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Increase of activated T-cells and up-regulation of Smad7 without elevation of TGF-beta expression in tonsils from patients with pustulosis palmaris et plantaris

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Title:

Increase of activated T-cells and up-regulation of Smad7 without elevation of TGF-beta expression in tonsils from patients with pustulosis palmaris et plantaris

Short title: T-cell activation of tonsillar lymphocytes from patients with PPP

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SUMMARY

Pustulosis palmaris et plantaris (PPP) is known to be skin disease related to tonsillitis, because the pustulosis often become exacerbated during acute tonsillitis and disappears after tonsillectomy. However, etiology of PPP remains unclear. In this study, we investigated the activation of tonsillar T-cell from PPP patients. Furthermore, we analyzed expressions of cytotoxic T-lymphocyte antigen-4 (CTLA4) that is a co-stimulatory molecule for inhibition of T-cell activation and of Smad7 that is a regulatory factor of TGF-beta intracellular signaling. For 47 Japanese patients with PPP who had tonsillectomy, the skin lesion was improved in 87 % of PPP patient at twelve month after tonsillectomy. In quantitative immunohistologic analysis, T-cell nodules on tonsillar tissues from PPP patients were more expanded than those from the patients with obstructive sleep apnea syndrome (OSAS) ($p=0.015$), and there was a positive correlation between the enlargement and clinical improvement ($r=0.422$, $p=0.021$). Flow cytometric analysis showed that the numbers of CD4+CD25+ and CD4+CD29+ cells in tonsils from PPP patients increased significantly compared to those from OSAS patients ($p=0.017$, $p=0.016$, respectively). Using reverse transcription-polymerase chain reaction (RT-PCR) and western blotting analyses with CD3+ tonsillar lymphocytes, we found that both expressions of Smad7 mRNA and protein were enhanced in PPP patients compared with OSAS patients ($p=0.03$, $p=0.02$, respectively), but expression of TGF-beta mRNA was not different between 2 groups. Although mRNA expression of CTLA4 was reduced in PPP patients compared with OSAS patients ($p=0.04$), the CTLA4 surface protein expression was not different between 2 groups. These data suggest that helper T-cells are frequently activated in tonsils from PPP patients, and this activation may be related to unresponsiveness of TGF-beta1 by overexpression of

Smad7. Such hyper-activation of T-cell may increase the risk of elicitation of self-reactive T-cell, being associated with pathogenesis of PPP.

INTRODUCTION

Pustulosis palmaris et plantaris (PPP) is the skin disease characterized by symmetrical erythematous and numerous non-bacterial small pustules mainly involving in the palms and soles. These skin lesions often get worse during acute tonsillitis [1, 2], and frequently improved after tonsillectomy [2-5]. Therefore, PPP is considered as one of the tonsil-related diseases. Although some reports supported that tonsillar autoimmune response was strongly correlated to pathogenesis of PPP, e.g. the serum anti-keratin antibodies and immune complexes were elevated in PPP patients, these parameters were decreased after tonsillectomy [4], and reconstitution of severe combined immunodeficiency (SCID) mice with tonsillar lymphocytes from PPP patients had PPP-like skin lesions [6], details of the pathogenesis remains unclear.

Tonsillar lymphocytes from PPP patients are known to be hypersensitive to indigenous bacteria of tonsil. In tonsillar lymphocytes from PPP patients, proliferation and immunoglobulin production increased in response to alpha-streptococcal antigens [7, 8]. The tonsillar lymphocytes produced anti-alpha-streptococcal antibodies at higher levels than those from obstructive sleep apnea syndrome (OSAS) patients not only in presence of alpha-streptococcal antigens but also spontaneously [7]. We previously showed that Tonsillar lymphocytes from PPP produced proinflammatory cytokine such as IFN- , TNF- , and IL-6 in response to *in vitro* alpha-streptococcal stimulus, but not in those from non-PPP patients [8]. These results suggest that a disorder of mucosal

immunological tolerance takes place in tonsil of PPP patients.

In peripheral blood of systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients, hyper activation of T-cells has been found [9, 10], and this activation was correlated with their clinical course [11, 12]. Moreover, activated T-cells reportedly take important part in pathogenesis of SLE by coordinating production of autoantibody with B-cell [13] and in RA by leading to chronic inflammation in synovial joint [14]. In a study about tonsillar T-cell status of PPP patients, Sakai, et al [15] reported that enlargement of T cell nodules was detected in the tonsils of PPP patients by immunohistology. Considering these results, tonsillar T-cells from PPP patients were supposed to be more activated and proliferated than that of non-PPP patients. However, the intimate examination about tonsillar T-cell status in PPP patients is not reported.

Although activation of T-cells is strictly regulated in normal immunity, the activated T-cells in lupus-prone mice were reported to avoid peripheral immunological tolerance [16]. One factor maintaining the tolerance through control of T-cells activation includes cytotoxic T-lymphocyte antigen-4 (CTLA4, CD152), which is a co-stimulatory molecule on T-cells. CTLA4 coordinates T-cell activation together with CD28 by binding to B7 on the antigen-presenting cells. CD28-B7 interaction enhances activation of T-cells, while CTLA4-B7 interaction diminish the activation [17, 18]. Therefore, CTLA4 plays an essential role in the prevention of uncontrolled T-cell activation, and it is not so difficult to imagine that some disorders of CTLA4 can lead to autoimmune disease. In fact, CTLA4 deficient mice are known to develop autoimmune diseases [19]. In T-cells of SLE patients, surface expression of CTLA4 is normal but its functional abnormality has been reported [20].

As another candidate, we focused on TGF-beta1 and Smad proteins. TGF-beta1 is well known as one of the regulators of oral tolerance [21]. The intercellular proteins belonging to Smad family are responsible for TGF-beta signals from the receptor to the nucleus. To date, nine different Smad proteins (Smad1-9) have been identified, and Smad7 acts as inhibitor of Smad pathway by occupying intracellular domain of activated TGF-beta receptor [22, 23]. In addition, recent study supported that TGF-beta had also important part in the prevention of hyper immune response to indigenous bacteria [24]. On the other hand, in intestinal mucosal T-cells of inflammatory bowel disease (IBD), which are also reported to hypersensitivity to indigenous bacteria as well as PPP, overexpression of Smad7 without alternation of TGF-beta expression has been reported [25-28]. Because TGF-beta is an inhibitory cytokine against T-cells activation [29], upregulation of Smad7 may leads to unresponsiveness of TGF-beta1, resulting in uncontrolled activation of T-cells and disorder of mucosal immunological tolerance.

In these studies, we reassessed areas of B-cell and T-cell nodules on tonsillar sections from PPP patients by quantitative immunohistochemical analysis, studied correlation between these values and rate of clinical improvement for the skin lesions of PPP patients, and investigated the characteristics of tonsillar T-cell subset from PPP patients by flow cytometry. Finally, to seek the factors inducing hyper-activation of tonsillar T-cells in PPP patients, we examined the CTLA4, TGF-beta1 and Smads expressions at mRNA and protein level in tonsillar T-cells from PPP patients.

MATERIALS AND METHODS

Patients and samples

All patients were treated at Asahikawa Medical College. PPP was diagnosed by dermatologists in our hospital on the basis of the findings characterized by symmetrical, erythematous, scaly plaques with numerous, sterile, pinpoint pustules restricted to the palms and soles. Our studies were based on comparison between the PPP patients and OSAS patients who underwent tonsilectomy. Patients with OSAS underwent uvulo-palate-pharyngoplasty together with tonsillectomy. Because the OSAS patients which we examined in these studies did not have other diseases including tonsil and skin disease, we used OSAS group as normal control. All patients signed informed consent for therapy and tissue studies. These studies were approved by the Institutional Review Board.

Clinical study

Forty-seven Japanese patients whose conditions were diagnosed as PPP and had tonsillectomy between 1990 and 2002 were available for this study. The patient age ranged from 21 to 74 years with 49 years of mean age. There were 14 male and 33 female. Clinical information and follow–up data were obtained from hospital charts for all patients with PPP. The degree of improvement was classified on a scale of one to ten by self-evaluation of each PPP patients. Zero was defined as no change of skin lesions before and after tonsillectomy, and ten was defined as disappearance of skin lesions after tonsillectomy. The skin condition thought to be over five scale was regarded as an improvement. The patients evaluated their skin conditions at the time of one, six, and

twenty month after tonsillectomy.

Quantitative immunohistologic study

Measurement of T and B-cell nodules was done by partially modified Sakai's method [6]. Briefly, serial sections from paraffin embedded tonsils were stained by anti-CD20 mouse monoclonal antibody, anti-CD3 mouse monoclonal antibody (DAKO, Glostrup, Denmark) or hematoxylin-eosin (HE). Central 5.5x4mm part of the sections stained by CD20 antibody was photographed by photomicrographic digitalcamera (DP-10; Olympus). On the computer, this part was plotted into B-cell nodules and T-cell nodules based on the staining patterns of three sections, and area of T-cell, B-cell nodules and whole tonsillar tissues were measured by NIH image software. Percentages of T-cell, and B-cell nodules to whole tonsillar tissues were calculated. These percentages of tonsil areas from PPP patients were compared to those of OSAS patients, and the relationship between these percentages and the degree of improvement was studied.

Flow cytometric study

Tonsils were manually cut into small pieces in phosphate buffered saline (PBS) with penicillin (100IU/ml) and streptomycin (100IU/ml), and cells were passed through a cell strainer (70u). Tonsillar mononuclear cells were purified following centrifugation on a Ficoll-Conray gradient (d=1.078) at 1400 g for 20 min. The cells were washed 5 times with sterile PBS. 10^6 tonsillar lymphocytes were incubated with each undiluted 10ul antibody, and analyzed on EPICS ELITE (Coulter Electronics Inc. Hialeah, FL, USA). The antibodies were anti human CD3-fluorescein isothiocyanate

(FITC), CD4-phycoerythrin (PE), CD25-FITC, CD29-FITC, CD45RA-FITC, CTLA4 (CD152)-PE (Pharmingen, San Jose, CA, USA), CD20-PE, IgM-FITC, IgA-FITC, and IgG-FITC (DAKO, Glostrup, Denmark). Isotype mouse IgG2 and rat IgM were used as a negative control.

Reverse transcription-polymerase chain reaction (RT-PCR) study

Tonsillar mononuclear cells were incubated with anti human CD3 antibody conjugated to magnetic beads (IgG1; Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min on ice. After washing, the CD3+ cells were separated by MACS columns (LS+; Miltenyi Biotec, Bergisch Gladbach, Germany). Total RNA was extracted from $5x10^6$ CD3+ tonsillar lymphocyte by Acid Guanidinium Phenol Chloroform (AGPC) method with Sepasol-RNA1 (NACALAI TESQUE, Tokyo, Japan) The first-strand cDNA was synthesized by MMLV reverse transcriptase (GenHunter Corporation, Nashville, USA), and subsequent PCR was performed. The primer sequences of TGF-beta1, IL-10, CTLA4, Smad3, 4, 7, and beta-actin are shown in Table 1. Reaction mixtures were subjected to 28 cycles (TGF-beta1, IL-10, and beta-actin) or 32 cycles (CTLA4 and Smad3, 4, and 7) of denaturation (94^0C, 1 min), annealing (50^0C, 1 min), and extension (72^0C, 1 min). Ten microliters of PCR products were electrophoresed in 1% agarose gel and visualized under ultraviolet light. The intensities of amplified DNA bands were measured using NIH image software, and the standardized amount of the gene transcripts was calculated by dividing the representation of each transcript by that of beta-actin gene.

Western blotting analysis

Western blotting analysis for Smad3 and Smad7 was performed on aliquots of the CD3+ cells tested by RT-PCR analysis. CD3+ cells were sonicated and the supernatant fractions were collected after centrifugation. Each protein sample was adjusted to 2 mg of protein per milliliter in SDS-PAGE sample buffer (60% glycerol; 50 mM Tris-HCl, pH 6.5; 2% SDS; 0.1% BPB and 0.02% 2-ME), and was denatured by boiling for 3 min. Ten micrograms of protein per sample were electrophoresed in 4-12% bis-tris gels (NuPAGE, Invitrogen Corp., Carlsbad, CA, USA) and transferred to PVDF membrane at 60 V for 1 hr. After blocking with 5% milk in PBS, membranes were incubated with rabbit polyclonal anti-human Smad3 and 7 (Santa Cruz Biotechnology, Inc. Santa Cruz, California, U.S.A. 1:2,500 dilution) overnight at room temperature. The blots were then incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit Ig antibody (Amersham, Arlington Heights, IL, 1:2,500 dilution). Immnoreactivity was visualized by chemilluminescent method using an ECL kit (Amersham, Arlington Heights, IL) according to the manufacturer's protocol, and protein bands were semi-quantified by NIH image software. The standardized amount of Smad protein expression was calculated by dividing the amount of protein by that of beta-actin.

Statistics

Two group comparisons were tested using nonparametric test procedures (Mann-Whitney U test and Spearman rank correlation coefficient).

RESULTS

Clinical finding after tonsillectomy

In 47 patients of PPP, average values of the degree of improvement at the time of one, six, and twenty month after tonsillectomy were 5.11, 6.81, and 8.09, respectively. The percentage of patients with improved skin lesion increased in a time dependent manner. Of 47 patients with PPP, 24 (51%), 38 (81%), and 41 (87%) showed the improvement of the skin at the time of 1, 6, and 12 months after tonsillectomy, respectively.

Determination of T-cell and B-cell nodules areas

Quantitative immunohistologic analysis was performed in tonsillar sections from 31 patients with PPP (12 males, 19 females; mean age 47 years, range 20-72 years) and from13 patients OSAS (8 males, 5 females; mean age 42 years, range 26-69 years). There was no difference of age distribution between PPP and OSAS groups ($p=0.46$).

In 31 tonsillar tissues of patients with PPP, T-cell nodule area ranged from 10% to 44.1% with a median of 29.5% and B-cell nodule area was ranged from 24.3% to 78.7% with a median of 47.4%. In 13 tonsillar tissues of patients with OSAS, T-cell nodule area was distributed from 12.1% to 45.4% with a median of 21.1% and B-cell nodule area was distributed from 36.0% to 65.4% with a median of 59.1%. T-cell area of tonsils from PPP patients was significantly larger than that from OSAS patients ($p=0.015$; Fig. 1a). On the other hand, B-cell nodule area from PPP patients was significantly smaller than that from OSAS patients ($p=0.021$; Fig. 1a). There was no correlation between B-cell nodule area and degree of skin improvement, but significant positive correlation between T-cell nodule area and degree of skin improvement at 6

months after tonsillectomy (r=0.422, p=0.021; Fig. 1b).

Surface marker expression on tonsillar lymphocytes

Flow cytometric analysis was performed in tonsillar mononuclear cells from tonsils of 20 patients with PPP (8 males, 12 females; mean age 42.5 years, range 23-62 years) and 15 patients with OSAS (9 males, 6 females; mean age 37 years, range 26-69 years). There was no difference of age distribution between PPP and OSAS groups (p=0.91).

The results are summarized in Table 2. In tonsillar lymphocytes from PPP patients, the percentages of CD3+, CD4+, CD4+CD25+, and CD4+CD29+ cells per total cell populations were significantly higher than those from OSAS patients (p=0.0037, p=0.0089, p=0.017, and p=0.016, respectively; Table 2) In addition, the percentage of CD4+CD25+ and CD4+CD29+ cells per CD4 positive cells also increased in PPP tonsils (p=0.049 and p=0.026, respectively; Table2) On the other hand, the percentages of surface IgA+ cells per CD20+ cells were significantly lower in PPP tonsils than in OSAS tonsils (p=0.046; Table2) There was no significant difference in CD4+ CD45RA+, CD20+, CTLA4+, surface IgM+ cell, and surface IgM+ cells, between tonsillar lymphocytes from PPP and those from OSAS patients (Table 2).

Expressions of T-cell activation regulators

RT-PCR and western blot analyses were performed in tonsillar CD3+ cells from 5 patients with PPP (3 males, 2 females; mean age 41 years, range 38-46 years) and from 5 patients with OSAS (3 males, 2 females; mean age 41 years, range 30-49 years). There was no difference of age distribution between PPP and OSAS groups

(p=0.92).

The expressions of TGF-beta, IL-10, Smad3, and Smad4 mRNA in CD3+ tonsillar lymphocytes from PPP patients were at same level as those in OSAS patients. Smad7 mRNA was strongly expressed in CD3+ tonsillar lymphocytes from PPP patients compared with those from OSAS patients (Fig. 2a). On the other hand, expression of CTLA4 mRNA was lower in PPP group than that in OSAS group. (Fig. 2a) Semiquantitative analysis with NIH image software also revealed that Smad7 mRNA expression was significantly higher and CTLA4 mRNA expression was significantly lower in CD3+ tonsillar lymphocytes from PPP patients than those from OSAS patients (p=0.03 and p=0.04, respectively; Fig. 2b).

In western blot analysis, the expression of Smad7 protein in CD3+ tonsillar lymphocytes from PPP patients was higher than those from OSAS patients (Fig. 3a). Semiquantitative analysis also confirmed that expression of Smad7 protein was significantly higher in CD3+ tonsillar lymphocytes from PPP patients than that from OSAS patients (p=0.02, Fig. 3b).

DISCUSSION

As described in introduction, Pustulosis palmaris et plantaris (PPP) is known to be associated with focal infection of the tonsils. Kataura et al [2] reported that in 88% of 289 PPP patients who underwent tonsillectomy, the skin lesion improved. We also detected that in 41 (89%) out of 47 patients with PPP, the lesions improved during one year after tonsillectomy. These results led us to believe the strong relationship between tonsillar immunity and pathogenesis of PPP.

Using manual quantification on tonsillar tissue sections stained with CD20

antibody, Sakai, et al [15] reported that enlargement of T-cell nodules and reduction of B-cell nodules were detected in the tonsils of PPP patients compared to that of patients with habitual tonsillitis. We found similar results by computer-assisted automatic quantification using normal tonsils obtained from OSAS patients. Moreover, the degree of improvement was positively correlated with enlargement of T-cell nodules. Considering these results, the T-cell nodule expansion may reflect specific autoimmune response involved in the pathogenesis of PPP.

Flow cytometric analysis revealed that the number of CD3+ cells increased in tonsillar lymphocytes from PPP patients. Moreover, the percentage of CD4+CD25+ and CD4+CD29+ cells increased in PPP tonsils as well. Although CD25 is well known as activation marker of T-cells [30], CD4+ CD25+ cells have been reported to have the function to suppress other T cells [31]. This suppressor T-cell population was reported to express CTLA4 [32, 33], but we did not find difference in surface CTLA4 expression between PPP and OSAS group. In addition, recent studies suggested that the subpopulation with strong expression of CD25 in CD4+CD25+ population took important part in the regulation of other T-cells [34]. Therefore, we speculated that the CD4+CD25+ populations in the present study include this regulatory population, but do not measure exactly their numbers. In addition, CD4+ CD29+ cells, also known as helper inducer T-cell subset, were thought to be one activation marker for chronic infection because of their frequent infiltration in chronic infection sites [11, 35]. Moreover, it was reported that tonsillar lymphocytes from PPP patients incorporated more 3H-thymidine than those from non-PPP patients even without any stimulation [36]. From these observations, T-cells in tonsils of PPP patients are thought to be activated

more frequently than those of OSAS patients. Similarly, the enrichment of these two populations has been also reported in synovial fluid from rheumatoid arthritis patients and in peripheral blood of SLE patients [10, 11, 37].

To investigate the factors that induce hyper-activation of T-cells in tonsils of PPP patients, we first studied CTLA4. Quantitative or functional reduction of CTLA4 has a possibility to induce uncontrolled T-cell activation, resulting in autoimmune disease [19]. In the present study, we found very few CTLA4+ cells in both PPP and OSAS groups, and no difference of CTLA4 expression between PPP and OSAS group. This result coincides with Hirashima's report about cell surface CTLA4 expression in SLE patients [20]. In addition, they also reported insufficient inhibitory effect of CTLA4 which may be caused by CTLA4 gene polymorphism in SLE patients [20]. There are many studies about CTLA4 gene polymorphism in several autoimmune diseases including SLE [38], and RA [39]. Interestingly, Ligers et al [40] noted that a polymorphism in the promoter region of CTLA4 gene altered CTLA4 mRNA expression but not cell surface expression. Our results showing decreased CTLA4 mRNA expression without alternation of cell surface CTLA4 expression in PPP group may be due to existence of CTLA4 gene polymorphism in PPP patients, causing functional abnormality of CTLA4. Further genetic and functional studies are needed to clarify the role of the polymorphism in pathogenesis of PPP.

Secondly, we examined Smad, especially Smad7 protein, which is a major inhibitory factor of TGF-beta signaling [22, 23]. TGF-beta1 is reported to have important part in mucosal immunological tolerance by controlling activation of T-cells [21]. In our studies, we detected up-regulation of both Smad7 mRNA and protein

without elevation of TGF-beta mRNA expression in tonsillar T-cells from PPP patients. These results are compatible with the reports about intestinal mucosal lymphocyte of inflammatory bowel disease patients [25-27]. We conjecture, on the basis of these findings, that overexpression of Smad7 enables T-cells to activate through blockage of TGF-beta intracellular signaling in tonsillar T-lymphocyte of PPP patients. In B-cell differentiation, TGF-beta1 is reported to induce IgA isotype switching through the Smad pathway in murine B lymphocytes [41]. In the present study, IgA positive cells were significantly diminished in tonsillar lymphocytes of PPP patients. Therefore, in B-cells, similarly to T-cells, unresponsiveness of TGF-beta1 by overexpression of Smad7 may take place. Although the regulation mechanisms of Smad7 are not fully known, recent studies have described that not only TGF-beta itself but also inflammatory cytokines such as TNF-alpha and IFN-gamma enhanced Smad7 expression [42-44]. Because expression of TGF-beta mRNA in PPP group was at same level as that in OSAS group in our study, we presume that negative feedback of TGF-beta does not strongly affects on the overexpression of Smad7. On the other hand, Murakata et al [8] reported that the production of TNF-alpha and IFN-gamma were more increased in the tonsillar lymphocytes stimulated with alpha-streptococci from PPP patients than those from OSAS patients. Therefore, TNF-alpha and IFN-gamma may take part in induction of Smad7 overexpression. Further studies about regulation mechanism of Smad7 including the inflammatory cytokines remain to be solved.

Cross-antigenicity between epithelium of tonsillar crypt and skin of the palm and sole through common antigen including keratine was reported in PPP patients [4]. Previous study about human skin/SCID mouse chimera indicated that T-cells originated from tonsillar lymphocytes migrate to the skin of the palm and sole in PPP patients [6,

45]. Additionally, restricted usage of T-cell receptor V beta subsets was found in peripheral blood mononuclear cells and in tonsillar tissue from PPP patients [46]. Recently, we analyzed expression of cutaneous lymphocyte associated antigen (CLA), which is a specific homing receptor that facilitates T-cell migration into skin, in tonsillar T-cell from PPP. We found that CLA expression of tonsillar T-cells in PPP patients significantly increased compared to that in non-PPP patients [47]. These results suggest that auto-reactive T-cells may take central part in pathogenesis of PPP, as well as of RA [14]. Although a few auto-reactive T-cells exist in lymphatic tissue, this activation is strictly controlled by several regulatory mechanisms [48]. Therefore, disorders of these mechanisms take important part in development of autoimmune diseases [48]. In the present study, we examined two regulators of T-cell activation, which are reportedly related to self-tolerance of T-cells. Our results suggest that Smad7 overexpression may be related to pathogenesis of PPP through uncontrolled T-cell activation.

In summary, we found enlargement of T-cell nodules in tonsillar tissues of PPP patients and a positive correlation between this enlargement and clinical improvement. In tonsillar lymphocytes of PPP patients, enrichment of CD4+ cells, especially activated CD4+ cells were shown. In tonsillar T-cells of PPP patients, overexpression of Smad7 mRNA and protein and downregulation of CTLA4 mRNA were found. These results suggested that tonsillar T-cells of PPP patients were hyper-activated, and overexpression Smad7 was involved in this activation.

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FIGURE REGEND

Figure 1

Quantitative immunohistologic analysis of B- and T-cell nodules. Tonsillar sections stained by anti-CD20 or anti-CD3 mouse monoclonal antibodies and the percentages of T-cell, and B-cell nodules to whole tonsillar tissues were measured by using NIH image software. a) The T-cell area of tonsils from pustulosis palmaris et plantaris (PPP) patients was significantly larger than that from obstructive sleep apnea syndrome (OSAS) patients ($p=0.015$). On the other hand, B-cell nodule area from PPP patients was significantly smaller than that from OSAS patients ($p=0.021$). b) There was a positive correlation between T-cell nodule area and degree of improvement at six month after tonsillectomy ($r=0.422$, $p=0.021$).

Figure 2

Reverse transcription-polymerase chain reaction (RT-PCR) profiles of TGF-beta, IL-10, CTLA4 and Smad3, 4, and 7 in tonsillar CD3+ lymphocytes from pustulosis palmaris et

plantaris (PPP) patients and obstructive sleep apnea syndrome (OSAS) patients. a) The PCR-electrophoretogram of each parameter was shown. b) Smad7 and CTLA4 mRNA expression in PPP patients were semiquantitatively compared with those in OSAS patients. The relative amount of the gene transcripts is expressed as the ratio of each gene to beta-actin gene. The median values are displayed as short bar (-). Mann-Whitney U test was used to determine p-value. The expression of Smad7 mRNA from PPP patients was expressed at higher level than those from OSAS patients ($p=0.03$), and expression of CTLA4 mRNA from PPP patients was at lower level than those from OSAS patients ($p=0.04$).

Figure 3

Western blot profiles of Smad7 and 3 in tonsillar CD3+ lymphocytes from pustulosis palmaris et plantaris (PPP) patients and obstructive sleep apnea syndrome (OSAS) patients. a) The Western blot-electrophoretogram of each parameter was shown. b) The expression of Smad7 protein in PPP patients was semiquantitatively compared with that in OSAS patients. The relative amount of the protein is expressed as the ratio of each protein to beta-actin protein. The median values are displayed as short bar (-). Mann-Whitney U test was used to determine p-value. The Smad7 protein from PPP patients was expressed at higher level than those from OSAS patients ($p=0.02$).

Figure 1

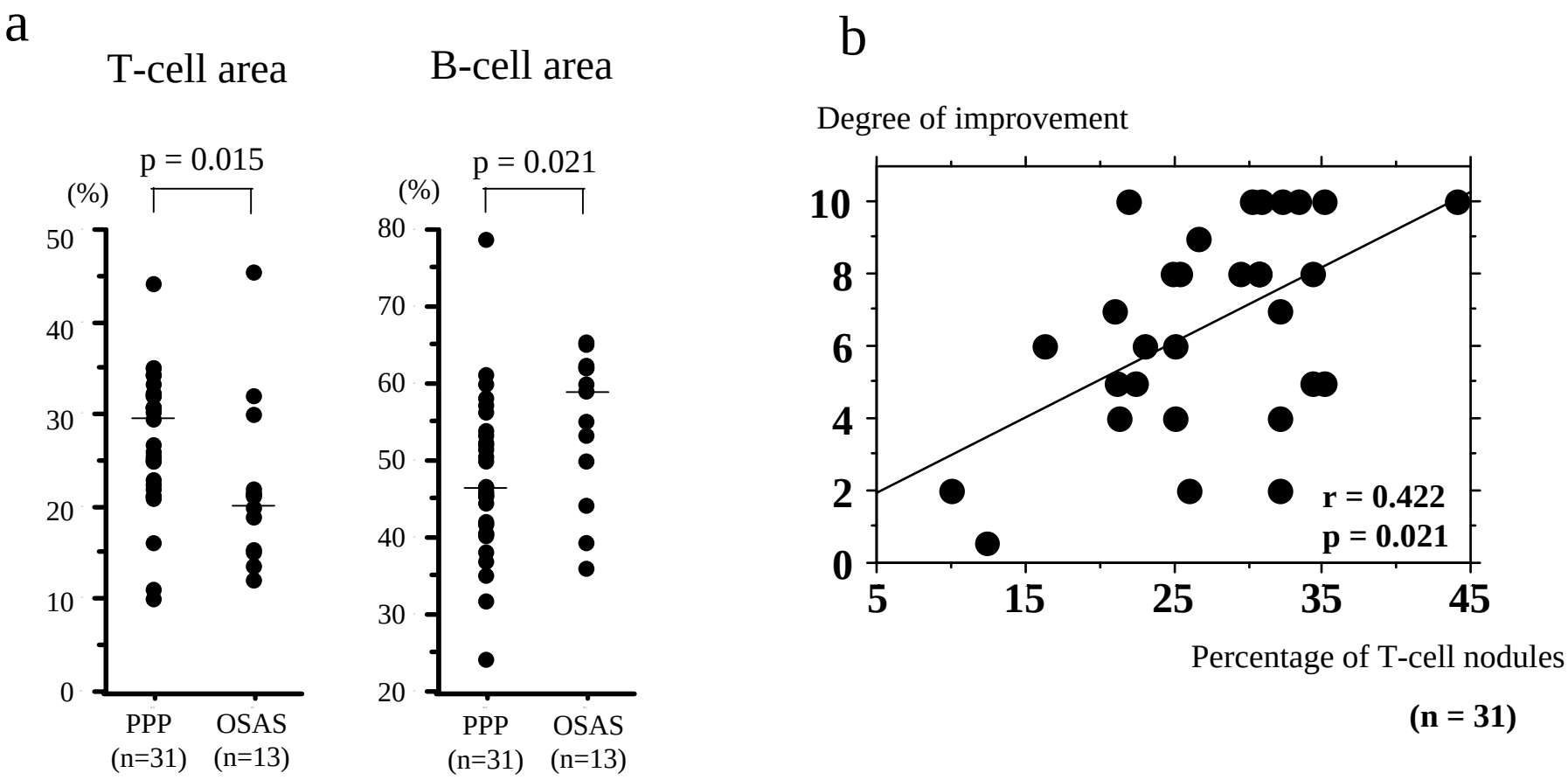
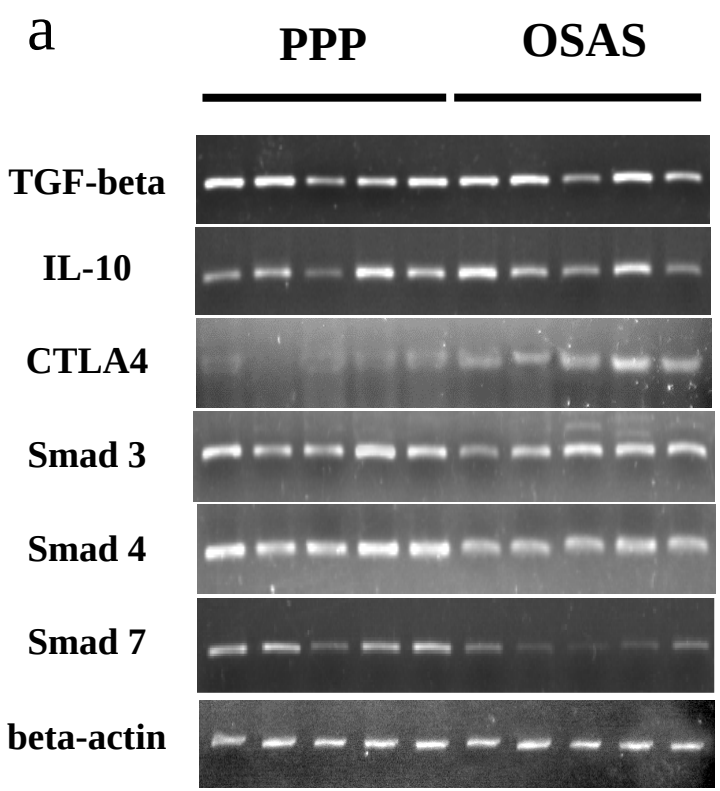

a
T-cell area
B-cell area
p = 0.015
p = 0.021
(%)
(%)
50
40
30
20
10
0
80
70
60
50
40
30
20
PPP
(n=31)
OSAS
(n=13)
PPP
(n=31)
OSAS
(n=13)
b
Degree of improvement
10
8
6
4
2
0
5
15
25
35
45
r = 0.422
p = 0.021
Percentage of T-cell nodules
(n = 31)

Figure 2

a


PPP
OSAS
TGF-beta
IL-10
CTLA4
Smad 3
Smad 4
Smad 7
beta-actin

b

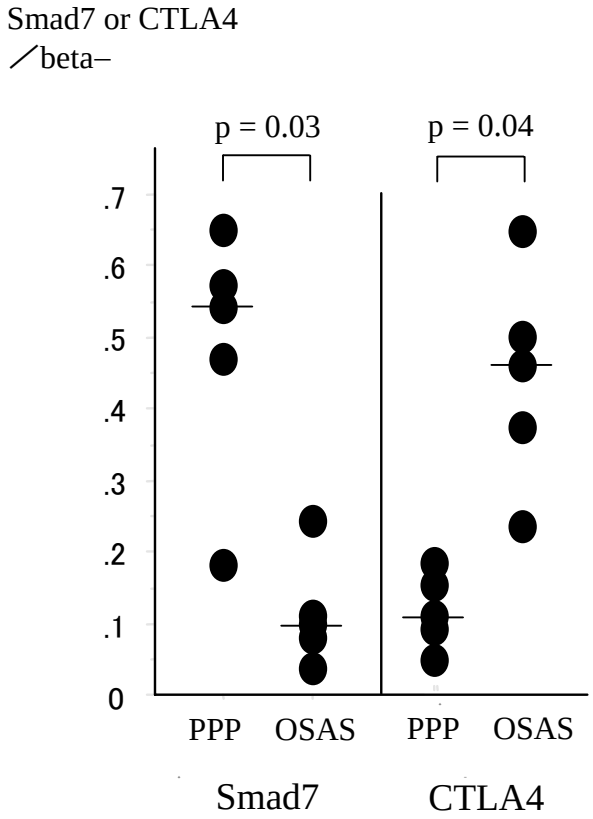
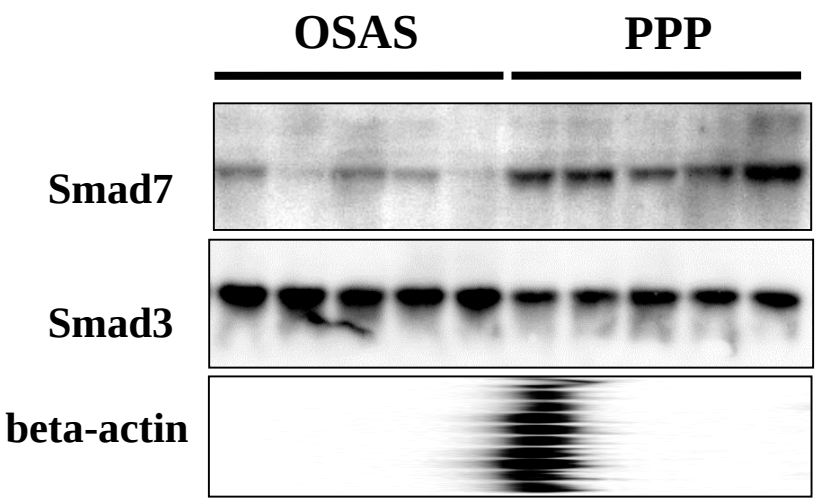
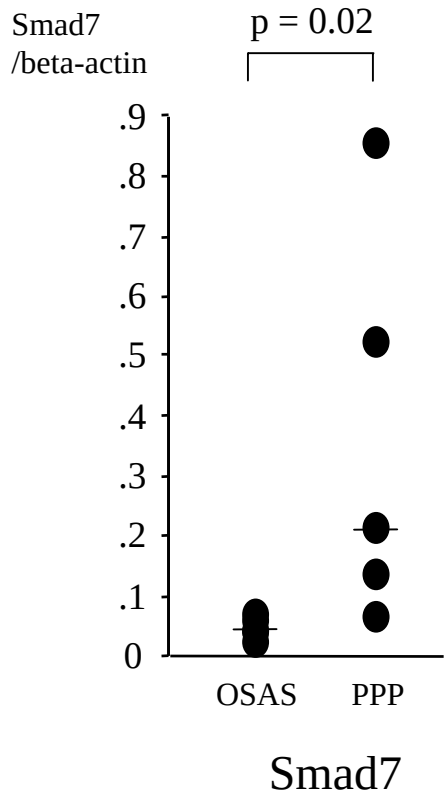

Smad7 or CTLA4
／beta−
p = 0.03
p = 0.04
.7
.6
.5
.4
.3
.2
.1
0
PPP
OSAS
PPP
OSAS
Smad7
CTLA4

Figure 3

a



b



Smad7

Table 1.

Sequences of primers with expected size of RT-PCR

primer	sequence	size of PCR product
TGF-beta1	5'-ACCAACTATTGCTTCAGCTC-3' 5'-TTATGCTGGTTGTACAGGG-3'	197bp
IL-10	5'-ATGCTTCGAGATCTCCGAGA-3' 5'-AAATCGATGACAGCGCCGTA-3'	269bp
CTLA4	5'-ATGGCTTGCCTTGGATTTCAG-3' 5'-TTCTGGATCAATTACATAAATCTGG-3'	464bp
Smad3	5'-CAGAACGTCAACACCAAGT-3' 5'-ATGGAATGGCTGTAGTCGT-3'	308bp
Smad4	5'-CCAGGATCAGTAGGTGGAAT-3' 5'-GTCTAAAGGTTGTGGGTCTG-3'	243bp
Smad7	5'-GCCCTCTCTGGATATCTTC-3' 5'-GCTGCATAAACTCGTGGTCA-3'	320bp
beta-actine	5'-CAAGAGATGGCCACGGCTGCT-3' 5'-TCAGGAGGAGCAATGATCTTGA-3'	330bp

Table2.

Flow cytometric analysis of tonsillar mononuclear cells

	PPP (n=20)	OSAS (n=15)	*p* -value
CD3+ / total cells	49.05 (42.5-55.1)	39.30 (34.4-43.4)	0.0037
CD4+ / total cells	41.65 (36.5-46.9)	33.40 (25.3-37.4)	0.0089
CD4+CD25+ / total cells	4.75 (2.7-10.3)	2.30 (1.1-4.0)	0.017
CD4+CD25+ / CD4+ cells	11.70 (7.5-22.3)	6.80 (4.3-12.6)	0.049
CD4+CD29+ / total cells	11.10 (8.8-16.7)	6.50 (5.9-8.8)	0.016
CD4+CD29+ / CD4+ cells	26.80 (23.7-37.5)	22.1 (17.9-24.2)	0.026
CD4+CD45RA+ / total cells	10.35 (8.5-13.4)	8.90 (7.1-10.9)	NS
CD4+CD45RA+ / CD4+ cells	26.90 (21.6-31.5)	27.40 (21.8-37.8)	NS
CD3+CTLA4+ / total cells	1.07 (0.7-1.2)	1.12 (0.6-1.4)	NS
CD20+ / total cells	49.60 (48.3-60.2)	61.40 (54.3-66.3)	NS
IgM+ / CD20+ cells	53.30 (49.5-62.0)	62.10 (49.5-69.4)	NS
IgA+ / CD20+ cells	12.50 (11.7-14.7)	16.00 (12.8-27.7)	0.046
IgG+ / CD20+ cells	31.20 (23.3-38.4)	24.70(19.2-32.0)	NS

The figures described above show median (25th-75th percentiles)

Mann-Whitney U test was used to determine p-value

ns: not significant