by autoantibodies of individual SLE patients was different. As individual patient has a specific autoantibody spectrum, detection of autoantibodies by peptide microarray is useful for diagnosis of SLE.

The use of autoantigens to detect autoantibodies is an important diagnostic technique for autoimmune diseases. However, all the current detection methods have limited by poor positive rates, sensitivity. Although the sensitivity of ANA in SLE diagnosis is as high as 97-100%, its specificity is only 10-40%. Moreover, when ANA is negative, it cannot rule out the SLE completely, suggesting the diagnosis by ANA should take account the clinical conditions. The specificity of anti-rRNP antibody in SLE is ~20-30% (4). The specificity of anti-SSA antibody in neonatal patients with lupus erythematosus is 100% (27,28). The anti-dsDNA antibody are closely associated with SLE, which shown a high specificity in the diagnosis of SLE (29). However, excessive free DNA antigens in the serum will influent the diagnosis of SLE in some patients since they could combine with anti-dsDNA antibodies (29,30). It was also demonstrated that the anti-Sm antibodies, anti-nucleosome antibody could use in the diagnosis of SLE. Although the positive rate of anti-Sm antibody for SLE was 98%, its sensitivity was only 20-30% (3,5,31,32). In addition, the detection of antibodies by ELISA is not only inefficient but also costly, which is becoming a major obstacle to the early diagnosis of SLE.

Compared with the intact antigen, we found that peptide microarray results were negative when the intact antigen detection was negative. If the intact antigen test result is positive, at least one peptide detection was positive. Zhu *et al* designed arrays containing synthetic peptides and molecular modified protein which being utilized for identification of autoantibodies targeting to special antigenic epitopes (33). Our results showed the sensitivity and specificity of the combination of 73 peptides in the diagnosis of SLEs can reach 96.9 and 93.8%, but any single index cannot meet the clinical needs. Therefore, the combined detection of multiple indictors by peptide microarray has important value in the detection of SLE.

In the present study, the linear epitopes of 14 antigens were predicted by antigen epitope prediction software and an optimal set of epitopes for SLE diagnosis was obtained. Although the current epitope prediction software could predict the spatial epitopes, short peptide could not be simulated due to the far away between amino acid in space epitope. Nowadays, the peptide microarray could not be used to detect the autoantibody by identified spatial epitope. This is a flaw in this project. In addition, we found that the spectrum of autoantibodies varied considerably among different SLE patients. Therefore, a large number of samples are needed to accurately calculate the positive rate of each locus, which is another defect of the present study. Constructing a map of each Person's epitope and analyzing his association with disease progression are our future research direction.

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