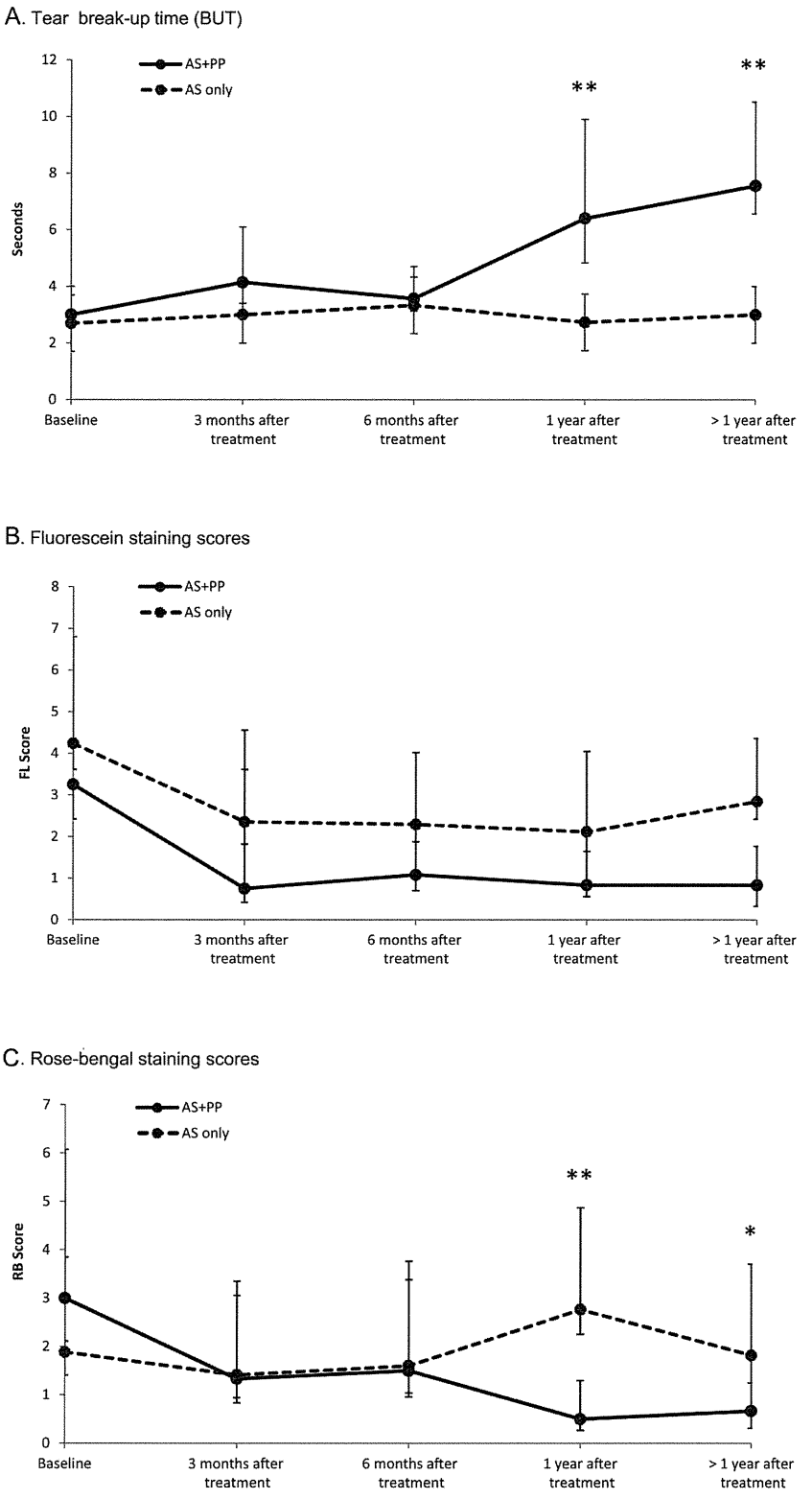

FIGURE 2. Comparison of dry eye parameters between the AS + PP group and AS only group at each follow-up time point. A, tBUT. B and C, FL staining scores and RB staining scores of the ocular surface (*$P <$ 0.05 and **$P < 0.01$).

studies investigating AS treatment, AS has been used to treat refractory dry eye and SS dry eye and to promote epithelial healing after alkali wounds.[5,13,16,19,20] A standard protocol has been reported, and AS treatment has been administered in many medical institutions.[5,21,22] The results of this study showed significant improvements of the tBUT, FL staining score, and RB staining score with the use of AS eye drops. Improvement in inflammation of the ocular surface was also observed. Five eyes did not benefit from AS eye drop treatment. Those 5 patients had severe dry eye associated with systemic lupus erythematosus, meibomian gland dysfunction, ocular pemphigus, and superior limbic keratitis, which lead to marked corneal staining.

AS eye drop therapy was established as a therapeutic approach to refractory dry eye. However, the long-term use of AS eye drops can raise the level of tumor growth factor β, which is a molecule that can suppress ocular surface wound healing, and increases the risk of infection.[23,24] Moreover, the effectiveness and safety of the long-term use of AS eye drops are not well understood. Pflugfelder[25] and Lee et al[22] showed that the effectiveness of the long-term application of AS in improving tear stability, vital staining scores of the ocular surface, and subjective improvement in many types of recalcitrant dry eye, but no data are available on the effectiveness of the treatment on SS dry eye. In this study, we followed the standard serum eye drop protocol[5] and witnessed no clinical evidence of ocular infection in any patient during the long-term treatment and follow-up. Thus, this study is the first to report the effectiveness and probable safety of the long-term application of AS eye drops for SS dry eye.

Our study compared the efficacy between patients treated with PPs and patients treated solely with eye drops in SS dry eye. In the AS + PP group, patients who experienced the PP dropping out underwent reinsertion. The mean tBUT, FL staining score, and RB staining score in the AS + PP group showed results superior to those of the AS only group. In some prospective studies, a minimum period of 3 months was required for AS treatment to be effective.[7,26] However, in this study, improvements in RB staining scores were not observed until after at least 3 months of treatment, which may be a result of the severe dry eye experienced by patients as improvement depends on the severity and underlying cause of dry eye.[9] Thus, for severe SS dry eye, the application period of AS eye drops may be required to be more than 3 months even if combined with PPs.[22,27–29] Moreover, there was no statistical significance in the improvement of tBUT, FL staining scores, or RB staining scores at any time point in the AS only group, which might be explained by the small sample size.

We clearly demonstrate that the use of the PP has an additive effect with AS treatment. Ogawa et al[14] reported that the use of PPs can prolong the retention time of AS on the ocular surface so that the active ingredients can be supplied more effectively. Roberts et al[30] also reported the effectiveness of topical cyclosporine combined with PPs for patients with severe dry eye. Both these studies support our results. These synergistic effects may be explained by PPs increasing the resident time of AS eye drops.

AS eye drops contain no preservatives; thus, patients are free from the risk of preservative toxicity. AS showed good tolerance and almost no serious complications in our study. In many cases, AS eye drops have been used in combination with artificial tears to take advantage of the former's diverse biological characteristics, including promoting mucin secretion in vitro, upregulating mucin expression in cultivated conjunctival cells, supplying nutritional elements, increasing conjunctive goblet cells, and causing corneal epithelial cell proliferation.[5,19,26,31] To minimize the possibility of infection, we need strict guidelines for the aseptic processing, preparation, and preservation of AS eye drops as well as clear explanations and education for patients about the correct application methods. Periodic container collection for bacterial cultivation should help monitor any signs of infection.[31,32]

Although the treatment in this study was effective for SS dry eye, the mechanism of antiinflammation and synergy of the combination therapy was not evaluated. Further studies are needed to assess the inflammatory cytokines in tears after long-term AS use and the changes in these cytokines after combining AS eye drops with PPs, which might be helpful to clarify the mechanism of action.

In conclusion, our results highlight not only the effectiveness and safety of the long-term application of AS eye drops but also the beneficial effects of the combination of AS eye drops with PPs. We observed improvements in both tear stability and ocular surface vital staining scores in patients with SS dry eye.

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Research article

Cytokeratin expression in mouse lacrimal gland germ epithelium

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ABSTRACT

Purpose: The lacrimal gland secretes tear fluids that protect the ocular surface epithelium, and its dysfunction leads to dry eye disease (DED). The functional restoration of the lacrimal gland by engraftment of a bioengineered lacrimal gland using lacrimal gland germ epithelial cells has been proposed to cure DED in mice. Here, we investigate the expression profile of cytokeratins in the lacrimal gland germ epithelium to clarify their unique characteristics.
Methods: We performed quantitative polymerase chain reaction (Q-PCR) and immunohistochemistry (IHC) analysis to clarify the expression profile of cytokeratin in the lacrimal gland germ epithelium.
Results: The mRNA expression of keratin (KRT) 5, KRT8, KRT14, KRT15, and KRT18 in the lacrimal gland germ epithelium was increased compared with that in mouse embryonic stem cells and the lacrimal gland germ mesenchyme, as analyzed by Q-PCR. The expression level of KRT15 increased in the transition from stem cells to lacrimal gland germ epithelium, then decreased as the lacrimal gland matured. IHC revealed that the expression set of these cytokeratins in the lacrimal gland germ epithelium was different from that in the adult lacrimal gland. The expression of KRT15 was observed in the lacrimal gland germ epithelium, and it segmentalized into some of the basal cells in the intercanulated duct in mature gland.
Conclusion: We determined the expression profile of cytokeratins in the lacrimal gland epithelium, and identified KRT15 as a candidate unique cellular marker for the lacrimal gland germ epithelium.

1. Introduction

Lacrimal glands secrete tear fluid, including tear water and tear proteins, thus maintaining the homeostatic microenvironment for a healthy ocular surface (Mathers, 2000; Mishima, 1965). A shortage of tear volume caused by lacrimal gland dysfunction leads to dry eye disease (DED) (Mathers, 2000). Systemic diseases and environmental exposures including Sjögren's syndrome, ocular cicatricial pemphigoid, aging, extensive usage of a visual display, the use of contact lenses and refractive surgery have all been reported as pathological causes of DED (Uchino et al., 2011; Tsubota and Nakamori, 1993; Toda et al., 2004). DED is the most common eye disease and results in ocular surface epithelial damage, ocular discomfort, significant loss of vision and a decrease in the quality of life (Kaido et al., 2011). Regenerative therapeutic approaches to restore lacrimal gland function have recently been suggested as a future curative approach for DED (Lemp, 1987; Hirayama et al., 2013a).

One of the goals of regenerative medicine is to generate the desired cells, tissues and organs from pluripotent stem cells to restore damaged organ function (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998; Takahashi and Yamanaka, 2006). Current studies of cell differentiation have increased our understanding of developmental biology, which provides information regarding the sequential requirements of the transcription factors, growth factors, signaling cascades, and cell–cell interactions necessary to optimize culture conditions (Murry and Keller, 2008; Snykers et al., 2009; Zaret and Grompe, 2008). For the generation of target cells, cell characterization, including the identification of cell markers such as surface membrane proteins that define the specific cell characteristics, is indispensable to verify the commitment to a cell lineage (Lombaert and Hoffman, 2010).

The lacrimal gland is organized according to the tubuloalveolar system, which consists of acini, ducts, myoepithelial cells and peripheral tissues such as nerves (Schechter et al., 2010). Such functional 3D architecture of the mature lacrimal gland is achieved through a epithelial–mesenchymal interaction during organogenesis (Makarenkova et al., 2000; Tsau et al., 2011). Recently, the concept of bioengineered lacrimal gland replacement therapy to cure DED has been demonstrated by the engraftment of a bioengineered lacrimal gland germ that was reconstituted by using

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0014-4835/

cells from mouse embryonic lacrimal gland germs (Hirayama et al., 2013a, b). Thus, lacrimal gland germ epithelial cells may be attractive candidates for regeneration using pluripotent stem cells (Hirayama et al., 2015). These cells may be useful for bioengineered organ replacement therapy to restore lacrimal gland function; therefore, the characterization of their attributes is required.

Here, we characterized cytokeratin expression in the mouse lacrimal gland germ epithelium, and our results provided evidence for the definition of the cells that constitute the epithelium.

2. Methods

2.1. Animals

C57BL/6 mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mouse embryonic stem cells (mESCs) were kindly provided by Prof. Minoru Ko, Department of Systems Medicine, Keio University (Nishiyama et al., 2009). The care and handling of the animals were performed in accordance with the NIH guidelines. All of the experimental protocols were approved by the Animal Care and Use Committee of the Keio University (Approval No. 09167).

2.2. Preparation of samples for quantitative PCR

Lacrimal gland germs that were isolated from embryonic day-16.5 male and female mice were treated with 50 U/ml dispase (BD, Franklin Lakes, NJ, USA) for 1.5 min at room temperature. The epithelial and mesenchymal tissues were separated surgically by using 23 G needles as previously reported (Hirayama et al., 2013a; Ogawa et al., 2013; Nakao et al., 2007). Adult lacrimal glands were obtained from 7-week-old mice.

2.3. Quantitative polymerase chain reaction (Q-PCR)

Total RNA was extracted from cells using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). The expression levels of the mRNAs in each RNA sample were determined using the Thermal cycler Dice Real Time System (TaKaRa Bio Inc., Otsu, Japan). QPCR was performed using a One Step SYBR PrimeScript PLUS RT-PCR Kit (TaKaRa Bio Inc.). The conditions were as follows: initial hold at 42 °C for 5 min; incubation at 95 °C for 10 s; 50 cycles at 95 °C for 5 s and then 60 °C for 31 s. The expression of mRNA was assessed by evaluating threshold cycle (CT) values. The CT values were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase, and the relative amount of mRNA specific to each of the target genes was calculated.

2.4. Immunostaining

Immunostaining for the lacrimal gland epithelium of E16.5 mouse (E16.5 LGE) was carried out using the fixed tissues on the dish. In short, 6 h after cultivation of the E16.5 LGE in AK-03 (Ajinomoto, Tokyo, Japan), the tissue was fixed with 4% paraformaldehyde (pH 7.0) in phosphate buffered saline (PBS) for 20 min at room temperature. After two rinses with PBS, the cells were incubated with 0.1% Triton X-100 in PBS for 15 min at room temperature and then washed three times with PBS for 5 min each. For the whole lacrimal glands from 7-week-old mice (7w LG), the tissues were fixed in Mildform 10 N (Wako, Osaka, Japan) overnight at 4 °C, and frozen sections (10 μm) were prepared and immunostained. Samples were then incubated with 10% bovine serum albumin (BSA) in PBS for 30 min at room temperature, followed by primary antibody incubation for 16 h at 4 °C. The primary antibodies used were the following: rabbit anti-cytokeratin 5 (1:250, Abcam, Cambridge, UK), rabbit anti-cytokeratin 8 (1:500, Abcam), rabbit anti-cytokeratin 8 (1:500, Abcam), rabbit anti-cytokeratin 14 (1:500, Abcam), rabbit anti-cytokeratin 15 (1:250, Abcam), rabbit anti-cytokeratin 18 (1:500, Abcam), goat anti-E cadherin antibody (1:250, Abcam). The secondary antibody reactions were carried out by incubation with the corresponding species-specific antibodies (1:500, Life Technologies) for 1 h at room temperature in the dark. After four washes with PBS for 5 min each, the specimens were nuclear-stained with DAPI (Life Technologies) and observed with an IX73 inverted microscope (Olympus, Tokyo, Japan).

3. Results

3.1. Gene expression profile of cytokeratin in mouse lacrimal gland germ epithelium

To investigate the levels of gene expression of cytokeratin in the mouse lacrimal gland epithelium, we first performed Q-PCR analysis. We used separated epithelial and mesenchymal tissues of E16.5 mouse-lacrimal gland germs, mouse ESCs, and 7w LG for the extraction of mRNA (Fig. 1a). We analyzed the expression levels of a series of cytokeratins. Q-PCR revealed that the relative mRNA levels of cytokeratin (KRT) 5, KRT8, KRT14, KRT15, KRT18 were higher in the E16.5 lacrimal gland germ epithelium (E16.5 LGE) than in mouse ESCs (Fig. 1b). The levels of KRT5 and KRT15 mRNA were expressed at high levels in E16.5 LGE. In the comparison among the E16.5 LGE, the E16.5 lacrimal gland germ mesenchyme (E16.5 LGM) and 7w LG, the relative RNA levels of these cytokeratins (KRT 5, 8, 14, 15, 18) were higher in E16.5 LGE than in E16.5 LGM and 7W LG. Interestingly, the level of KRT15 mRNA was much higher only in E16.5 LGE (Fig. 2). These results indicated that the relative RNA levels of KRT5, KRT8, KRT14, KRT15, KRT18 were higher in E16.5 epithelium than in E16.5 mesenchyme, and KRT15 was more highly expressed in embryonic developmental stages than in mature lacrimal gland tissue. These results indicated that the expression level of KRT15 increased in the differentiation from stem cells to lacrimal gland germ epithelium, then decreased as the lacrimal

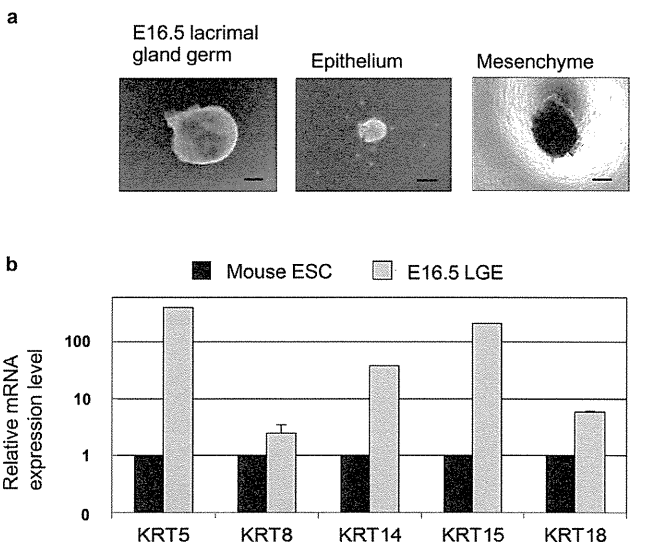

Fig. 1. mRNA expression in the lacrimal gland cells compared with that of mouse embryonic stem cells. **a.** Phase-contrast microscopic images of E16.5 mouse lacrimal gland germ. Images of whole (left), separated epithelium (center) and separated mesenchyme (right) are shown. Scale bar, 100 μm. **b.** Expression profiles of cytokeratins in E16.5 LGE and mouse ESCs. The error bars represent the mean ± standard deviation (SD) of three samples. LGE; lacrimal gland germ epithelium, ESCs; embryonic stem cells.

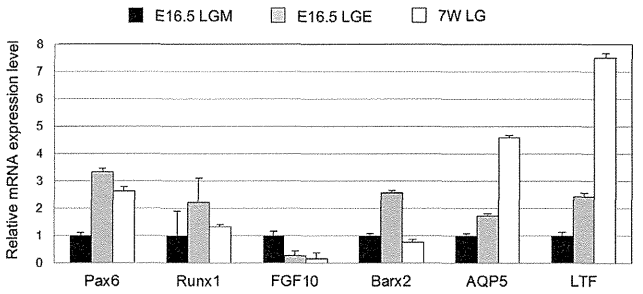

Fig. 2. Analysis of mRNA expression of cytokeratin in the lacrimal gland epithelium. The expression profiles of cytokeratins in E16.5 LGE, E16.5 LGM and 7w LG. The error bars represent the mean ± standard deviation (SD) of three samples. LGE; lacrimal gland germ epithelium, LGM; lacrimal gland germ mesenchyme, 7w LG; 7 week old mouse lacrimal gland.

gland matured. Next, we analyzed the level of mRNA expression of other markers of lacrimal gland development (Fig. 3). The relative expression of Pax6, which is necessary for lacrimal gland development, was higher in E16.5 LGE and 7w LG. The expression of Runx1, one of the epithelial markers, was much higher in E16.5 LGE. The mRNA expression of branching morphogenesis factors such as fibroblast growth factor (Fgf) 10, which is a growth factor facilitating branching in the mesenchyme, and Barx2, which is expressed in the epithelium during branching in the embryonic stage, were higher in E16.5 LG than in 7w LG. The expression level of aquaporin (Aqp) 5, a water channel, and lactoferrin (Ltf), a tear protein, were higher in E16.5 LGE than in E16.5 LGM and were much higher in the mature gland (Fig. 3). These results indicated that the change in expression levels of KRT15 is correlated with other markers highly expressed in the embryonic stage of lacrimal gland development such as Fgf10 and Barx2.

3.2. Protein expression pattern of cytokeratin in mouse lacrimal gland germ epithelium

To clarify the protein expression pattern of cytokeratin in the lacrimal gland germ epithelium, we next performed immunohistochemical (IHC) analysis of the mouse lacrimal gland germ epithelium in *in vitro* culture. IHC analysis revealed that the protein expression of KRT5, KRT8, KRT14, KRT15, and KRT18 could be observed throughout the mouse lacrimal gland germ epithelium (Fig. 4).

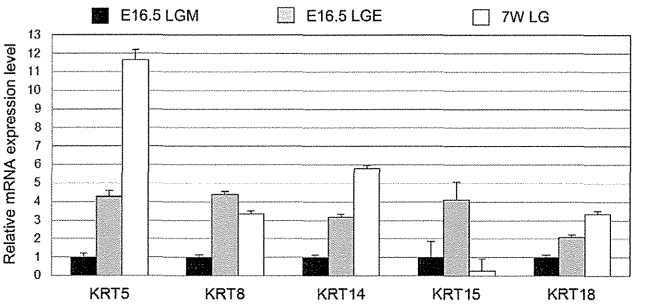

Fig. 3. Analysis of mRNA expression of other markers of lacrimal gland development. The expression profiles of other lacrimal gland markers in E16.5 LGE, E16.5 LGM and 7w LG. The error bars represent the mean ± standard deviation (SD) of three samples. LGE; lacrimal gland germ epithelium, LGM; lacrimal gland germ mesenchyme, 7w LG; 7 week old mouse lacrimal gland.

3.3. Protein expression pattern of cytokeratin in 7-week-old mouse lacrimal glands

We further investigated the protein expression pattern of cytokeratin in the 7w LG to determine changes during lacrimal gland development. IHC analysis revealed that KRT5 was expressed in myoepithelial cells, KRT14 was expressed in myoepithelial cells and duct cells, and KRT8 and KRT18 were expressed only in duct cells. In 7W LG, KRT15 was localized in some of the E-cadherin positive basal, intermediate cells in the intercanulated duct, which was reported as a location of tissue stem cells in mammary glands and salivary glands, in contrast with high expression in the lacrimal gland germ epithelium (Fig. 5). Together, our results indicated that the different expression patterns of cytokeratin were observed in the lacrimal gland epithelium according to developmental stages and that KRT15 may be unique differentiation marker for the lacrimal gland germ epithelium.

4. Discussions

In this study, we successfully determined the cytokeratin expression profiles of the lacrimal gland epithelium during its development. Our findings indicate that the lacrimal gland epithelium has different expression patterns according to developmental stages, and KRT15 is highly expressed in the lacrimal gland germ epithelium during lacrimal gland development. Our study thus determined several characteristics of the lacrimal gland epithelium and provided evidence of epithelial markers for cell differentiation in lacrimal gland tissue.

The organogenesis of the lacrimal gland involves a reciprocal interaction between the epithelial and mesenchymal cells at the embryonic ocular surface mucosa (Schechter et al., 2010). The developmental process of the epithelium during branching morphogenesis, such as proliferation, differentiation, and cell death, is regulated by various growth factors and undergoes a series of cell fate decisions to differentiate into different cell types, including acini, ducts, and myoepithelial cells (Lemp, 1987; Zaret and Grompe, 2008). Previous studies on bioengineered lacrimal gland organ replacement have reported that lacrimal gland germ epithelial cells can develop into mature secretory glands *in vitro* and *in vivo* (Hirayama et al., 2013a). The lacrimal gland germ epithelium may be generated from pluripotent stem cells for the functional restoration of the gland to cure DED (Hirayama et al., 2013a). However, few studies on lineage tracing, which define the lacrimal gland germ epithelium, have been reported (Kobayashi et al., 2012).

One of the useful approaches to identify the cell-lineage of the lacrimal gland epithelium is by evaluating the cytoskeleton keratin expression profiles of cells (Stasiak et al., 1989). The cytokeratin, which exists as heterodimer pairs of type1 and type2 keratin, is expressed in a tissue-specific manner (Lombaert and Hoffman, 2010). Mutation of cytokeratin is associated with diseases in several organs, such as the intestine, pancreas and liver (Toivola et al., 2015). The expression profile of cytokeratin has been used to characterize several cell types, including basal, intermediate, and luminal cells in many epithelial tissues and secretory glands (Lombaert and Hoffman, 2010; Stasiak et al., 1989; Purkis et al., 1990; Brembeck and Rustgi, 2000; Knox et al., 2010; Zeineldin and Hudson, 2006; Rebustini et al., 2007; Walker et al., 2008). In this study, the expression of KRT5, KRT8, KRT14, KRT15, and KRT18 was observed throughout the E16.5 lacrimal gland germ epithelium, and the expression changed during the further differentiation into a mature secretory gland structure. The expression set of cytokeratins in E16.5 LGE was different from that of other secretory glands previously reported (Table 1) (Lombaert and Hoffman,

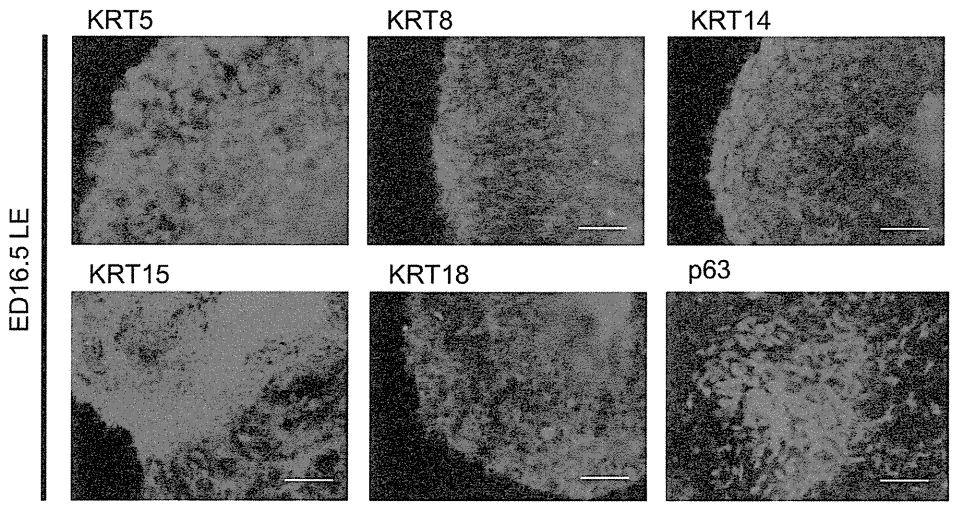

Fig. 4. Immunohistochemical Analysis of the lacrimal gland epithelium. Immunohistochemical analysis of the lacrimal gland germ epithelium with antibodies against cytokeratins (green). The DAPI (blue) and control panels (p63) are shown (lower right). Scale bar, 100 μm.

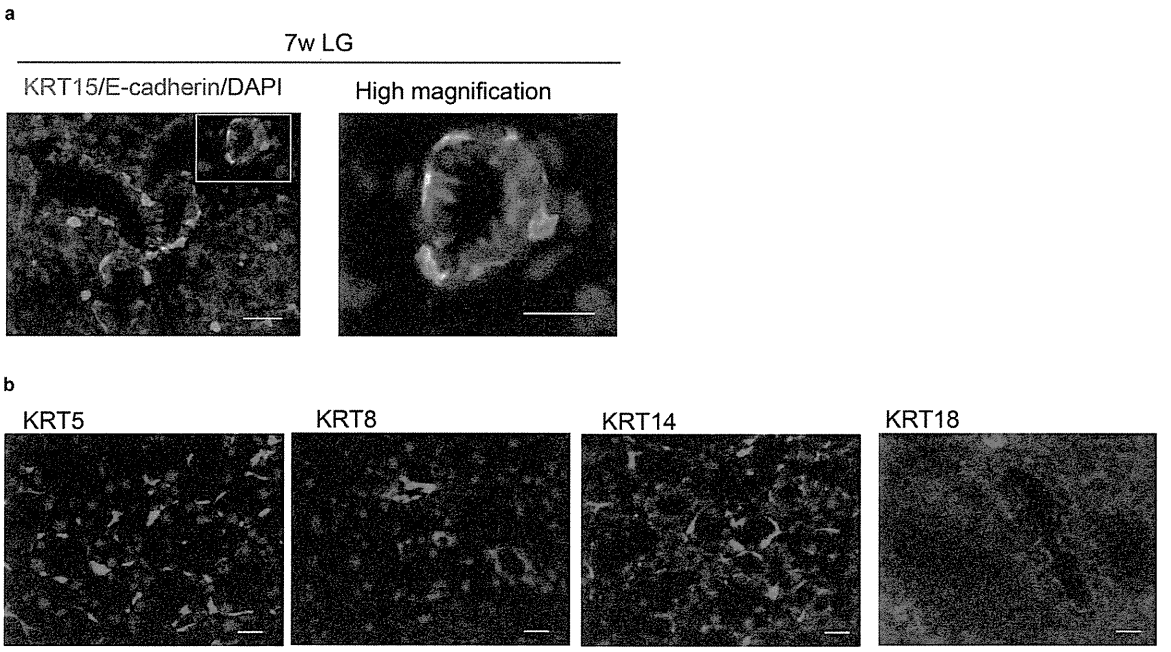

Fig. 5. Immunohistochemical Analysis of 7 week old mouse lacrimal glands. **a.** Immunohistochemical analysis of the lacrimal gland germ epithelium with antibodies against KRT15 (green) and E-cadherin (red). DAPI (blue), Scale bar, 100 μm. **b.** Immunohistochemical analysis of the lacrimal gland germ epithelium with antibodies against cytokeratins (green or red) and DAPI (blue). Scale bar, 100 μm.

2010). Strikingly, the expression level of KRT15 was higher in E16.5 LGE compared with that of stem cells, whereas it clearly decreased in 7w LG. These results indicated that KRT15 is a candidate cell marker for the lacrimal gland germ epithelium during lacrimal gland epithelial development.

Identification of tissue stem/progenitor cells in the lacrimal gland has been of interested due to the potential therapeutic application of the lacrimal gland regeneration to replace damaged tissue by cell transplantation therapy (Kobayashi et al., 2012; Zoukhri, 2010; Feng et al., 2009; Sumita et al., 2011; You et al., 2011). Previous studies have indicated that tissue stem cells are found in different niches within an organ, similarly to skin stem cells, hair follicles, sebaceous glands, and the neural crest (Ambler and Maatta, 2009). Regarding salivary gland cytokeratins, KRT5 and KRT8 were found as embryonic stem cell markers, and their expression decreased or was segmentalized into limited area such as the basal region of the acini and duct. Our study may suggest that KRT15-positive cells in the ducts of adult LG are a candidate for

Table 1
Summary of expression profile of cytokeratin in lacrimal gland and other epidermal tissues in our and previous studies.

	E16.5 LGE (Our study)	Adult LG (Our study)	Salivary gland	Other secretory glands	Others
KRT5	Throughout	Duct cells	E13 epithelium (especially in basal duct and end bud), decrease in adult (Knox et al., 2010)	Epithelial basal layer, contains tissue stem cells in prostate and mammary gland (Purkis et al., 1990)	Epithelial basal layer, contains tissue stem cells in skin bulge (Purkis et al., 1990)
KRT8	Throughout	Duct cells, cultivated lacrimal gland epithelium (Kobayashi et al., 2012)	Throughout E13 epithelium (Rebustini et al., 2007)	Luminal prostate and mammary gland cells (Purkis et al., 1990)	
KRT14	Throughout	Duct cells, Myoepithelial cells	Increase in E15 epithelium (also expressed in E13 and adult) (Lombaert and Hoffman, 2010)	Epithelial basal layer, contains tissue stem cells in prostate and mammary gland (Purkis et al., 1990)	Epithelial basal layer, contains tissue stem cells in skin bulge (Purkis et al., 1990)
KRT15	Throughout	Basal, intermediate cells in duct		Mammary and salivary duct cells (limited to basal intermediate cells) (Stasiak et al., 1989)	Oral epithelium (Stasiak et al., 1989)
KRT18	Throughout	Luminal	Luminal (Hudson et al., 2001)	Luminal prostate and mammary gland cells (Purkis et al., 1990)	

tissue stem cells in LG. In this study, we successfully determined the cytokeratin expression profile of the early lacrimal gland, which has not been thoroughly characterized, and traced cytokeratin expression during development, which will provide useful information to detect tissue stem cells in the lacrimal gland.

In conclusion, the current study provides novel evidence for the cytokeratin expression profile of the lacrimal gland epithelium in early and adult developmental stages. These findings may be the first step for characterizing the regeneration of cells and identifying tissue stem/progenitor cells for future functional bioengineered organ replacement therapy for DED.

Author contributions

K.T., T.K., S.S. and M.H. designed the research plan; M.H. and Y.L. performed the experiments; M.H. and T.K. developed new assay methods and discussed the results; M.H. and Y.L. analyzed the data; M.H. , Y.L. and T.K. wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

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ORIGINAL ARTICLE

Saliva as a potential tool for diagnosis of dry mouth including Sjögren's syndrome

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OBJECTIVES: Recently, the use of saliva as a diagnostic tool has gained considerable attention because it is non-invasive and easy to perform repeatedly. In this study, we focused on soluble molecules in saliva to establish a new diagnostic method for xerostomia.

MATERIALS AND METHODS: Saliva was obtained from 90 patients with Sjögren's syndrome (SS), 22 patients with xerostomia associated with neurogenic/neuropsychiatric disorders and drugs (XND), 30 patients with radiation-induced xerostomia (RX), and 36 healthy controls. Concentrations of helper T (Th) cytokines in saliva were measured by flow cytometric analysis. Concentrations of secretory IgA (SIgA) and chromogranin A (CgA) were measured by ELISA.

RESULTS: Unstimulated and stimulated whole saliva from patients with SS, XND, and RX was significantly reduced compared with controls. Th1 and Th2 cytokines from SS patients were significantly higher than controls. Furthermore, Th2 cytokines were closely associated with strong lymphocytic accumulation in salivary glands from SS patients, while Th1 and Th17 cytokines were negatively associated. SIgA levels were not significantly different between all patient groups and controls. CgA levels from XND patients were significantly higher than controls.

CONCLUSIONS: The measurement of cytokines, CgA, and SIgA in saliva is suggested to be useful for the diagnosis of xerostomia and also to reveal disease status.

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Keywords: xerostomia; Sjögren's syndrome; saliva; cytokine; secretory IgA; chromogranin A

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Introduction

Recently, heightened interest in oral health has led to an increase in patients complaining of dry mouth (xerostomia) (Navazesh and Ship, 1983; Greenspan, 1996; Guggenheimer and Moore, 2003). Xerostomia can be classified into the following three groups based on the cause: (1) xerostomia caused by dysfunction of the salivary gland, which can be due to Sjögren's syndrome (SS) and radiation-induced xerostomia (RX); (2) xerostomia associated with neurogenic/neuropsychiatric disorders and drugs (XND); and (3) xerostomia associated with systemic diseases or metabolic disorders such as diarrhea, dehydration, thyroid hyperfunction, diabetes mellitus (DM), kidney malfunction, and anemia (Bahn, 1972; Ettinger, 1981; Spielman *et al*, 1981; Billings *et al*, 1996; Greenspan, 1996; Nakamura *et al*, 1997; Guggenheimer and Moore, 2003; Matear and Barbaro, 2005; Scully and Felix, 2005; Ivanovski *et al*, 2012; Nonzee *et al*, 2012; Rahman *et al*, 2013). However, it is difficult to make a differential diagnosis of xerostomia objectively. Consequently, the diagnostic criteria for xerostomia remain to be established except SS. The criteria for SS are determined from the results of relatively complicated examinations including sialography, salivary gland scintigraphy, and labial salivary gland (LSG) biopsy, which are invasive and only performed in special facilities. Recently, the use of saliva as a diagnostic tool has gained considerable attention, as it is non-invasive and easy to perform repeatedly. Bigler *et al* reported that tumor markers could be detected in saliva from subjects with oral, lung, or pancreatic cancer (Zhang *et al*, 2012; Lau *et al*, 2013; Wang *et al*, 2013). In addition, secretory immunoglobulin A (SIgA) and chromogranin A (CgA) in saliva were recently reported the usability as stress-related substances (Bosch *et al*, 1998). In our previous studies, we focused on the involvement of Th cytokines in the pathogenesis of SS (Ohyama *et al*, 1996, 1998; Tsunawaki *et al*, 2002; Maehara *et al*, 2012a,b; Moriyama *et al*, 2013). In this study, we thus examined the diagnostic utility of saliva from patients with xerostomia including SS, RX, and XND.

Materials and methods

Patients

Ninety patients with SS (84 women and 6 men; mean age ± standard deviation (s.d.), 61.9 ± 12.6 years), 30 patients with XND (25 women and 5 men; mean age ± s.d., 69.4 ± 10.5 years), 22 patients with RX (7 women and 15 men; mean age ± s.d., 67.3 ± 9.8 years), and 36 healthy subjects (21 women and 15 men, mean age ± s.d., 42.4 ± 15.1 years) were referred to the Department of Oral and Maxillofacial Surgery, Kyushu University Hospital between 2011 and 2013 and were included in the study. All patients with SS met the diagnostic criteria proposed by both the Research Committee on Sjögren's Syndrome of the Ministry of Health and Welfare of the Japanese Government (Fujibayashi *et al*, 1993) and the criteria proposed by the American–European Consensus Group criteria for SS (Vitali *et al*, 2002). The degree of lymphocytic infiltration in the specimens was determined by focus scoring (Greenspan *et al*, 1974; Daniels and Whitcher, 1994). One standardized score indicates the number of focal inflammatory cell aggregates containing 50 or more mononuclear cells in each 4-mm^2 area of salivary gland tissue (Szodoray *et al*, 2005). None of the patients had other autoimmune diseases and were not being treated with steroids or other immunodepressants. XND was diagnosed according to the following criteria: (1) failure to fulfill each of the above diagnostic criteria for SS; (2) taking drugs or diagnosed with depression in the Department of Psychosomatic Medicine; (3) decreased unstimulated whole salivary flow rate (UWS) (<1.5 ml 15 min^{-1}) or stimulated whole salivary flow rate (SWS) (<2.00 g 2 min^{-1}). XND patients were taking anxiolytics (n = 13), sleeping drugs (n = 11), antidepressant drugs (n = 1), and antihypertension drugs (n = 2). RX was diagnosed according to the following criteria: (1) failure to fulfill each of the above diagnostic criteria for SS; (2) having a history of radiotherapy to head and neck; and (3) decreased UWS (<1.5 ml 15 min^{-1}) or SWS (<2.00 g 2 min^{-1}). The disease duration was defined as the period from the initial observation of dry mouth to the first visit. The controls consisted of 36 healthy subjects who had no sicca or clinical or laboratory evidence of systemic disease. This study design was approved by the Ethics Committee of Kyushu University, Japan, and informed consent was obtained from all patients and healthy controls (IRB serial number: 25–287).

Saliva sample (stimulated)

Study participants were asked to refrain from smoking, eating, and drinking for at least 2 h prior to collection of samples. Patients rinsed their mouth with distilled water just before their saliva was taken. Stimulated whole saliva was collected by Salisoft tubes (Sarstedt, Nümbrecht, Germany) using polypropylene-polyethylene polymer swabs from the subjects' mouths following 2 min of chewing. Swabs with absorbed saliva were returned to Salisoft tubes and centrifuged for 5 min at 780 ***g,*** yielding a clear saliva sample. Saliva was transferred to a microtube and stored at −80°C immediately.

Salivary flow rate

Stimulated whole salivary flow rate was measured by the Saxon test. This test was performed by having subjects chew Surgeon® Type IV Gauze Sponge (Hakuzo Medical Corporation, Osaka, Japan) once a second for 2 min and measuring the weight of the gauze. If the increase in weight of the gauze was <2 g, the subject was classified as 'decreased' (Kohler and Winter, 1985). UWS was measured by a spitting test. This test was carried out by asking subjects to spit saliva into a paper cup for 15 min. The amount of saliva in the unstimulated condition (sitting on a chair and not moving) was measured. If the volume of saliva was <1.5 ml, the subject was classified as 'decreased' (Vitali *et al*, 2002).

Concentrations of cytokines in saliva by flow cytometry

The amount of saliva that could be taken from the xerostomia patients was very small, so we used Human High sensitivity Flex Sets of BDTM Cytometric Beads Array (CBA) system that allowed the measurement of many molecules at the same time from 50 μl of saliva (BD Biosciences San Jose, CA, USA). Saliva samples were thawed and centrifuged again, and the supernatant was measured. Soluble molecules including Th1, Th2, and Th17 cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF, IFN-γ, and IL-17A) in saliva were quantitatively determined using antibodies from the multiplex Flex Set Cytometric Bead system® (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Serial dilutions (1/2 v/v) of the standards were prepared; saliva samples were (1/4 v/v) diluted using assay diluent and transferred to a 96-well plate. Then, 50 μl of mixed capture beads was transferred to each well. After a 3-h incubation period at RT, 50 μl PE detection reagent was added and samples were incubated for another hour at RT. 96-well plate was washed with wash buffer, and after final centrifugation at 800 rpm for 10 min, 150 μl wash buffer was added. Flow cytometric analysis was performed using the BD FACSVerse™ and CBA analysis FCAP software (BD Biosciences San Jose, CA, USA), which allowed measurement of cytokines only after passing the quality-control (QC) test including performance QC. In this study, % coefficient of variation (CV) of all samples was acceptance value (%CV was <3%) (Cook *et al*, 2001; LaFrance *et al*, 2008).

Measurement of SIgA and CgA in saliva by enzyme-linked immunosorbent assay

The concentrations of SIgA and CgA were measured by immunoreactivity in a double-sandwich enzyme-linked immunosorbent assay (ELISA) using a Salivary Secretory IgA Enzyme Immunoassay KIT® and YK070 Human Chromogranin A EIA KIT, as reagents to detect these molecules by cytometric bead array are not available. Saliva total protein concentration was measured using the QuickStart™ Bradford Protein Assay Kit (Bio-Rad, CA, USA). Quantity of CgA was calculated by dividing the concentration of CgA with the concentration of the saliva total protein. Concentrations of cytokines, SIgA, and CgA were evaluated by a researcher (O M) who was blinded to sample information.

Statistical analysis
The statistical significance of differences between groups was determined using the chi-square test, Kruskal–Wallis test followed by *post hoc* Steel's test, Mann–Whitney *U*-test, and Spearman's rank correlation. Values of $P < 0.05$ were considered statistically significant.

Results

Clinical findings
Table 1 shows the clinical characteristics (mean age, sex, disease duration, UWS, and SWS) of the patient and control groups. The mean amount of UWS from all patient groups was lower than the standard value, while the SWS of patients with SS and RX was lower than the standard value. In comparison with healthy subjects, UWS from all patient groups was significantly lower than that from healthy subjects. SWS from SS and RX patients was significantly lower than that from healthy subjects (Figure 1).

Cytokine and stress-related substances in saliva
Our previous studies demonstrated that Th1 cytokines were essential for the induction and/or maintenance of SS, whereas Th2 cytokines might be involved in disease progression, especially local B-cell activation (Ohyama *et al*, 1996, 1998; Tsunawaki *et al*, 2002; Maehara *et al*, 2012a, b; Moriyama *et al*, 2013). We thus focused on soluble molecules such as cytokines and examined their concentrations in saliva from patients with xerostomia. As cytokines and stress-related substance are soluble factors secreted in the salivary glands, we examined these molecules in the saliva using flow cytometry and ELISA. The concentrations of Th1 cytokines (IFN-©, IL-1®, TNF, IL-2, IL-6, IL-8, IL-12p70), Th2 cytokines (IL-4, IL-5, IL-10), and Th17 cytokine (IL-17) in the saliva from SS patients were significantly higher than the controls (Figure 2).

Secretory immunoglobulin A is the dominant immunoglobulin in external secretions that bathe mucosal surfaces (respiratory, intestinal, and reproductive), and salivary glands, where it plays a role in the immune system 'first line of defense' against microbial invasion. SIgA is reported to be a stress-related substance, and concentrations of SIgA are increased in patients with sialadenitis (Bosch *et al*, 1998). CgA is produced by salivary epithelial cells and is generally used as a novel mental stress marker (Nakane *et al*, 1998). Although the concentrations of SIgA from SS and RX patients were higher than in the controls, this was not statistically significant. Quantities of CgA in saliva from patients with XND were significantly higher than in the controls (Figure 3).

Association of saliva cytokines with the degree of lymphocytic infiltration in LSGs from SS patients
Sjögren's syndrome patients were divided into two groups: (1) those with weak lymphocytic infiltration of LSGs (focus scores ranging from 1 to 6, 55 women and 1 man; mean age, 48.7 ± 13.9 years) and (2) those with strong lymphocytic infiltration (focus scores ranging from 7 to 12, 29 women and 5 men; mean age, 62.9 ± 11.7 years). The concentration of IL-17 had no relationship with the degree of lymphocytic infiltration in the LSGs from SS patients. The concentrations of a non-specific inflammatory cytokine (IL-1®) and Th1 cytokine (IL-12p70) were significantly higher in LSGs with weak lymphocytic infiltration in comparison with those with strong lymphocytic infiltration. In contrast, the concentrations of Th2 cytokines (IL-4, IL-5) were significantly higher in LSGs with strong lymphocytic infiltration in comparison with those with weak lymphocytic infiltration (Figure 4). Furthermore, we examined the correlation between the focus score and cytokines with significant difference in Figure 4 and found that IL-4 showed significant positive correlation, while IL-1® and IL-12p70 had a negative correlation (Figure 5).

Discussion

Xerostomia is caused by numerous factors and complications. It manifests with various symptoms including mucosal atrophy, multiple caries, periodontitis and dysphagia because saliva serves a variety of functions in the oral cavity such as dissolving substances important for taste and acting as a lubricant for speaking, chewing, and swallowing. Saliva has buffering functions that neutralize acids formed during bacterial carbohydrate metabolism and contains minerals for tooth remineralization [1–12]. Saliva also contains bactericidal substances such as lysozyme,

Table 1 Patient profiles and clinical findings

	SS (n = *90*)	*XND* (n = *30*)	*RX* (n = *22*)	*Control* (n = *36*)
Men/women	6:84	5:25	15:7	21:15
Age (mean ± s.d. years)	61.9 ± 12.6	69.4 ± 10.5	67.3 ± 9.8	42.4 ± 15.1
Disease duration (mean ± s.d days)	52.8 ± 12.5	32.6 ± 10.2	18.4 ± 4.5	
UWS by spitting method [median (25th–75th percentiles) ml/15 min]	0.5 (0.1–1.0)	0.7 (0.2–1.0)	0.8 (0.5–2.0)	3.8 (2.9–5.1)
SWS by Saxon's test [median (25th–75th percentiles) g 2 min^{-1}]	1.22 (0.58–2.17)	2.11 (1.14–3.40)	1.98 (1.04–3.06)	5.06 (4.57–6.12)

SS, Sjögren's syndrome; XND, xerostomia associated with neurogenic/neuropsychiatric disorders and drugs; RX, radiation-induced xerostomia; SWS, stimulated whole salivary flow rate; UWS, unstimulated whole salivary flow rate; s.d., standard deviation.

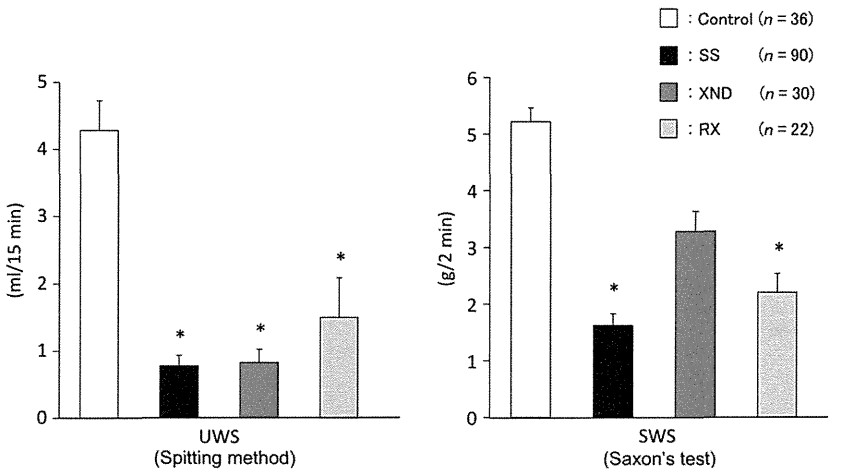

Figure 1 Salivary function of patients with xerostomia. SWS, stimulated whole salivary flow rate; UWS, unstimulated whole salivary flow rate; the bar shows the median value ± interquartile range; $*P < 0.01$ (Kruskal–Wallis test followed by *post hoc* steel's test)

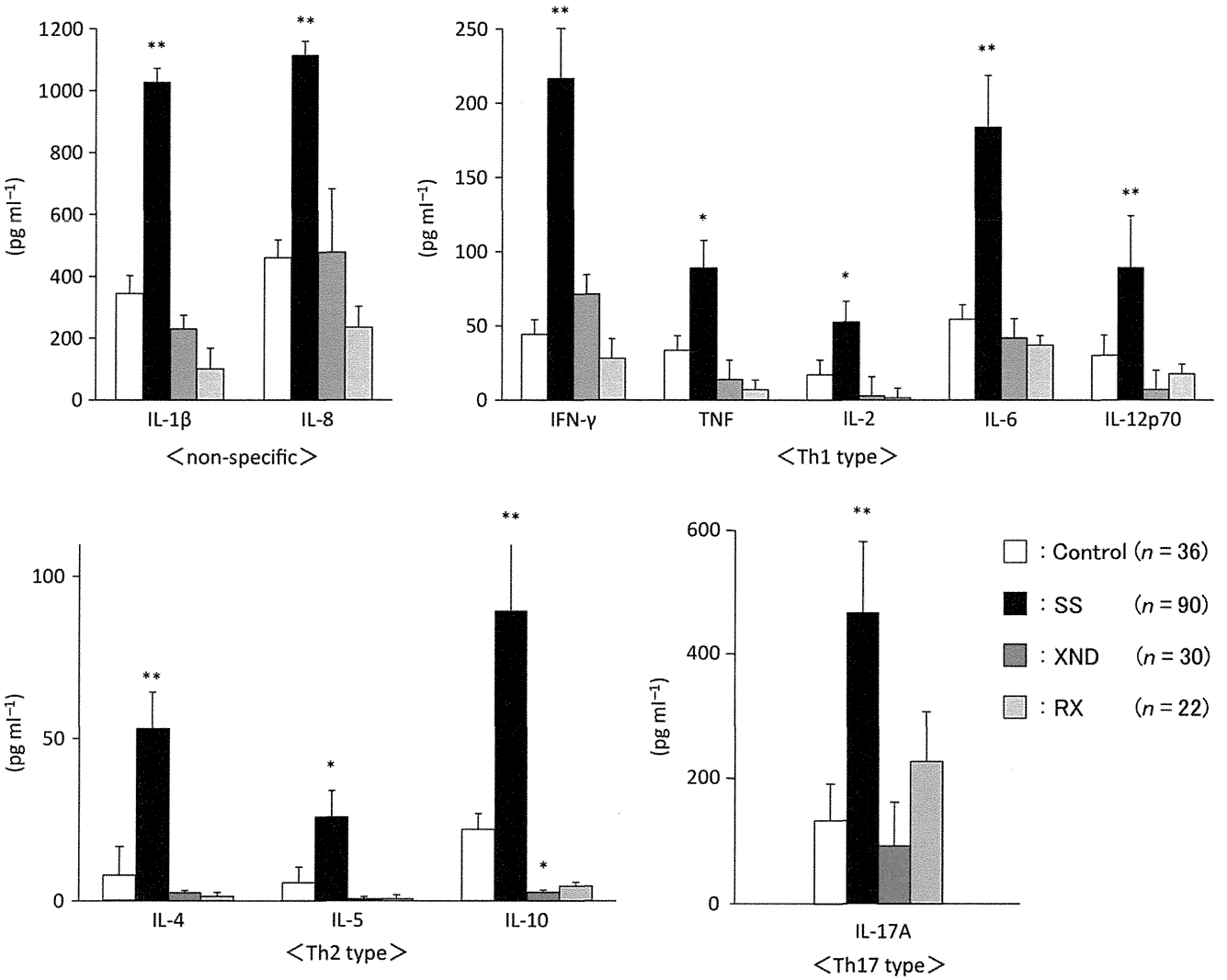

Figure 2 Cytokine in saliva from patients with xerostomia. Th T-helper cell; the bar shows the mean value ± standard deviation (s.d.); $*P < 0.05$, $**P < 0.01$ (Kruskal–Wallis test followed by *post hoc* Steel's test)

lactoferrin, and antibodies, and SIgA is especially important for the defense systems of mucosal membranes. In this study, we first focused on the differences of salivary flow rates (SWS and UWS) among each patient group including SS, XND, and RX. The SWS from all patient groups was significantly lower than in controls, while the

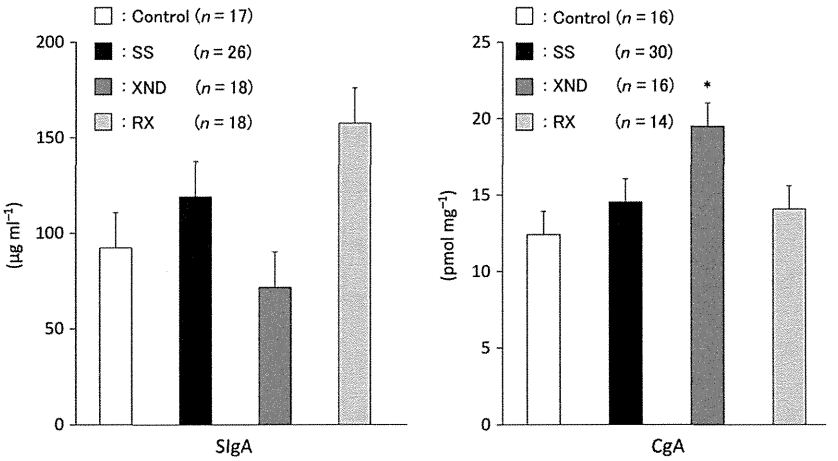

Figure 3 Secretory immunoglobulin A (SIgA) and chromogranin A (CgA) in saliva from patients with xerostomia. SIgA, secretory immunoglobulin A; CgA, chromogranin A; the bar shows the mean value ± s.d.; $*P < 0.01$ (Kruskal–Wallis test followed by *post hoc* steel's test)

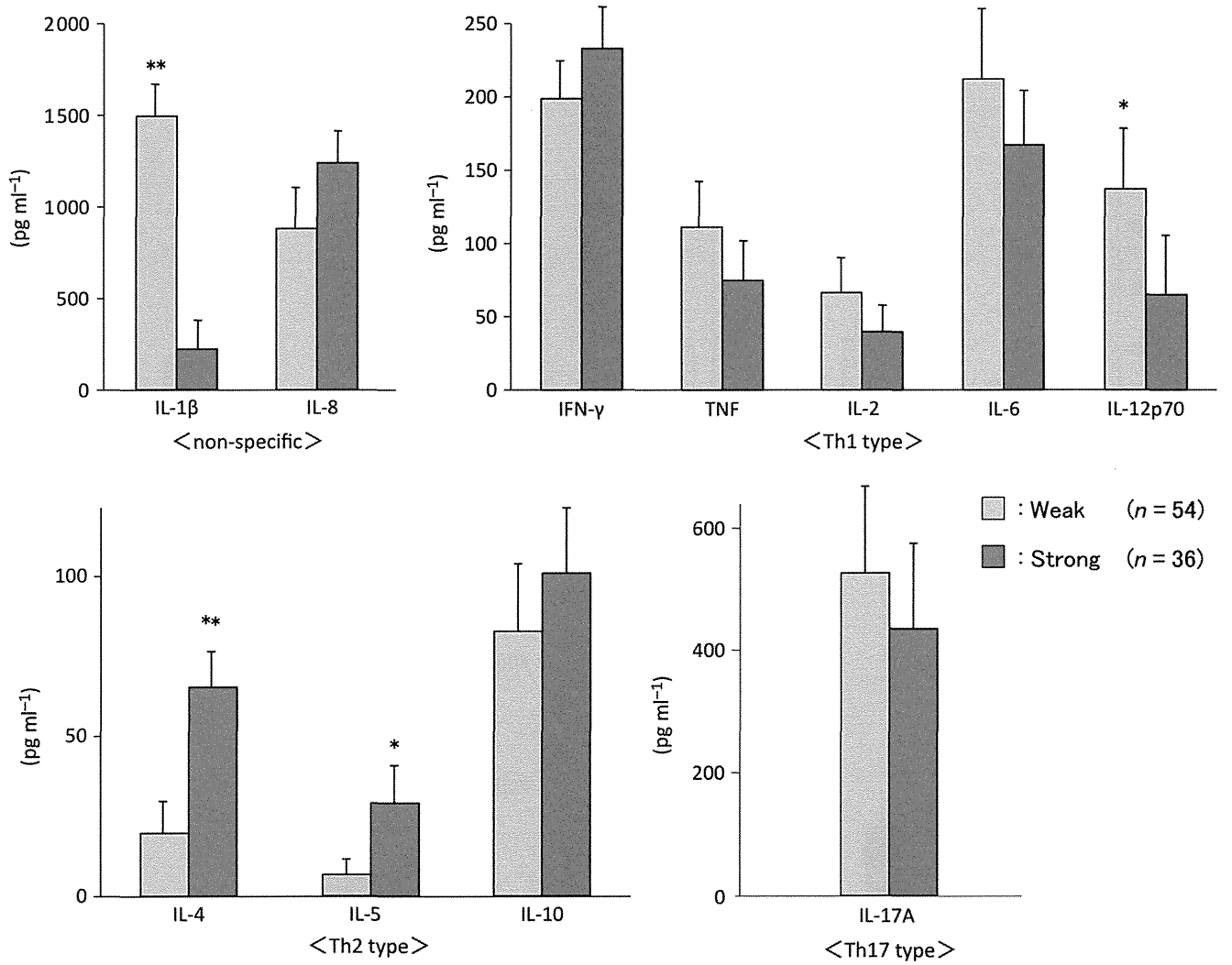

Figure 4 Association between cytokines and degree of lymphocytic infiltration in labial salivary glands (LSGs) from patients with Sjögren's syndrome (SS). The degree of lymphocytic infiltration in LSGs was graded from 1 to 12 by focus scoring and was then divided into groups: (i) those with focus scores ranging from 1 to 6 were categorized as having weak infiltration and (ii) those with scores from 7 to 12 were categorized as having strong infiltration; the bar shows the mean value ± s.d.; $*P < 0.05$, $**P < 0.01$ (Mann–Whitney *U*-test)

UWS from SS and RX patients was significantly lower than in controls. However, patients with XND showed no statistically significant difference with the controls. Several studies indicated that the decrease in SWS and UWS in SS and RX patients was caused by the physical destruction of salivary glands and that the decrease in UWS

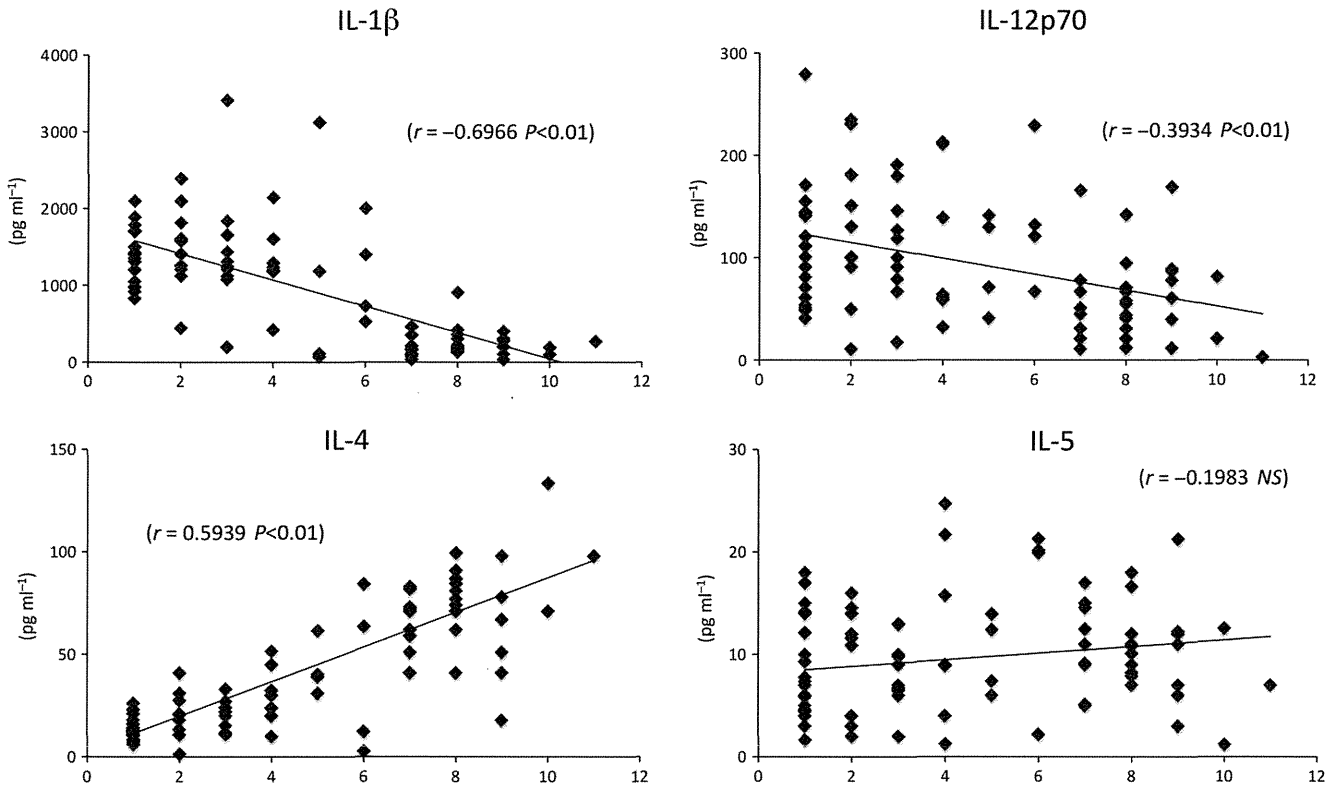

Figure 5 Correlation between cytokines and focus score in LSGs from patients with SS. Statistical significance of the differences between groups was determined by Spearman's rank correlation; NS, not significant

alone in XND patients was caused by suppression of the salivary secretory nerve system (Matear and Barbaro, 2005; Scully and Felix, 2005; Nonzee *et al*, 2012). When considering normal SWS in patients with XND, this suppression might be inhibited by stimulation of food intake (Matear and Barbaro, 2005; Scully and Felix, 2005; Nonzee *et al*, 2012). The results of the present study concerning salivary flow rates are consistent with these reports. However, it is difficult to diagnose xerostomia only using SWS and UWS because measurements of salivary flow rates often vary depending on the patient's condition and thus lack accuracy. In addition, the Saxon test often causes nausea. To overcome these issues, we examined soluble proteins such as cytokines and stress-related substances in saliva samples. However, it was difficult to collect the saliva required for analyses of multiple cytokines, especially in patients with xerostomia. Therefore, we used the 'CBA flex system' to measure many soluble factors from a single small-volume sample with high reproducibility. It is generally accepted that cytokines produced by Th cells play a crucial role in the pathogenesis of SS (Dhabhar *et al*, 2009). The results of the present study concerning cytokine concentration in the saliva from SS patients were consistent with this model. However, little is known concerning the association of cytokines in saliva with RX and XND. In this study, there was no significant difference in the levels of all tested cytokines between patients with RX and controls, indicating salivary RX dysfunction was caused by radiation-induced destruction without autoimmune reaction via cytokines. Only levels of IL-10 from XND patients were significantly lower than controls. Although it is unlikely that anti-inflammatory cytokines such as IL-10 are related to the inhibition of salivary secretion nervous system, Dhabhar *et al* (2009) reported that patients with depression had decreased serum IL-10, consistent with the results of this study.

Also important in clinical settings is the long-term follow-up for SS patients because SS is progressive and causes the gradual reduction of salivary functions. However, it is extremely difficult to perform serial LSG biopsy for patients with high invasiveness. We thus examined cytokine concentrations in the saliva, which could be collected repeatedly. Our previous studies of salivary glands suggested that Th1 and Th17 cells are involved in the early stages of SS, while Th2 and follicular T cells are associated with germinal center formation in the late stages of disease (Maehara *et al*, 2012a,b; Moriyama *et al*, 2012). In this study, we found that the concentrations of Th2 cytokines in saliva were closely associated with strong lymphocytic infiltration, which is consistent with previous reports in salivary glands.

We also focused on stress-related soluble proteins in saliva such as SIgA and CgA. SIgA is a soluble immunoglobulin produced by plasma cells and is increased during salivary gland inflammation by physical and emotional stress via sympathetic reaction of the sympathetic–adrenal–medullary axis system (Spangler, 1997; Deinzer *et al*, 2000; Ring *et al*, 2000; Bosch *et al*, 2002). In this study, we observed no significant difference in salivary SIgA levels between the patient groups and controls, whereas SIgA levels in RX and SS patients were higher than in controls. SIgA in RX and SS patients might be produced

by salivary gland ductal epithelium via inflammation caused by the physical destruction of salivary glands. However, the accumulation of cases and further examinations are required to elucidate the involvement of SIgA production in SS and RX. In contrast, CgA is localized in the salivary gland ductal epithelium and released into the saliva by autonomic nerve stimulation, indicating CgA might be useful as a novel mental stress marker (Kanno *et al*, 1998; Nakane *et al*, 1998, 2002). This study demonstrated that the quantity of salivary CgA from patients with XND was significantly higher than in the controls. Twenty-five of 30 patients with XND took anxiolytic, antidepressant or sleep-inducing drugs for a long period. These results are in accordance with a previous report that salivary CgA was secreted owing to chronic psychological stress.

In conclusion, this study indicated that the measurement of cytokines, CgA, and SIgA in saliva can be useful for the differential diagnosis of xerostomia and could be used to monitor pathological states in SS by collecting saliva over time. However, evaluating greater numbers of saliva samples is required to set the reference value of these molecules, which might eventually lead to diagnostic tool for xerostomia.

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Conflict of interest

The authors declare that they have no conflict of interests.

Author contributions

KO and MM designed the study; KO, SS, SF, MO, and YI collected samples; KO, AT, and TM performed the experiments; KO, MM, JNH, and AT analyzed the data; MM and SN drafted the paper.

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RESEARCH ARTICLE Open Access

Effectiveness of imaging modalities for screening IgG4-related dacryoadenitis and sialadenitis (Mikulicz's disease) and for differentiating it from Sjögren's syndrome (SS), with an emphasis on sonography

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Abstract

Introduction: The aim of this study was to clarify the effectiveness of various imaging modalities and characteristic imaging features in the screening of IgG4-related dacryoadenitis and sialadenitis (IgG4-DS), and to show the differences in the imaging features between IgG4-DS and Sjögren's syndrome (SS).

Methods: Thirty-nine patients with IgG4-DS, 51 with SS and 36 with normal salivary glands were enrolled. Images of the parotid and submandibular glands obtained using sonography, 2-[^{18}F]-fluoro-2-deoxy-D-glucose positron emission tomography/computed tomography (FDG-PET/CT), computed tomography (CT) and magnetic resonance imaging (MRI) were retrospectively analyzed. Six oral and maxillofacial radiologists randomly reviewed the arranged image sets under blinded conditions. Each observer scored the confidence rating regarding the presence of the characteristic imaging findings using a 5-grade rating system. After scoring various findings, diagnosis was made as normal, IgG4-DS or SS, considering all findings for each case.

Results: On sonography, multiple hypoechoic areas and hyperechoic lines and/or spots in the parotid glands and obscuration of submandibular gland configuration were detected mainly in patients with SS (median scores 4, 4 and 3, respectively). Reticular and nodal patterns were observed primarily in patients with IgG4-DS (median score 5). FDG-PET/CT revealed a tendency for abnormal ^{18}F-FDG accumulation and swelling of both the parotid and submandibular glands in patients with IgG4-DS, particularly in the submandibular glands. On MRI, SS had a high score regarding the findings of a salt-and-pepper appearance and/or multiple cystic areas in the parotid glands (median score 4.5). Sonography showed the highest values among the four imaging modalities for sensitivity, specificity and accuracy. There were significant differences between sonography and CT ($p = 0.0001$) and between sonography and FDG-PET/CT ($p = 0.0058$) concerning accuracy.

Conclusions: Changes in the submandibular glands affected by IgG4-DS could be easily detected using sonography (characteristic bilateral nodal/reticular change) and FDG-PET/CT (abnormal ^{18}F-FDG accumulation). Even inexperienced observers could detect these findings. In addition, sonography could also differentiate SS. Consequently, we recommend sonography as a modality for the screening of IgG4-DS, because it is easy to use, involves no radiation exposure and is an effective imaging modality.

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Introduction

Immunoglobulin G4 (IgG4)-related dacryoadenitis and sialadenitis (IgG4-DS), Mikulicz's disease, has recently been recognized as being an independent entity from Sjögren's syndrome (SS), because of its clinical and serological features [1–3]. Yamamoto et al. [2, 3] stated that these features include persistent gland swelling in IgG4-DS, while gland swelling in SS is periodic. Moreover, salivary function is either normal or improved with the administration of glucocorticoid in IgG4-DS, while it decreases and is not affected by treatment in SS. A further difference is marked elevation of serum IgG4 level in IgG4-DS, while SS show a normal serum IgG4 level. As for anti-SS-A and/or anti-SS-B antibodies, they test negative in IgG4-DS, but have a high positive rate in SS patients. Histopathologically prominent infiltration involving IgG4-positive plasmacytes has been observed using immunostaining in IgG4-DS, while no IgG4-positive plasmacytes have been seen in SS. Additionally, no punctate or globular sialectasis have been observed on sialograms in IgG4-DS, while they are generally observed in SS. Consequently, they considered IgG4-DS to be an entity independent of SS, although it is now known that there are patients who have active disease but normal serum IgG4 levels [4].

Regarding imaging diagnosis of IgG4-DS, most of the studies have been case reports, and we could only find a few which analyzed the effectiveness of the imaging modality involving ≥9 cases as follows: two studies on ^{67}Ga scintigraphy; two on 2-[^{18}F]-fluoro-2-deoxy-D-glucose positron emission tomography/computed tomography (FDG-PET/CT); three on sonography; and two on computed tomography (CT) and magnetic resonance imaging (MRI). Ishii et al. analyzed 13 cases with IgG4-related disease (IgG4-RD) including IgG4-DS using ^{67}Ga scintigraphy, and showed that there was significant accumulation in the lacrimal glands in seven cases and in the salivary glands in seven cases [5]. In another study, they also found differences in accumulation patterns of ^{67}Ga between 27 cases of sarcoidosis and 16 cases of IgG4-RD [6]. Some authors have analyzed and verified the usefulness of FDG-PET/CT for staging and follow-up, and have demonstrated abnormal ^{18}F-FDG uptake in sites involved with IgG4-RD [7, 8]. Some reports have described the characteristic sonographic features of the involved glands, which might be efficient for screening IgG4-DS [9–11]. Sonography is also a useful imaging modality for the follow-up of this disease after corticosteroid therapy [9, 11]. In contrast, CT and MRI have shown enlarged glands with rather nonspecific findings regarding IgG4-DS [12, 13]; although in autoimmune pancreatitis (AIP), one of the IgG4-RD types shows very characteristic findings on both CT and MRI, which has therefore been adopted as one of the diagnostic criteria for AIP [14]. Because these studies have involved only one or two imaging modalities and comparison among modalities are not sufficient, it remains unclear as to which modality is the most efficient in detecting IgG4-DS; the differences in imaging features between IgG4-DS and SS also remain unclear.

The purposes of the present retrospective study were to analyze the imaging features of IgG4-DS using various imaging modalities, and to clarify which imaging modality is the most effective for screening this disease. We also showed the differences in imaging characteristics between IgG4-DS and SS.

Methods

Patients

We enrolled 39 patients with IgG4-DS (21 females and 18 males) with a mean age of 59.9 years, between 1999 and 2014. IgG4-DS was serologically and histopathologically confirmed using the following diagnostic criteria: persistent symmetrical swelling involving >2 lacrimal and major salivary glands; an elevated serum level of IgG4 (>135 mg/dl); and infiltration of IgG4-positive plasma cells (percentage of IgG4-positive cells to IgG-positive cells >40 %) on immunostaining [15]. We also analyzed 51 patients with SS (47 females and 4 males) with a mean age of 55.6 years, between 2006 and 2013. SS was diagnosed using both the revised Japanese criteria [16] and the American-European Consensus Group criteria [17]. Additionally, in 2013, 36 patients suffering from oral carcinoma, but with normal salivary glands (14 females and 22 males) with mean age of 63.3 years, were evaluated. This study design was approved by the Ethics Committee of Kyushu University, Japan, and written informed consent was obtained from all of the patients (IRB serial number: 25–287).

Image preparation

We performed sonography on 30 patients with IgG4-DS, 38 with SS and 36 with normal salivary glands. Sonographic images were taken using a diagnostic unit (Logiq 7: GE Healthcare, Tokyo, Japan) with a center frequency of 12 megahertz (MHz). We extracted B-mode longitudinal images of both the parotid glands (parallel to the retromandibular plane) and the submandibular glands (parallel to the submandibular plane) for this study. Because insufficient Doppler images could be provided for the normal submandibular glands, we only used B-mode images, although we also recorded in the Doppler mode at the time of examination for IgG4-DS and SS. FDG-PET/CT was undertaken in 20 patients with IgG4-DS, 19 with SS and 21 with normal salivary glands. From the superimposed images, where the standardized uptake value (SUV) was reflected as graded colors, we extracted images at the parotid and submandibular gland level, not including the

lacrimal gland level. Similarly, CT images at the parotid and submandibular gland level of 24, 38 and 32 patients with IgG4-DS, SS and normal salivary glands, respectively and MRI of 5, 16 and 19 patients with IgG4-DS, SS and normal salivary glands, respectively, were also extracted for this study. Regarding MRI, besides plain axial T1- and T2-weighted images, we also extracted gadolinium-enhanced T1-weighted images and coronal images of parotid and/or submandibular glands (if any were present). We did not include images of the lacrimal glands. The reasons for this were: 1) we could not undertake sonography of the lacrimal glands for safety reasons regarding the eyes; and 2) when salivary gland tumors were suspected in the patients, lacrimal glands were often not included in the scanning range on CT/MRI.

Image analysis

Six oral and maxillofacial radiologists (with 2–26 years of diagnostic experience) randomly reviewed the arranged image sets under blinded conditions. The observation order involved sets of: 1) sonographic images; 2) FDG-PET/CT images; 3) CT images; and 4) MRI images. Observations were performed twice with a 3-week interval. Each observer was required to score the confidence rate for the presence of the characteristic imaging findings using a 5-grade rating system (1–5) in the answer sheet as follows: when written characteristic imaging findings were definitely present, 5; when written characteristic imaging findings were definitely absent, 1; presence of a swollen gland, 5; and presence of an atrophied gland, 1. After scoring each finding, diagnosis was made as normal, IgG4-DS or SS, taking into consideration all of the findings for each case.

Figure 1 shows the characteristic findings for IgG4-DS using sonography. They were multiple hypoechoic areas and a reticular pattern on the parotid and submandibular glands, and a nodal pattern on the submandibular glands [9–11]. The characteristic findings for SS were multiple hypoechoic areas, hyperechoic lines and/or spots, and a reticular pattern (a mixture of hypoechoic areas and hyperechoic lines) on both the parotid and submandibular glands [18, 19]. Obscuration of the gland configuration was only assessed in the submandibular glands [19].

On FDG-PET/CT, abnormal ^{18}F-FDG accumulation in the parotid and submandibular glands was assessed as one of the characteristic findings of IgG4-DS [7, 8] (Fig. 2), when the gland exhibited bright warm colors; red corresponded to an SUV ≥6, orange corresponded to an SUV of around 5, and yellow to bright green corresponded to an SUV of around 4. Gland size was also assessed in the case of both the parotid and submandibular glands.

On CT and MRI (Fig. 2), there are nonspecific findings regarding IgG4-DS [12, 13]. From the images in the references [5, 12, 13] and empirically, we chose parotid and submandibular gland swelling and superficial enhancement of the parotid glands, and the presence of a septum-like structure on the submandibular glands as characteristic CT findings. In relation to characteristic MRI findings, we only chose gland swelling regarding IgG4-DS. In contrast, patients with SS exhibit the following characteristics: atrophic parotid and submandibular glands; heterogeneity of gland parenchyma, which is called a "salt-and-pepper appearance" [20, 21];

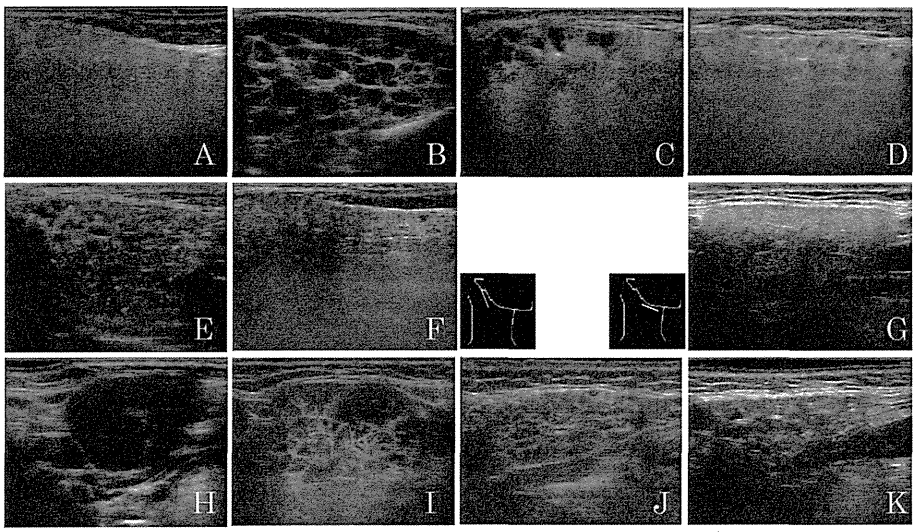

Fig. 1 Characteristic findings on sonograms. **a** Normal parotid gland. **b-d** Parotid glands of a patient with IgG4-DS. **e, f** Parotid gland of a patient with SS. **g** Normal submandibular gland. **h, i** Submandibular glands of a patient with IgG4-DS. **j, k** Submandibular glands of a patient with SS. Multiple hypoechoic areas (**b-f, i-k**), a reticular pattern (**b, e, i**), and a nodal pattern (**h**) can be seen. Hyperechoic lines and/or spots can also observed (**b-f, i-k**). *IgG4-DS* IgG4-related dacryoadenitis and sialadenitis, *SS* Sjögren's syndrome