
Fingerprinting of anti-alpha enolase antibodies in systemic sclerosis

G. Perconti¹, F. Pratesi², F. Angelotti², M.L. Manca², I. Puxeddu²,
P. Rubino¹, C. Maranto¹, A. Giallongo¹, P. Migliorini²

¹Institute for Biomedical Research and Innovation, National Research Council, Palermo;

²Clinical Immunology and Allergy Unit, Department of Clinical and Experimental Medicine, University of Pisa, Italy.

Giovanni Perconti, PhD*

Federico Pratesi, PhD*

Francesca Angelotti, MD

Maria Laura Manca, PhD

Ilaria Puxeddu, MD, PhD

Patrizia Rubino, PhD

Cristina Maranto, BSc

Agata Giallongo, PhD**

Paola Migliorini, MD**

*These authors share first authorship

**These authors share senior authorship.

Please address correspondence to:

Paola Migliorini,

Dipartimento di Medicina

Clinica e Sperimentale,

Università di Pisa,

Via Roma 67,

56126 Pisa, Italy.

E-mail: paola.migliorini@med.unipi.it

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ABSTRACT

Objective. Anti-alpha enolase antibodies have been detected in systemic sclerosis (SSc), but little is known on their fine specificity and their predictive value on single disease manifestations. The aim of this work is to perform an epitope mapping of alpha enolase by means of truncated recombinant proteins and to analyse the clinico-serological correlations of anti-alpha enolase antibodies in SSc patients.

Methods. Thirty-eight SSc patients were recruited and fully clinically and serologically characterised. Plasmids encoding full length and truncated polypeptides of alpha enolase were generated; the polypeptides were purified under native conditions and used in dot blot to test sera from SSc patients and controls. The densitometric values obtained on all the polypeptides with anti-IgG subclass specific antibodies were analysed by cluster analysis and partial least square regression.

Results. Anti-alpha enolase antibodies (mostly IgG1 and IgG2) are detected in 47% of SSc patients. IgG1 target the amino terminal region of alpha enolase, while IgG2 are more restricted to the central portion of the molecule. Anti-alpha enolase antibodies are not associated with disease-specific antibodies or with interstitial lung disease and do not identify patients affected by the limited vs. diffuse form.

Conclusion. Anti-alpha enolase antibodies are very frequent in SSc but are not associated with clinical or serological features of the disease. Further studies on larger cohorts of patients are necessary to define their possible contribution in defining specific subsets of the disease.

Introduction

Systemic sclerosis (SSc) is a rare fibrotic disorder affecting multiple organs,

with a chronic and progressive course. Two clinical subsets, a limited and a diffuse form, can be identified on the basis of organ involvement: distal cutaneous sclerosis, esophageal involvement and pulmonary hypertension are predominant in the limited form while diffuse skin fibrosis, interstitial lung disease and intestinal involvement characterise the diffuse form (1, 2).

Several autoantibodies have been detected in SSc sera: their pathogenic role has been only recently explored while their association with clinical subsets of the disease and specific clinical manifestations is well known (3, 4).

Anti-centromere antibodies (ACA), directed against CENP-B and CENP-A, are associated with the limited form, while anti-topoisomerase I antibodies (ATA) are detected more frequently in the diffuse form. ACA and ATA are mutually exclusive and the presence of both specificities in the same serum is a rare event. Several other autoantibodies can be detected in SSc sera, in lower frequency than ACA and ATA and with less stringent clinical associations. Anti-RNA polymerase III are associated with scleroderma renal crisis (5) and higher cancer risk (6, 7); anti-PMScl-75 and PMScl-100 are detected mostly in overlap syndromes (8). Anti-fibrillarin, Th/To, NOR90 and PDGFR antibodies are found at a very low frequency (9, 10). Anti-alpha enolase antibodies have also been detected in the sera of SSc patients (11, 12).

Alpha enolase is a moonlighting protein that can perform several functions in addition to its major glycolytic role, thus participating in numerous biological and pathophysiological processes. Biochemical, protein structural and evolutionary analyses have unraveled almost all the characteristics of alpha enolase as an enzyme and as a receptor for plasminogen on the cell surface. Moreover,

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several studies have elucidated its role as a transcription factor, which is exerted in the cell nucleus by a shorter form that originates by alternative translation of the mRNA (reviewed in Didiasova *et al.* 2019, ref 13). Localisation of alpha enolase on the cell surface (14) has been associated with cell migration and invasion, as expected from the capacity of plasminogen binding, as well as with recognition by circulating antibodies in a number of cancer diseases and autoimmune pathologies, including SSc.

Recombinant alpha enolase or synthetic peptides have been widely used to monitor autoantibodies, and it has been reported that both N- and C-terminal fragments of the protein are target of autoantibodies in diseases such as CAR, endometriosis, Hashimoto's encephalopathy, membranous nephropathy (15-18).

Little is known on the fine specificity of anti-alpha enolase antibodies in SSc, on their predictive value on single disease manifestations and on their associations with other autoantibodies.

In this paper we performed an epitope mapping of alpha enolase by means of truncated recombinant proteins and analyse the clinico-serological correlations of anti-alpha enolase autoantibodies.

Patients and methods

Patients

We analysed 38 patients (34 women, 4 men) affected by systemic sclerosis followed at the Clinical Immunology and Rheumatology Units of the University of Pisa, Italy. Mean age at the time of diagnosis was 57 years (range 29-87) and the average duration of illness before diagnosis was 97 months (range 10-240). All the patients underwent a clinical and serological workup at the time of blood sampling.

Sera from 22 age- and sex-matched controls were also tested.

The study was approved by the local ethics committee (protocol 14914).

Fifty-eight percent of the patients were treated with immunosuppressants (monotherapy in 19, association of 2 drugs in 3), most frequently mycophenolate (13 patients out of 22), followed by hydroxychloroquine (3/22) and cyclosporine (2/22). Other drugs used (by

1 patient out of 22) are azathioprine, tacrolimus, methotrexate, leflunomide, cyclophosphamide, etanercept and imatinib. Forty seven percent of the patients used calcium channel blockers.

Production of recombinant histidine-tagged alpha-enolase polypeptides

The construction of the pRSET-Eno plasmid, expressing a nearly full-length alpha enolase (aa 11-434, numbering the translation initiator Methionine as 1) fused in frame with a poly-histidine coding sequence (His), has been previously described (19). Plasmids expressing truncated alpha-enolase polypeptides, aa11-275, 48-275 and 275-434, were generated by restriction enzyme cleavage and subcloning in the appropriate pRSET vector, whereas PCR amplification with *ad hoc* designed oligomers was employed for the construction of the expression vector encoding aa 97-275. This last construct was sequenced to verify the open reading frame. Recombinant alpha-enolase polypeptides were overexpressed in the *E. Coli* strain BL21(DE3)/pLysS (Thermo Fisher Scientific) upon induction with 1 mM IPTG at 30°C and purified on Ni₂⁺ affinity column using the Probond Purification System supplied by Thermo Fisher Scientific. Three out of five truncated proteins (11-434, 11-275, 275-434) were isolated using hybrid conditions as previously described in details (19), while 48-275 and 97-275 polypeptides were purified under native conditions. All the proteins were eluted in elution buffer pH 8.0 containing 350 mM imidazole. The eluted fractions were dialysed against 20mM Tris-HCl pH7.6, 150mM NaCl, 2mM EDTA and 10% glycerol, than protein content was determined by the Bradford method using a commercially available kit (Bio-Rad). All recombinant proteins were diluted at 0.15 ug/ul and stored at -80°C after adding 0.02% n-dodecyl-D-maltoside (DDM), 5% Trehalose and 100mM L-Arginine to stabilise protein structures (20, 21).

Detection of autoantibodies

ACA are detected by immunofluorescence on Hep2 cells. ATA (anti-Sc170), anti-CENP-A, anti-CENP-B, anti-

RP11, anti-RP155, anti-fibrillarin, anti-NOR90, anti-Th/To, anti-PM-Sc1100, anti-PM-Sc175, anti-Ku, anti-PDGFR antibodies are evaluated by line immunoassay (EUROLINE Systemic Sclerosis Profile, Euroimmun AG, Lübeck, Germany).

Dot blot

Recombinant proteins (150ng) were spotted in duplicate onto nitrocellulose membrane using an Automation Workstation (Biomek® 3000, Beckman Coulter). Commercially available His-tagged alpha enolase (Sino Biological Inc., 11554-H07E) was used as a positive control and Bovine Serum Albumin (BSA) to monitor unspecific binding of antibodies. Odyssey western blotting reagents from Li-COR were used following the manufacturers' instructions. Briefly, the membrane was incubated with blocking reagent at room temperature for two hours, than with patient serum at a dilution of 1:100 overnight at 4°C in the presence of 0.1% Tween. After three washes in TBS containing 0.1% Tween, the membrane was incubated with Alexa Fluor 647-conjugated mouse monoclonal antibodies to human IgG1, IgG2, IgG3, IgG4 (Southern Biotech, clone E3, clone HP6014, clone HP6050, clone HP6025, respectively) at a dilution of 1:5000 in blocking reagent-Tween 0.05%. After three washes, secondary antibodies on the membrane were visualised using the Odyssey infrared imaging system (LI-COR Biosciences) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed utilising IBM® SPSS® Statistics, v. 20, and Addinsoft, XLSTAT statistical and data analysis solution (2019) Long Island, NY, USA, for Mac OsX.

After the application of a test for normality for continuous variables, data with normal distribution were presented as mean and standard deviation, and as median and interquartile range otherwise. The first elaboration has been conducted using a k-means clustering, by choosing a number of two clusters. Briefly, clustering is the process of separating a group of data points into

Table I. Reactivity of positive SSc sera with alpha-enolase polypeptides.

IgG Subclass	a-Enolase	S A M P L E S																		
		S1	S2	S7	S8	S11	S14	S15	S16	S17	S18	S19	S20	S23	S24	S29	S33	S34	S35	
IgG ₁	Eno ₁₁₋₄₃₄																			
	Eno ₁₁₋₂₇₅																			
	Eno ₄₈₋₂₇₅																			
	Eno ₉₇₋₂₇₅																			
	Eno ₂₇₅₋₄₃₄																			
IgG ₂	Eno ₁₁₋₄₃₄																			
	Eno ₁₁₋₂₇₅																			
	Eno ₄₈₋₂₇₅																			
	Eno ₉₇₋₂₇₅																			
	Eno ₂₇₅₋₄₃₄																			
IgG ₃	Eno ₁₁₋₄₃₄																			
	Eno ₁₁₋₂₇₅																			
	Eno ₄₈₋₂₇₅																			
	Eno ₉₇₋₂₇₅																			
	Eno ₂₇₅₋₄₃₄																			
IgG ₄	Eno ₁₁₋₄₃₄																			
	Eno ₁₁₋₂₇₅																			
	Eno ₄₈₋₂₇₅																			
	Eno ₉₇₋₂₇₅																			
	Eno ₂₇₅₋₄₃₄																			

The densitometric signals obtained with all the recombinant proteins using a single serum were subtracted of the values relative to antibody binding to BSA and normalised with respect to the commercial full length enolase signal to which was assigned the value 1. Reactivity of SSc sera was considered positive when higher than the 97th percentile of normal controls. Grey cells represent positivity of each serum with the correspondent form of alpha enolase.

a small number of k-clusters, aimed to assign a cluster to each data point.

Data were also analysed to identify the serological markers mainly contributing to set division of sample based on the following outcome variables: i. diffuse *versus* limited form of the disease, ii. presence of interstitial lung involvement. To this purpose, partial least square (PLS) models were created (one for each outcome variable), and the markers with a VIP ("variable importance in projection", representing a measure of a variable's relevance in the model) greater than 1.25 were considered significant for the group separation. Moreover, the Chi Square test for the categorical data, and the Mann-Whitney test for continuous variables were used to compare two subgroups. When

necessary, a multiple testing correction was used.

A *p*-value of 0.05 was used as the cut-off for significance in all statistical analyses.

Results

Clinical features of SSc patients

Nineteen patients (50%) are affected by the limited variant of the disease and the remaining 19 by the diffuse form. Sclerodactyly was observed in 30 of 38 patients. Raynaud's phenomenon is present in 37 patients, with an average duration before diagnosis of 129 months (range 10-444). Oesophageal involvement has been detected in 30 patients and calcinosis in only 1 patient.

As far as cardio-pulmonary involvement is concerned, pulmonary arterial

hypertension (PAP >25 mmHg at rest) is present in 15 of the 26 patients examined (57.7%). Interstitial lung disease (ILD) is shown by high-resolution computer tomography (HRCT) in 29 patients out of 38. From the functional point of view, 23 patients had a diffusing capacity for carbon monoxide (DLCO) value lower than 80% of predicted (mean 65.4%, range 29-123%); 19 patients out of 23 presented lung interstitial disease by HRCT.

Serologically, increased C reactive protein (CRP) levels are detected in 9 patients out of 34, low C3 levels in 5 and low C4 in 2.

Autoantibodies

ATA antibodies are detected in 17/38 patients and ACA in 13/38.

Out of the 19 patients affected by the limited variant, 11 are ACA + /ATA-, 4 ACA- / ATA -, 3 ACA- / ATA + and 1 patient is ACA + / ATA +. Out of the 19 patients with the diffuse form, 13 are ACA- / ATA +, 4 ACA- / ATA -, 1 ACA + / ATA - and none are double-positive. ACA positive patients react with both CENP-A and CENP-B polypeptides. Reactivity with CENP-A only was observed in 2 ATA positive patients. Other autoantibodies were rarely detected. Anti-RP155 antibodies are detected in 4 patients, 3 affected by the diffuse and one from the limited form; one out of four is negative for ATA and ACA. One patient (limited form) is positive for anti-NOR90 antibodies, one for anti-PM-Scl100 (diffuse) and one for anti-PM-Scl75 (limited).

Reactivity with alpha enolase was explored on the full length protein and on recombinant truncated forms, detecting IgG1, IgG2, IgG3 and IgG4 antibodies. The densitometric signals obtained with all the recombinant proteins using a single serum were subtracted of the values relative to antibody binding to BSA and normalised with respect to the commercial full length enolase signal to which was assigned the value 1. Reactivity of SSc sera was considered positive when higher than the 97th percentile of normal controls

Accordingly, anti-alpha enolase antibodies were detected in 18 patients (47%) (Table I); IgG1 antibodies are present in 8 patients as only subclass and in 5 together with IgG2. IgG2 in the absence of IgG1 are detected in 5 patients. IgG1 circulating antibodies from 4 patients react with 11-434 polypeptide, 7 with 11-275, 7 with 48-275, 5 with 97-275 polypeptide.

Regarding IgG2, antibodies from 1 patient react with 11-434 polypeptide, 4 with 11-275, 10 with 48-275, 9 with 97-275 and 1 with 275-434 polypeptide.

IgG3 targeting 48-275 and 97-275 are detected in one patient containing also IgG1 and IgG2 antibodies; IgG4 of the same specificity in one patient only, containing also IgG2 of the same specificity.

Overall the major target of IgG1 circulating antibodies is the amino terminal region of alpha enolase, while IgG2

epitopes are more restricted to the central portion of the molecule (aa 97-275).

Autoantibodies and clinical features of the disease

The relation between anti-enolase antibodies and other clinical and serological parameters of the disease was explored by cluster analysis. The results obtained with IgG1 anti-eno using k-means clustering show that the 2 identified clusters contain 9 and 29 patients, respectively. Patients in cluster B are characterised by higher disease duration, higher levels of NT-proBNP and higher titres of antibodies to 11-275 polypeptide, but do not differ from cluster A under any other clinical or serological parameter. We then analysed the data by a "partial least squares" regression, to identify the variables that maximally contribute to distinguish diffuse and limited form of the disease. The only autoantibodies identified are anti-topoisomerase antibodies with a VIP of 2,10 and anti-centromere antibodies with a VIP of 2,17. When we searched for the variables that maximally contribute to identify the patients affected by ILD, no autoantibody gave a significant contribution and only clinical variables were selected. Namely, DLCO, pulmonary pressure and esophageal involvement are the relevant variables

Discussion

This study reports a comprehensive analysis of the immune response to alpha enolase in patients affected by SSc. The results indicate that anti-enolase antibodies are frequently produced, being detected in roughly half of the patients. On the whole, sera react preferentially with the N-terminal portion of the molecule: reactivity with the C-terminal region is never observed in the absence of binding to the N-terminal polypeptides, suggesting a spreading from N to C terminal sequences. This epitope spreading, as often observed in the immune response to autoantigens, probably takes place in the very early phases of the disease. In fact, no relation can be established between disease duration and the number of epitopes recognized on the molecule, probably because in our cohort of patients dis-

ease duration is long enough to blur any possible evidence of epitope spreading. Moreover, the major reactivity observed against the N-terminal portion of alpha enolase is in agreement with the *in silico* prediction of linear epitopes by the web-tool ElliPro (<http://tools.iedb.org/ellipro/>; PDB-ID: 2PSN).

Several immunoreactive regions were identified in alpha enolase and despite a prevalent recognition of the aminoterminal portion, no dominant epitope could be detected. This pattern is very frequent in the immune response to autoantigens. Alpha enolase is a very conserved molecule, expressed also on the surface of different bacteria and fungi (22); thus, it is likely that exposure to infectious antigens may induce the production of antibodies against multiple epitopes of alpha enolase, shared with the human molecule. In the present work, immunogenicity of alpha enolase was explored by means of recombinant truncated proteins, that contain linear epitopes and probably also conformational ones. However, a bacterial vector was used for protein expression, limiting the possibility to detect post-translational modifications. Thus, we cannot exclude that autoantibodies to post-translationally modified alpha enolase do exist, and might define an immunodominant region of the enzyme.

The immune response to alpha enolase has no relation with the production of anti-centromere or anti-topoisomerase antibodies. Recently, the co-expression of anti-alpha enolase and anti-thyroid antibodies in Hashimoto's encephalopathy has been explained on the basis of sequence homology between enolase and thyroid antigens (23). On the contrary, no sequence homology can be detected between alpha enolase and CENP B or topoisomerase I, thus explaining the independent production of these autoantibodies in SSc.

Subclass distribution of anti-eno antibodies reflects the relative concentration of IgG subclasses in serum, being skewed to IgG1 (the most frequent) and IgG2 (present in 10 patients), while IgG3 and IgG4 are detectable in only one patient each. IgG subclasses differ substantially in the ability to interact with receptors and mediate effector re-

sponses; their relative concentration is dependent on the stimulating antigen and the cytokine environment where B cells mature. IgG1 are considered Th1-dependent and IgG4 Th2-dependent. In anti-phospholipid syndrome, IgG1, IgG2 and IgG3 antibodies to beta2glycoprotein are detected, while IgG3 dominate in the immune response to the first domain of the molecule (24). In rheumatoid arthritis, antibodies to citrullinated antigens display a different profile: IgG1 is the dominant subclass, followed by IgG4, while IgG3 and IgG2 are low or undetectable (25). Other rheumatoid arthritis specific antibodies as anti-PAD antibodies show the same subclass distribution. In systemic lupus, autoantibodies of different specificity display an individual subclass distribution and relation with disease activity (26, 27). As far as anti alpha enolase antibodies are concerned, serum IgG2 anti enolase are strongly associated with active lupus nephritis (28, 29).

Thus, antigen-related factors as well as disease-specific features seem to dictate the IgG subclass distribution of autoantibodies. In SSc, no data are available on the IgG subclass distribution of other disease-specific or disease-associated autoantibodies.

Anti-enolase antibodies are not associated with clinical or serological features of the disease and do not contribute to the distinction of diffuse *versus* limited form of the disease. Moreover, they display no association with disease-specific antibodies. Under this respect, it is of interest that in the present study the same technique (immunoblot) has been employed for the detection of all the different autoantibody specificities in patients sera. Cluster analysis identified ATA and ACA as the only autoantibodies that distinguish the limited from the diffuse form of the disease.

Despite the high number of disease-specific autoantibodies described in SSc, autoantibody negative patients still exist and new serological parameters are needed to better stratify patients. Under this respect, the contribution of anti-alpha enolase antibodies to the definition of specific subsets of the disease should be addressed in further studies on larger cohorts of patients.

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