
A clinical and histopathological analysis of the anti-centromere antibody positive subset of primary Sjögren's syndrome

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ABSTRACT

Objectives. ACA-positive/primary Sjögren's syndrome (pSS) represents a distinct overlapping entity with intermediate features in between limited systemic sclerosis (lSSc) and pSS. Few data are available on their general risk for lymphoproliferative complications, specifically regarding adverse predictors at the level of minor salivary gland (MSG) histology.

The objectives of this work are: a) to characterise, through a detailed immunohistochemistry study, the organisation of the lymphomonocytic infiltrates in ACA-positive/pSS patient vs. ACA-negative/pSS patients focusing on the presence of GC-like structures in minor salivary gland biopsies; b) to compare the frequency of traditional clinical and serological risk factors for lymphoma between the two subgroups.

Methods. We analysed 28 MSG samples from ACA-positive/pSS patients and 43 consecutive MSGs from ACA-negative/pSS, using sequential IHC staining for CD3, CD20 and CD21 in order to define the T/B cell segregation within the periductal infiltrates and presence of ectopic GC-like on the detection of GC-like structures. Clinical and serological data of all the patients were retrieved and analysed.

Results. Ectopic lymphoid structures (ELS) with GC-like structures were observed in 7 out of 28 ACA-positive/pSS patients (25%) and in 13 out of 43 ACA-negative/pSS patients (30.2%). Similarly, no statistical significant difference was found between the two groups as far as the classical pSS risk factors for lymphoproliferative complications was concerned (i.e. salivary gland enlargement, purpura, low C4, leukocytopenia, clonal gammopathy). Finally, the 3 cases of non-Hodgkin's lymphoma observed were equally distributed between the two subsets.

Conclusion. Overall, this study indicates that ACA-positive/and ACA-negative pSS patients apparently present a similar risk for lymphoproliferative complications as suggested indirectly by the analogies between the two groups observed at the histopathology level.

Introduction

Sjögren's syndrome (SS) is a common, systemic autoimmune disease that mainly affects the exocrine glands leading to clinical symptoms of dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca) (1). The disease can occur alone as primary SS (pSS) or in association with other systemic autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and scleroderma (SSc) (i.e. secondary Sjögren's syndrome-sSS) (2). Although the clinical presentation of SS is generally dominated by the progressive hypofunction of the salivary and lachrymal glands, a certain percentage of patients also develop severe extra-glandular disease manifestations, including malignant lymphoproliferative disorders (3-6). In order to fulfill the diagnostic criteria (American European Consensus Group Criteria-AECG 2002), patients are required to present the typical periductal lymphomononuclear infiltrates in the minor salivary glands biopsy (MSGB) or high serum titres of anti-Ro/SSA and/or anti-La/SSB autoantibodies (7).

However, the existence of a subgroup of pSS which displays a different serological profile characterised by the presence of anti-centromere autoantibodies (ACA) has been reported and is often associated with the lack of anti-Ro/SSA and anti-La/SSB autoantibodies (8, 9). According to several retrospective studies, the ACA-positive subset of pSS accounts to an estimated proportion of 3-5% of all patients attending to a Sjögren's clinic and may be considered

Competing interests: none declared.

as a distinct clinical entity showing intermediate feature between limited cutaneous scleroderma (lSSc) and pSS (8, 10, 11). In these patients the clinical course of SSc has been generally reported as milder with a lower frequency of severe disease phenotype when compared to “classical SSc patients”. Accordingly, although Raynaud’s phenomenon is a common early manifestation of ACA-positive/pSS, in the long term these patients tend to either remain stable or evolve towards a less severe scleroderma clinical expression characterised by uncommon internal organ involvement and milder microvascular dysfunction with lower frequency of active nailfold capillaroscopy pattern, sclerodactily and digital ulcers (12, 13). By contrast, ACA-positive/pSS patients display a full-blown pSS phenotype including sicca symptoms, extraglandular manifestations and evolution towards lymphoproliferative complications. Interestingly, several literature datasets reported that, in this subgroup, the prevalence of lymphoma is comparable or even higher than the frequency in the “classical” ACA-negative/pSS patients (8, 14).

In recent years, together with the classical clinical and laboratory predictors of lymphoma evolution (*i.e.* salivary gland swelling, lymphadenopathy, cryoglobulinaemia, low complement C4 etc) (15) salivary gland histopathology has emerged as a potential important tool to identify patients at higher risk of lymphomagenesis. Both a high focus score (FS>3) in the minor salivary glands (16) and the presence of ectopic germinal centres like structures (GC-like), also named ectopic lymphoid structures (ELS), have been linked with the evolution towards lymphoma in pSS patients (17, 18) although conflicting data are also present in the literature (19, 20). ELS are detectable in 30–40% of salivary gland biopsy with FS>1 and are characterised by periductal aggregates of T and B lymphocytes often presenting T/B cell segregation, development of high endothelial venules (HEV) and follicular dendritic cell network (FDC) which support a GC response. Intriguingly, in pSS patients, these structures seem to act as functional niches where

autoreactive B cell response takes place leading to autoantibody production and to the selection and proliferation of malignant clones that in the long term might promote MALT lymphoma development (17).

The main purpose of the present work was to characterise, through a detailed immunohistochemistry study, the degree of lymphoid organisation of the lymphomonocytic infiltrates in the specific subgroup of ACA-positive/pSS patient. In particular, we compared the frequency of GC-like structures in ACA-positive/pSS patients *versus* ACA-negative/pSS patients. Finally, we compared clinical and serological data of the two groups enrolling specifically on the presence of traditional risk factors for lymphoma.

Material and methods

Patients

A total of 28 MSGBs samples from ACA-positive/pSS patients were available from biopsy material stored either at University of Pisa, Italy or at the EMR Biobank at Queen Mary University of London QMUL, UK. In addition, 43 consecutive patients with an established diagnosis of ACA-negative/pSS and a FS>1 recruited between 2010 and 2013 were re-analysed for the main purpose of this study. The diagnosis of pSS was made according to the American European consensus criteria (AECG) whereas in ACA-positive/pSS patients the diagnosis of SSc followed the LeRoy *et al.* “early SSc criteria”. Demographic, clinical and immunological data were collected for all patients of each group. In particular, clinical evaluation for the SS component consisted in the registration of relevant clinical parameters including parotid gland swelling, and extra-glandular manifestations. Salivary gland ultrasonography was scored according to a modified version of the De Vita score (21). Diagnosis of lymphoma required histological confirmation. For the SSc component we collected data on the presence of Raynaud’s phenomenon, sclerodactily, digital ulcerations, telangiectasia, and internal organ involvement such as oesophageal involvement, lung interstitial fibrosis and pul-

monary hypertension. Similarly, laboratory and immunological variables were collected including antinuclear antibodies (detected by indirect immunofluorescence), anti Ro/SSA, La/SSB antibodies (by counterimmunoelectrophoresis), ACA (detected by indirect immunofluorescence) and Rheumatoid factor (by nephelometry). As regards to other laboratory tests, white blood cell count <4000/mm³, C3 <80 mg/dl, C4 < 10 mg/dl and IgG globulin >1.6 g/dl were considered abnormal. The study was approved by the local Ethics Committee and was performed according to the Helsinki Declaration.

Histology and immunohistochemistry

H&E stained paraffin-embedded MSGBs with FS >1, obtained at the time of diagnosis from all patients included, was re-evaluated by light microscopy in order to detect the presence of GC-like structures. Sequential sections from each group were stained for CD3 (T cell marker) and CD20 (B cell marker) in order to analyse T/B cell segregation within the periductal infiltrates. Briefly, after deparaffinisation and hydration, antigen retrieval was performed with 45 minutes incubation in Target Retrieval Solution (pH=6) Dako preheated at 95°C. Sections were washed in Tris Buffer Saline, peroxidase and protein blocked and then incubated 1 hour at room temperature with CD3 (Clone F7.2.38-Dako, M7254) or CD20 (Clone L26-Dako, M0755). DAKO EN VISION+ System-HRP (DAB) was used as secondary antibody and developing system in both immunohistochemistry staining.

On the samples displaying features of focal sialadenitis and GC-like structures an immunohistochemistry confirmation was obtained through the CD21 (Follicular dendritic cell marker-FDC) staining. Three µm-thick paraffin-embedded tissue sections were deparaffinised, hydrated and incubated with pre-warmed protein kinase K solution (Dako) at 37°C for antigen retrieval. After washing in Tris Buffer Saline, slides were incubated with peroxidase block, then with Dako Protein Block Serum Free and finally, left overnight at 4°C with CD21 antibody (Clone 1F8-Dako M0784).

All the sections were then examined on an Olympus microscope (4x and 10x microscopic fields).

Statistical analysis

Data were expressed as absolute frequencies and percentages for nominal variables and as the mean (standard deviation) for continuous variables. Comparisons were made using parametric Student's *t*-test and non-parametric Mann-Whitney U-test as applicable. Dichotomous variables were compared using contingency table analysis and Fisher's Exact test. Statistical analysis was carried out using SPSS 13 (SPSS Inc., Chicago IL, USA)

Results

Demographic, laboratory and clinical features of the ACA-positive vs. ACA-negative/pSS subsets

Table I summarises the demographic, laboratory and clinical features of the ACA-positive and ACA-negative/pSS subgroups. No differences were detected between ACA-positive and ACA-negative/pSS patient in terms of demographic characteristic. Specifically, the two subgroups did not differ for sex ratio, age at the diagnosis, age at the inclusion and follow up length.

Anti-Ro/SSA and anti-La/SSB autoantibodies were detected in 29% and 3.6% of ACA-positive/pSS patients, compared to 67% ($p=0.003$) and 35% ($p=0.001$) ACA-negative/pSS patients, respectively. Moreover, ACA-negative/pSS patients presented higher frequency of hypergammaglobulinaemia when compared to ACA-positive/pSS subgroup (54% and 13%, respectively, $p=0.002$). Rheumatoid factor prevalence was higher in the ACA-negative subgroup, although this difference was not significant. Antinuclear antibody positivity, presence of monoclonal gammopathy and complement levels did not differ in the two subgroups.

No statistical significant difference was found as far as the typical pSS manifestations was concerned (glandular and extra-glandular manifestations including lymphoproliferative disorders). Regarding glandular involvement, the two groups were comparable in terms of dryness severity. Moreover, at the

Table I. Demographics, clinical and serological features of ACA-positive and ACA-negative pSS patients.

Epidemiologic features	ACA pos/pSS (n=28)	ACA neg/pSS (n=43)	p-value
Sex	28 F:0 M	43 F:0 M	ns
Age at the inclusion (mean \pm SD, years)	63.6 \pm 12.5	59.11 \pm 15.9	ns
Age at diagnosis (mean \pm SD, years)	59.64 \pm 13.6	55.2 \pm 15.1	ns
Length of follow-up (mean \pm SD, years)	3.93 \pm 4.5	3.9 \pm 4.4	ns
Xerostomia	25/28 (89%)	40/43 (93%)	ns
Xerophthalmia	27/28 (96%)	38/43 (88%)	ns
Positive ocular test	24/28 (86%)	35/43 (81.4%)	ns
Parotid gland swelling	9/28 (32%)	14/43 (34%)	ns
Arthralgias	19/28 (68%)	30/43 (70%)	ns
Arthritis	5/28 (18%)	5/40 (12.5%)	ns
Raynaud's phenomenon	24/28 (86%)	15/40 (37.5%)	0.0001
Haematological manifestations	5/28 (18%)	12/43 (28%)	ns
Internal organ involvement	20/28 (71.4%)	20/43 (46%)	0.05
Skin involvement/SS type	2/28 (7%)	11/43 (26%)	0.06
Purpura	2/28 (7%)	8/43 (19%)	0.29
Skin involvement/ISSC type	7/28 (25%)	none	0.0002
Gastroesophageal involvement	11/28 (39%)	2/38 (5%)	0.0002
Pulmonary involvement	5/28 (18%)	none	0.008
Peripheral neuropathy	2/28 (7%)	8/43 (18%)	ns
Lymphoproliferative disorders	2/28 (7%)	1/43 (2.3%)	ns
ANA	28/28 (100%)	43/43 (100%)	ns
Ro/SSA antibodies	8/28 (29%)	29/43 (67%)	0.002
La/SSB antibodies	1/28 (3.6%)	15/43 (35%)	0.003
Rheumatoid Factor	9/28 (32%)	22/43 (51%)	ns
Hypergammaglobulinaemia	3/28 (11%)	23/43 (53%)	0.0003
Monoclonal gammopathy	4/27 (15%)	1/41 (2.4%)	0.07
Low C3	4/23 (17.4%)	2/33 (6%)	ns
Low C4	2/23 (9%)	5/33 (15%)	ns
HCQ	6/28 (21.4%)	22/43 (51.2%)	0.01
Steroids	10/28 (35.7%)	3/43 (7%)	0.003
Immunosuppressant drugs	4/28 (14.3%)	none	0.02

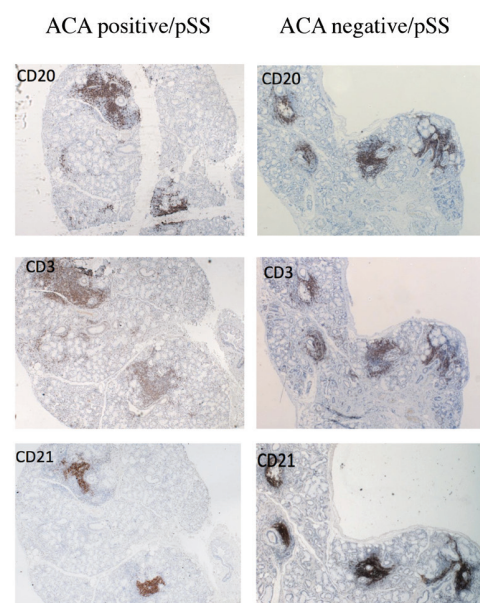


Fig. 1. Frequency of GC-like positive biopsies in ACA-positive/pSS and ACA-negative/pSS groups: immunohistochemical characterisation of periductal inflammatory infiltrates. (CD20-B cells; CD3-T cells; CD21-FDC network).

salivary gland ultrasonography examination a score ≥ 2 (based on the hyperechoic areas number and distribution) was observed in a similar percentage of ACA positive and ACA-negative pSS patients (11/17 (65%) vs. 25/33 (77%), $p=n.s.$).

Three cases of parotid MALT lymphoma were detected: two in the ACA-positive/pSS group and one in the ACA-negative. Moreover, no differences were observed between the two groups regarding the prevalence and distribution of conventional risk factors for

lymphoma (*i.e.* salivary gland swelling, vasculitic manifestation, low C4, clonal gammopathy) (Table I). ACA-negative patients tended to present an increased frequency of palpable purpura, even though this difference was not statistically significant (Table I).

When compared to ACA-negative/pSS, ACA-positive/pSS patients presented increased frequency of Raynaud phenomenon (37.5% vs. 89%, $p=0.0001$) and internal organ involvement (46% vs. 71% $p=0.048$) specifically due to SSc related manifestation such as oesophageal dysmotility disorder (5% vs. 46%, $p=0.0002$) and pulmonary fibrosis (0% vs. 18% $p=0.0075$). None of the patient of the two groups presented features of pulmonary hypertension. Additionally, ACA-positive/pSS patients displayed higher prevalence of ISSc-related skin involvement including sclerodactily and teleangiectasia. Considering the whole ACA positive subgroup, only one patient presented digital ulcers while melanoderma and skin calcinosis were absent in our cohort. None of the patient included in our study had history of kidney, heart or central nervous system involvement. Regarding the therapeutic strategies adopted in the two subgroups, the use of hydroxychloroquine was more common in the ACA-negative/pSS than in the ACA-positive subgroup (87.5% vs. 62.3% $p=0.03$ and 51.2% vs. 21.4% $p=0.01$, respectively). On the contrary, the percentage of ACA-positive/pSS patients requiring steroids or immunosuppressant drugs was significantly higher when compared to the ACA-negative subgroup (37.5% vs. 7% $p=0.03$; 14.3% vs. 7% $p=0.02$).

Detection of the prevalence of GC-like structures

We performed a systematic re-evaluation of MSGBs taken at time of diagnosis using sequential IHC staining for CD3, CD20 and CD21 in order to define the presence of ELS with features of ectopic GC-like structures. As depicted in representative Figure 1A-B, lymphocytic aggregates characterised by typical features of ELS such as segregation of CD3⁺ T and CD20⁺ B cells in separate areas and differentiation of CD21⁺ FDC networks within the B cell

rich areas were detected in both ACA-positive/pSS and ACA-negative pSS. Specifically, ELS with GC-like structures were observed in 7 out of 28 ACA-positive/pSS patients (prevalence 25%) and in 13 out of 43 ACA-negative/pSS patients (prevalence 30.2%, $p=NS$, Fig. 1C). Overall, this suggests that ectopic GCs from with similar frequencies in the two subsets, thus conferring a histopathology-related risk of lymphomagenesis in ACA-positive/pSS patients similar to the one described in the “classical” ACA-negative pSS subset.

Discussion

In the present study we described the histopathological, serological and clinical features of a specific subset of “overlap pSS-ISSc patients”, previously named as ACA-positive/pSS subset. According to our MSGBs re-evaluation and immunohistochemistry characterisation, no difference was found in terms of periductal inflammatory infiltrates organisation between ACA-positive and ACA-negative/pSS subgroups. In particular, the prevalence of highly organised, CD21⁺ GC-like structures in the ACA-positive/pSS MSGBs was comparable to the frequency reported in literature (and confirmed in our cohort) for the “classical” ACA-negative/pSS patients (25% vs. 30%) (22). Interestingly, in our cohort and in previous reports, lymphoma prevalence was also not inferior in ACA-positive/pSS with respect to ACA-negative/pSS patients. In keeping with this evidence, no statistically significant difference was detected when examining the frequency of known lymphoproliferative risk factors (*e.g.* low C4 levels, parotid gland swelling, cutaneous vasculitis, peripheral neuropathy) in the two groups. Noteworthy, newly identified histological markers of lymphomagenesis such as GC-like structures in the MSGBs did not show a different prevalence in the two groups as well. Taken together this evidence supports the hypothesis that the occurrence of lymphoproliferative complications in this specific ACA-positive/pSS subset is actually comparable with the rest of pSS population.

As far as the serological profile is concerned, our results confirmed the lower

prevalence of the typical pSS autoantibodies anti-Ro/SSA and anti-La/SSB in the ACA positive subgroup, as reported in previous literature datasets. From a clinical prospective, the ACA-positive/pSS patients seem to display a classical pSS disease phenotype with no significant difference in the prevalence of glandular and extraglandular manifestation when compared to ACA-negative/pSS subgroup (8, 9).

On the other hand, ACA-positive/pSS patients usually present with a polymorphic clinical picture enriched by all the spectrum of ISSc-related manifestations including Raynaud’s phenomenon, cutaneous, oesophageal and pulmonary involvement and required more commonly steroids and immunosuppressant drugs, in comparison to the ACA-negative subgroup. This might imply that antibodies control some clinical features regardless of the disease setting, influencing the disease phenotypes and shedding new light on possible targeted therapy in specific disease patterns. However, the absence of severe clinical manifestations of ISSc (*i.e.* lack of digital ulcers and pulmonary hypertension) suggest that ACA-positive pSS patients are dominated clinically by pSS-related glandular and extraglandular manifestations and represent a true subset of pSS. Although the main limitations of the present study are its retrospective nature and the relatively low number of patients enrolled due to the low frequency of the ACA-positive patients routinely attending a SS clinic (around 5%), we can conclude that ACA-positive/pSS patients represent a distinct overlapping subset in which ISSc-related manifestations are frequently mild and slowly progressing, while pSS-related glandular and systemic manifestations are indistinguishable from the “classical” anti-SSA/SSB+ subsets of pSS including clinical, laboratory and histopathological risk factors of lymphomagenesis. These patients require a tailored management focused on both ISSc and pSS related manifestations with a particular attention to the early treatment of lymphoproliferative complications. The long-term relevance of our data should be addressed in future studies on larger multicentric and prospective cohorts.

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