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Research paper

Detection of anti-mitochondrial antibodies by immunoprecipitation in patients with systemic sclerosis

Angela Ceribelli[a], Natasa Isailovic[a], Maria De Santis[a], Elena Generali[a], Minoru Satoh[b,1], Carlo Selmi[a,c,*,1]

[a] Division of Rheumatology and Clinical Immunology, Laboratory of Autoimmunity and Metabolism, Humanitas Research Hospital, Rozzano, Milan, Italy
[b] Department of Clinical Nursing, University of Occupational and Environmental Health, Kitakyushu, Japan
[c] BIOMETRA Department, University of Milan, Italy

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ABSTRACT

Objective: To describe a new immunoprecipitation pattern identified in Italian patients affected by systemic sclerosis (SSc), corresponding to the pyruvate dehydrogenase antigen complex recognized by anti-mitochondrial antibodies (AMA) in primary biliary cholangitis (PBC).
Methods: Autoantibodies in sera from 85 patients with SSc were tested by protein- and RNA-immunoprecipitation. Immunoprecipitation-Western blot was used to determine the identified proteins, and medical records re-evaluated for liver function tests and PBC.
Results: In 13/85 (15%) SSc sera, a unique set of 75-50-40-34 kD proteins that had not been previously reported, was noted. The four proteins were identified as the proteins X/E3BP, E1α, E1β, and E2/E3 of the pyruvate dehydrogenase antigen complex by immunoprecipitation-Western blot. From clinical record evaluation, 9/13 (69%) SSc patients with this new pattern were positive for AMA by routine indirect immunofluorescence, and 7/13 (54%) had a diagnosis of PBC, while 4/13 (31%) manifested no biochemical signs of cholestasis. Twelve of 13 patients with SSc and AMA by immunoprecipitation have a limited cutaneous form of SSc and anti-centromere antibodies.
Conclusions: We describe a pattern of 4 proteins in 15% of SSc patients, identified for the first time by protein-immunoprecipitation. This pattern corresponds to serum AMA against the pyruvate dehydrogenase antigen complex and it must be considered in the interpretation of protein-immunoprecipitation results.

1. Introduction

A large proportion of patients with systemic sclerosis (SSc) manifest a coexisting autoimmune condition, including primary biliary cholangitis (PBC) (Assassi et al., 2009; Rigamonti et al., 2006). As immunoprecipitation (IP) is currently one of the most sensitive techniques to detect new and known rare autoantibodies, a clear understanding of common patterns is necessary for proper interpretation of the results. Anti-mitochondrial autoantibodies (AMA) are the hallmark of PBC (Gershwin et al., 2005; Selmi et al., 2014; Selmi et al., 2004) and in clinical practice they may predate the clinical onset of disease (Gershwin et al., 2000; Kaplan & Gershwin, 2005) when tested with routine indirect immunofluorescence performed on tissue slides, while the use of alternative techniques such as ELISA for mitochondrial antigens still is not routinely used and it has unclear clinical significance (Cavazzana et al., 2011). PBC is associated with other autoimmune diseases in about 30% of patients, with SSc found in 7–12% of cases (Assassi et al., 2009; Rigamonti et al., 2006), even though in clinical practice a higher percentage of SSc patients may have biochemical liver abnormalities without clinical significance (Norman et al., 2009).

We herein used IP to test new and uncommon serum autoantibodies in SSc and in this screening analysis we observed that 15% of SSc sera manifest a novel IP pattern, comprising a set of 4 proteins corresponding to the E1α, E1β, protein X/E3BP, and E2/E3 subunits of the pyruvate dehydrogenase complex (PDC) recognized by AMA (McHugh et al., 1990; Miyachi et al., 1980; Fregeau et al., 1990). The prevalence of AMA by IP outnumbers what is observed in routine tests in our cohort of SSc patients and this new IP pattern should be known when interpreting IP data in SSc sera.

2. Patients and methods

2.1. Subjects

Eighty-five consecutive patients with SSc attending the outpatient Rheumatology clinic at Humanitas Research Hospital (Rozzano, Milan,

* Corresponding author at: Division of Rheumatology and Clinical Immunology, Humanitas Research Hospital, via A. Manzoni 56, 20089 Rozzano, Milan, Italy.
E-mail address: carlo.selmi@unimi.it (C. Selmi).
[1] These authors contributed equally to the work.

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Italy) between 2012 and 2016, were enrolled to the study. Controls included sera from 74 healthy subjects, 49 patients with polymyositis/dermatomyositis (PM/DM), 32 patients with undifferentiated connective-tissue disease (UCTD), and 2 patients with established PBC previously AMA-positive and negative for serum antinuclear antibody (ANA) without signs of rheumatic diseases. Internationally accepted criteria were used for the diagnosis of SSc (van den Hoogen, 2013), PM/DM (Targoff et al., 1997), UCTD (Mosca et al., 2014), and PBC (Bowlus & Gershwin, 2014) and we collected clinical and laboratory data at enrollment. SSc patients who did not fulfill the ACR/EULAR criteria were defined as sine SSc (Poormoghim et al., 2000) and very early diagnosis of SSc (VEDOSS) (Minier et al., 2014). Liver laboratory tests included in our study are aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (gammaGT), alkaline phosphatase (ALP), bilirubin (total, direct) and, when available, liver histology was also evaluated. The study was approved by the Institutional Review Board of the Humanitas Research Hospital and a signed informed consent was obtained from all subjects in accordance with the Declaration of Helsinki and its subsequent modifications.

3. Methods

3.1. Protein- and RNA-immunoprecipitation (IP)

Sera were obtained from whole blood through centrifugation at 2000*g* for 15 min, and then stored in − 20 °C freezer until use. Serum autoantibodies were screened by protein-IP using ^{35}S-methionine-labeled K562 cell extract followed by SDS-PAGE and autoradiography, and by RNA-IP using unlabeled K562 cell extract followed by urea-PAGE and silver staining (Ceribelli et al., 2012; Ceribelli et al., 2010). Autoantibodies were analyzed using reference sera obtained from the Autoantibody Standardization Committee (www.autoab.org) and from internal controls, and they were used for the correct interpretation of protein-IP bands for known ANA specificities. These reference sera help in determining protein-IP specificities for ANA in connective tissue diseases and are used also for SSc patients as in our cohort.

3.2. IP-Western Blot (IP-WB)

Sera with a novel IP pattern of a set of 4 proteins were tested by IP-WB. In detail, 50 μl of candidate sera were cross-linked with protein-A Sepharose beads and then immunoprecipitated with cell extract from 5×10^6 K562 cells/sample. Proteins were then fractionated by 8% SDS-PAGE and transferred to a nitrocellulose filter, probed with 1:500 of mouse polyclonal anti-human PDH E1α antibody (Novus Biologicals, Littleton, CO, USA) for a 41 kD protein identification, followed by horseradish peroxidase (HRP) goat anti-mouse IgG (1: 10,000 dilution; ThermoFisher, Waltham, MA, USA). The same procedure was used to identify the other bands of the complex: mouse anti-human PDH E1β (1: 500 dilution; Novus Biological, Littleton, CO, USA) for the protein of 34 kD; mouse anti-human PDH protein X/E3BP (1: 1000 dilution; Novus Biological, Littleton, CO, USA) for the 54 kD; mouse anti-human PDH E2/E3 proteins of 58 kD and 74 kD (1: 10,000 dilution; Abcam, Cambridge, UK) followed by goat anti-mouse IgG (ThermoFisher, Waltham, MA, USA). Development was performed by Immobilon Western Chemiluminescent HRP substrate (Millipore, Darmstadt, Germany) and acquired using ChemiDoc (Bio-Rad, California, USA).

3.3. Indirect immunofluorescence (IIF)

Antinuclear and cytoplasmic antibodies were tested by IIF on HEp-2 ANA slides (INOVA Diagnostics, San Diego, CA, USA) using a 1:80 dilution of human sera of patients and controls, followed by AlexaFluor488 AffiniPure F(ab′)2 fragment goat anti-human IgG, Fcγ fragment specific (Jackson Immunoresearch Europe Ltd., Suffolk, UK) as previously described (Ceribelli et al., 2012). Images were acquired on Olympus BX53 Upright fluorescence microscope. No immunofluorescence analysis on tissue slides was performed for AMA identification.

3.4. ELISA

Positive serum AMA identified by protein-IP ($n = 13$) were tested for confirmation by ELISA using the QUANTA Lite M2 EP (MIT3) IgG ELISA (INOVA Diagnostics, Inc., San Diego, USA) with the patented pMIT3 antigen, which consists of a recombinant protein containing the immunodominant epitopes of the 3 major AMA targets (Moteki et al., 1996), currently limited to research settings. ELISA was performed according to the manufacturer instructions, and results were shown as Units.

3.5. Statistical analysis

All comparisons were performed by Fisher's exact test using Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). All analyses were two-tailed and p values < 0.05 were considered as statistically significant.

4. Results

4.1. Demographic and laboratory data of the SSc cohort

We included 85 patients (81 women, mean age ± standard deviation 66 ± 15 years) with SSc and their sera were studied by IP. The age at SSc onset was 53 ± 15 years, and the mean follow-up was 106 months (range 4 to 408). From clinical record retrospective evaluation, nine patients with limited cutaneous SSc had a previous diagnosis of PBC with available liver biopsy, and in one patient an overlap with ANA-positive autoimmune hepatitis was found. In 5/9 (56%) PBC cases, the onset of chronic cholestasis preceded the diagnosis of SSc (range 1–24 years), while in 4/9 (44%) the diagnosis of PBC followed SSc by 3–24 years. Additional features of our SSc cohort are described in Table 1. Fourteen cases defined as sine SSc (Poormoghim et al., 2000) ($n = 4$) and very early diagnosis of SSc (VEDOSS, (Minier et al., 2014), $n = 10$) are not included in the statistical evaluation shown in Table 1, but two AMA-positive IP cases were identified in sine SSc patients defined by ACA-positivity, Raynaud's phenomenon and capillaroscopy alterations but no signs of skin fibrosis.

Table 1
Main features of the limited and diffuse cutaneous SSc cohort analyzed for AMA identification by protein-IP. Only p values < 0.05 are reported. Fourteen cases defined as sine SSc ($n = 4$) and VEDOSS ($n = 10$) are not included in the present table.

	Limited cutaneous SSc ($n = 59$)	Diffuse cutaneous SSc ($n = 12$)	p values
PBC cases, % (n.cases)	13 (8)	8 (1)	ns
Interstitial lung disease, % (n.cases)	25 (15)	67 (8)	0.014
Pulmonary hypertension, % (n.cases)	24 (14)	42 (5)	ns
AMA by IP, % (n.cases)	17 (10)	8 (1)	ns
ACA, % (n.cases)	59 (35)	33 (4)	ns
ANA nucleolar, % (n.cases)	10 (6)	0	ns
ANA homogeneous, % (n.cases)	8 (5)	0	ns
Anti-topo1/Scl-70, % (n.cases)	5 (3)	50 (6)	< 0.001
Anti-RNAPIII, % (n.cases)	0	8 (1)	ns

Abbreviations: ACA = anti-centromere antibodies; AMA = anti-mitochondrial antibodies; ANA = anti-nuclear antibodies; IP = immunoprecipitation; PBC = primary biliary cholangitis; RNAPIII = RNA polymerase III.

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