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Increased Expression of the lncRNA NRON Along With NFATc1/PIM-1 in Labial Salivary Glands of Sjögren's Syndrome Patients

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Abstract: The aim of our study was to analyze the expressions of nuclear factor of activated T cells (NFAT)-related substances including long noncoding RNA NRON which participates in pathophysiology of Sjögren's syndrome (SS), and to assess the histologic findings in individuals with SS. In this study, the expressions of NRON, NFATc1, CD3/CD4, and proviral integration site for Moloney murine leukemia virus (PIM)-1 were examined by in situ hybridization, immunohistochemical analysis, and immunofluorescence in labial salivary glands (LSGs) obtained from 16 patients with SS and five controls. The microcell count method has been applied to calculate the NFATc1-positive area/infiltrating cell area in LSGs, and we compared those results to the infiltrating cell area, focus score, serum immunoglobulin G, and the European League Against Rheumatism Sjögren's Syndrome Disease Activity Index. The NRON expression in the nuclei of cell-infiltration lesions of the SS patients were prominent. The NFATc1 expression was strong in the cytoplasm of infiltrating mononuclear cells and weak in ducts of both SS and controls. In SS, the NFATc1-positive area/infiltrating cell area was positively correlated with the infiltrating cell area and focus score. CD3/CD4 was expressed in infiltrating mononuclear cells, and PIM-1 colocalized with NFATc1 in the cytoplasm. These results suggest NRON along with NFATc1/PIM-1 in SS LSGs might participate in SS pathophysiology.

Key Words: long noncoding RNA, nuclear factor of activated T cells (NFAT)c1, NRON, proviral integration site for Moloney murine leukemia virus (PIM)-1, Sjögren's syndrome

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Sjögren's syndrome (SS) is a rheumatic disease characterized by sicca symptoms because of the lymphocytic infiltration of exocrine glands. Salivary glands (SGs) are the sites for production of anti-SS-A antibody, the typical autoantibody to SS, and are the main target organ in SS.[1] Although the pathogenesis of SS has not been fully clarified, the regulation of nuclear factor of activated T cells (NFAT) in SGs has been suggested to take part in the pathophysiology of SS.[2] In recent years, long noncoding RNAs (lncRNAs), which refers to a class of RNAs with more than 200 nucleotides that are not transcribed into proteins,[3] have been reported to have modulating effects in cell functions. Also, lncRNAs draw the attention of scientists through their roles in pathogenesis of various diseases including SS.[3–8] However, the expressions of specific lncRNAs in SGs have not been histologically investigated, and the relationship between epigenetic alterations and clinical manifestations remains to be studied. Noncoding repressor of NFAT (NRON) is an lncRNA that regulates the activity of NFAT by altering its intracellular localization.[9] The localization of NFAT is also regulated by kinases that control phosphorylation signaling. Proviral integration site for Moloney murine leukemia virus (PIM)-1 is a serine/threonine kinase that participates in phosphorylation of NFATc1 but enhances its transcriptional activity.[10] We hypothesized that the above-mentioned

substances which compose NFAT-regulation mechanisms work together in SS labial SGs (LSGs): among lncRNAs, NRON would be a potentially increased in SS LSGs. We conducted the present study to analyze the expressions of NRON and related substances (eg, NFATc1 and PIM-1) in LSGs of SS patients and healthy controls, and we assessed the pathologic findings and serum/clinical manifestations of the SS patients.

PATIENTS AND METHODS

The Patients With SS

The study population consisted of patients who had been admitted to the Department of Immunology and Rheumatology, Nagasaki University Hospital. We selected 16 patients with SS and five controls for in situ hybridization (ISH) and/or immunohistochemical analyses of NFATc1. All of the patients with SS fulfilled the 2002 American-European Consensus Group (AECG) SS Classification Criteria.[11] We excluded the patients having other connective tissue diseases such as rheumatoid arthritis and systemic lupus erythematosus, and the patients positive for anticentromere antibodies, which reportedly exist in LSGs of SS with different characteristics from typical anti-SS-A antibody positive SS.[1] The controls had xerophthalmia, xerostomia, or both but did not fulfill the AECG SS Classification Criteria. LSGs were obtained from each patient by an incisional biopsy at the time of initial SS diagnosis.

All of the patients with SS revealed focal lymphocytic sialadenitis with a focus score (FS) ≥ 1 focus/4 mm^2 in the LSG biopsy, whereas no aggregates of lymphocytes which were consistent with FS ≥ 1 focus/4 mm^2 were detected in any of the 5 controls. FS in LSGs were determined as previously described.[12] Briefly, the FSs in LSGs were determined through the following 3 steps. First, the surface area of the glandular tissue was measured in a single tissue section containing multiple glands sectioned through their midportion. Second, the number of lymphocytic foci in the entire tissue section was counted. Foci with 50 or more tightly aggregated lymphocytes that were adjacent to normal-appearing mucous acini were counted. Third, the number of foci was divided by the total glandular surface area of the section and then multiplied by 4 to yield the FS (number of foci/4 mm^2). The histopathologic diagnosis of SS was evaluated by Japan College of Rheumatology-certified rheumatologists (Y.H., H.N., T.S., and A.K.) and confirmed by expert pathologists.

Blood specimens were obtained from all of the patients in this study for serological analysis and were used to measure anti-SS-A antibody and immunoglobulin G (IgG). All of the patients with SS were positive for anti-SS-A antibodies, whereas none of the controls were positive. For the assessment of the clinical disease activity of the SS patients, their European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI)[13] at the diagnosis of SS were carefully evaluated by rheumatologists certified by the Japanese College

of Rheumatology. Written informed consent to participate in the study and have their data published was obtained from all of the SS patients and control subjects, and the protocol was approved by the Institutional Ethics Committee of Nagasaki University (No. 09102822-4) and the experiments were conducted in accordance with the Declaration of Helsinki.

ISH

For the ISH, the paraffin-embedded biopsy specimens were sent to the Tokushima Molecular Pathology Institute (TMPI), where the ViewRNA probe designed and manufactured by Affimetrix (part #61905, catalog no. VA6-11880; Santa Clara, CA) was used to detect NRON. The ISH was performed at the TMPI according to the probe manufacturer's instructions.

Briefly, the biopsy sections were incubated at 60°C for 60 minutes. After deparaffinization, the sections were processed in a 1:100 dilution pretreatment quantitative fluorescence at 40°C for 10–20 minutes and digested with 1:100 dilution protease quantitative fluorescence solution at 40°C for 10 minutes. After the inactivation of intrinsic alkaline phosphatase with 0.2 M HCl and 0.3 M NaCl at room temperature (RT), the sections were hybridized with the ViewRNA probe set for 120 minutes at 40°C, followed by series of posthybridization washes. The sections were then processed with PreAmplifier Mix QT at 40°C for 25 minutes, then with Amplifier MIX QT at 40°C for 15 minutes, with Label Probe 6-AP at 40°C for 15 minutes, with AP enhancer for 30 minutes, and lastly with Fast Red substrate at 40°C for 30 minutes (all, Affimetrix). The slides were postfixed in 10% formalin at RT for 5 minutes. The nuclei were stained blue with Hoechst for 5 minutes.

Immunohistochemical Analyses of NFATc1

We performed immunohistochemical staining to determine the in vivo expression of NFATc1. Briefly, biopsy sections were blocked with 3% bovine serum albumin/phosphate-buffered salts for 30 minutes, followed by incubation with NFATc1 mouse monoclonal antibodies (dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes at RT. After incubation, all sections (including the negative control sections) were treated with secondary antibodies for 30 minutes (EnVision+ System-HRP Labeled Polymer; Dako, Glostrup, Denmark). The color was developed by incubating the sections in 3.3′-diaminobenzidine for 5 minutes followed by counterstaining with hematoxylin solution. For the quantification of NFATc1-positive cells within each focus composed of mononuclear cells (MNCs) in a single LSG section, we used the microcell count method and a fluorescence microscope (BZ-X710; Keyence, Osaka, Japan) to capture the precise 3.3′-diaminobenzidine signal as described.[14]

Immunofluorescence

We performed an immunofluorescence examination to determine the localization of CD3-, CD4-positive T cells and PIM-1. Endogenous peroxidase was inactivated by

incubation with a 3% H_2O_2 solution after microwave epitope retrieval. Sections were then blocked with 5% bovine serum albumin/phosphate-buffered salts for 60 minutes, followed by incubation with NFATc1 mouse monoclonal antibody (dilution 1:100; Santa Cruz Biotechnology) and either CD3 rabbit polyclonal antibody (dilution 1:100; Proteintech, Chicago, IL), CD4 rabbit polyclonal antibody (dilution 1:100; Proteintech) or PIM-1 rabbit polyclonal antibody (dilution 1:50; GeneTex, Irvine, CA) for 60 minutes at RT.

After the incubation with primary antibodies, the sections from LSGs were reacted with secondary antibodies including donkey anti-mouse IgG conjugated with fluorescein isothiocyanate antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), donkey anti-rabbit IgG conjugated with tetramethylrhodamine isothiocyanate (TRITC) antibody (Jackson ImmunoResearch), and Hoechst dye 33258 (Sigma-Aldrich, St. Louis, MO) for 45 minutes at RT in the dark. The sections were then mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Deconvolution Technique

Deconvolution was applied to the high-resolution images obtained with the BZ-X710 microscope on sections of the LSGs.

Statistical Analysis

Any differences between the two groups were determined by the Mann-Whitney U test or the Fisher t test, and P-values of < 0.05 were considered statistically significant.

The correlations between pairs of results from the microcell count method and other data associated with SS were calculated using the Pearson correlation test and values of $P < 0.05$ were considered significant (Microsoft Excel).

RESULTS

Clinical and Laboratorial Features of the Study Patients

Clinical and laboratorial features of the patients with SS and the controls were shown in Table 1. There were no significant differences in age, sex. There were statistically significant differences in FS ($P < 0.0001$), anti-SS-A antibody ($P < 0.0001$), and IgG levels ($P = 0.01$).

Expression of NRON in LSGs

We analyzed the expression of NRON in the LSGs of the 5 patients with SS and 5 controls by ISH. NRON was expressed strongly in ducts and weakly in alveoli in the nuclei of LSGs from both the SS and control groups. The NRON expression in cell-infiltration lesions from the SS patients was particularly prominent (Fig. 1).

Immunohistochemical Detection and Quantitative Analyses of NFATc1 in the Labial Salivary Glands (LSGs)

For the immunohistochemical staining, LSGs from 8 of the patients with SS and 4of the controls were used.

TABLE 1. Clinical and Laboratorial Features of the 16 Patients With SS and the 5 Controls With Xerophthalmia, Xerostomia, or Both

	SS (n = 16)	Control (n = 5)	P
Age, years	60.5 (median, 16-79)	64 (median, 36-70)	0.78
Female (n)	14	5	1.00
FS	4.0 (median, 1.3-10.4)	0	< 0.0001
Anti-SS-A antibody (U/mL)	122.0 (median, 57.5-145.0)	0.7 (median, 0.3-3.6)	< 0.0001
IgG (mg/ld.)	2008.5 (median, 1151-3282)	1370 (median, 859-1539)	0.01
ESSDAI	6 (median, 0-23)		

ESSDAI indicates European League Against Rheumatism Sjögren's Syndrome Disease Activity Index; FS, focus score; IgG, immunoglobulin G.

The expression of NFATc1 was strong in infiltrating MNCs and weak in ducts from both the patients with SS and the controls. Ductal epithelial destruction adjacent to infiltrating MNCs was rarely observed, which was consistent with typical histopathologic findings of SS by Sjögren's International Collaborative Clinical Alliance (Fig. 2).[15]

We further examined the NFATc1-positive areas in infiltrating cells of the 13 SS patients (Fig. 3A). A positive correlation was observed between the infiltrating cell area and the NFATc1-positive area/infiltrating cell area ($r = 0.61$, $P = 0.03$) (Fig. 3B). Also, a significant correlation was observed between the FS values and the NFATc1-positive area/infiltrating cell area ($r = 0.59$, $P = 0.03$) (Fig. 3C). We observed a slight positive but insignificant correlation in each of between the IgG values and the NFATc1-positive area/infiltrating cell area values, between the FS and IgG values, and between the ESSDAI results and the NFATc1-positive area/infiltrating cell area values (Figs. 3D–F).

Immunohistochemical Detection of CD3/CD4 and PIM-1in the LSGs

In the immunofluorescence double staining for NFATc1 and CD3 of the 13 SS patients, CD3 was present in the surfaces of infiltrating lymphocytes whereas few were expressed in ducts (Fig. 4). Similar findings of colocalization of NFAT and CD3 were also observed in controls (Supplementary Figure S1, Supplemental Digital Content 1, http://links.lww.com/AIMM/A308). In the immunofluorescence double staining for NFATc1 and CD4 of the 13 SS patients, CD4 was not expressed in ducts but was present in infiltrating lymphocytes (Supplementary Figure S2, Supplemental Digital Content 1, http://links.lww.com/AIMM/A308). PIM-1 was colocalized with NFATc1 in the cytoplasm of both infiltrating cells and ducts (Fig. 5).

DISCUSSION

This is the first study revealing the intracellular localizations in LSGs of the lncRNA NRON and NFATc1/PIM-1, which is involved in the pathophysiology of SS.
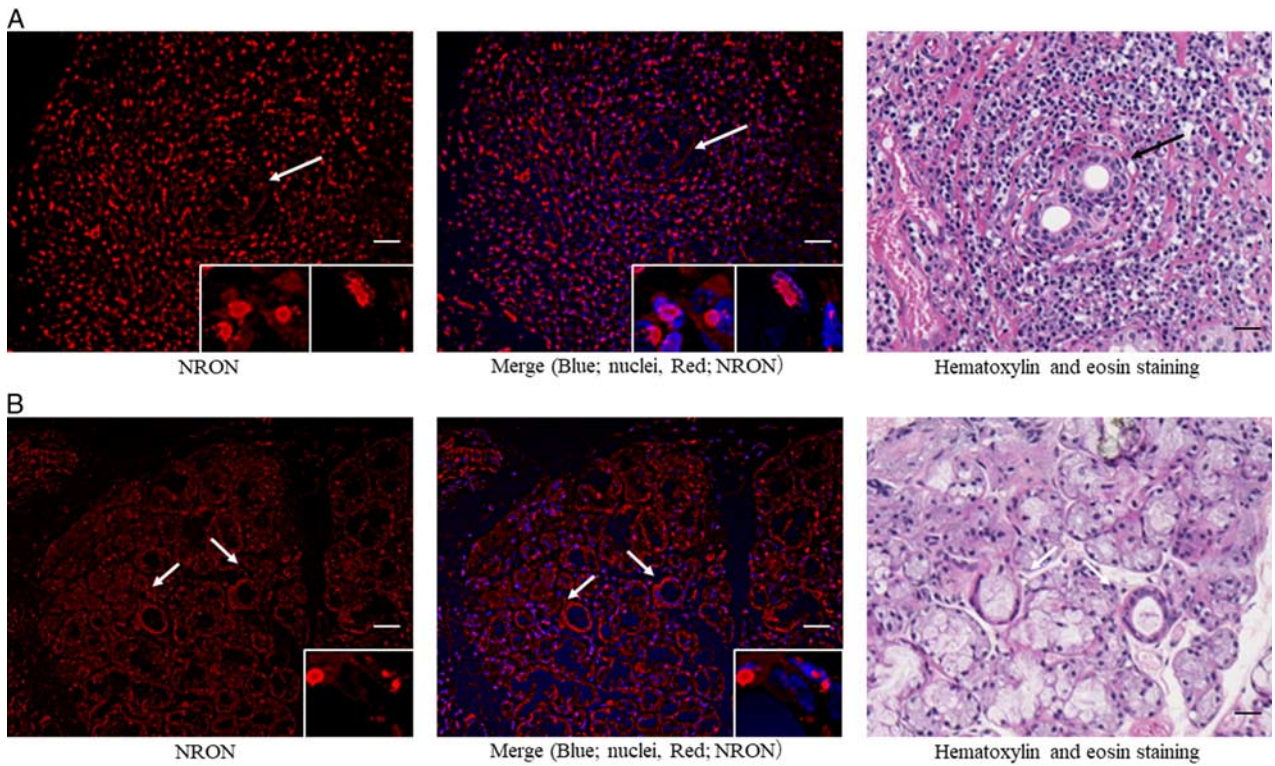
FIGURE 1. Representative images reflecting the detection of noncoding repressor of NFAT (NRON) on labial salivary glands by in situ hybridization. (A) From an Sjögren's syndrome patient and (B) a controls. In each picture, whole images of labial salivary glands are shown and insets show infiltrating MNCs (left in panel A) or nuclei of ducts (right in panels A and B). Each arrow indicates a duct. Bar: 20 μM. MNCs indicates mononuclear cells.

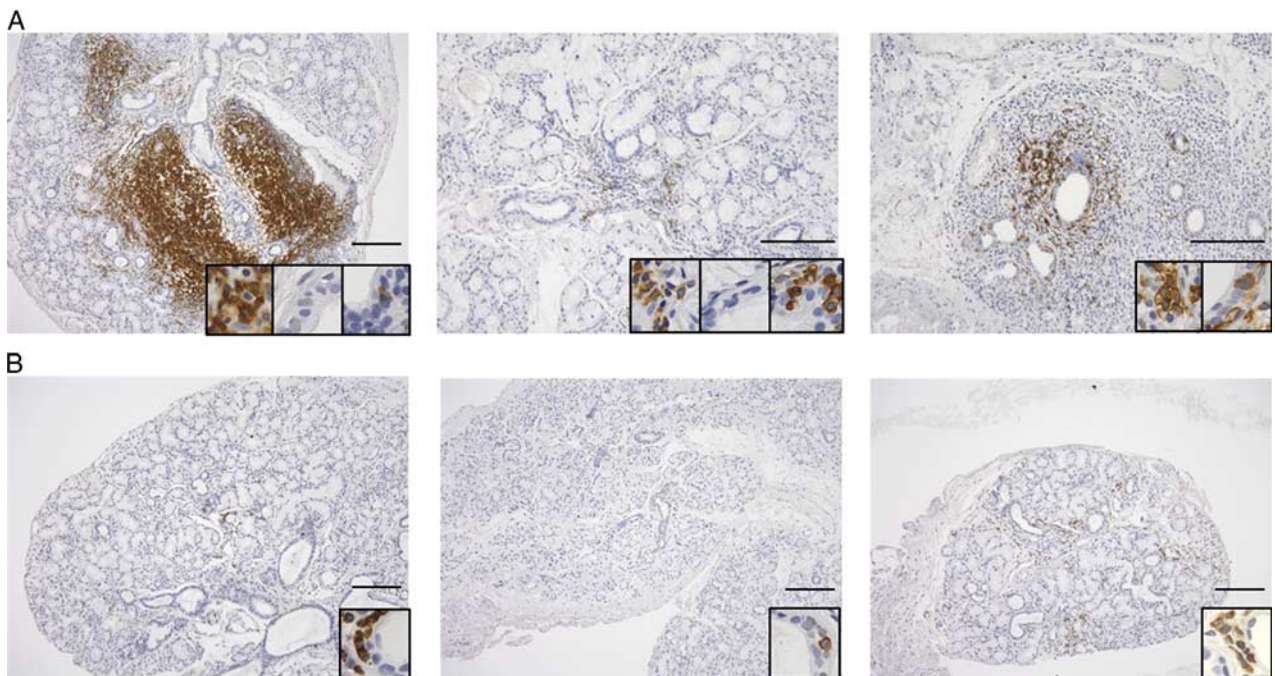**FIGURE 2.** Representative immunostaining images of NFATc1 on labial salivary glands (A) from an Sjögren's syndrome patient and (B) a control (×100). In each picture, whole images of labial salivary glands are shown and insets show infiltrating MNCs (left and/or center in panel A) or ducts (right or rightmost in panels A and B). Bar: 100 μM. MNCs indicates mononuclear cells.
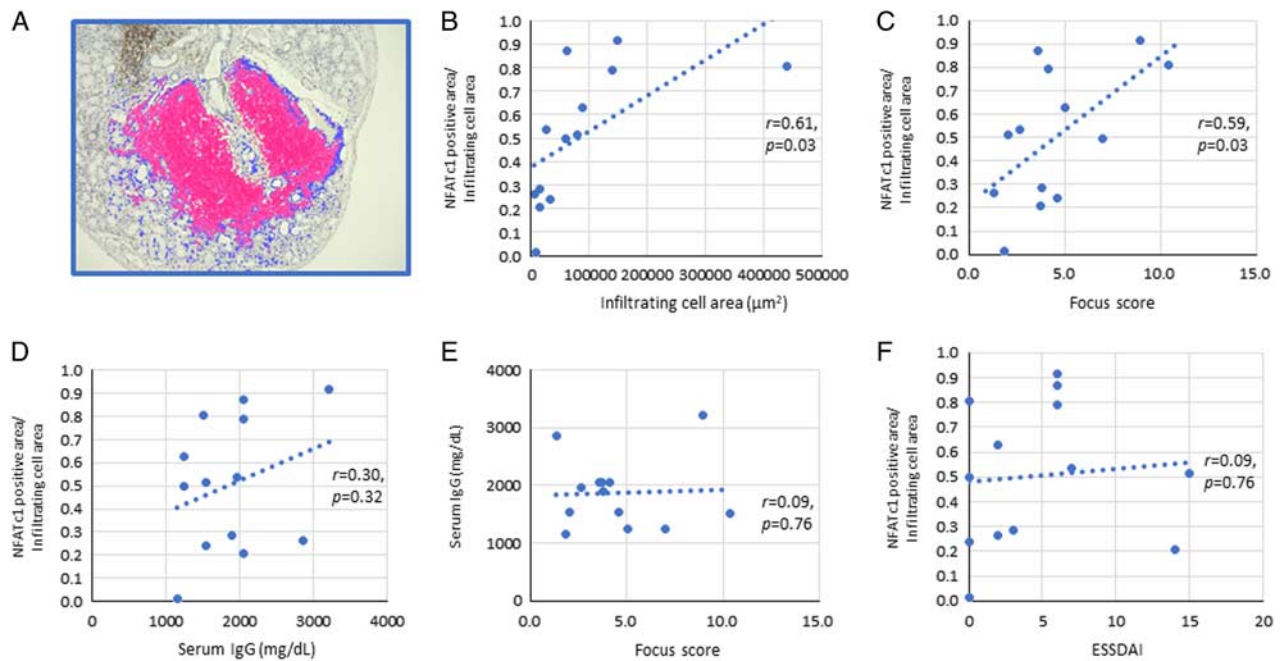
FIGURE 3. The NFATc1-positive area/infiltrating cell area, serum marker, and European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI) results. The NFATc1-positive areas in infiltrating cells from the Sjögren's syndrome patients were calculated by the microcell count method. A, Blue, infiltrating cell area; red, NFATc1-positive area. B, The infiltrating cell area and the NFATc1-positive area/infiltrating cell area. C, The focus score values and the NFATc1-positive area/infiltrating cell area. D, The immunoglobulin G values and the NFATc1-positive area/infiltrating cell area. E, The focus score and immunoglobulin G values. F, The ESSDAI results and the NFATc1-positive area/infiltrating cell area values.
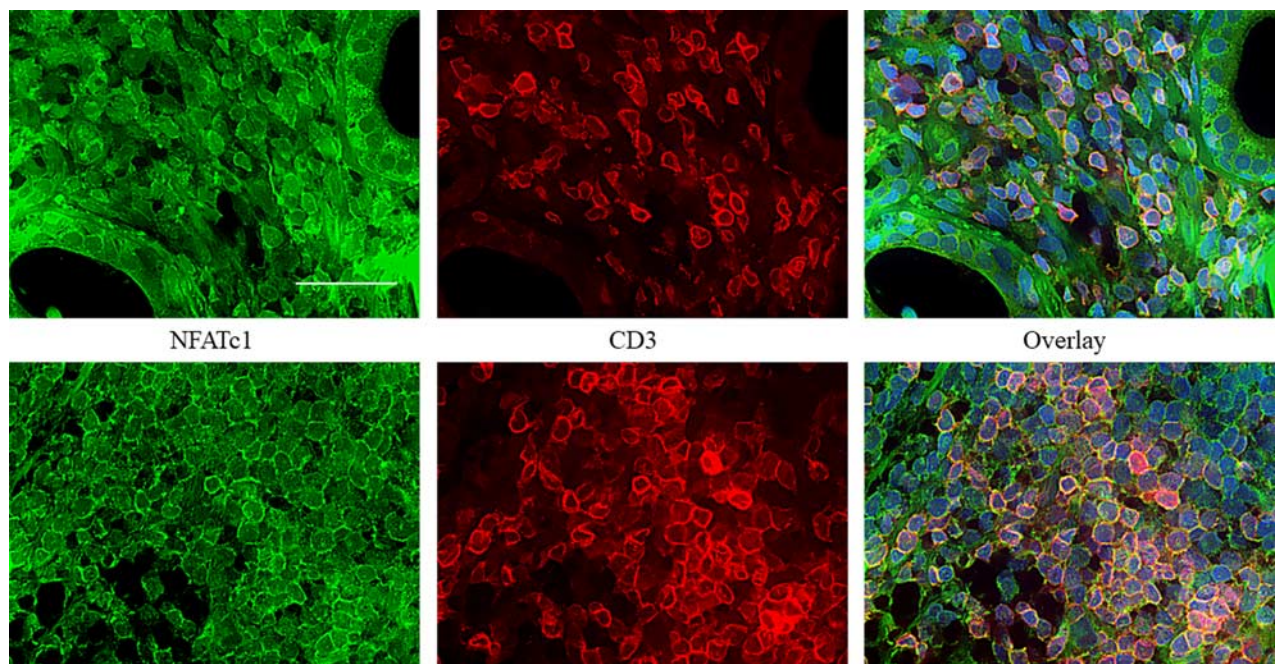
FIGURE 4. Representative immunofluorescent images of NFATc1 and CD3 on labial salivary glands from an Sjögren's syndrome patient. In each picture, infiltrating MNCs surrounding ducts were highlighted. Signals of NFATc1 are depicted by fluorescein isothiocyanate (green); those of CD3 by tetramethylrhodamine (red), and those of nuclei by Hoechst (blue). CD3 was present in the surfaces of infiltrating lymphocytes (upper row) whereas few were expressed in ducts (lower row). Bar: 20 μM. MNCs indicates mononuclear cells.
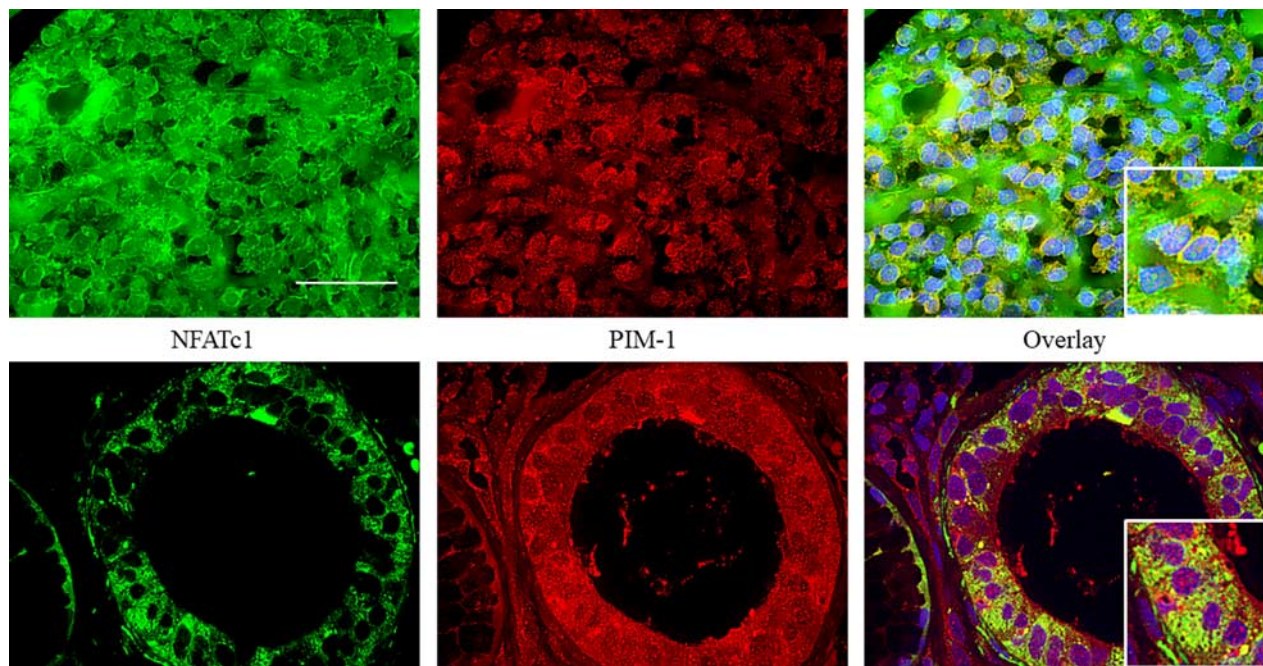
FIGURE 5. Representative immunofluorescent images of NFATc1 and PIM-1. Infiltrating MNCs surrounding ducts were highlighted in the upper row and ducts were highlighted in the lower row. Signals of NFATc1 are depicted by fluorescein isothiocyanate (green); those of PIM-1 by tetramethylrhodamine (red), and those of nuclei by Hoechst (blue). The insets of the rightmost pictures show PIM-1 colocalized with NFATc1 in infiltrating cells (upper row) and ducts (lower row). Bar: 20 μM. MNCs indicates mononuclear cells.

In 2005, Willingham et al[9] reported that NRON is a regulator of NFAT trafficking that interacts with multiple proteins. Our present findings suggest that NRON might also exist as a factor affecting the localization of NFATc1 in the LSGs of individuals with SS.

We previously reported higher Foxp3 positive cell area/infiltrating cell area in LSGs from adult T-cell leukemia patient and HTLV-1-associated myelopathy-SS patients compared with those of HTLV-I asymptomatic carrier and HTLV-I-seronegative patients, which was calculated by the microcell count method.[14] We also applied the microcell count method to observe NFATc1 expression in LSGs in the present study, and obtained an interesting observation that the higher ratios of NFATc1-positive areas corresponding to larger infiltrating cell areas and the FS. This result may indicate that the size of the infiltrating lymphocyte area and the number of infiltrating lymphocyte areas — accounts for the regulation of the NRON-NFATc1 activation mechanism. Higher FSs are reported to have associations with widespread manifestations of SS, such as lymphoma[16] and respiratory complications.[12] The aforementioned reports and the present findings suggest NRON-NFATc1 activation mechanism might not only play roles in glandular involvements of SS, but also be predictive of extraglandular involvements. The correlation between the ESSDAI and the NFATc1-positive area/infiltrating cell area values was not ascertained as statistically correlated in the study, the result of which might be attributed to the homogenous population of SS. Quartuccio et al[17] analyzed upregulation of serum B-lymphocyte stimular (s-BLyS) levels in SS with or without lymphoproliferative disorders: SS with lymphoproliferative disorders, which was not included in the present study, showed higher s-BLyS levels than SS without lymphoproliferative disorders, and s-BLyS levels significantly correlated with ESSDAI. It is presumable the correlation between the ESSDAI and the NFATc1-positive area/infiltrating cell area values failed to reach statistical significance because of confounding factors such as s-BLyS.

We observed CD3-positive T cells as well as CD4-positive T cells in the areas of infiltrating cells, but not in ducts; this reflects the possibility that the ducts expressed NFATc1 themselves, and it precludes the possibility that the cells in the ducts were the result of ductitis caused by an invasion of infiltrating cells. Further histologic studies on affected organs are necessary to elucidate the NFATc1 activity underlying different clinical subsets of SS.

PIM-1 is a type of serine/threonine kinase. It localizes with and phosphorylates NFATc1 without preventing nuclear entry, which leads to the production of interleukin-2.[10] The immunofluorescence examination in our present study demonstrated that PIM-1 resided with NFATc1 in the cytoplasm of infiltrating cells, with a similar observation in the ducts. This result may indicate that PIM-1 does not work as an NFATc1 activator under the existence of NRON, and that PIM-1 was kept in the cytoplasm with NFATc1. Sharma et al[18] described a mechanism that phosphorylated NFAT1 in resting cells consisted of a large cytoplasmic RNA-protein scaffold complex with NRON, an IQ motif containing GTPase activating protein, and three NFAT kinases in Jurkat and CD8$^+$ T cells. In this

study, we attempted to locate NRON in biopsied human LSG cells by ISH. 'Z-probe,' applied in the ISH, enables detection of a small amount of NRON in formalin-fixed paraffin-embedded tissues.[19] Recent findings revealed lncRNAs tend to be localized in the nuclei, which was compatible with the location of the NRON in this study.[20] The findings in this study might be attributed to the differences in kinds of cells and kinases. As mentioned above, PIM-1 has phosphorylation-dependent regulatory mechanism targeting NFATc1 which is different from other serine/threonine kinases,[10] that is, in SS LSGs, NRON might affect NFATc1 localization through PIM-1 in a different manner from other serine/threonine kinases. Further analyses on modes of action of NRON in LSG cells are needed.

There are limitations to this study. We could not analyze the expressions of all the targeted NFAT-related substances in all of the enrolled patients because of exhausting of specimens in the course of this study. Causal links between the expression of NRON in ISH and NFATc1 in immunohistochemical staining of LSGs of individuals with SS have not been established. Precise intervening mechanisms between NRON in the nuclei and NFATc1/PIM-1 in the cytoplasm are to be clarified in future studies. The significance of the NFATc1 expression in ducts also remains to be clarified.

CONCLUSION

This is the first study investigating the expression of an lncRNA in LSGs of SS patients. Our observations suggest a link between NRON and the location of NFATc1 associated with PIM-1in LSGs of SS patients.

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