Histopathological and immunohistochemical profile in primary Sjögren’s syndrome

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Abstract

Sjögren’s syndrome (SS) is an autoimmune disease characterized by hypofunction of the salivary and lachrymal glands. Main clinical features of SS are sicca symptoms, due to the altered glandular function. Also, in advanced stages, bilateral swelling of the parotid glands can be noted, indicative of severe glandular involvement. Phenotypic expression of various mononuclear cells present in the affected tissue offers additional insight into cellular proliferation, survival, migration, antibody secretion and also the potential of forming tertiary lymphoid tissue, i.e., germinal centers. The main objective of the present study was to evaluate various autoimmune activity patterns present in salivary glands by means of immunohistochemistry (IHC) analysis. The study group comprised of 10 primary SS patients, with various degrees of lymphocytic infiltrates confirmed on minor salivary gland (MSG) biopsy. We could identify both morphological changes, i.e., ductal system abnormalities or increased interstitial fibrosis, and immunological patterns associated with SS pathogenesis. CD3+ T-cells displayed a more intense reaction in specimens with mild to intermediate focus score (FS) grade. Specimens with important CD20+ B-cell component of lymphocytic infiltrate were associated with intermediate and severe FS grade. Specimens showing degrees of CD68+ cells, with more intense IHC reactions in slides displaying a more advanced mononuclear infiltration. Immunoreactivity was strong for both MMP-2 and MMP-8 matrix metalloproteinases, throughout the gland, in areas of acini, without it being linked with proximity of mononuclear cell infiltration. We could also establish some correlations between the degree of lymphocytic infiltration and clinical profile.

Keywords: Sjögren’s syndrome, sialadenitis, immunohistochemistry, autoimmune activity.

Introduction

Sjögren’s syndrome (SS) is an autoimmune disease characterized by hypofunction of the salivary and lachrymal glands. Depending on the clinical context, it can be classified either as primary (pSS) form or secondary form (sSS), if a co-existing autoimmune disease is confirmed [1]. Main clinical features of SS are sicca symptoms, due to the altered glandular function. Also, in advanced stages, bilateral swelling of the parotid glands (PGs) can be noted, indicative of severe glandular involvement. Being a multisystemic disease, the clinical presentation is usually complex, displaying also extra-glandular features, such as arthritis, peripheral neuropathy, pulmonary infiltrate, renal tubular acidosis or purpura [1]. Other terms associated with SS are autoimmune exocrinopathy or autoimmune epithelitis, both stemming from the core pathological processes that develops, i.e., chronic inflammation of the epithelium lining the exocrine glands [2, 3]. Estimates of prevalence range from 0.2% to 2.7% depending on study protocols, with a strong female propensity from 9:1 to as high as 20:1 [4]. Mean age of onset is usually in the 4th to 5th decade, with juvenile forms being a very rare occurrence [5].

The current diagnostic approach to SS is based on the American–European Consensus Group (AECG) and American College of Rheumatology (ACR) criteria [6, 7]. Although initial diagnostic criteria were continuously revised in order to standardize the clinical approach, histological assessment of the lymphocytic infiltration through biopsy of the minor salivary glands (MSGs) remained crucial in establishing the severity of glandular involvement. The characteristic anatomopathological feature of this disease is the autoimmune sialadenitis (AS), characterized by mononuclear cell infiltration of exocrine glands. Chronic T-cell mediated inflammation leads to proliferation of autoantibody producing B-cells, resulting in an overall glandular atrophy and deficient function [8]. Specific autoantibodies associated with SS target ribonucleoprotein particles SSA/Ro and SSB/La. Initial studies of MSG biopsies were centered on the quantification of lymphocytic aggregates by calculating the focus score (FS) [9]. Recently, research interest is shifted to the characterization of lymphocytic subpopulations within the MSG infiltrate. Although the FS in-itself represents a significant prognostic factor, the phenotypic expression of various mononuclear cells present in the affected tissue offers additional insight into cellular proliferation, survival, migration, antibody secretion and also the potential of forming tertiary lymphoid tissue, i.e., germinal centers (GCs) [10–12].

The main objective of the present study was to evaluate various autoimmune activity patterns present in salivary
glands by means of immunohistochemistry analysis. The study group comprised of 10 primary SS patients, with various degrees of lymphocytic infiltrates confirmed on MSG biopsy. Immunohistochemistry (IHC) assessment was performed using various antibodies targeting both lymphocytic subpopulations, and also other markers linked to autoimmune activity. We aim to obtain a distribution pattern of various IHC markers within the MSG tissues and to establish a potential correlation with the disease severity.

**Patients, Materials and Methods**

We conducted our study on a small group of 10 patients diagnosed with pSS, based on the AECG criteria [6]. The clinical assessment of each patient was focused on acquiring subjective data regarding sicca symptoms, which were further examined using established clinical tests and questionnaires [e.g., Schirmer’s test, The Xerostomia Inventory (XI), Ocular Surface Disease Index (OSDI)] [13, 14], and also on examining the presence of salivary gland enlargement or development of extra-glandular manifestations. The laboratory panel consisted of a complete blood count (CBC), liver and kidney tests, inflammatory markers (e.g., erythrocyte sedimentation rate, fibrinogen, C-reactive protein), complemented by screening of specific diagnostic and prognostic markers, such as positivity for anti-Ro/SSA and anti-La/SSB autoantibodies, complement C3, C4 levels, high rheumatoid factor (RF) defined as RF >14 IU/mL, hypergammaglobulinemia defined as >20.3% of total serum proteins and a qualitative cryoglobulinemia test.

Every MSG biopsy specimen was obtained from the inner surface of the lower lip following the Daniel’s technique [15]. Thus, between four and five MSGs were extracted from each subject. For the histological assessment, specimens obtained were fixed in paraffin blocks, from which 3-μm cross-sections were cut and stained with Hematoxylin–Eosin (HE) and Goldner–Székely green light trichrome (GLT) technique. An area of 10×10 mm was evaluated by light microscopy. Quantitative analysis of the focal lymphocytic sialadenitis was performed using the FS, through which the number of foci, i.e., an aggregate of more than 50 lymphocytes/4 mm², is specified. Further staging of the infiltration was made using the Tarpley et al. classification [16], through which three stages were defined, as follows: mild – class 1, intermediate – class 2 and severe – class 3+. For the IHC assessment, the paraffin-included material was sectioned to 3-μm thick histological cross-sections. The slides obtained were then kept for 24 hours in the thermostat, at 37°C. Next steps consisted of de-waxing, hydration, incubation in 1% hydrogen peroxide solution and unmasking of the antigen through boiling in pH 6 citrate solution. After the washing in phosphate-buffered saline (PBS) solution and blocking by endogenous peroxidase, the cross-sections were incubated overnight with primary antibodies at 4°C. The secondary antibodies were added the next day with polymer-based peroxidase, and the signal was detected by 3,3'-Diaminobenzidine (DAB). Cross-sections were stained with the following antibodies: CD3, CD4, CD8, CD20, CD68, CD34, matrix metalloproteinase (MMP)-2, MMP-8 and tryptase. The slides were examined using an Eclipse 55i Nikon microscope connected to a Ds-fi1 microscope C-mount camera and then stored in digital format.

**Results**

The study group included in our research consists of 10 female patients, with a mean age of 49.7 years (±6.7 years) and mean history of disease prior to study inclusion of 5.8 years (±2.2 years). Sicca symptoms were present in the majority of patients, with prevalence of xerophthalmia (9/10 patients) higher than xerostomia (6/10 patients). Parotidomegaly was observed in half of patients, being associated with severe salivary hypofunction and longer disease history. Main extraglandular manifestations (EGMs) observed within our study group consisted of arthritis (4/10 patients) and purpura (3/10 patients). One patient with longer disease history (nine years) associated besides arthritis also renal involvement and peripheral neuropathy. Analysis of the immunological panel revealed a higher prevalence of anti-SSA autoantibodies (9/10 patients) than anti-SSB (6/10 patients). Both autoantibodies were positive in half of the patients. High titers of RF (4/10 patients) were associated with articular manifestations. Other immunological markers, such as low C3 or C4, hypergammaglobulinemia and cryoglobulinemia were noted in isolated cases.

Histopathology analysis revealed structural changes in the glandular parenchyma in relation to the degree of chronic inflammatory infiltration (Figure 1). Main features observed were focal lymphocytic sialadenitis (FLS), secondary ductal abnormalities and non-specific desmoplastic reaction. Morphological changes of intraglandular ductal system varied from sialectasis to mild deterioration or destruction of ducts (Figure 2). Distribution of lymphocytic infiltrates was predominantly periductal, leading to the aforementioned defects. Another feature often noted was hyperplasia of ductal epithelium that accompanied the FLS (Figure 1).

We observed varying degrees of lymphocytic infiltrate (Figure 1) assessed through semi-quantitative analysis, i.e., FS. Of the 10 specimens, half-displayed focus score of 2 and only two specimens were assessed as 3+, i.e., severe form. These intermediate and severe forms were associated with ductal deterioration and increased expression of B-cells in IHC. Although the lymphoid infiltrate often displayed a follicular pattern, we did not observe any aggregates with features suggestive of GCs. Interstitial fibrosis due to chronic inflammatory status was observed in some specimens (Figure 3), regarded also as a secondary phenomenon, in relation with increased age.

Some correlations were noted between the degree of lymphocytic infiltration and clinical profile (Table 1).

Seven patients with intermediate and severe FS grades featured severe oral and ocular symptoms, confirmed by Schirmer’s test, and high titer of anti-SSA/SSB antibodies. Both patients with severe forms and two patients with intermediate forms of lymphocytic infiltration featured parotidomegaly. EGMs were limited to arthritis in three patients with intermediate FS grade and one with severe FS grade, purpuric eruption in two patients with intermediate FS grade and one with severe FS grade; one patient with severe FS grade also featured renal involvement and peripheral mononeuropathy.
Figure 1 – Focal lymphocytic sialadenitis (FLS) comprised of mononuclear cell infiltrate throughout the parotid gland parenchyma, between glandular acini and adjacent to salivary ducts: (a) Mild FLS, HE staining, ×200; (b) Severe FLS and hyperplasia of ductal epithelium (arrowheads), GLT staining, ×200.

Figure 2 – Morphological changes of intraglandular ductal system: (a) Marked sialectasis; (b) Destruction and merging of adjacent salivary ducts developing a moniliform aspect. GLT staining, ×100.

Figure 3 – Varying degrees of desmoplastic reaction in parotid gland: (a) Moderate; (b) Marked interstitial fibrosis. GLT staining, ×200.
Table 1 – Study group clinical and histopathological features

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Gender</th>
<th>Age [years]</th>
<th>Disease history [years]</th>
<th>Extraglandular manifestations</th>
<th>Specific auto-antibodies</th>
<th>Clinical and serological profile</th>
<th>Histological and immunohistochemical patterns</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>FS</td>
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<tr>
<td>1.</td>
<td>F</td>
<td>55</td>
<td>7</td>
<td>Purpura</td>
<td>Anti-SSA, anti-SSB</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>2.</td>
<td>F</td>
<td>61</td>
<td>9</td>
<td>Arthritis</td>
<td>Anti-SSA, anti-SSB</td>
<td>2</td>
<td>++</td>
</tr>
<tr>
<td>3.</td>
<td>F</td>
<td>47</td>
<td>2</td>
<td>none</td>
<td>Anti-SSA</td>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>4.</td>
<td>F</td>
<td>49</td>
<td>5</td>
<td>Purpura</td>
<td>Anti-SSA</td>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td>5.</td>
<td>F</td>
<td>50</td>
<td>6</td>
<td>Arthritis</td>
<td>Anti-SSB</td>
<td>2</td>
<td>++</td>
</tr>
<tr>
<td>6.</td>
<td>F</td>
<td>38</td>
<td>4</td>
<td>none</td>
<td>Anti-SSA, anti-SSB</td>
<td>1</td>
<td>++</td>
</tr>
<tr>
<td>7.</td>
<td>F</td>
<td>42</td>
<td>6</td>
<td>Arthritis, purpura</td>
<td>Anti-SSA</td>
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<td>8.</td>
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<td>56</td>
<td>9</td>
<td>Arthritis, renal involvement, peripheral neuropathy</td>
<td>Anti-SSA, anti-SSB</td>
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<td>nd</td>
</tr>
<tr>
<td>9.</td>
<td>F</td>
<td>50</td>
<td>6</td>
<td>none</td>
<td>Anti-SSA, anti-SSB</td>
<td>2</td>
<td>+++</td>
</tr>
<tr>
<td>10.</td>
<td>F</td>
<td>46</td>
<td>4</td>
<td>none</td>
<td>Anti-SSA</td>
<td>1</td>
<td>++</td>
</tr>
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FS: Focus score; MMP: Matrix metalloproteinase; F: Female; SSA: Sjögren’s syndrome antigen A; SSB: Sjögren’s syndrome antigen B. Qualitative immunohistochemical evaluation: (+) mild; (++) moderate; (+++) severe; nd – not detected.

Examination in IHC offered further characteristic of T-cell and B-cell subpopulation patterns and also presence of macrophages, mast cells or expression of MMPs. The vast majority of infiltrating cells were comprised of T- and B-lymphocytes, with variable frequencies depending on the grade of infiltration. Specimens were analyzed for patterns of T-cells using CD3, CD4 and CD8 antibodies. CD3+ T-cells displayed more intense reaction in specimens with mild to intermediate FS grade (Figure 4). CD4+ and CD8+ T-cell reactions were scarcely noted, regardless of the severity of lymphocytic infiltration (Figure 5). Patterns of B-cell population were analyzed by CD20+ immunostaining (Figure 6). Specimens with important CD20+ B-cell component of lymphocytic infiltrate were associated with intermediate and severe FS grade. A correlation between overall lymphocytic infiltration severity and CD20+ B-cell population could be established. Thus, B-cell proliferation was linked with disease stage, positive serology, marked glandular hypofunction and also EGM. Both CD3+ T-cells and CD20+ B-cells displayed a predominantly periductal distribution (Figure 7).

The mononuclear cell infiltrates also displayed CD68-positive IHC reactions, due to the increased macrophage activity (Figure 8). Although all specimens showed varying degrees of CD68+ cells, IHC reactions were more intense in slides displaying a more advanced mononuclear infiltration, resulting also in a positive correlation with the CD20+ B-cell population. CD68+ cells were scattered throughout the glandular parenchyma or aggregated in lymphoepithelial lesions around the salivary ducts.

Mast cell activity was examined using tryptase antibodies (Figure 8b). Very few specimens were positive for tryptase reactions. The overall intensity of IHC reactions for tryptase in these specimens was weak, regardless of the degree of desmoplastic activity or histopathology grading. Mast cells are one of the main mediators of chronic inflammations, thus being linked with stimulation of angiogenesis. In our study, occurrence of increased vascularity associated with MSG lesions, was observed on some specimens (Figure 9). A direct correlation between mast cell activity and pattern of vascular neoformation could not be established, in part because the total number of mast cell-positive specimens was low and also because some slides with increased vascularity did not display positive tryptase reactions.

Figure 4 – T-cell CD3 immunostaining (a, ×200), with detailed features of highlighted area (b, ×400).
Figure 5 – Weak reaction on T-cell CD4 (a, ×200) and CD8 (b, ×200) immunostaining; (b) Follicular feature of lymphocytic infiltrate (arrowheads).

Figure 6 – B-cell CD20 immunostaining, ×200: distribution of B-cells between glandular acini (a) and periductal area (b).

Figure 7 – Periductal distribution of both CD3+ T-cells (a, ×200) and CD20+ B-cells (b, ×200) associated with marked sialectasis and altered ductal morphology.
Figure 8 – Immunolocalization of macrophages using CD68 antibodies (a, ×200); tryptase immunostaining for mast cell patterns displaying a weak reaction (b, ×200).

Figure 9 – Increased vascularity features (arrowheads) associated with chronic inflammatory status: (a) HE staining, ×200; (b) GLT staining, ×200.

IHC analysis was completed with the immunolocalization of MMPs, using MMP-2 and MMP-8 antibodies (Figure 10). Immunoreactivity was strong for both enzymes, throughout the gland, in areas of acini, predominantly in the basal region of acinar cells. Strong reactions were also seen in specimens with mild FS class. Thus, the degree of MMPs activity was not linked with proximity of mononuclear cell infiltration.

Figure 10 – Immunostaining for MMP-2 (a, ×200) and MMP-8 (b, ×200) activity.
Discussion

Diagnosis of pSS requires the presence of a combination of subjective and objective criteria [6], of which salivary gland biopsy (SGB) is considered an essential, if not mandatory assessment tool. The importance of SGB resides in its disease specificity, yielding information about nature and extent of the disease process. The histopathological hallmark of pSS is the FLS in exocrine glands. The predominant components of infiltrating mononuclear cells in pSS are T-cells, B-cells, macrophages, interdigitating and follicular dendritic cells, and natural killer (NK) cells [17]. Similar to other autoimmune diseases, multifactorial etiological processes initiate the pathophysiological events, which broadly consist of four stages: exogenous factor initiation, glandular epithelial cell disruption, T-lymphocyte migration with lymphocytic infiltration and finally B-lymphocyte hyper-reactivity with production of autoantibodies [18]. Other studies also support a possible neurological involvement in the exocrine hypofunction through loss of innervation or inadequate function of the autonomic nervous system [19, 20].

Initially, the mononuclear aggregates are found surrounding the salivary ducts, but as the disease progresses, it extends throughout the glandular parenchyma, leading to the loss of acinar cells and acinar-cell atrophy [17]. As seen in our MSG specimens, mononuclear infiltration is often accompanied by sialectasis, merging and eventual destruction of salivary ducts. Proportional increase in salivary gland fibrous tissue was described in our study and, although it is a common feature of chronic inflammatory status, it is considered non-specific, with no significant diagnostic contribution. We also noted on some slides features of increased vascularity, which could be attributed to the local inflammatory status. Our observations were of descriptive value, lacking any relevant correlations. Changes in the vascular component of PGs are described in SS mainly through ultrasound studies of the post-stimulation peak systolic velocity or the overall vascularity index [21, 22].

One important weakness of SGB is the inconsistent histopathology assessment and lack of adherence to an established protocol [23]. False negative results can occur mainly if the biopsy sample does not contain the required surface (4 mm²) or number of glands (≥50). A correct FLS should include one or more dense aggregates of ≥50 lymphocytes, usually with a periductal or perivascular distribution. An essential feature of FLS is the presence of normal-appearing mucous acini in adjacent gland lobules, lacking sialectasia and containing a small proportion of the infiltrating cells [23]. These aspects are to be taken into account when differentiating FLS from other pathologies of the salivary gland, such as: non-specific chronic sialadenitis (NSC), chronic sialadenitis (CS), granulomatous inflammation, hepatitis C, acquired immunodeficiency syndrome, pre-existing lymphoma, graft-vs-host disease, or sialadenosis [17]. Typical features NSCS and SCS, which is considered an advanced stage of the former, are focal or scattered mononuclear infiltrates and the lack of normal-appearing adjacent acini [23]. Several studies focused on the sialadenitis lesions in the setting of hepatitis C infection. Pawlotsky et al. described similar lesions to pSS in 50% of hepatitis C virus (HCV)-infected patients, but with intact duct walls and predominantly pericapillary distribution of cellular infiltrate [24].

Through quantification and grading of FS, we classified the patients in three histopathological groups and could link some clinical and serological features with the severity of glandular involvement. In accordance to other studies, a more advanced FS was associated with abnormal serological tests, ocular involvement and EGM [23, 25]. There are some discrepancies between studies concerning the relationship between histopathological grading and phenotypic features. It has been hypothesized that the lack of correlation found by some authors may reside in the dynamic characteristics of lymphocytic infiltrates and the fact that involvement of major and minor SG is not simultaneous [25]. Most advanced lesions can also contain tertiary ectopic lymphoid structures, which can develop into GC. Presence of GC is not only linked to greater disease severity, but also increased risk of malignant lymphoma development [26]. Of the two specimens in our series, which displayed severe FS grade, none featured CG-like structures.

Our IHC analysis offered some insight into the characteristics and cellular distribution of the mononuclear cell infiltrate, presence of mast cells and also local protease activity through MMP staining. The complementary analysis through the examination of IHC patterns is an essential tool in establishing the severity of the disease, prognosis [11, 12, 17, 27] and also malignant development, of which pSS patients are at high risk [28, 29]. Some studies also point to the value of IHC essay in differentiating between pSS and sSS by analyzing the immunoglobulin (Ig) expression pattern [10]. It has been suggested that the presence of plasma cells containing increased proportion of IgG in relation to IgA, is more sensitive and specific than histopathology analysis, thus strengthening the diagnosis of pSS [10, 30].

We characterized the lymphocytic distribution through positive CD3 and CD20 reactions, and noted that a more intense CD20+ B-cell is linked to more severe MSG infiltrate. This observation is in accordance to the majority of studies [11, 12], which associate later stages of the disease with hyperactivity of B-cells. Curiously, immuno-reactivity for CD4+ and CD8+ T-cell subpopulation was very weak. In one IHC study on 39 pSS patients, Christodoulou et al. [12], described some key differences between CD4+ and CD8+ T-cells in relation to disease severity. They concluded that the CD4+ subpopulations vary according to grade of glandular infiltrate, while CD8+ T-cells do not display significant changes.

Immunolocalization of CD68+ macrophages was noted in the majority of studied specimens, with more intense reactions on advanced FLS. In one study focused on occurrence of antigen presenting cells (APCs) in pSS biopsies and in situ cytokine expression, Manoussakis et al. [31] demonstrated the co-localization of CD68+ cells and IL-18, a pleiotropic proinflammatory cytokine. Mast cell activity, although scarcely noted in our study can be associated to the local increase in fibrotic tissue formation. Interaction of mast cells with fibroblast and their contribution to collagen synthesis has been shown in many diseases [32], as arthritis or rheumatoid nodules.
[33]. In one study on SS patients, mast cells in MSG were strongly associated with fibrosis and fatty infiltration, but not with lymphoid sialadenitis [34].

Presence of MMPs in patients with pSS has been investigated both in MSG tissue and saliva samples, specifically the MMP-9 subgroup [35, 36]. Our tests did not include MMP-9 markers, but we could observe increased in situ expression on MMP-2 and MMP-8, although lacking a comparative control reference. In the classical pathogenesis paradigm of SS, lymphocytes occupy a central role and glandular destruction is related to the increased number of immune cells and higher expression of cytokines favoring catabolic processes of extracellular matrix (ECM). Studies on MMP activity stemming from exocrine epithelial cells established that these enzymes lead to metabolic alteration of the cells, with loss of nuclear polarity, detachment from the basal lamina, inhibition of proliferation, differentiation and regeneration [37, 38]. Also, a relationship between mast cells and MMP activity has been cited, in which enzymes released by the former can proteolytically activate some MMPs [36].

IHC studies on salivary gland biopsies of pSS patients are also of increased interest in regard to the follow-up of B-cell depletion therapy using the anti-CD20 monoclonal antibody Rituximab (RTX) [39, 40]. A study on 12-week post-RTX follow-up showed significant reduction in PG B-cell population, absence of GC and regain of normal salivary duct morphology [39].

Conclusions

Through our study, we could identify both morphological changes and immunological patterns associated with SS pathogenesis. Major limitations of our study reside in the low number of patients enrolled and lack of control group. The findings were in general accordance with the body of knowledge reported in the literature, which supports the value of IHC analysis. Its implications in SS patients range from diagnostic and prognostic use to implementation in therapy follow-up strategies.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

Ştefan-Cristian Dinescu and Mircea-Cătălin Fortošoiu contributed equally to the study design and manuscript proofing.

References


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