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IgV peptide mapping of native Ro60 autoantibody proteomes in primary Sjögren's syndrome reveals molecular markers of Ro/La diversification

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ABSTRACT

We have used high-resolution mass spectrometry to sequence precipitating anti-Ro60 proteomes from sera of patients with primary Sjögren's syndrome and compare immunoglobulin variable-region (IgV) peptide signatures in Ro/La autoantibody subsets. Anti-Ro60 were purified by elution from native Ro60-coated ELISA plates and subjected to combined *de novo* amino acid sequencing and database matching. Monospecific anti-Ro60 Igs comprised dominant public and minor private sets of IgG1 kappa and lambda restricted heavy and light chains. Specific IgV amino acid substitutions stratified anti-Ro60 from anti-Ro60/La responses, providing a molecular fingerprint of Ro60/La determinant spreading and suggesting that different forms of Ro60 antigen drive these responses. Sequencing of linked anti-Ro52 proteomes from individual patients and comparison with their anti-Ro60 partners revealed sharing of a dominant IGHV3–23/IGKV3–20 paired clonotype but with divergent IgV mutational signatures. In summary, anti-Ro60 IgV peptide mapping provides insights into Ro/La autoantibody diversification and reveals serum-based molecular markers of humoral Ro60 autoimmunity.

1. Introduction

Autoantibodies directed against the 60-kD Ro (Ro60)/SSA ribonucleoprotein (RNP) particle are the major target of humoral autoimmunity in patients with primary Sjögren's syndrome (SS) and are associated with more severe exocrine gland hypofunction, extraglandular complications and neonatal lupus [1,2]. Ro60 protein is a ring-shaped structure complexed with small non-coding RNAs termed Y RNAs and involved in degradation of misfolded RNAs, with many of the autoepitopes on Ro60 overlapping regions involved in RNA binding [3,4]. Interest in the pathogenic importance of anti-Ro60 has been heightened by the recent discovery that Ro60 also binds Alu-containing RNA retroelements, and that anti-Ro60-positive patients with systemic lupus erythematosus (SLE) have high levels of Alu transcripts in their serum and IgG fractions than controls [5]. In brief, a model of anti-Ro60-mediated tissue injury in SS, SLE and neonatal lupus is emerging in which complexes of anti-Ro60, Ro60 protein and associated Y RNA and Alu RNA formed from apoptotic cells ligate TLR7/TLR8 in macrophages or plasmacytoid dendritic cells and initiate inflammation, with a feed-forward inflammatory loop as type I interferon drives Y RNA and Alu expression [5–7].

Despite advances in our understanding of the structure, epitopes and RNA-binding properties of Ro60 autoantigen, and the unravelling of the pathogenic properties of the autoantibody in models of maternal anti-Ro60 antibody-mediated injury in congenital heart block, little is known of the anti-Ro60 immunoglobulin variable-region (IgV) repertoire in terms of clonality and IgV gene usage at the end-point response of the secreted (serum) proteome. A recent study showed Ro60 polyreactivity for recombinant monoclonal antibodies derived from salivary glands in primary SS, with binding to other antigens in addition to Ro60, but single-cell techniques involve a selection bias and their relevance to the monospecific anti-Ro60 serum repertoire is unclear [8]. Mass spectrometric sequencing of a subpopulation of Ro60 autoantibodies selected from anti-Ro/La polyclonal serum identified a IGHV3–23/IGKV3–20 paired clonotypic autoantibody specific for an apical peg-like epitope on Ro60, but autoantibody proteomes against full-length native Ro60 protein are likely to be more complex and comprise multiple clonotypes with different molecular signatures [9,10]. Given the potential value of serum clonotypic IgV peptides as novel

Abbreviations: BCR, B-cell receptor; CDR, complementary determining regions; CIEP, counterimmunoelectrophoresis; D, diversity; EBV, Epstein Barr virus; FR, framework region; H, heavy; IgV, immunoglobulin variable-region; IMGT, ImMunoGeneTics; J, joining; L, light; MS, mass spectrometry; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome.

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biomarkers of Ro60 humoral autoimmunity, it is important to determine whether complete anti-Ro60 proteomes are dominated by restricted sets of public (shared) clonotypes and share V-region peptide signatures in unrelated patients, as reported recently for anti-Ro52, anti-SmD and anti-ribosomal P autoantibodies [11–13].

While linked anti-Ro52 and anti-Ro60 responses in patients with primary SS are known to express subsets of shared clonotypic structures, the IgV peptide signatures of mixed anti-Ro52/Ro60 responses in the same patients have not been analysed at the amino acid level [10,13]. Furthermore, anti-Ro60 are frequently linked with anti-La responses in primary SS, consistent with a HLA-dependent mechanism of T-cell-dependent Ro/La intermolecular epitope spreading driven by Ro and/or Ro/La RNPs [14]. This assumes different pathways of monospecific anti-Ro60 *versus* mixed anti-Ro60/La responses that may be reflected in different IgV-region mutational signatures. Recent advances in high-resolution mass spectrometric sequencing now allow serological features of humoral Ro/La autoimmunity to be resolved for the first time at a molecular level. In the present study, we use a novel streamlined proteomic workflow to sequence complete precipitating Ro60 autoantibody proteomes in patients with seropositive primary SS [15]. The findings reveal a more complex pattern of public and private clonotypic IgGs than has hitherto been observed for systemic autoantibodies and identify distinct patterns of mutated IgV peptides in different autoantibody subsets.

2. Materials and methods

2.1. Human samples

Sera were collected from eight patients with primary SS whose sera were positive for anti-Ro60 by native Ro60 protein ELISA. Patients with primary SS fulfilled at least four of the six American–European Consensus Group Criteria [16]. The primary SS patients in this study were not treated with any immunomodulatory medications. The study was approved by the Clinical Ethics Committee of the Flinders Medical Centre. Control sera were obtained from three SLE patients with anti-Ro60, two asymptomatic subjects with anti-Ro60 and four healthy donors. Demographic and serological characteristics are shown in Supplementary Table 1.

2.2. Isolation and specificity analysis of anti-Ro60 and anti-Ro52 autoantibodies

Serum anti-Ro60 or anti-Ro52 Igs were purified from ELISA plates (Maxi-Sorp; Nunc, Roskilde, Denmark) coated with either 1 μg/ml purified bovine native Ro60 or 1 μg/ml recombinant full-length Ro52 (Arotec Diagnostics, New Zealand) by a low pH elution method [11], after incubation with anti-Ro or anti-Ro/La sera or control sera. The activity and specificity of purified Igs for Ro60 or Ro52 were determined by testing starting sera (diluted 1:100), unbound fractions (normalized to each starting serum), and eluted Igs (2.5 μg/ml) for reactivity against native Ro60, recombinant Ro52 and native La (Arotec Diagnostics) by ELISA. Purified anti-Ro60 Igs were also tested for reactivity with human Ro60 antigen by indirect immunofluorescence of HEp-2 cells (Immunoconcepts, Sacramento, USA) and by counterimmunoelectrophoresis (CIEP) using human K562 extract and anti-Ro60 and anti-RNP reference controls.

2.3. Mass spectrometry (MS)

The purity of affinity isolated Igs were verified by reduced SDS-PAGE. Heavy (H)- and light (L)-chain gel bands were excised and digested with Pierce trypsin protease (Thermo Fisher Scientific, USA). Tryptic peptides were analysed using a TripleTOF 5600+ mass spectrometer (AB Sciex, USA) coupled to an Ekspert NanoLC 400 HPLC (Eksigent) as detailed previously [11], with the following alterations: dynamic accumulation and rolling collision energy were employed for MS/MS scans. Purification of anti-Ro60 or anti-Ro52 Igs from individual patient sera was carried out on at least two independent occasions, and the eluted Igs from each purification were run for MS at two technical replicates.

2.4. Protein sequence data analyses

MS data file was firstly analysed using Protein Pilot (AB Sciex, USA), and post-calibrated Mascot generic file exported for *de novo* analysis by Peaks Studio v7.5 software (Bioinformatics Solution Inc., Canada) using the combined ImMunoGeneTics (IMGT) (http://www.imgt.org), NCBI and Uniprot 2015-08 databases. Unique peptides to individual V-region gene family were identified by an in-house programme against IMGT database. The parameters for database searches, data refinement, IgV gene family assignments have been described previously [11–13]. In brief, a maximum of two missed cleavages; precursor mass/charge tolerance of <10 ppm; product ion error tolerance of 0.02 Da; precursor charge state of +2 to +4.

3. Results

3.1. Purification of precipitating anti-Ro60 IgGs from anti-Ro and anti-Ro/La sera

The first step for mass spectrometric sequencing of a secreted autoantibody repertoire involves purification of antibodies specific for the autoantigen of interest from the starting serum [15]. Accordingly, Igs bound to native bovine Ro60-coated ELISA plates were eluted by low pH buffer after incubation with anti-Ro52/Ro60/La-positive sera from eight subjects with primary SS. Anti-Ro60 monospecificity was confirmed by testing starting sera, unbound and eluted fractions on individual Ro60, Ro52 and La ELISAs (Fig.1A, B). Purified anti-Ro60 Igs also bound full-length human Ro60 expressed in HEp-2 Ro60 transfectants

Table 1
IgV gene usage of precipitating anti-Ro60 autoantibody proteomes in primary SS.

Patients with primary SS		IGHV	IGHJ	IGKV	IGKJ	IGLV	IGLJ
Anti-Ro60	SS1	**1–18**, **3–23**, **3–74**, 5–10	**3**, **5**, **6**, 1	**1–5**, **3–20**	**2**, **3**, **4**	**3–19**	**2**
	SS2	**1–18**, **3–23**, **3–74**, 5–10, 1–2, 3–7	**3**, **5**, **6**	**1–5**, **3–20**	**2**, **4**	**3–19**	**2**
	SS3	**1–18**, **3–23**, **3–74**, 3/OR16–10	**3**, **5**, **6**	**1–5**, **3–20**	**2**, **3**, **4**	**3–19**, 3–10	**2**
	SS4	**1–18**, **3–23**, **3–74**	**3**, **5**, **6**	**1–5**, **3–20**	**2**, **3**, 5	**3–19**	**2**
Anti-Ro60/La	SS5	**1–18**, **3–23**, **3–74**	**3**, **5**, **6**	**1–5**, **3–20**	**2**, **4**	**3–19**	**2**
	SS6	**1–18**, **3–23**, **3–74**, 3–15, 3–64, 3–7	**3**, **5**, **6**, 1	**1–5**, **3–20**	**2**, **3**, **4**	**3–19**, 3–21	**2**, 1
	SS7	**1–18**, **3–23**, **3–74**, 3–21	**3**, **5**, **6**	**1–5**, **3–20**	**2**, **3**, **4**	**3–19**	**2**
	SS8	**1–18**, **3–23**, **3–74**	**3**, **5**, **6**	**1–5**, **3–20**	**2**, **3**, 5	**3–19**	**2**

SS, Sjögren's syndrome; IGHV, IgG heavy chain variable region; IGHJ , IgG heavy chain joining region; IGKV, IgG kappa chain variable region; IGLV, IgG lambda chain variable region; IGKJ, IgG kappa chain joining region; IGLJ, IgG lambda chain joining region; public gene families (shared in 4 out of 8 patients) are in bold.

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