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ORIGINAL PAPER

Interferon gamma-inducible protein 16 (IFI16) and anti-IFI16 antibodies in primary Sjögren's syndrome: findings in serum and minor salivary glands

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SUMMARY

The interferon (IFN) signature, namely the overexpression of IFN-inducible genes is a crucial aspect in the pathogenesis of primary Sjögren's syndrome (pSS). The IFN-inducible IFI16 protein, normally expressed in cell nuclei, may be overexpressed, mislocalized in the cytoplasm and secreted in the extracellular milieu in several autoimmune disorders including pSS. This leads to tolerance breaking to this self-protein and development of anti-IFI16 antibodies. The aim of this study was to identify pathogenic and clinical significance of IFI16 and anti-IFI16 autoantibodies in pSS.

IFI16 and anti-IFI16 were assessed in the serum of 30 pSS patients and one-hundred healthy donors (HD) by ELISA. IFI16 was also evaluated in 5 minor salivary glands (MSGs) of pSS patients and 5 MSGs of non-pSS patients with sicca symptoms by immunohistochemistry.

Normal MSGs do not constitutively express IFI16. Conversely, in pSS-MSGs a marked expression and cytoplasmic mislocalization of IFI16 by epithelial cells was observed with infiltrations in lymphocytes and peri/intra-lesional endothelium. pSS patients display higher serum levels of both IFI16 and anti-IFI16 autoantibodies compared to HD.

Our data suggest that IFI16 protein may be involved in the initiation and perpetuation of glandular inflammation occurring in pSS.

Key words: *Sjögren's syndrome; interferons; IFI16.*

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INTRODUCTION

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disorder mainly affecting exocrine glands. The clinical spectrum of pSS ranges from mild symptoms of mucosal dryness to severe extraglandular manifestations. Non-Hodgkin's lymphoma is the most severe complication (1). The histological hallmark of pSS is a periductal/perivascular mononuclear cell infiltrate of at least 50 lymphocytes (focus). Although the pathogenesis of pSS is not entirely clarified, growing evidence points to the central role of interferon (IFNs) in the initiation and perpetuation of the disease (2, 3). Type I IFNs are early mediators of the innate immune response that foster the adaptive immune response through direct and indirect effects on dendritic cells, T and B cells, and natural killer cells. Increased expression of IFN-inducible genes, namely the type I IFN signature, has been reported in systemic lupus erythematosus, systemic sclerosis (SSc), rheumatoid arthritis and pSS (4-7). Among IFN-inducible genes, the interferon gamma-inducible protein 16 (IFI16), a member of the HIN200/Ifi200 family has been recently investigated in several autoimmune diseases including

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pSS (8-12). IFI16 is normally expressed in cell nuclei of hematopoietic cells, particularly lymphocytes, vascular endothelial cells and keratinocytes (13).

IFI16 aberrant overexpression drives early steps of the inflammatory response through nuclear factor kappa B mediated secretion of proinflammatory molecules such as intercellular adhesion molecule 1, E-selectin, interleukin-8 and monocyte chemoattractant protein-1 (12, 14, 15). It is worth noting that an overexpression and mislocalization in the cytoplasm was described in target organs of autoimmune diseases, such as the skin in SSc (16).

IFI16 is also increased in serum samples of patients with autoimmune diseases (8-12). Such events, namely the extracellular spreading of IFI16, lead to the breaking of tolerance to this self-protein and eventually to the development of anti-IFI16 autoantibodies (17).

On this basis, it is reasonable to speculate that IFI16 may act as an autoantigen in pSS and therefore be involved in the development of autoimmunity (14). The aim of this study was to investigate possible pathogenic, diagnostic and prognostic significance of IFI16 protein and anti-IFI16 autoantibodies in patients with pSS.

MATERIALS AND METHODS

Patients

Thirty consecutive patients with pSS, classified according to the European-American criteria (18), were enrolled. One-hundred sex- and age-matched healthy donors (HD) were selected as controls. Five pSS minor salivary glands (MSGs), collected at the time of diagnosis, were also retrospectively evaluated. Five normal MSGs obtained from subjects with sicca symptoms, but without any clinical and serological features of pSS, were used as controls. The whole study was approved by the local Ethics Committee and written informed consent was obtained in accordance with the declaration of Helsinki.

Determination of extracellular IFI16 protein by capture ELISA

A capture ELISA was used to measure the circulating extracellular IFI16 protein with a procedure described below (10).

Determination of antibody titres towards human recombinant IFI16 by ELISA

To detect anti-IFI16 antibodies, polystyrene micro-well plates (Nunc-Immuno MaxiSorp; Nunc, Roskilde, Denmark)

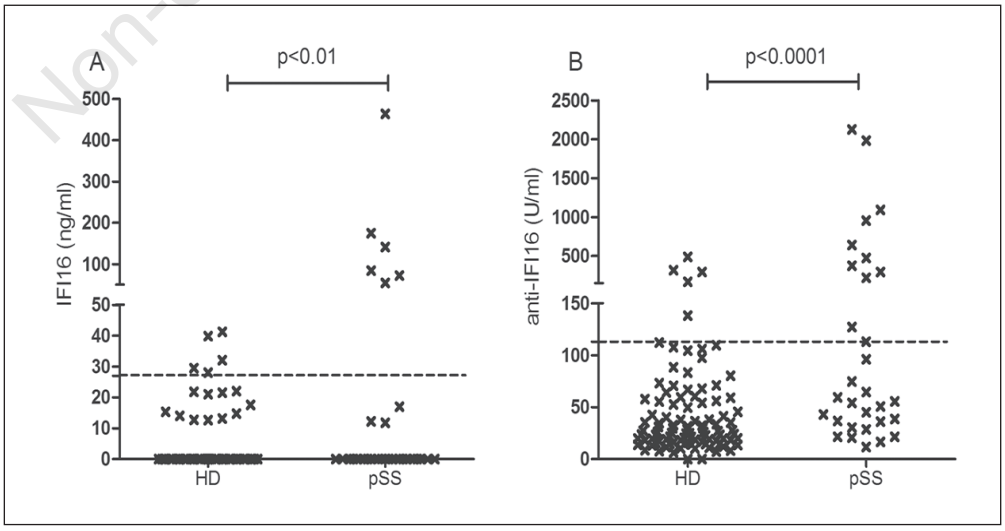

Figure 1 - Expression of IFI16 protein (A) and anti-IFI16 antibodies (B) in healthy donors (HD) (n. 100) and primary Sjögren's syndrome (pSS) patients (n. 30). Dotted lines mark cut-off levels of positivity as determined as the 95th percentile of the control population. P values were calculated with Mann Whitney U test.

were coated with a solution of recombinant IFI16 in phosphate-buffered saline and, after blocking, sera were added in duplicate, as previously described (11, 12).

Histological analysis of minor salivary glands biopsies

Labial MSG specimens were scored using hematoxilin-eosin staining sections (focus score, number of foci in 4 mm^2 of tissue) (19, 20).

IFI16 expression was evaluated using standard immunohistochemistry, as already described in a previous study (17). Images were acquired using an Olympus BX53 fluorescence microscope with CellSens software (Olympus America Inc., Center Valley, PA, USA).

Statistical analysis

Data analysis was performed using GraphPad 5.0 software. Mann Whitney U test or Chi square test were used to compare variables among subgroups. The significance level was two sided and set at $p<0.05$.

RESULTS

Serum IFI16 protein and anti-IFI16 antibody are highly expressed in primary Sjögren's syndrome

Cut-off values were established according to the 95th percentile of the control population, being 27 ng/mL and 113 U/mL for IFI16 protein and anti-IFI16 autoantibodies, respectively.

As shown in Figure 1A and B, serum levels of both IFI16 protein and anti-IFI16 autoantibodies were higher in pSS than in HD ($p<0.01$ and $p<0.0001$, respectively). IFI16 was significantly more prevalent in pSS compared to HD as it was detectable in 6/30 patients (21%) and in 5/100 (5%) ($p<0.05$). Similarly, a significantly higher prevalence in pSS was confirmed also for anti-IFI16 antibodies that were present in 10/30 pSS patients (33%) and in 5/100 HD (5%) ($p<0.001$).

Table I summarizes clinical and serological features in pSS patients. We failed to observe any clinical or serological differences according to the positivity/concentration of IFI16 or positivity/titer of antiIFI16 autoantibodies.

Table I - Clinical and serological features of patient cohort.

Number of patients	30
Female	30 (100%)
Age (mean ± SEM)	58±2,3
Age at diagnosis (mean ± SEM)	49±2,4
Disease duration (mean ± SEM)	8,8±1
Xerostomia	28 (93)
Xerophtalmia	28 (93)
Salivary swelling	17 (57)
Articular involvement	18 (60)
Purpura	2 (7)
Raynaud's phenomenon	3 (10)
Visceral involvement	8 (27)
Lymphadenopathy	2 (7)
Lymphoma	1 (3)
Hypocomplementemia	7 (23)
Leukopenia	14 (47)
Hypergammaglobulinemia	17 (57)
Monoclonal component	3 (10)
Autoantibodies	
Neither anti-SSA nor anti-SSB	4 (13)
Anti-SSA only	9 (30)
Both anti-SSA and anti-SSB	17 (57)
Rheumatoid factor	22 (73)
Cryoglobulins	1 (3)
Topical drugs	23 (77)
Hydroxicloroquine	16 (53)
Methotrexate	6 (20)

Unless specified, values are displayed as number of patients (percentage). SEM, standard error of the mean.

IFI16 protein is highly expressed and mislocalized in the cytoplasm at glandular level in primary Sjögren's syndrome

Figure 2A and B display a representative normal and pSS MSG stained with hematoxilin and eosin. The histological analysis of normal MSGs revealed that IFI16 protein was not constitutively expressed by the glandular tissue (Fig. 2C). Conversely, in pSS biopsies IFI16 was highly expressed

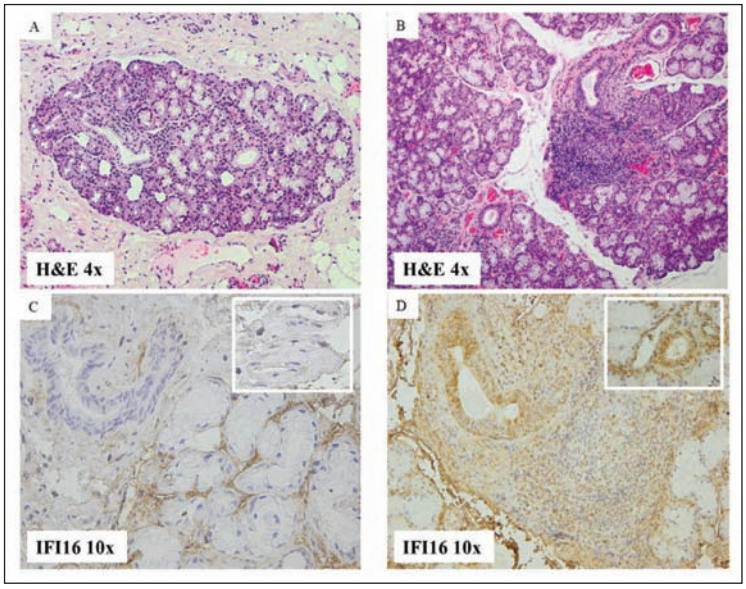

Figure 2 - Expression of IFI16 in minor salivary glands (MSGs). Hematoxilin and eosin staining of normal MSG (A) and primary Sjögren's syndrome (pSS) MSG (B). Immunohistochemical analysis for IFI16 in normal MSG (C) and pSS-MSG (D). Inserts in C and D depict a detail of endothelial cells from the corresponding panel.

in the nuclei of both ductal and acinar epithelial cells. It is worth noting that IFI16 is aberrantly expressed in the cytoplasm of ductal epithelial cells (Figure 2D). IFI16 nuclear and cytoplasmic staining was also observed in the inflammatory cells infiltrating the tissue (Fig. 2D). Finally, the constitutional expression of IFI16 in the nuclei of endothelial cells that could be detected in normal MSGs was more pronounced in pSS peri- and intra-lesional endothelium (Fig. 2C-D, inserts).

DISCUSSION AND CONCLUSIONS

A large body of evidence supports the pathogenic role of the IFN signature in several autoimmune diseases including pSS (6, 21). However several aspects of this pathway as well as the role of each IFN-inducible molecule are not fully elucidated. Our study demonstrated that the IFN inducible protein IFI16 is aberrantly expressed in sera and target organs of patients with pSS. In addition we confirmed that pSS patients display circulating autoantibodies towards IFI16. Therefore, it is reasonable to postulate that IFI16 protein may be involved in the induction and maintenance of glandular inflammation and, more generally, in triggering the autoimmune response.

Previous studies pointed out that IFI16 is involved in the regulation of cell growth, differentiation, and angiogenesis. In fact, its overexpression leads to decreased cell proliferation with a block in the cell cycle progression at the G1-S phase transition of cancer cell lines, and to an impairment of tube morphogenesis and proliferation of human endothelial cells (22, 23). IFI16 is expressed in myeloid precursor cells and such expression remains stable throughout the lymphoid development. In addition, the histological evaluation of a variety of normal tissues revealed that IFI16 expression is highly clustered. As far as epithelial cells are concerned, IFI16 expression was described in skin, gastrointestinal tract, urogenital tract, and glands and ducts of breast tissues (13, 24). This tissue-specific physiological expression of IFI16 may indicate that this molecule is involved in the early phases of inflammation.

In this study we demonstrated that, unlike other epithelial cells, those of normal MSGs do not constitutively express IFI16 protein. Conversely, a marked expression of this molecule by acinar and ductal epithelial cells as well as infiltrating lymphocytes and peri/intra-lesional endothelium could be observed during pSS. Hence, IFI16 expression appears to be induced de novo in the target tissue of this disease.

Recently, it has been hypothesized that salivary gland epithelial cells are actively involved in the initiation and perpetuation of pSS (25) and IFN appears to be crucial in this scenario. In fact, IFN binding to toll-like receptors aberrantly expressed by activated salivary gland epithelium leads to an up-regulation and mislocalization in the cytoplasm of nuclear proteins. Besides the well-known Ro52 and Ro60 nuclear proteins, our findings suggest that also IFI16 may undergo this fate in pSS-MSGs. In de-

tail, IFN over-expression occurring early in the disease may induce salivary epithelial cells to express IFI16, which is normally absent in these cells, to upregulate it at pathological levels and to miscolocalize it in the cytoplasm.

The abnormal release in the extracellular milieu of nuclear proteins normally hidden to the immune system, including IFI16, leads to tolerance breaking, antigen-specific B-cell activation and, eventually, autoantibody production. IFI16, therefore, may act as an autoantigen in pSS being involved in the disease pathogenesis. In addition the evidence that IFI16 is also overexpressed and mislocalized in the cytoplasm of infiltrating lymphocytes further underscores its possible pathogenic role. Finally, the consistent presence of IFI16 in pSS peri/intra-lesional endothelial cells fits with the recent observation that IFI16 overexpression in endothelial cells in another pebble in the mosaic of inflammation (14, 15).

The aforementioned observation, together with the evidence that only a subgroup of pSS patients display consistent levels of circulating IFI16, allow us to speculate that the main pathogenic role of IFI16 is exerted at glandular tissue level. Similarly, since IFI16 may exert its role of autoantigen mainly at tissue level, again only a subgroup of patients display circulating anti-IFI16 autoantibodies.

In conclusion, our study provides some bases to understand the pathogenic role of IFI16 and anti-IFI16 autoantibodies in pSS. Larger and prospective studies are required to identify the potential diagnostic and/or prognostic role of IFI16 protein anti-IFI16 autoantibodies in pSS and eventually provide the rationale for their detection in clinical practice.

Contributions: AA and VC equally contributed to this study.

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