RHEUMATOLOGY

Rheumatology 2013;52:276–281
doi:10.1093/rheumatology/kes180
Advance Access publication 9 August 2012

Original article

BLyS upregulation in Sjögren's syndrome associated with lymphoproliferative disorders, higher ESSDAI score and B-cell clonal expansion in the salivary glands

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Abstract

Objective. Primary SS is characterized by an increased risk of lymphoma in patients with prelymphomatous manifestations (i.e. myoepithelial sialadenitis or mixed cryoglobulinaemia). Serum B-lymphocyte stimulator (s-BLyS) levels in SS-related B-cell lymphoproliferative disorders were studied by integrating the results with the disease activity score and with molecular analyses of B-cell expansion in the salivary glands.

Methods. Seventy-six primary SS patients (with or without lymphoma or prelymphomatous manifestations), 56 HCV-related cryoglobulinaemic vasculitis patients and 55 controls were studied. s-BLyS and molecular analyses of B-cell expansion in the salivary gland tissues were performed. Patients with SS and persistent parotid swelling underwent parotid biopsy.

Results. s-BLyS differed between SS subgroups, higher levels being documented in patients with lymphoma or prelymphomatous manifestations *vs* SS without [1.85 (0.45–4.12) ng/ml *vs* 1.12 (0.56–1.98) ng/ml; $P < 0.0001$]. s-BLyS levels significantly correlated with the European League Against Rheumatism (EULAR) SS disease activity index ($r = 0.62$, $P < 0.0001$, Spearman's test). Clonal B-cell expansion in the salivary glands, but not polyclonal B-cell expansion, was associated with higher s-BLyS levels [1.98 (0.45–4.12) ng/ml *vs* 1.15 (0.56–3.25) ng/ml; $P = 0.013$)].

Conclusion. Higher s-BLyS levels and tissue clonal B-cell expansion characterize SS with B-cell lymphoproliferative disorders, even at prelymphomatous stages. This subgroup of SS patients showed the highest EULAR SS disease activity index scores. This represents a biologic rationale for targeting both clonal B-cell expansion and s-BLyS overproduction in SS.

Key words: SS, BLyS, B-cell activating factor, salivary glands, lymphoma.

CLINICAL SCIENCE

Introduction

SS is an autoimmune and lymphoproliferative disorder primarily involving the salivary and lachrymal glands, leading to glandular damage, dysfunction and sicca syndrome [1]. Risk factors for lymphoma evolution in SS include, in particular, mixed cryoglobulinaemia, persistent parotid swelling and, recently, the presence of ectopic germinal centre-like structures in labial salivary glands [2, 3].

Molecular analyses of B-cell expansion in SS-associated myoepithelial sialadenitis (MESA) detected a higher risk in those cases with more aggressive pathological features and evidence of tissue-persistent monoclonal B-cell expansion [4]. Furthermore, the overproduction of serum B-lymphocyte stimulator (s-BLyS), which is a known antiapoptotic cytokine driving B-cell autoreactive

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Submitted 22 December 2011; revised version accepted 28 May 2012.

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clonal expansion, may represent an additional risk factor for SS-related lymphoproliferation [5–8].

The aim of this study was to explore the association between s-BLyS levels and lymphoproliferation in SS, focusing on SS patients with overt lymphoma or non-neoplastic lymphoproliferation [i.e. MESA or cryoglobulinemic vasculitis (CV)]. The association between s-BLyS levels and the pattern of B-cell expansion in salivary tissue samples was also explored, as BLyS upregulation, with respect to the evidence B-cell overexpansion in mucosa-associated lymphoid tissue sites, has been never investigated in SS. If present, such an association would further support the role of BLyS in SS-related B-cell expansion. Finally, the new European League Against Rheumatism SS disease activity index (ESSDAI) [9] was calculated and related to s-BLyS levels.

Materials and methods

Study participants

Seventy-six Caucasian primary SS patients [68/76 females (89.5%), mean (S.D.) age 49.7 (14.1) years; Table 1] were retrospectively studied; 56 consecutive HCV-related CV patients [76.8% females, mean (S.D.) age 61.2 (11.5) years] and 55 blood donors [45.5% females, mean (S.D.) age 41.3 (12.7) years] were included in the study as control groups. The SS patients met the revised 2002 American–European consensus research classification criteria for primary SS [10], and the CV patients met the preliminary classification criteria for CV [11]. The median ESSDAI score was 10, ranging from 0 to 37 [9], and it was registered at the time of detection of s-BLyS levels. B-cell lymphoproliferative disorder in the course of SS was defined as the presence of overt lymphoma, or a prelymphomatous manifestation, i.e. CV, or persistent parotid swelling with histopathological diagnosis of MESA. All the SS patients underwent a standard protocol with a careful clinical examination, an abdomen ultrasonography, thoracic X-rays and laboratory analyses to detect a possible lymphoproliferative disorder. All the patients with persistent major salivary gland enlargement underwent major salivary biopsy.

The 76 SS patients belonged to the following four groups: (i) SS with overt lymphoma ($n = 14$), (ii) SS with persistent major salivary gland swelling and histopathological evidence of MESA ($n = 14$), (iii) SS with CV ($n = 14$) and (iv) SS with none of these processes (i.e. without any lymphoproliferative disorder) ($n = 34$). The SS patients in the last group were consecutively selected, while the SS patients included in the other three groups were all the patients currently referred to our clinic who showed an adequate follow-up.

Patients with SS were taking neither immunosuppressors nor glucocorticoids at the time of detection of s-BLyS levels, and 24/76 (31.6%) patients were taking chloroquine or HCQ, whereas 14/56 (25%) patients with HCV-CV were taking low to medium doses of glucocorticoids, which do not seem to affect s-BLyS levels [12]. s-BLyS levels were investigated in SS patients with overt lymphoma at the time of the diagnosis of lymphoma, before chemotherapy or B-cell depleting therapy.

The study was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee (Ethics Committee of Azienda Ospedaliera Universitaria 'Santa Maria della Misericordia' di Udine), and all patients gave written informed consent. Clinical

TABLE 1 Clinical and serological characterization of SS patients and association or correlation with s-BLyS levels

Features	SS patients ($n = 76$)	P value
Age at diagnosis [mean (S.D.), median (range)]	49.7 (14.1), 50.0 (10–75)	0.94[b]
Sex (F/M)	68/8	0.37[c]
Objective dry mouth, *n* (%)	68 (89.5)	0.13[c]
Objective dry eyes, *n* (%)	70 (92.1)	0.11[c]
Parotid swelling, *n* (%)	27 (35.5)	<0.0001[c]
Extraglandular involvement[a], *n* (%)	40 (52.6)	0.67[c]
Autoimmune thyroiditis, *n* (%)	22 (28.9)	0.25[c]
Primary biliary cirrhosis, *n* (%)	4 (5.3)	0.47[c]
ANA, *n* (%)	70 (92.1)	0.06[c]
ANA titre, median (range)	1 : 640 (0–1 : 5120)	0.03[b]
Anti-Ro (SSA), *n* (%)	63 (82.9)	0.009[c]
Anti-La (SSB), *n* (%)	52 (68.4)	<0.0001[c]
RF, *n* (%)	53 (69.7)	0.03[c]
Low C4 (<10 mg/dl), *n* (%)	28 (36.8)	0.002[c]
Presence of serum cryoglobulins, *n* (%)	28 (36.8)	0.01[c]
Serum monoclonal component, *n* (%)	29 (38.2)	0.01[c]
Levels of gammaglobulins [mean (S.D), median (range)], g/l	17.2 (8.0), 15.3 (5.9–40.9)	0.84[b]
Levels of serum β_2-microglobulin [mean (S.D), median (range)], mg/dl	2.9 (1.4), 2.6 (1.1–7.7)	0.87[b]

[a]Arthritis and/or pulmonary involvement (interstitial pneumonia) and/or renal involvement (interstitial nephritis or renal vasculitis) and/or cutaneous vasculitis and/or peripheral neuropathy. [b]Spearman correlation; [c]Mann–Whitney test. F: female; M: male.

data regarding glandular and extraglandular features and serological characterization of SS patients were reported in Table 1, together with the associations with s-BLyS levels (Table 1).

Serology

Serum levels of ANA, Ro/La autoantibodies, C4, cryoglobulins gammaglobulins and IgM RF (nephelometry) were measured as part of standard diagnostic procedure. Anti-Ro/La autoantibody specificity was determined by ELISA using recombinant Ro60, Ro52 and La proteins, and sera from patients with anti-La were further tested by counterimmunoelectrophoresis, as previously described [13]. The s-BLyS was measured by a quantitative sandwich enzyme immunoassay (Quantikine Human BAFF Immunoassay; R&D Systems, Minneapolis, MN, USA) using automated instruments and procedures (Dynex Technologies ELISA processor) [12].

Molecular analysis of B-cell clonality

The pattern of B-cell expansion (clonal or polyclonal) in the salivary glands (parotid or minor salivary glands) was evaluated by seminested PCR using an upstream primer directed to the third framework variable (V) region of the IgH gene and downstream primers directed to the joining (J) region, as previously described elsewhere [14].

Salivary gland samples were available for the analysis of B-cell clonality in 48/76 SS patients (63.2%). Patients with SS and persistent parotid swelling underwent parotid biopsy, after giving and signing the informed consent.

Statistics

Continuous variables were described as the mean and standard deviation or median and range. Categorical variables were summarized using frequencies and percentages. Data were tested for normality distribution using the Kolmogorov–Smirnov test. Analysis of variance or Kruskall–Wallis test was used to compare s-BLyS levels among groups, as appropriate. Mann–Whitney or *t*-test was used to compare continuous variables between two groups. Bonferroni's correction was used for multiple comparisons. Chi-square or Fisher's exact test was used to compare categorical variables. The correlation between continuous variables was assessed by Pearson's or Spearman's test, as required. The receiver operating characteristic curve was used to estimate the accuracy of s-BLyS in discriminating the presence of lymphoma in SS patients.

Univariate and multivariate stepwise logistic analyses were done to explore the association between the presence of lymphoma and s-BLyS levels, the presence of RF, cryoglobulins, low complement C4 and the associations between the presence of B-cell lymphoproliferative disorders, in general, and the same covariates. Data were analysed with SPSS software version 13.1. Results were considered statistically significant when $P < 0.05$.

Results

s-BLyS levels and SS-related B-cell lymphoproliferative disorders

s-BLyS levels differed among SS patient subgroups ($P < 0.0001$), higher levels being documented in the group of SS patients with a B-cell lymphoproliferative disorder (i.e. overt lymphoma, or MESA, or CV) as compared with SS patients without any lymphoproliferative disorder [1.85 (0.45–4.12) ng/ml *vs* 1.12 (0.56–1.98) ng/ml; $P < 0.0001$] (Fig. 1). In addition, there were increased s-BLyS levels, going from SS patients without any B-cell lymphoproliferative disorder [1.12 (0.56–1.98) ng/ml] to SS patients with CV [1.62 (0.45–3.25) ng/ml], to SS patients with MESA [1.85 (0.57–4.12) ng/ml] and finally, SS patients with overt lymphoma [2.06 (1.1–3.96) ng/ml], even if a significant difference was observed only between SS with lymphoma and SS without any lymphoproliferative disorder ($P = 0.001$) (Fig. 2) (SS with MESA vs SS with CV, $P = 0.46$, SS with MESA vs SS with lymphoma, $P = 0.15$, SS with MESA vs SS without any lymphoproliferative disorder, $P = 0.14$, SS with CV vs SS without and lymphoproliferative disorder, $P = 0.22$).

The 1.75 ng/ml cut-off value for serum concentration of s-BLyS distinguished SS patients with lymphoma from SS patients without lymphoma with a sensitivity of 78.6% (95% CI 49.2, 95.3) and a specificity of 77.4% (95% CI 65.0, 87.1; $P < 0.0001$; area under the curve = 0.817, standard error = 0.06, 95% CI 0.71, 0.89). Positive and negative predictive values were 22% (95% CI 6.9, 45.9) and 97.8% (95% CI 89.7, 99.9), respectively.

By univariate analyses, s-BLyS [$P = 0.001$; odds ratio (OR) = 3.2, 95% CI 1.6, 6.4], low C4 ($P = 0.02$; OR = 4.1, 95% CI 1.2, 13.8) and the presence of cryoglobulins ($P = 0.006$; OR = 6.1, 95% CI 1.7, 22.0) were associated with

Fig. 1 s-BLyS levels (ng/ml) in SS patients with B-cell lymphoproliferative disorders (lymphoma or prelymphomatous manifestations; $n = 42$) or without lymphoproliferative disorders ($n = 34$).

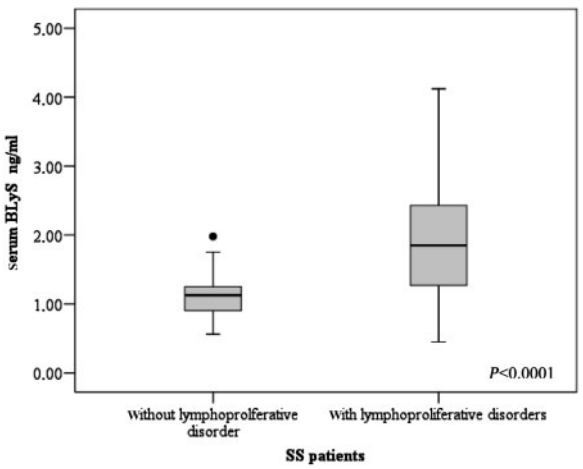
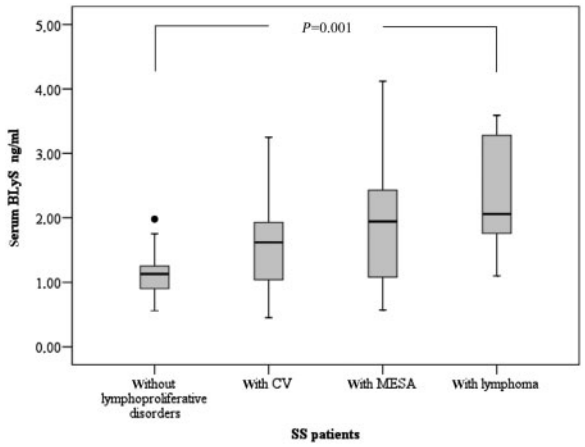
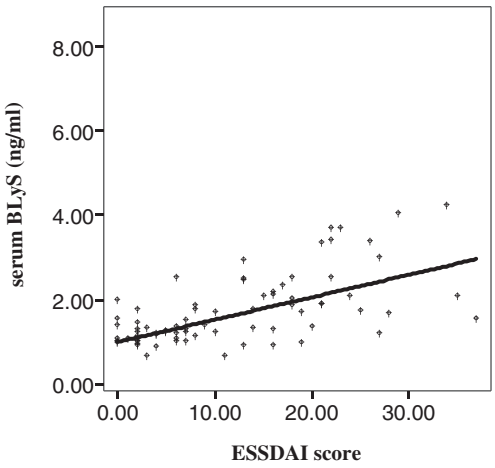


FIG. 2 s-BLyS levels (ng/ml) in the four groups of SS patients.



From left to right: (i) SS without a B-cell lymphoproliferative disorder ($n = 34$), (ii) SS with CV ($n = 14$), (iii) SS with MESA ($n = 14$) and (iv) SS with overt lymphoma ($n = 14$).

FIG. 3 Correlation between s-BLyS levels (ng/ml) and ESSDAI scores in SS patients.



lymphoma, whereas the presence of RF was not ($P = 0.21$). When considering the larger group of SS with a lymphoproliferative disorder (i.e. lymphoma or prelymphomatous manifestations), s-BLyS ($P = 0.0003$; OR = 8.5, 95% CI 2.7, 27.1), the presence of RF ($P = 0.001$; OR = 6.8, 95% CI 2.1, 21.6), low C4 ($P = 0.0003$; OR = 9.2, 95% CI 2.7, 30.6) and the presence of cryoglobulins ($P < 0.0001$; OR = 23.7, 95% CI 5.0, 112.3) were associated with the presence of a B-cell lymphoproliferative disorder by the same analyses.

Multivariate analyses showed that s-BLyS was independently associated with both lymphoma ($P = 0.02$; OR = 2.8, 95% CI 1.2, 6.6) and with the presence of a B-cell lymphoproliferative disorder (lymphoma or prelymphomatous manifestations; $P = 0.003$; OR = 9.4, 95% CI 2.2, 40.6). Also, the presence of serum cryoglobulins was independently associated both with lymphoma ($P = 0.04$; OR = 4.2, 95% CI 1.1, 16.9) and with B-cell lymphoproliferative disorders (lymphoma or prelymphomatous manifestations; $P = 0.001$; OR = 16.4, 95% CI 2.9, 90.9).

s-BLyS levels were higher in SS patients than in healthy controls [1.29 (0.45–4.12) ng/ml *vs* 0.65 (0.41–2.52) ng/ml; $P < 0.0001$], whereas they were not different in HCV-related CV patients [1.29 (0.45–4.12) ng/ml *vs* 1.37 (0.18–8.6) ng/ml; $P = 0.56$], confirming previous results both in SS and in HCV-positive CV patients [6, 12].

No age or sex distribution differences were observed between SS patients with or without lymphoproliferative disorders (data not shown). As observed in a previous study [5], s-BLyS was associated with ANA titre and with positivity of anti-Ro, anti-La, RF, cryoglobulins and serum monoclonal bands in our series (Table 1).

s-BLyS levels and disease activity

s-BLyS levels significantly correlated with the ESSDAI score ($r = 0.62$, $P < 0.0001$, Spearman's test) (Fig. 3). When exploring the different ESSDAI domains, s-BLyS levels significantly correlated with the constitutional domain ($r = 0.53$, $P < 0.0001$, Spearman's test), the lymphoadenopathy domain ($r = 0.53$, $P < 0.0001$, Spearman's test), the glandular domain ($r = 0.52$, $P < 0.0001$, Spearman's test) and the biological domain ($r = 0.24$, $P = 0.04$, Spearman's test).

s-BLyS levels and B-cell clonal expansion in SS salivary tissue

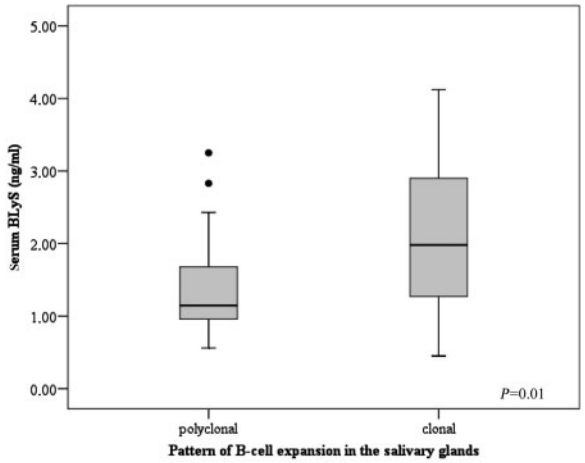
Forty-eight salivary gland tissues were available for clonal analyses, 25/48 from major salivary glands and 23/48 from minor salivary glands. Fourteen lymphomas (13 marginal zone lymphomas of salivary gland and 1 diffuse large B-cell lymphoma) and 14 MESA were collected. A clonal pattern of B-cell expansion in the salivary glands was observed in 26/48 SS patients (54.2%), and it was distributed in the four groups as follows: 13/14 in the lymphoma group, 8/14 in the MESA group, 4/8 in the CV group and 1/11 in the group of SS without any of these processes.

Notably, s-BLyS levels were significantly higher in SS patients showing a clonal B-cell expansion in the salivary glands than in SS patients with a polyclonal B-cell expansion [1.98 (0.45–4.12) ng/ml *vs* 1.15 (0.56–3.25) ng/ml; $P = 0.013$)] (Fig. 4).

Discussion

SS is an autoimmune B-cell disorder with non-malignant B-cell overexpansion [1]. Evolution into B-cell lymphoma occurs in ~5% of patients [2, 15], whereas prelymphomatous manifestations, i.e. parotid MESA and mixed cryoglobulinaemia, are more frequent than lymphoma [2]. B-cell overexpansion is a dominant feature of SS, as it is represented by the presence of specific autoantibodies

FIG. 4 s-BLyS levels (ng/ml) in SS patients with or without clonal B-cell expansion in the salivary glands.



and hypergammaglobulinaemia and the presence of RF and serum cryoglobulins [1, 2]. A significant number of B cells and plasma cells are also present in the inflamed tissue, where they can lead to the formation of ectopic germinal centre-like structures, recently associated with lymphoma development in SS [3]. Several cellular and humoral factors may contribute to clonal B-cell expansion and to the selection of high-affinity self-reactive B cells in SS salivary lesions. One of them is BLyS, a key survival factor for B-cell maturation [16]. High BLyS levels have been found in SS in serum, in saliva and in the salivary glands [5–8].

BLyS transgenic mice develop autoimmune diseases resembling SLE and SS [17] and are predisposed to marginal zone lymphoma when they are also knocked out for TNF [18]. Indeed, BLyS has been implicated in the formation of ectopic germinal centres in SS [19, 20], highlighting the possible role of this cytokine in lymphoproliferation at a prelymphomatous stage. BLyS may therefore represent a link between autoimmunity and lymphoma evolution in SS, as suggested in HCV-related CV [12].

The present study dissected BLyS upregulation with regard to both the presence of a B-cell lymphoproliferative disorder in SS (malignant or non-malignant) and the occurrence of tissue B-cell clonal expansion. Interestingly, for the first time, s-BLyS was also correlated with disease activity in SS, as recently quantified by the ESSDAI score [9]. Notably, s-BLyS correlated with disease activity also in B-cell non-Hodgkin lymphoma [21]. This result is also in agreement with the recently published observation of an association between increased levels of β_2-microglobulin and the ESSDAI score [22], pointing out the relationship between lymphocyte activation and disease activity in SS patients. Overall, s-BLyS overproduction seems more linked to a subset of SS characterized by more advanced stages of lymphoproliferation and higher disease activity. Thus the link between BLyS upregulation and SS-related B-cell lymphoproliferation can be better supported. BLyS may then represent an important therapeutic target in SS.

Interestingly, similar increased s-BLyS levels were noticed also in HCV-related CV, which is another autoimmune disease characterized by the presence of pathogenic RF-positive B-cell clones. Strikingly, SS represented the first cause of HCV-unrelated CV in a recent large multicentre survey [11]. Biologic similarities between the two disorders, possibly implicating similar mechanisms for BLyS upregulation, may be hypothesized. However, different pathogenic mechanisms may exist [23], and B-cell depleting therapy may be more effective in HCV-related CV [24] than in SS [25]. As BLyS upregulation was strictly related to clonal expansion of B cell in the salivary glands, the presence of BLyS in the local salivary microenvironment [8] may play a pivotal role for B-cell proliferation in SS, and may also cause resistance to B-cell depleting therapy with rituximab [14].

The design of this study, i.e. a retrospective analyses rather than a prospective study, represents a limitation. Prospective studies are needed to explore the hypothesis that increased s-BLyS levels could be predictive for lymphoma or non-malignant lymphoproliferative disorder evolution in SS. As higher s-BLyS levels characterize not only SS-related lymphoma but also SS-related lymphoproliferation in general, BLyS may be implicated in B-cell lymphoproliferation in this disease before lymphoma evolution. BLyS overproduction in the local microenvironment may support the persistence of RF-positive B-cell clones in tissue lesions and the emergence of a neoplastic clone [26, 27].

In conclusion, while increased s-BLyS levels are detected in SS patients in general, BLyS upregulation appears more pronounced in SS patients with a B-cell lymphoproliferative disorder (i.e. B-cell lymphoma or CV or MESA) and with B-cell clonal expansion in the salivary glands. Anti-BLyS treatment may therefore prove useful in SS. Furthermore, as in the mucosa-associated lymphoid tissue microenvironment, BLyS may provide local resistance to B-cell depletion [15, 27, 28] and BLyS blockade might prove effective in facilitating direct targeting of B cells in SS.

Rheumatology key messages

- BLyS is upregulated in SS associated with lymphoproliferative disorders and higher ESSDAI score.
- BLyS upregulation is associated with B-cell clonal expansion in the target tissue of SS.
- BLyS is a good therapeutic target in SS.

Disclosure statement: The authors have declared no conflicts of interest.

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