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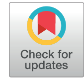
Autoantibodies targeting TLR and SMAD pathways define new subgroups in systemic lupus erythematosus



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Objectives: The molecular targets of the vast majority of autoantibodies in systemic lupus erythematosus (SLE) are unknown. We set out to identify novel autoantibodies in SLE to improve diagnosis and identify subgroups of SLE individuals.
Methods: A baculovirus-insect cell expression system was used to create an advanced protein microarray with 1543 full-length human proteins expressed with a biotin carboxyl carrier protein (BCCP) folding tag, to enrich for correctly folded proteins. Sera from a discovery cohort of UK and US SLE individuals ($n = 186$) and age/ethnicity matched controls ($n = 188$) were assayed using the microarray to identify novel autoantibodies. Autoantibodies were validated in a second validation cohort (91 SLE, 92 controls) and a confounding rheumatic disease cohort ($n = 92$).
Results: We confirmed 68 novel proteins as autoantigens in SLE and 11 previous autoantigens in both cohorts (FDR<0.05). Using hierarchical clustering and principal component analysis, we observed four subgroups of SLE individuals associated with four corresponding clusters of functionally linked autoantigens. Two clusters of novel autoantigens revealed distinctive networks of interacting proteins: SMAD2, SMAD5 and proteins linked to TGF-β signalling; and MyD88 and proteins involved in TLR signalling, apoptosis, NF-κB regulation and lymphocyte development. The autoantibody clusters were associated with different patterns of organ involvement (arthritis, pulmonary, renal and neurological). A panel of 26 autoantibodies, which accounted for four SLE clusters, showed improved diagnostic accuracy compared to conventional antinuclear antibody and anti-dsDNA antibody assays.
Conclusions: These data suggest that the novel SLE autoantibody clusters may be of prognostic utility for predicting organ involvement in SLE patients and for stratifying SLE patients for specific therapies.
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1. Introduction

Although first described in 1957, anti-nuclear antibodies (ANA) and anti-double-stranded DNA (dsDNA) antibody assays remain the primary diagnostic tests for systemic lupus erythematosus (SLE) [1,2]. Following the development of assays for extractable nuclear antigens (ENA) Ro, La, Sm and U1-RNP, there have been no significant improvements in diagnostic assays for SLE for many years [3]. In contrast, the identification of citrullinated proteins as autoantigen epitopes in rheumatoid arthritis (RA) led to a marked improvement in RA diagnostic tests with the development of anti-cyclic citrullinated peptide (CCP) assays. Although numerous SLE-associated autoantibodies have been described [4], they have not significantly improved upon the diagnostic and biomarker abilities of conventional ANA, dsDNA and ENA tests, and in many cases the

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true molecular targets remain undefined. Initial protein microarrays used to detect autoantibodies in SLE sera were largely based on existing autoantigens, but have identified several glomerular proteins and serum factors including B cell-activating factor (BAFF) as SLE autoantigens [5–10]. Microarrays utilising large scale *de novo* synthesis of thousands of proteins have detected autoantibodies in cancer and other diseases [11,12], but only identified a single SLE autoantigen [13]. Older protein microarrays may have failed to identify autoantibodies due to poor protein conformation caused by misfolding or lack of post-translational modification.

We used a novel protein microarray utilising 1543 distinct proteins chosen from multiple functional and disease pathways, to identify novel autoantigens in SLE. Our aim was to identify previously undiscovered autoantibodies that might act as SLE biomarkers to improve diagnostic (and potentially prognostic) performance over existing clinical assays and to determine whether subgroups of SLE patients with different autoantibody repertoires existed. Full-length human proteins bound to the microarray were expressed in a baculovirus-insect cell expression system with a biotin carboxyl carrier protein (BCCP) folding tag. The BCCP tag enriches for correctly folded proteins, conserving protein epitopes in their native conformation, which may be necessary for high affinity antibody binding (Fig. 1A) [14]. In this study, we used this newer design of protein microarray to elucidate the underlying nature of autoantigens in SLE.

2. Materials and methods

2.1. Study population

Serum samples from SLE individuals were collected from multiple UK institutions and USA (Seralabs). Serum samples from age/ethnicity matched controls for UK individuals were obtained from the TwinsUK resource (part of the National Institute for Health Research (NIHR) BioResource) and for USA individuals from Seralabs. SLE and control samples were randomly assigned 2:1 to the Discovery cohort (186 SLE and 188 controls) and the Validation cohort (91 SLE and 92 controls). SLE patients were almost all female reflecting the sexual dimorphism of SLE, while healthy controls were exclusively female. All SLE patients fulfilled the 1997 revised American College of Rheumatology (ACR) criteria for classification of SLE. The validation cohort was compared with a Confounding/interfering disease cohort included patients with the following conditions: systemic sclerosis ($n = 12$), primary Sjögren's syndrome ($n = 6$), polymyositis ($n = 3$) and mixed connective tissue disease ($n = 3$) sourced from USA (Seralabs), and rheumatoid arthritis (RA) ($n = 68$) obtained from multiple UK institutions. RA patients fulfilled the 2010 ACR-EULAR (European League against Rheumatism) criteria for diagnosis of RA. Ethical approval was granted by the Independent Investigational Review Board Inc. (4/16/2008) and the UK National Research Ethics Service London (reference numbers MREC98/2/06, 06/MRE02/9 and 07/H0718/49).

2.2. Protein microarray

Protein microarray assay using the Discovery Array v3.0 protein microarray is described in the Supplementary Methods. The microarray data are available at ArrayExpress accession E-MTAB-5900.

2.3. Serum autoantibody measurement

Anti-dsDNA and ANA titres were measured in all serum samples by ELISA (Inova Diagnostics, San Diego, USA), according to the manufacturer's instructions. ANA and dsDNA positive/negative results were defined using thresholds determined by the manufacturer, and were not based on historical case record results.

2.4. Statistical analysis

Statistical analysis, protein-protein interaction analysis, genotyping, HLA imputation and analysis, and predictive models are described in detail in the Supplementary Methods. The STARD checklist was completed and is available in the online supplement.

3. Results

3.1. Identification of novel SLE-associated autoantigens by microarray

Serum samples from a discovery cohort of 186 SLE patients and 188 age/ethnicity matched healthy controls (Table S1) were analysed for IgG autoantibody levels against 1543 correctly folded, full-length human proteins using a custom protein microarray (Oxford Gene Technology, UK) (Fig. 1A). Samples were assayed by ELISA for ANA and anti-dsDNA antibodies for comparison. Normalised autoantibody levels were compared between SLE individuals and healthy controls in the discovery cohort, using a linear regression model adjusting for age, gender, ethnicity and country. A total of 226 autoantibodies, which were increased in the SLE individuals compared to controls in the discovery cohort at FDR-corrected $P < 0.05$, were investigated in a validation cohort of 91 SLE individuals and 92 age/ethnicity matched controls. Demographics for the discovery and validation cohorts are shown in Table S1. Of 226 autoantigens observed in the discovery cohort, a total of 79 autoantibodies were also significantly increased in SLE individuals in the validation cohort at FDR<0.05 (Fig. 1C, Table S2). The well-known SLE autoantigens TROVE2 (Ro60) and SSB (La) showed the most significant difference between SLE and control groups in both cohorts. The array validated a further nine previously reported SLE autoantigens (Fig. 1D, Table S2). A total of 68 novel autoantigens were validated by the microarray, with the most statistically significant four novel autoantibodies shown in Fig. 1E.

A post-validation meta-analysis was performed using a regression model adjusting for age, gender, ethnicity and country. Suggestive evidence at $FDR_{meta}<0.01$ was found for a further 41 autoantibodies (Table S3), of which 38 were novel. Nine of the validated autoantigens have been shown to be implicated in SLE pathogenesis through immunological studies, but were not previously known to be autoantigens: CREB1, ZAP70, VAV1, PPP2CB, IRF4, IRF5, EGR2, PPP2R5A and LYN [15–19], while TEK (Tie2 receptor) was identified in the meta-analysis [20]. Five novel autoantigens are the products of SLE susceptibility genes: *IRF5*, *LYN*, *PIK3C3*, *NFKBIA* and *DNAJA1* [21–25]. In summary, 26 of 120 autoantigens (79 validated and 41 identified in the meta-analysis) have a previously identified link to SLE, either as known autoantibody targets or directly implicated in SLE pathogenesis.

In a secondary analysis of the discovery cohort, autoantibodies from the array were ranked by positivity in SLE patients, defined as autoantibody levels >2 SD of the control population, and tested for statistical significance using Fisher's exact test, corrected for multiple testing. Autoantibodies with FDR-corrected $P < 0.05$ were analysed for positivity in the validation cohort. A total of 60 autoantibodies showed a significant increase in antibody positivity in both discovery and validation cohorts (Fig. 2A). The most prevalent autoantibodies were the known SLE autoantigens Ro60 (overall prevalence 37.5%), SSB/La (35.4%), HNRNPA2B1 (29.6%) and PSME3/Ki (23.8%). The most prevalent novel autoantibodies were LIN28A (22.4%), IGF2BP3 (21.7%) and HNRNPUL1 (21.3%). SLE patients tended to be simultaneously positive for multiple autoantibodies in

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