

Figure 1. Microscopic findings of the primary tumor. HE and immunostaining with mAbs against CD68, HLA class I, and HLA-A2 are shown. Original magnification is $\times 100$ for HE and $\times 40$ for immunostaining sections. Small squares indicate $\times 100$ magnification fields.

HLA-*A0206 cDNA was transfected into MFH2004 cells using LipofectAMIN2000 (Invitrogen Corp., Carlsbad, CA). After selection in IMDM containing puromycin (2 $\mu\text{g}/\text{mL}$), the transfectant was obtained and designated MFH2004-A2.

Flow Cytometry

MFH2004, MFH2004-A2, and B2004-EBV were incubated with an anti-HLA-A2 monoclonal antibody (mAb; BB7.2), anti-HLA-A24 (C7709A2.6), and anti-HLA-class I (W6/32), respectively, for 30 min on ice. Then the cells

were incubated with FITC-labeled second antibody and analyzed by flow cytometry as described elsewhere.^{17,19} Hybridomas for C7709A2.6 were donated by D. G. Coulie (Christian de Duve Institute of Cellular Pathology, University of Louvain, Brussels) and those for BB7.2 and W6/32 were purchased from the American Type Culture Collection (Manassas, VA).

In experiments to evaluate the effects of interferon ($\text{IFN-}\gamma$), MFH2004 cells were cultured with 100 U/mL $\text{IFN-}\gamma$ (a gift from Shionogi & Co., Ltd., Osaka, Japan) for 48 h at 37°C prior to incubation with the primary antibodies.

Development of Cytotoxic T-Lymphocyte Clones

CTLs against autologous MFH cells were induced by the method described before.¹⁷ Briefly, CD8+ T cells (5×10^6) collected from peripheral blood of the patient were incubated with 5×10^6 cells of 100 Gy-irradiated MFH2004 cells and irradiated MFH2004-A2 cells in AIM-V medium (GIBCO), respectively. The following day 100 U/mL recombinant human interleukin-2 (rhIL-2; a gift from Takeda Industries, Ltd., Osaka, Japan) was added. Stimulation of the T cells was repeated at intervals of 7 to 10 days using irradiated MFH cells and a reduced concentration (50 U/mL) of rhIL-2. After the fifth stimulation, cytotoxicity of CTLs was assessed by the standard ⁵¹Cr release assay and cytokine release assay as described below.

Cytokine and ⁵¹Cr Release Assays

Immune responses of CD8+ T cells and CTL clones against target cells were evaluated by IFN- γ release assay¹⁷ and the standard 6-h ⁵¹Cr release assay^{17,19-21} as previously described. MFH2004, MFH2004-A2, B2004-EBV, and K562 cells were used as targets. In blocking experiments, anti-HLA-A2 (BB7.2), anti-HLA-A24 (C7709A2.6), anti-HLA-B&C (B1.23.2), anti-HLA-class I (W6/32), and anti-HLA-class II (L243) mAbs were used. Hybridomas for B1.23.2 and L243 were purchased from the American Type Culture Collection.

RESULTS

Establishment of MFH and Peripheral Blood B-Cell Lines

To investigate the immune response against autologous tumor cells, we first established two cell lines from the primary MFH tumor and peripheral blood B cells as the target. A cell culture obtained from the primary tumor was maintained for over 1 year and designated MFH2004. Peripheral blood B cells were immortalized by infection with Epstein-Barr virus (B2004-EBV). Subcutaneous inoculation of MFH2004 cells into NOD-SCID mice produced tumors with histological and immunophenotypic features similar to the primary tumor. The xenotransplanted tumors were stained with the anti-human HLA class I mAb, proving their human origin. The tumors also showed positive staining for vimentin and CD68, but were negative for desmin, cytokeratin, S-100, HHF35, caldesmon, EMA, and CD34.

Haplotype Loss of HLA Class I Molecules in MFH2004 Cells

We then determined the HLA genotypes of MFH2004 and B2004-EBV cells. The HLA class I genotype of B2004-EBV cells was A*0206/2402, B*4006/4601, and C*0102/0801. In contrast,

MFH2004 cells possessed only HLA-A*2402, B*4601, and C*0102. Consistent with these profiles, flow cytometric analysis (Fig. 2) showed positive cell surface expression HLA-A2 and HLA-A24 in B2004-EBV cells, whereas HLA-A2 expression was not detectable in MFH-2004 cells. Expression of HLA-A2 on MFH2004 cells was not restored by IFN- γ treatment despite there being increased expression of HLA class I and HLA-A24 with this treatment. We also found in the primary tumor sections that tumor cells were stained positively with the anti-HLA-class-I mAb but not with the anti-HLA-A2 mAb (Fig. 1), even though the anti-HLA-A2 mAb stained normal interstitial tissue in the same section. These findings indicated haplotype loss of HLA class A*0206, B*4006, and C*0801 in MFH-2004 cells.

Induction and Development of Cytotoxic T-Lymphocyte Clones

Subsequently, we attempted to induce autologous CTLs from peripheral T cells against MFH2004 cells. Neither of two attempts with T-cell stimulation using MFH2004 cells succeeded in inducing CTLs. We reasoned that these failures might have been caused by haplotype loss of HLA class I molecules in MFH2004 cells. Therefore we cloned HLA-A*0206 cDNA from B2004-EBV cells and transfected it into MFH2004 cells. The resultant transfectant, designated MFH2004-A2, showed expression of HLA-A2 on the surface along with HLA-24 in flow cytometric analysis (Fig. 2).

Using MFH2004-A2, we again attempted CTL induction. This time, both of two attempts successfully induced CTLs against MFH2004-A2 cells. Using limiting dilution procedures, we obtained two CTL clones, CTL2004-c6 and CTL2004-c17. As representatively shown in Figure 3A, cytokine release assays revealed a response of CTL2004-c17 against MFH2004-A2, but not MFH2004 or B2004-EBV cells. In blocking experiments, the anti-HLA class I mAb and anti-HLA-A2 mAb inhibited cytokine release of CTL2004-c17 against MFH2004-A2 cells (Fig. 3B). In contrast, anti-HLA-A24, anti-HLA B&C, and anti-HLA class II mAbs did not show such marked inhibitory effects, indicating that the T-cell response was restricted by HLA-A2. ⁵¹Cr release assay also showed specific cytotoxicity of CTL2004-c17 against MFH2004-A2 cells (Fig. 4). CTL2004-c6 exhibited similar cytotoxic activities with CTL2004-c17 (data not shown). These results indicated that CTL2004-c6 and CTL2004-c17 recognized antigens on MFH2004-A2 presented by the retrieved HLA-A2.

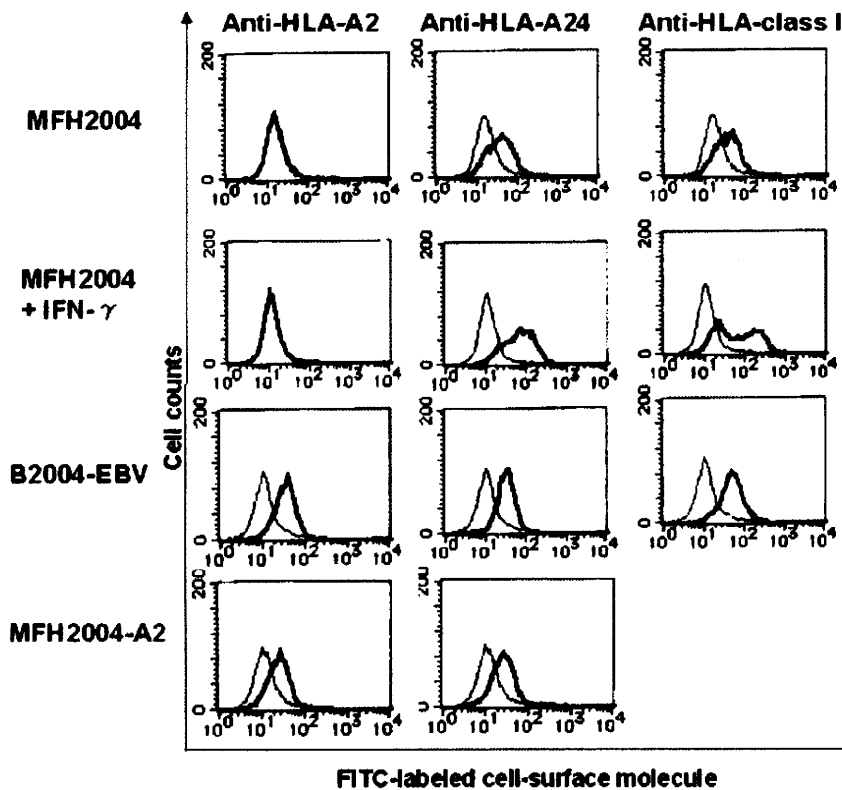


Figure 2. FACS analysis of cell-surface HLA class I molecules.

DISCUSSION

With the goal of identifying efficacious therapeutic antigens in bone and soft tissue sarcomas, we have conducted a series of studies with the strategies referred to as forward immunology^{16,17,21} and reverse immunology.^{19,20} The present study was designed with the forward immunology strategy, which is based on the establishment of an autologous pair of tumor cells and tumor cell-reactive CTLs.^{9,22,23} In this study, our initial two attempts at autologous CTL induction using MFH2004 cells resulted in failure, likely due to tumor escape mechanisms from the host immune surveillance such as haplotype loss of HLA class I antigens. Retrieval of a lost HLA class I antigen, HLA-A*0206, in MFH2004 cells then led to the development of CTL clones, which specifically lysed HLA-A*0206-transfected MFH2004 cells (MFH2004-A2) in an HLA-A2-restricted manner. These findings indicated that CD8⁺ T cells had previously been primed with the antigen presented by HLA-A*0206 on the MFH tumor during tumor development and had eradicated HLA-A*0206-positive tumor cells in the immune selection process.

An alternative interpretation for the present findings would be that the parental MFH tumor was composed entirely of tumor cells with haplotype loss of HLA class I antigens since the early stage of tumor development. In this regard, MFH represents a tumor with complex karyotypes.^{24,25} Thus, it is unlikely that one genetic abnormality, including a regional defect of chromosome 6p21 that encodes the HLA class I heavy chains,¹¹ accounts for the tumorigenesis of MFH. Another interpretation would be that the tumor cell population with HLA haplotype loss might have been more tumorigenic than the others and thus dominated them during tumor development. Loss of heterozygosity at chromosome 6p21 might have simultaneously caused loss of both HLA class I and other genes located nearby such as a tumor suppressor gene *WAF1*.²⁶ We are not able to exclude this possibility without conducting microsatellite analysis.

In our previous immunohistochemical study,¹² loss or downregulation of HLA class I on tumor tissues was significantly associated with poor survival of patients with osteosarcoma. In contrast, such prognostic significance of HLA class I loss was not found in patients with MFH.¹² These findings

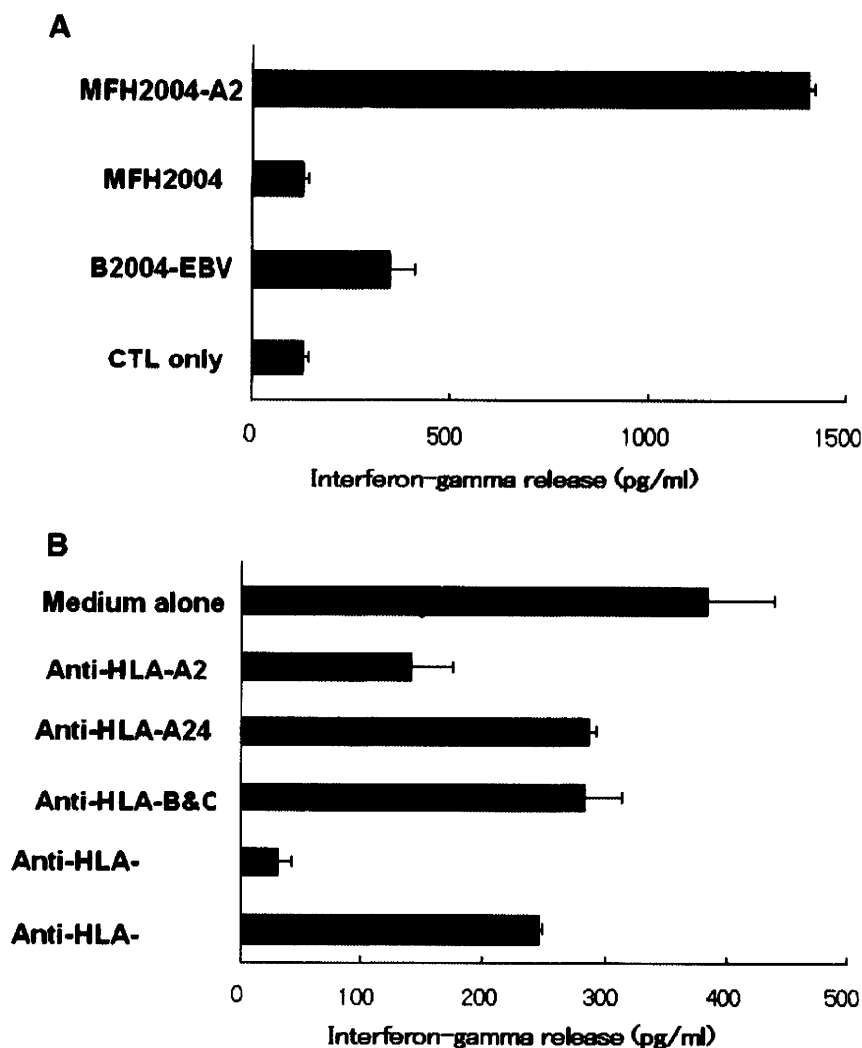


Figure 3. HLA-restricted recognition of CTL2004-c17 against autologous cell lines. (A) Immune responses of CTL2004-c17 against MFH2004-A2, MFH2004, and B2004-EBV were assessed by IFN- γ release assay at a 1:1 of effector:target ratio. (B) Effects of blocking mAb were examined by cytokine release assay. MFH2004-A2 cells were incubated with CTL2004-c17 in the presence or absence of anti-HLA-A2, anti-HLA-A24, anti-HLA-B&C, anti-HLA-class-I, and anti-HLA-class-II mAbs. The effector:target ratio was set at 1:1.

suggest that antigens presented by HLA class I in the established MFH tumors are less immunogenic and play a less important role in immune surveillance of the host than those in osteosarcoma. To define antigens in the established MFH tumors, certain enhancement of tumor cell immunogenicity such as exogenous expression of costimulatory B7-1 molecules is thus required, as we did in a previous study.¹⁷ Identification of immunogenic antigens on MFH cells would require retrieval of the HLA class I, which was lost with the eradicated tumor cells during development, as in the present study.

The present findings support the concept of immunologic sculpting¹³ in bone and soft tissue sarcomas. HLA-A*0206 in MFH2004-A2 cells likely presents a tumor-rejection antigen that differs from other tumor-associated antigens defined in established tumors. Clinical application of the present findings requires identification of the antigen and subsequent evaluation of its immunogenicity. We are currently in the process of antigen identification by expression cloning.

In conclusion, we demonstrated clonal T-cell responses against autologous pleomorphic MFH antigens presented by retrieved HLA-A*0206.

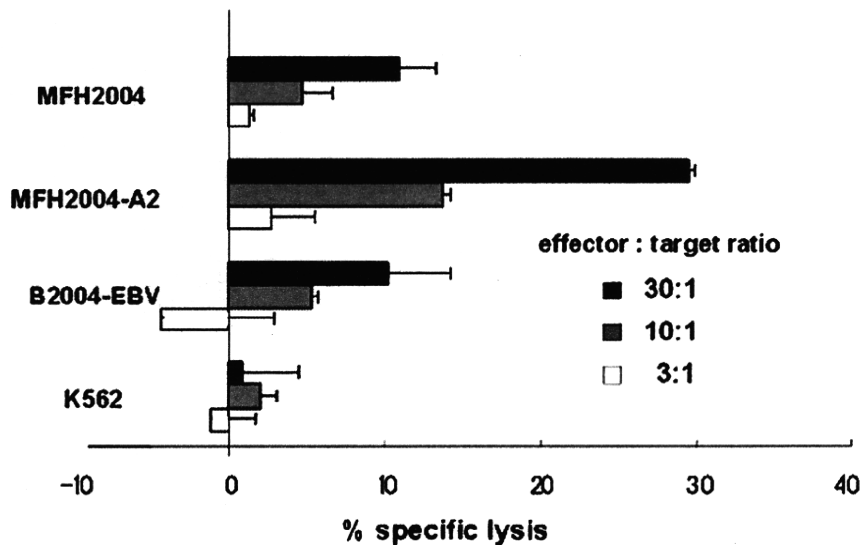


Figure 4. Cytotoxicity of CTL2004-c17 against MFH-2004-A2. Cytotoxicity of CTL2004-c17 was assessed by ^{51}Cr release assay using MFH2004, MFH1004-A2, B2004-EBV, and K562 as targets at the indicated effector:target ratio.

MFH2004-A2 and autologous CTL clones are useful for subsequent identification of putatively immunogenic and therapeutic antigens presented by HLA-A*0206.

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Overexpression of papillomavirus binding factor in Ewing's sarcoma family of tumors conferring poor prognosis

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Abstract. Ewing's sarcoma family of tumors (ESFT) is comprised of highly malignant bone and soft tissue tumors in children and young adults. Despite intensive treatments for patients with ESFT, disease which presents with metastatic spread or relapses after primary treatment remains incurable in the majority of cases, indicating the importance of efforts to develop new treatment modalities, including immunotherapy. The present study was designed to examine the expression profile of papillomavirus binding factor (PBF), which we previously defined as an osteosarcoma-associated antigen, and its prognostic significance for patients with ESFT. Biopsy specimens from 20 ESFT were stained with an anti-PBF antibody. Survival was estimated using Kaplan-Meier plots and the prognostic significance of several variables, including the expression status of PBF, on disease-free and overall survival was determined by univariate analysis using the log-rank test. Of 20 specimens, 18 (90%) reacted positively to the anti-PBF antibody. Fifteen specimens (75%) were graded as PBF overexpression. Of the 11 variables analyzed, stage III disease, inadequate surgical margins and PBF overexpression were significantly associated with decreased disease-free and overall survival. None of the other variables, including age, gender, origin of tumor, tumor site or levels of LDH, ALP, CRP and ESR, showed any significant association. These findings indicate that the overexpression of PBF is a factor indicative of poor prognosis in ESFT. PBF may also serve as a putative target antigen in immunotherapy for patients with ESFT that have a poor prognosis and PBF overexpression.

Introduction

Ewing's sarcoma family of tumors (ESFT) is comprised of highly malignant bone and soft tissue tumors in children and young adults (1). Although systemic adjuvant chemotherapy has significantly improved the prognosis for patients with ESFT, disease which presents metastatic spread or relapses after primary treatment remains incurable in the majority of cases (2,3).

After a nearly 30-year interval from the initial immunotherapeutic trials for osteosarcoma (4,5), immunotherapy has recently re-emerged as a targeted therapy for bone and soft tissue sarcomas, including ESFT (6,7). In a clinical trial with ESFT patients, peptides derived from the junction regions of EWS-FLI1 fusion genes were used as ESFT-specific vaccines (6). However, clinical response was seen in only 1 of the 12 patients enrolled, suggesting the importance of finding further efficacious antigens and of developing antigenic peptide vaccines (8).

Recently, papillomavirus binding factor (PBF) was identified as an autologous cytotoxic T-lymphocyte-defined osteosarcoma antigen (9). It was originally defined as a transcriptional regulator of genomic DNA of human papillomavirus type 8 (10). The antigenic and oncologic roles of PBF in ESFT remain uncertain. In this study, with the aim of gaining basic information on these aspects of PBF, we examined its expression in 20 cases of ESFT and analyzed its prognostic significance.

Materials and methods

This study was approved according to institutional guidelines for the use of human subjects in research. Patient specimens were analyzed after informed written consent was obtained from the patients or their families.

Patients and samples. Between 1979 and 2005, 20 consecutive patients with ESFT were treated at Keio University hospital. The clinical picture is summarized in Table I. There were 12 male and 8 female patients, with an average age at diagnosis of 23.9 years (range, 1-63 years). Twelve tumors arose from bone and the remaining 8 from soft tissue. Eight tumors were located in the trunk and 13 in the extremities. Fusion genes, including

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Table I. Clinical characteristics and expression status of PBF in patients with ESFT.

Patient	Age (years)	Gender	Location	Stage ^a	Fusion gene	Treatment	PBF status	Event-free survival (mos)	Overall survival (mos)	Prognosis
Bone tumor										
1	1	M	Right tibia	IIB	ND	Chx (VAC) + Amp + Rx	++	179	179	CDF
2	36	F	Right femur	IIB	ND	Chx (A-VAC) + Amp+ Rx	-	174	174	CDF
3	16	M	Right humerus	IIB	ND	Chx (A-VAC) + WE + Rx	+	250	250	CDF
4	13	M	Left tibia	IIB	ND	Chx (CYVADIC) + WE + Rx	+++	166	166	CDF
5	17	M	Thoracic spine	IIB	EWS/ERG	Chx (T11) + ME + Rx	+++	8	45	DOD
6	16	M	Sacrum	IIB	ND	Chx (T11) + WE + Rx	++	99	99	CDF
7	16	F	Left tibia	IIIB	EWS/FLI-1	Chx (KS-1) + WE	+++	0	16	DOD
8	22	F	Right fibula	IIB	EWS/FLI-1	Chx (KS-1) + WE	+++	108	108	CDF
9	18	M	Left 5th rib	IIB	EWS/FLI-1	Chx (KS-1) + WE +Rx	+++	105	105	CDF
10	18	M	Right fibula	IIB	EWS/FLI-1	Chx (KS-1) + WE +Rx	+++	7	21	DOD
11	24	M	Left tibia	IIB	EWS/FLI-1	Chx (KS-1) + WE +Rx	-	15	15	CDF
12	23	M	Pelvis	IIIB	ND	Chx (KS-1) +Rx	+++	0	17	DOD
Soft tissue tumor										
13	29	M	Right thigh	IIIB	EWS/FLI-1	Chx (KS-1) + ILE +Rx	+++	0	12	DOD
14	63	F	Paraspine	IIB	ND	Chx (KS-1) + ILE +Rx	+++	0	71	DOD
15	20	F	Right tibia	IIB	EWS/FLI-1	Chx (KS-1) + ME +Rx	+++	9	14	DOD
16	56	F	Right forearm	IIIB	ND	Chx (CYVADIC + KS-1) + WE	+++	0	11	DOD
17	7	M	Paraspinal cord	IIB	Not detected	Chx (KS-1) + ME	+++	14	16	DOD
18	11	F	Paraspinal cord	IIB	EWS/FLI-1	Chx (KS-1) + ILE +Rx	+++	17	22	DOD
19	35	M	Left femur	IIB	ND	Chx (KS-1) + WE	+++	126	126	CDF
20	36	F	Right upper arm	IIB	Not detected	Chx (KS-1) + WE	+++	101	101	CDF

^aStage was determined according to Enneking's surgical staging system. ND, not determined; Chx, chemotherapy; Rx, radiotherapy; ILE, intraliesional resection; ME, marginal excision; WE, wide excision; CDF, continuous disease free; DOD, death of the disease.

EWS/FLI-1, EWS/ERG, EWS/ETV1 and EWS/E1AF, were determined by RT-PCR (11) in 9 of the 11 cases for which frozen biopsy specimens were available. According to Enneking's surgical stage (12), 16 patients were in stage IIB and 4 in IIIB. Treatment consisted of chemotherapy and surgery for 6, chemotherapy, surgery, and radiotherapy for 13, and chemotherapy and radiotherapy for 1. The chemotherapy protocols used were VAC (13), A-VAC (14), CYVADIC (15), T11 (16) and KS1, which is a modified protocol of New A3 (17). Radiotherapy (50-60 Gy) was instituted postoperatively. The average follow-up period after diagnosis was 78.4 months (range, 11-250 months).

Immunohistochemistry. Polyclonal antibody against PBF was generated previously (9). Formalin-fixed paraffin-embedded

sections of biopsy specimens were boiled for 20 min in a microwave oven for antigen retrieval. Sections were blocked with 1% non-fat dry milk and stained with streptavidin-biotin-complex (Nichirei), followed by hematoxylin staining as previously described (9). The reactivity of the anti-PBF polyclonal antibody was determined by staining the nuclei. The expression status of PBF was estimated based on the number of tumor cells according to Ahmed *et al* (18) (Fig. 1): the presence of ≤5% of positively-stained tumor cells was represented by a minus (-), 6-25% by a plus (+) and 26-60% by ++. Overexpression, a number of positive tumor cells >60%, was represented by +++.

Clinicopathological analysis. Survival was estimated using Kaplan-Meier plots. Univariate analysis with the log-rank test

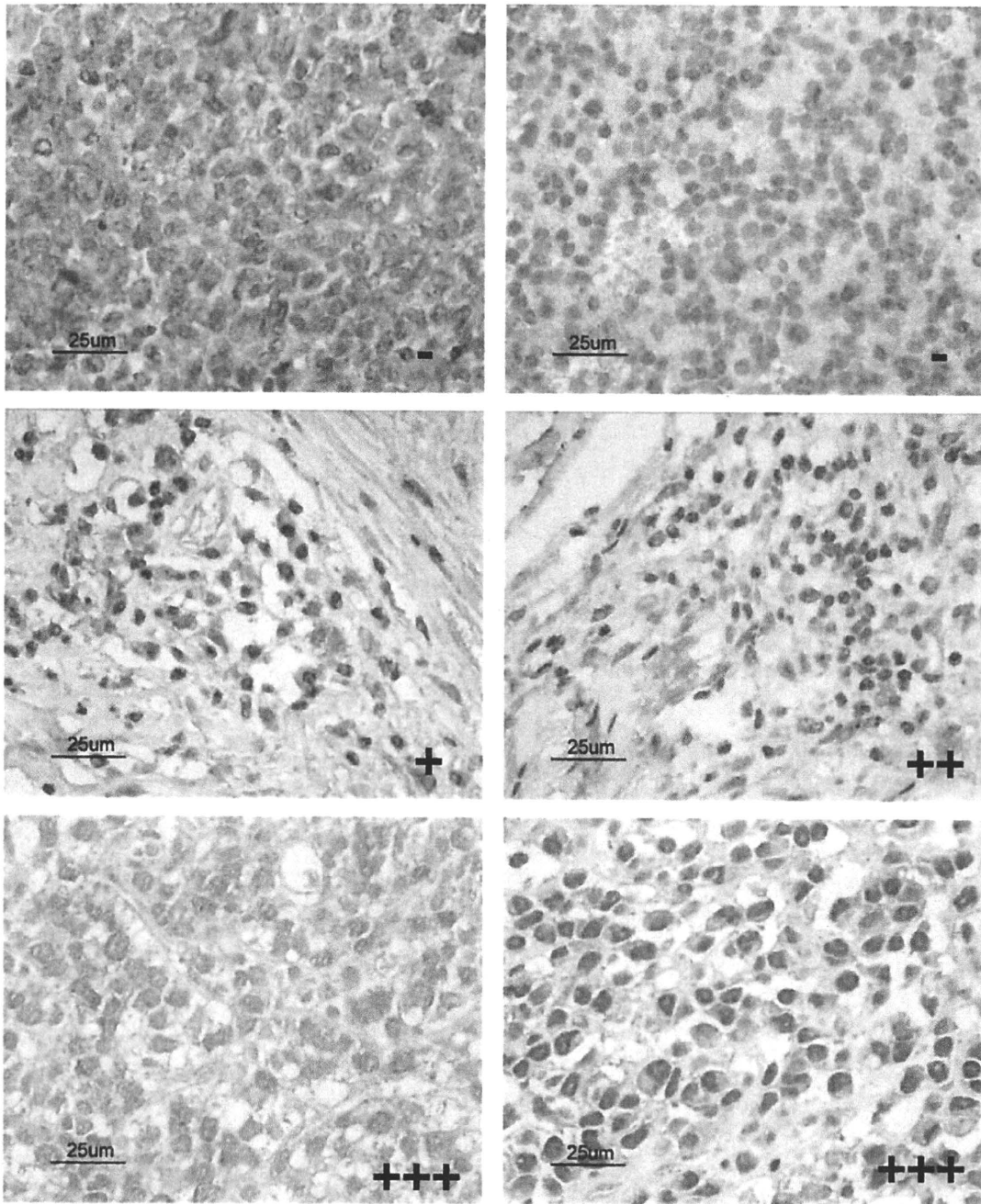


Figure 1. Immunohistochemical grading of PBF expression. Representative sections of ESFT specimens stained with anti-PBF antibody. Tumor cells showing positive reactivity in the nuclei were counted: -, $\leq 5\%$ positive cells; +, 6-25% positive cells; ++, 26-60% positive cells; +++, $>60\%$ positive cells.

(19) was used to determine the prognostic significance of the following variables for disease-free and overall survival: age (≥ 30 or <30), gender (female or male), tumor site (trunk or limb), origin of tumor (bone or soft tissue), stage (I, II or III), laboratory parameters (within or higher than normal range), surgical margin (adequate or inadequate) and PBF expression status (-, +, ++ or +++). Laboratory parameters included LDH, alkaline phosphatase (ALP), C-reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) at 1 h. Analysis of the surgical margins was performed in 16 stage IIB patients. Wide excision and amputation were regarded as adequate margins, whereas intralesional and marginal excision were regarded as inadequate. A probability of <0.05 was considered to be statistically significant.

Results

PBF expression in ESFT. To determine the expression profiles of PBF in ESFT, we stained 20 ESFT biopsy specimens with anti-PBF antibody. Of these, 18 (90%) reacted positively to the anti-PBF antibody where the nuclei of tumor cells were stained (Fig. 1 and Table I). Two specimens were grade ++ and 1 was +. Fifteen specimens (75%) were graded as +++, indicating PBF overexpression.

Prognostic significance of PBF expression in ESFT. We then analyzed the prognostic significance of several variables, including the overexpression of PBF (grade +++). Overall survival rates of the 20 patients with ESFT were 53.1 and

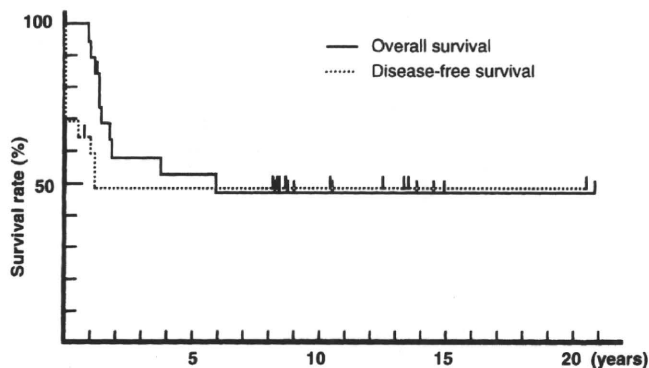


Figure 2. Overall survival of 20 patients with ESFT. Overall survival was estimated using Kaplan-Meier plots. The date of histological diagnosis was used as time 0.

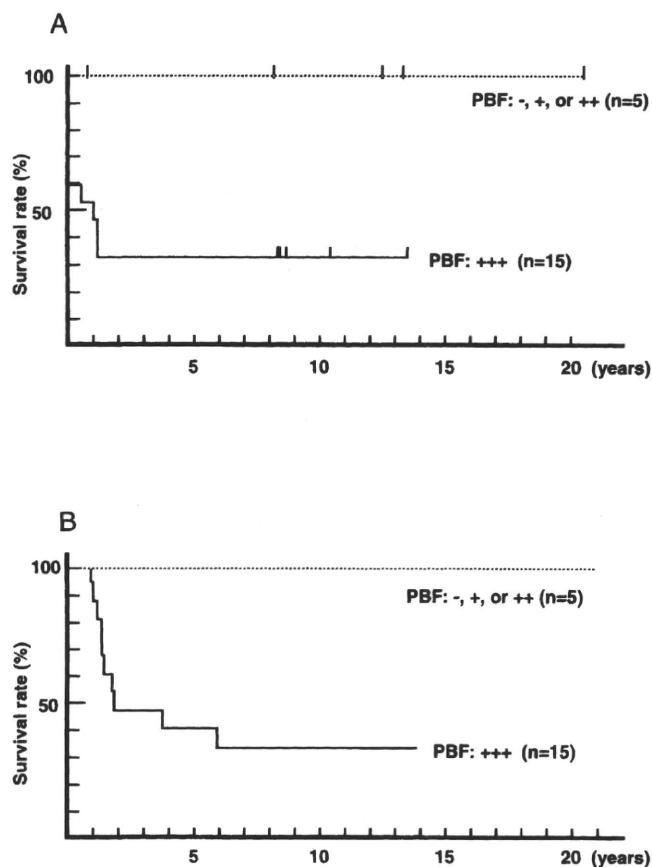


Figure 3. Survival curves of 20 patients with ESFT stratified by PBF expression status. (A) Disease-free survival curve. (B) Overall survival curve. Patients were divided according to PBF expression status into 2 groups (PBF expression of -, +, and ++, n=5; PBF expression of +++, n=15). Survival was estimated using Kaplan-Meier plots.

47.8% at 5 and 10 years, respectively (Fig. 2). Of the 11 variables analyzed, stage III, an inadequate surgical margin and PBF overexpression were significantly associated with decreased disease-free and overall survival (Fig. 3 and Table II). Of note, the overall survival of the 15 patients with PBF overexpression was 33.3% at 10 years, whereas 5 patients remained continuously disease free during the entire follow-up period. None of the other variables, including age,

Table II. Univariate analysis of potential unfavorable prognostic factors.

Factor	No. of patients	P-value	
		Disease-free survival	Overall survival
Age (≥ 30 years)	5	0.71	0.54
Male sex	12	0.31	0.45
Soft tissue tumor	8	0.09	0.06
Trunk tumor	7	0.24	0.47
Stage III	4	0.006	P<0.001
High ALP	7	0.61	0.56
High LDH	8	0.34	0.51
High CRP	8	0.27	0.35
High ESR	8	0.30	0.46
Inadequate surgical margin ^a	6	P<0.001	P<0.001
PBF +++	15	0.04	0.04

^aMarginal excision and intralesional excision were regarded as inadequate surgical margin and 16 stage IIB patients having undergone surgical treatment were analyzed.

gender, origin of tumor, tumor site and levels of LDH, ALP, CRP and ESR, showed a significant association to disease-free or overall survival.

Discussion

By staining 20 biopsy specimens of ESFT treated at a single institute with an antibody against PBF we found i) that PBF was expressed in 18 ESFT specimens (90%), including 15 specimens (75%) with grade +++ overexpression, and ii) that PBF overexpression was significantly associated with the decreased disease-free and overall survival of patients. These findings indicate that the overexpression of PBF is a factor of poor prognosis for ESFT. PBF, which was originally defined as an osteosarcoma-associated antigen (9), may also serve as a putative target antigen in immunotherapy for patients with ESFT and PBF overexpression, which confers a poor prognosis.

Compared to malignant melanoma and epithelial cancers, there is a marked delay in the identification of tumor-associated antigens in bone and soft tissue sarcomas (7,8). In ESFT, antigens proven to have specific T cell responses have been limited to EWS-FLI1 fusion gene products (6,20,21). More recently, cancer-testis antigens (also termed cancer-germline genes) were defined in 11 of 18 ESFT specimens and included MAGE-A3, A4, A6, A10, A12, C2 and GAGE-1, -2 and -8 (22). However, their expression levels were lower than those of other sarcomas, including osteosarcoma and rhabdomyosarcoma (22).

Among the tumor-associated antigens identified in malignant melanoma, some were later found in solid tumors in a significant association with poor prognosis (Table III). These include cancer-testis antigens MAGE-3 (23,24), MAGE-4

Table III. Tumor-associated antigens showing prognostic significance in solid tumors.

Antigen	Tumor	No. of samples (% positive)	Detection procedure	Refs.
Cancer testis				
MAGE-A3	NSCLC	523 (55.2)	RT-PCR	23
	Pancreatic cancer	57 (44.0)	qRT-PCR	24
MAGE-A4	NSCLC ^a	19 (36.8)	RT-PCR	25
	Squamous cell lung carcinoma	153 (56.9)	IHC	26
	Ovarian carcinoma	53 (57.0)	IHC	27
	Bladder carcinoma	908 (4.0) ^b	IHC	28
NY-ESO-1	NSCLC	523 (26.6)	RT-PCR	23
Overexpression				
PRAME	Neuroblastoma	95 (33.7) ^b	qRT-PCR	29
WT1	Soft tissue sarcomas	52 (32.7) ^b	qRT-PCR	30
	Osteosarcoma	37 (27.0) ^b	IHC	31
PBF	ESFT	20 (75.0) ^b	IHC	Present study

NSCLC, non-small cell lung carcinoma; qRT-PCR, quantitative real-time RT-PCR; IHC, immunohistochemistry. ^aAdvanced stage cancers. ^bPercentage of samples with overexpression.

(25-28) and NY-ESO-1 (23), and an overexpression antigen, PRAME (29). Apart from melanoma-derived antigens, it has been reported that the overexpression of WT1 is associated with poor prognosis in bone and soft tissue sarcomas (30,31) (Table III). Though WT1 was originally defined as the tumor-suppressor gene responsible for Wilms' tumor, antigenic peptides derived from it have recently been used as vaccines for hematopoietic malignancies and solid cancers (32). PBF is classified as an overexpression antigen as it is detected in some normal tissues by RT-PCR (9). In addition to ESFT, expression of PBF was found to be significantly associated with poor prognosis in patients with osteosarcoma, with statistical significance (Tsukahara *et al.*, unpublished data).

The antigenic role of PBF in patients with ESFT remains to be defined by T cell responses specific to PBF-derived peptides. To this end, we recently developed a limiting dilution/mixed lymphocyte peptide culture/tetramer/cytotoxicity assay by which the frequency and anti-tumor cytotoxicity of peripheral T lymphocytes directed against PBF were determined in patients with osteosarcoma (Tsukahara *et al.*, unpublished data). This approach is also applicable to patients with ESFT. Another limitation of the present study is the small number of samples used, due mainly to the rare occurrence of ESFT in the Japanese population. It is, however, based on a consecutive series of patients treated at a single institute for more than 25 years.

In conclusion, the present analysis serves as a pilot study showing the prognostic significance of PBF for patients with ESFT. Large-scale analyses need to be conducted to verify the present findings if PBF-targeted immunotherapy for patients with ESFT is to be developed.

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Original Paper

Tonsillar crypt epithelium of palmoplantar pustulosis secretes interleukin-6 to support B-cell development via p63/p73 transcription factors

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Abstract

Palmoplantar pustulosis (PPP) is an autoimmune disease characterized by psoriasis-like erythematous lesions on palms and/or soles due to an abnormal humoral immune response. Tonsillectomy is effectively employed for the treatment of PPP; however, how tonsils are involved in the aetiology of PPP remains unclear. Here we analysed surgically resected palatine tonsils from 36 cases of PPP as well as usual recurrent tonsillitis (RT) as a control. Histological examination revealed that a unique lesion, with lymphoid follicles surrounded by reticular crypt epithelial cells, was more frequently observed in tonsils of patients with PPP than in those with RT ($p < 0.0001$; PPP vs RT). Interestingly, crypt epithelial cells in primary cultures derived from PPP tonsils showed marked production of interleukin-6 (IL-6). Moreover, these epithelial cells from PPP tonsils expressed p53-related transcription factors in their nuclei that were found to contribute to the up-regulation of *IL-6* gene expression. These findings suggest that, at least in part, the specialized lymphoepithelial symbiosis of PPP tonsils, under the control of p53-related factors, may be relevant to the generation of the impaired micro-environment underlying the aberrant production of autoantibodies.

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Introduction

Focal infection of palatine tonsils may cause palmoplantar pustulosis (PPP, synonymously recognized as palmoplantar pustular psoriasis) [1,2]. Patients with PPP display chronic relapsing inflammatory changes, with erythematous and scaly plaques on the palms and/or soles bilaterally, so that PPP has a profound impact on their quality of life. According to reports describing putative mechanisms leading to PPP, autoantibodies against a certain antigen(s) specifically presented by the epithelia of the palms and/or soles probably appear in certain lymphoid tissue(s) [3,4]. Once the autoantibodies have an opportunity to bind to epithelial cells of the palms and/or soles, subsequent cytopathic effects, including local activation of the complement system, might cause aseptic epidermal lesions in palmoplantar areas. To date, surgical resection of palatine tonsils combined with steroid therapy is considered to be one of the most effective therapeutic approaches for the treatment of PPP [5–7]. Thus, palatine tonsils have been considered to be a lymphoid tissue possibly responsible for generating such unusual immune responses in patients with PPP, although the

mechanistic details in the patients' tonsils have not been fully investigated.

Tonsillar crypt epithelial cells (TCECs) constitutively form a unique reticular meshwork, which is often infiltrated by small lymphocytes such as B cells [8–11]. Such an association of TCECs and lymphocytes is specifically called lymphoepithelial symbiosis (LES), further characterizing the physiological features of palatine tonsils. Functional properties of the lymphocytes of PPP tonsils have been the focus of several studies, although knowledge about the function of LES in PPP tonsils is still limited in lymphocytes [12,13]. The biological significance of TCECs as another component of LES in the pathogenesis of PPP remains elusive.

The aim of this study was to shed light on TCECs of PPP tonsils and explore the role of TCECs in the pathogenesis of PPP. Our results provide the first evidence that TCECs of PPP tonsils may take part in the specialized formation of lymphoid follicles by secreting a high amount of interleukin-6 (IL-6), which is required for the development of B cells and plasma cells [14,15]. Moreover, we show that TCECs

of PPP tonsils abundantly express the epitheliotropic transcription factors p63 and p73, p53-related factors, in their nuclei. Surprisingly, p63 seems to be involved in the up-regulation of IL-6 at the transcriptional level through the NF- κ B promoter of the *IL-6* gene. These results may open the way to the development of a new modality for the treatment of PPP as an alternative to surgical resection of the tonsils, in which quenching the activity of IL-6 produced by TCECs may be required.

Materials and methods

Patients of this study

A total of 74 patients were enrolled, as summarized in Table 1, including 36 with PPP, 36 with recurrent

tonsillitis (RT), and two with IgA nephropathy (IgAN). All patients in this study underwent tonsillectomy at Sapporo Medical University Hospital in Japan from 1989 to 2006. Sole skin biopsies were performed in two patients with PPP and sera were collected from the same patients. All tissue was obtained with informed consent and the approval of the institutional review board.

Assessment of clinical status in patients with PPP

For determination of the clinical status of skin lesions in patients with PPP, the physician's global assessment (PGA) of change was used to assess treatment efficacy in this study. The PGA of change was categorized according to seven ratings: 'cleared' (100% improvement from baseline); 'excellent' (75–99%); 'good' (50–74%); 'fair' (25–49%); 'slight' (1–24%);

Table 1. Summary of PPP, RT, and IgAN cases in this study

PPP						RT			IgAN		
Case No	Age (years)	Sex	PGA (Palm)	PGA (Sole)	IP (months)	Case No	Age (years)	Sex	Case No	Age (years)	Sex
1	34	F	Unchanged	Unchanged	—	1	65	F	1	24	F
2	21	F	ND	ND	ND	2	53	M	2	20	F
3	50	F	ND	ND	ND	3	40	M	Average	22.0	
4	52	F	Cleared	—	1.5	4	40	M			
5	27	F	ND	ND	ND	5	52	F			
6	41	M	Cleared	Cleared	1	6	41	F			
7	46	M	Unchanged	Unchanged	—	7	44	F			
8	19	M	ND	ND	ND	8	54	M			
9	70	F	ND	ND	ND	9	53	F			
10	43	F	Cleared	Cleared	12	10	46	M			
11	32	F	ND	ND	ND	11	54	M			
12	58	F	Cleared	Cleared	3	12	46	F			
13	47	F	Good	Good	24	13	58	F			
14	50	F	Cleared	Cleared	1	14	9	M			
15	17	F	ND	ND	ND	15	5	M			
16	36	M	ND	ND	ND	16	24	F			
17	50	F	Cleared	Cleared	60	17	47	M			
18	49	M	Cleared	Cleared	6	18	5	M			
19	20	F	ND	ND	ND	19	25	F			
20	39	F	ND	ND	ND	20	41	F			
21	36	M	Cleared	Cleared	1	21	24	M			
22	51	F	Unchanged	ND	—	22	36	F			
23	55	F	Cleared	Cleared	1.5	23	5	F			
24	61	M	ND	ND	ND	24	31	M			
25	55	F	Cleared	Fair	1	25	5	F			
26	54	F	Cleared	Cleared	12	26	38	F			
27	51	F	Fair	Unchanged	2.5	27	53	F			
28	58	M	Cleared	Cleared	12	28	53	M			
29	43	F	Cleared	Cleared	2	29	37	F			
30	55	M	Cleared	—	1	30	53	F			
31	59	F	Unchanged	—	—	31	44	M			
32	48	F	Cleared	Excellent	18	32	26	M			
33	58	F	Unchanged	—	—	33	26	F			
34	62	F	Cleared	Cleared	3	34	53	F			
35	34	M	Cleared	Cleared	7	35	19	M			
36	31	F	ND	ND	ND	36	30	F			
Average	44.8				8.9	Average	37.1				

Patients with PPP ranged from 17 to 70 years old (median = 44.8 years) and the gender ratio was 1:2.6 (male:female). Patients with RT ranged in age from 5 to 65 years (median = 37.1 years) and had a gender ratio of 1:1.3. Patients with IgAN ranged in age from 20 to 22 years (median = 22.0 years) and were all female. The status of 12 cases ('ND' in the table) in the PPP group could not be surveyed after surgery. PGA = physician's global assessment; IP = improvement period; ND = not determined.

'unchanged' (no change in clinical signs and symptoms from baseline); and 'worse' (deterioration of clinical signs and symptoms from baseline).

Tissue and cell culture

For primary culture of TCECs, the surface epithelial cell layers were initially removed from the tonsil with micro-scissors, to prevent contamination of surface epithelial cells, under a research microscope (SZX7; Olympus). The procedures used for primary culture of TCECs were applied with modifications, as previously reported for primary culture of thymic epithelial cells [16]. Human HaCaT and HSC-3 epithelial cells were maintained in modified DMEM supplemented with 10% heat-inactivated bovine calf serum, 50 µg/ml streptomycin, and 100 units/ml penicillin. All cells were cultured at 37 °C in a humidified atmosphere in 5% CO₂.

Antibodies and immunohistochemistry

The antibodies used were a mouse anti-cytokeratin monoclonal antibody (MAb) (KL-1; Immunotech), rabbit anti-p63 polyclonal antibody (PAb) (H-137; Santa Cruz Biotechnology), rabbit anti-p73 PAb (ST-2G [17]), and mouse anti-IL-6 MAb (6708; Dako). The procedures for immunofluorescence have been previously described [16]. Signals were detected under an immunofluorescence microscope (IX71, Olympus) or a confocal laser microscope (R2100AG2, Bio-Rad).

RT-PCR and real-time PCR analyses

RT-PCR was conducted to detect certain transcripts, as previously reported [18]. Primer pairs were selected using Primer3 software on mRNA sequences based on the NCBI database. Sequences of the primer pairs were finally determined after reference to the original genomic organization presented in the Ensembl database (Sanger Centre), as summarized in Supplementary Table 1 (available online at <http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2266.html>). Real-time PCR was performed as described in the manufacturer's protocol for Assays-on-Demand Gene Expression products (Applied Biosystems). To compare the levels of expression of IL-6, the $\Delta\Delta\text{CT}$ method was employed to analyse triplicate specimens according to the manufacturer's instructions.

Cytokine assays

Primary TCECs were seeded on collagen type I-coated dishes at a density of 5×10^6 cells for 3 days. The concentrations of cytokines in the culture supernatants were analysed in triplicate with ELISA kits, as described in the manufacturer's protocol (Biosource).

Luciferase activity assay

Human IL-6 promoter-reporter constructs in luciferase pGL3-basic vector were employed as previously described [19]. HSC-3 cells were seeded in 12-well plates at a density of 2×10^5 cells per well. After 24 h, 250 ng of a reporter gene plasmid, 250 ng of pcDNA3.1 harbouring cDNA encoding p63 (pcDNA3.1-p63), and 25 ng of pSV- β -galactosidase were transiently transfected using Lipofectamine 2000 reagent (Invitrogen). Transfections were performed in triplicate and cells were harvested after 48 h. Luciferase activity was measured according to the manufacturer's protocol (Promega) and normalized for transformation efficiency with the β -galactosidase assay as a relative luciferase unit (Stratagene).

Statistical analysis

The computer program Statview (Abacus Concepts) was used for all calculations. Values were expressed as the mean \pm SD for each group. Comparisons between the two groups (PPP and RT) were performed with the Mann-Whitney *U*-test. Significance was assigned to $p < 0.05$. Spearman's rank-order correlations were used to test for a monotonic association of the CLG/GC (circular lymphoepithelial lesion with germinal centre/germinal centre) ratio and the improvement period.

Results

Clinical assessment after tonsillectomy in PPP patients

In 24 cases in the group with PPP, clinical responsiveness after tonsillectomy was categorized into the five classes of the physician's global assessment (PGA; see the Materials and methods section). Patients classified as 'cleared', 'excellent' or 'good' showed favourable recovery with noticeable improvements on the palms and soles in 18/24 (75.0%) and 15/19 (78.9%), respectively.

The sera of patients with PPP reacting to sole epidermis

We initially studied how the serum was involved in the development of the symptoms and signs of PPP as described in previous reports [3]. Immunofluorescence studies demonstrated strong signals in the epithelial cells of the sole tissue sections when using the patient's serum (Figure 1). This evidence indicated that the serum from the patient with PPP contained autoantibodies capable of binding to a sole epidermal cell antigen(s). Thus, it was thought that aberrant humoral immunity might induce the clinical manifestations of PPP. A possible candidate antigen might be a certain subtype of cytokeratin specifically expressed on palms and soles [20].

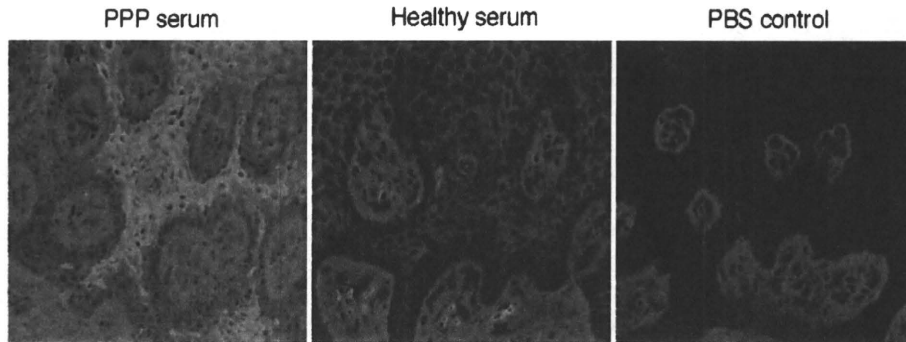


Figure 1. Immunofluorescence analysis of sole skin using the serum of a patient with PPP. The left panel shows high-level autoantibodies in the serum (PPP case 36) reacting to antigens in the sole epidermis. The middle and right panels show results using healthy serum and PBS, respectively, as controls. Original magnification: $\times 400$

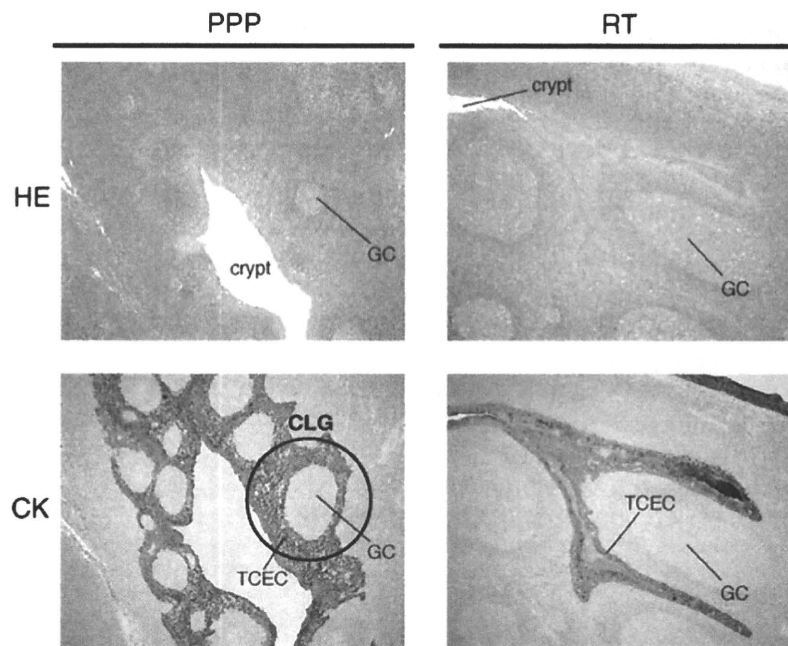


Figure 2. Histopathology focusing on relationships of the structures of lymphoid follicles and TCECs in tonsils of patients with PPP and RT. PPP tonsils (case 6) shown on the left contain lymphoid follicles with relatively small germinal centres (GC) along crypts, some of which were recognized as circular lymphoepithelial lesions with germinal centres (CLG) demarcated by TCECs, as indicated in the circle. In contrast, RT tonsils (case 19) on the right had lymphoid follicles with larger GCs that were infrequently surrounded by epithelial cells. PPP tonsils had thicker lymphoepithelial symbiosis (LES) than that of RT tonsils. Upper panels show haematoxylin and eosin (HE) staining of the tissue sections of tonsils. Lower panels depict immunostaining with the anti-cytokeratin (CK) MAb KL-1 of the respective areas of the upper panels. Original magnification: $\times 200$

Histopathological characteristics of tonsils of PPP

We next investigated the features of surgically resected tonsils in PPP and RT cases as a control. In the tissue sections of PPP tonsils, we often found small lymphoid follicles along the crypts (Figure 2). In contrast, those around the crypts of RT tonsils seemed to be organized in larger and wider forms. Immunostaining analysis using anti-cytokeratin MAbs showed that lymphoid follicles with germinal centres in PPP tonsils were often surrounded by epithelial cells contiguous to the crypts (ie TCECs), whereas such structures were infrequently observed in RT tonsils. We designated this type of histological finding in PPP tonsils as 'a circular lymphoepithelial lesion with a germinal centre (CLG)'. To evaluate the functional significance of CLG in PPP cases, we counted the number

of CLGs and also the total number of lymphoid follicles with a germinal centre (GC) in each case of PPP and RT (Table 2). Statistical analysis revealed that the raw number of GCs in PPP tonsils (average 60.0) was not significantly different from that of RT tonsils (average 75.5; $p = 0.305$). These probably accounted for the chronic inflammation similarly occurring in tonsils of both PPP and RT. In contrast to the situation for GCs, the raw number of CLGs in PPP tonsils (average 7.2) was larger than that in RT tonsils (average 2.1; $p < 0.001$). We therefore considered CLG to be a structure unique to the chronic inflammatory responses in PPP tonsils. To verify the results in terms of artefacts during the preparation of the tissue sections, the ratio of the raw numbers of CLGs to the total numbers of GCs (CLG/GC ratio)

Table 2. The number of GCs and CLGs in each case of PPP and RT

Case No	PPP			Case No	RT		
	GC (R No)	CLG (R No)	CLG/GC (%)		GC (R No)	CLG (R No)	CLG/GC (%)
1	24	1	4.2	1	21	0	0.0
2	130	10	7.7	2	86	1	1.2
3	23	0	0.0	3	19	0	0.0
4	14	5	35.7	4	71	2	2.8
5	101	6	5.9	5	22	0	0.0
6	160	54	33.8	6	180	4	2.2
7	110	40	36.4	7	18	1	5.6
8	68	9	13.2	8	46	0	0.0
9	21	5	23.8	9	40	3	7.5
10	20	0	0.0	10	52	3	5.8
11	135	8	5.9	11	82	2	2.4
12	49	8	16.3	12	24	1	4.2
13	92	16	17.4	13	44	0	0.0
14	16	7	43.8	14	132	1	0.8
15	67	4	6.0	15	21	0	0.0
16	98	8	8.2	16	108	0	0.0
17	6	0	0.0	17	32	1	3.1
18	21	4	19.0	18	56	1	1.8
19	70	12	17.1	19	69	0	0.0
20	42	3	7.1	20	90	9	10.0
21	123	11	8.9	21	38	1	2.6
22	106	4	3.8	22	18	0	0.0
23	25	1	4.0	23	49	1	2.0
24	9	0	0.0	24	118	13	11.0
25	40	5	12.5	25	128	5	3.9
26	46	1	2.2	26	116	4	3.4
27	35	6	17.1	27	71	0	0.0
28	120	8	6.7	28	262	2	0.8
29	18	2	11.1	29	3	0	0.0
30	47	3	6.4	30	56	0	0.0
31	44	2	4.5	31	73	1	1.4
32	23	0	0.0	32	118	1	0.8
33	12	0	0.0	33	106	0	0.0
34	97	12	12.4	34	225	11	4.9
35	95	2	2.1	35	15	0	0.0
36	53	2	3.8	36	108	6	5.6
Average	60.0	7.2	11.0	Average	75.5	2.1	2.3

CLG/GC ratio (%) means the raw number of CLGs per raw number of GCs. R No = raw number; GC = germinal centre; CLG = circular lymphoepithelial lesion with germinal centre.

was further examined in every case of PPP and RT. Interestingly, it was found that the CLG/GC ratio of PPP was clearly higher than that of RT tonsils, in agreement with the comparison of the raw numbers of CLGs in PPP and RT tonsils (Figure 3A). Collectively, these data implied that CLG, as a unique form of lymphoepithelial interaction, would markedly contribute to the inflammatory process of PPP tonsils rather than that of RT tonsils. Based on these results, the relationship of the CLG/GC ratio to the improvement period was investigated to determine the clinicopathological significance of CLG in PPP cases with a favourable prognosis for a PGA change to be 'cleared', 'excellent' or 'good'. We found, to our surprise, that the CLG/GC ratio was inversely related to the improvement period after tonsillectomy (Figure 3B), further indicating the active involvement of CLG in the pathogenesis of PPP, possibly in association with the preservation of abnormal humoral immune responses.

Functional characteristics of epithelial cells constituting CLG of tonsils of PPP

To address the question of whether there was any difference in the inflammatory process related to the formation of CLGs in PPP tonsils, we established primary cultures of TCECs derived from PPP and RT (PPP-TCECs and RT-TCECs, respectively) to investigate their functions (Figure 4A). We then examined the transcription levels of various cytokines including interleukins in these primary TCECs by using specific PCR primers. High levels of the transcripts of IL-6 were detected in primary PPP-TCECs in comparison to the levels of IL-6 in primary RT-TCECs (Figure 4B). Accordingly, quantitative PCR analysis of primary PPP-TCECs indicated levels of IL-6 transcripts higher than those of primary RT-TCECs (Figure 4C). These findings were further supported by ELISA of the culture supernatants of primary TCECs of PPP and RT tonsils (Figure 4D). Immunohistochemical analysis

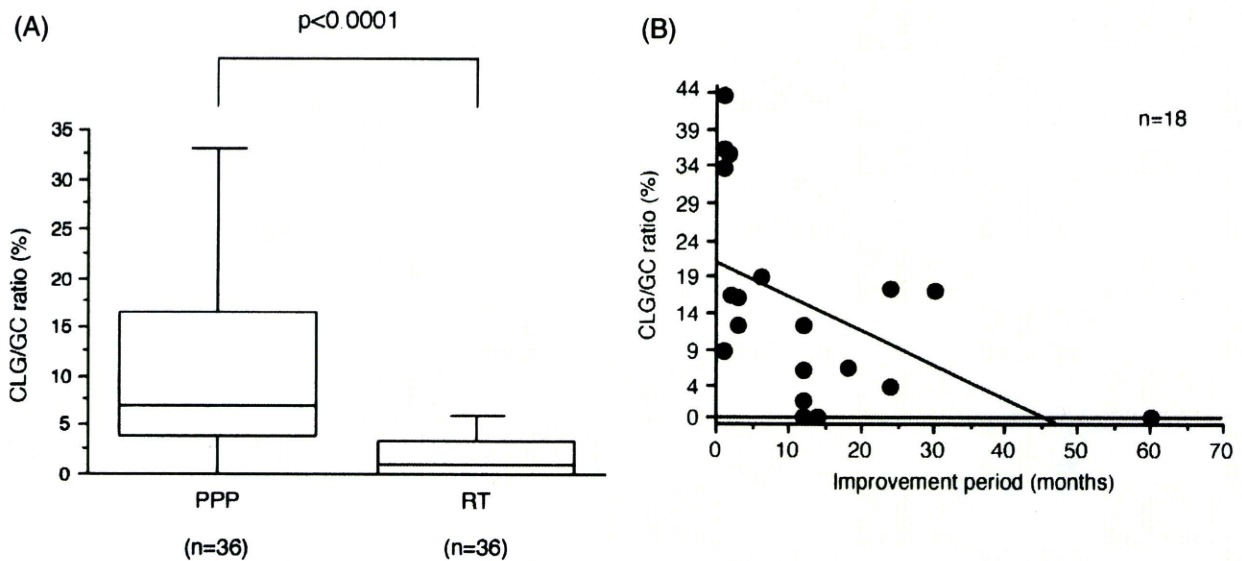


Figure 3. Involvement of tonsillar CLG in the pathogenesis of PPP. (A) The CLG/GC ratio of PPP tonsils was greater than that of RT tonsils, with statistical significance ($p < 0.0001$). Median values are shown by horizontal lines; the box represents values between the 25th and the 75th percentiles; and the lower and upper bars indicate the tenth and 90th percentiles, respectively. (B) The CLG/GC ratio was inversely related to the improvement period after surgical resection of tonsils (correlation coefficient: -0.511 , $p < 0.024$). Favourable cases of patients with PPP were analysed ($n = 18$)

also showed high expression of IL-6 when compared with that of RT tonsils (Figure 4E). Thus, TCECs of PPP tonsils would secrete IL-6 to foster reacting B cells within CLGs. Factors affecting lymphoid follicle formation such as IL-7 and IL-11 were not significantly increased in PPP-TCECs (Figure 4B). The levels of other factors influencing Th2 responses such as IL-4 and IL-5 also showed insignificant differences between PPP- and RT-TCECs.

Role of p53-related transcription factors in TCECs in PPP tonsils

We further investigated regulatory factors determining the functional properties of TCECs of PPP tonsils. In recent investigations, the p53-related molecules p63 and p73 have been studied as epitheliotropic transcription factors [21]. When we examined the expression of p63 and p73 in the tissue sections of tonsils, we found high expression of these factors in the nuclei of epithelial cells around CLGs in PPP tonsils (Figures 5A and 5B). On the other hand, p63 and p73 were scattered more faintly and were localized in the nuclei of epithelial cells in RT tonsils. When transcription analysis was employed on primary TCECs, transcripts of p63 and p73 were clearly detected in PPP-TCECs rather than in RT-TCECs under PCR conditions with a low number of cycles (25 cycles) (Figure 5C). These findings suggested that p63 and p73 of TCECs might have a role in the histogenesis of CLG in PPP tonsils.

To determine the regulatory roles of p63 and p73 in the expression of IL-6, we studied two different types of human epithelial cells into which cDNA encoding p63 or p73 was transiently introduced. Intriguingly, both p63 and p73 had the capacity to up-regulate the transcription levels of IL-6 in these epithelial cells

(Figure 5D). These findings were compatible with our previous results showing that when p63 was transfected into thymic epithelial cells, a large amount of IL-6 was produced in the culture supernatant [16]. We next conducted reporter analysis of the *IL-6* gene, which indicated that p63 could up-regulate the transcription levels of IL-6 through sites including NF- κ B recognition sequences (Figure 5E). Taken together, these results suggest that p63 and p73 may have a role in the up-regulation of the level of IL-6 in epithelial cells.

Discussion

The present study is, to the best of our knowledge, the first to describe the particular tissue structure of CLG and the histogenesis of LES in PPP tonsils. The CLG of the PPP tonsil is considered to represent a unique form of lymphoid follicle in the TCEC meshwork. This situation of lymphoid follicles in PPP tonsils may reflect their appearance, with a relatively smaller conformation than those of RT tonsils. To facilitate the formation of lymphoid follicles within the restricted area of the TCEC meshwork, B cells probably require a stimulating factor(s) in the PPP tonsil. It is well known that various epithelial cells secrete IL-6 [22]. In this context, we could identify the surrounding TCECs as a potential producer of IL-6, an important cytokine for B-cell maturation, by employing primary culture techniques. Such B cells might be related to the production of autoantibodies reacting to a certain antigen(s) specifically presented by palmoplantar epithelial cells. T cells are considered in the pathogenesis of PPP tonsils. IL-6 is also thought as an inducer of certain T-cell populations including T_H -17 cells which

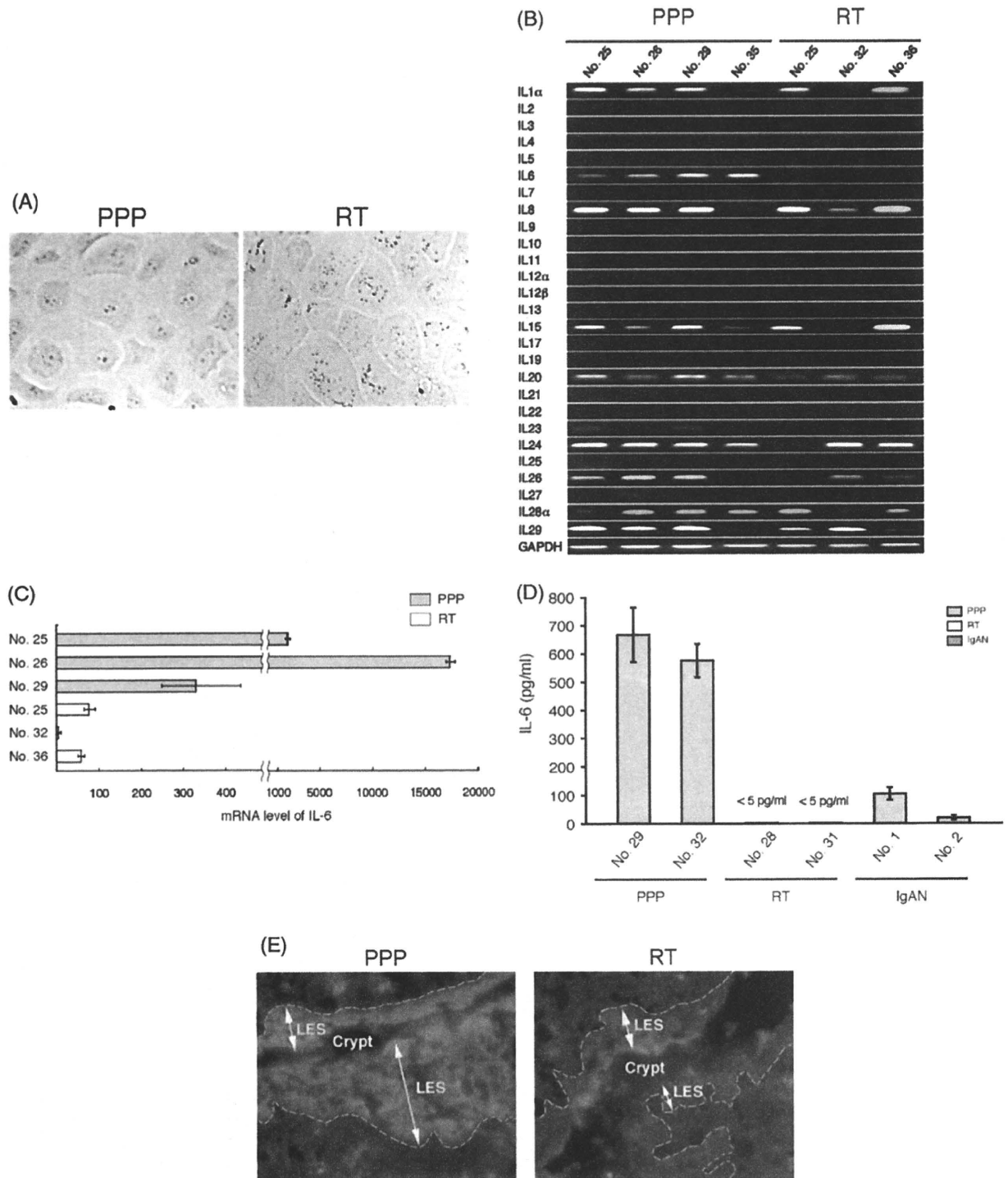


Figure 4. Expression and production of IL-6 in TCECs of PPP tonsils. (A) Representative images of PPP-TCECs (case 26) and RT-TCECs (case 32) obtained from day 5 after the initial primary culture are shown on the left and right, respectively. The morphology of PPP- and RT-TCECs of the primary culture was not significantly different, both showing flat and polygonal shaped cells with round nuclei. Original magnification: $\times 450$. (B) Expression of various types of interleukin in primary cultures of PPP- and RT-TCECs as assessed by RT-PCR analysis. PCR was performed for 35 cycles and GAPDH was used as a control. (C) Quantification of IL-6 at the transcription level in PPP- and RT-TCECs by real-time PCR analysis. IL-6 transcripts were detected in PPP-TCECs at higher levels than in RT-TCECs. (D) Quantification of IL-6 at the protein level in the culture supernatants of PPP- and RT-TCECs as assessed by ELISA. Two cases of TCECs from IgAN tonsils were also employed as a control. (E) Immunohistochemistry of frozen sections of PPP (case 30) and RT (case 33) tonsils after staining with anti-IL-6 MAbs. IL-6 was detected in TCECs of LES of PPP tonsil rather than in those of RT tonsil. Original magnification: $\times 400$. Case numbers are depicted for PPP and RT cases in B, C, and D

are thought to serve autoimmunity [23,24]. In fact, enlargement of the T-cell-dependent area in PPP tonsils has been previously reported [25]. In accordance,

our flow cytometric analysis showed that the number of CD4⁺ T-cell populations was larger in the PPP tonsil than in the RT tonsil (data not shown).

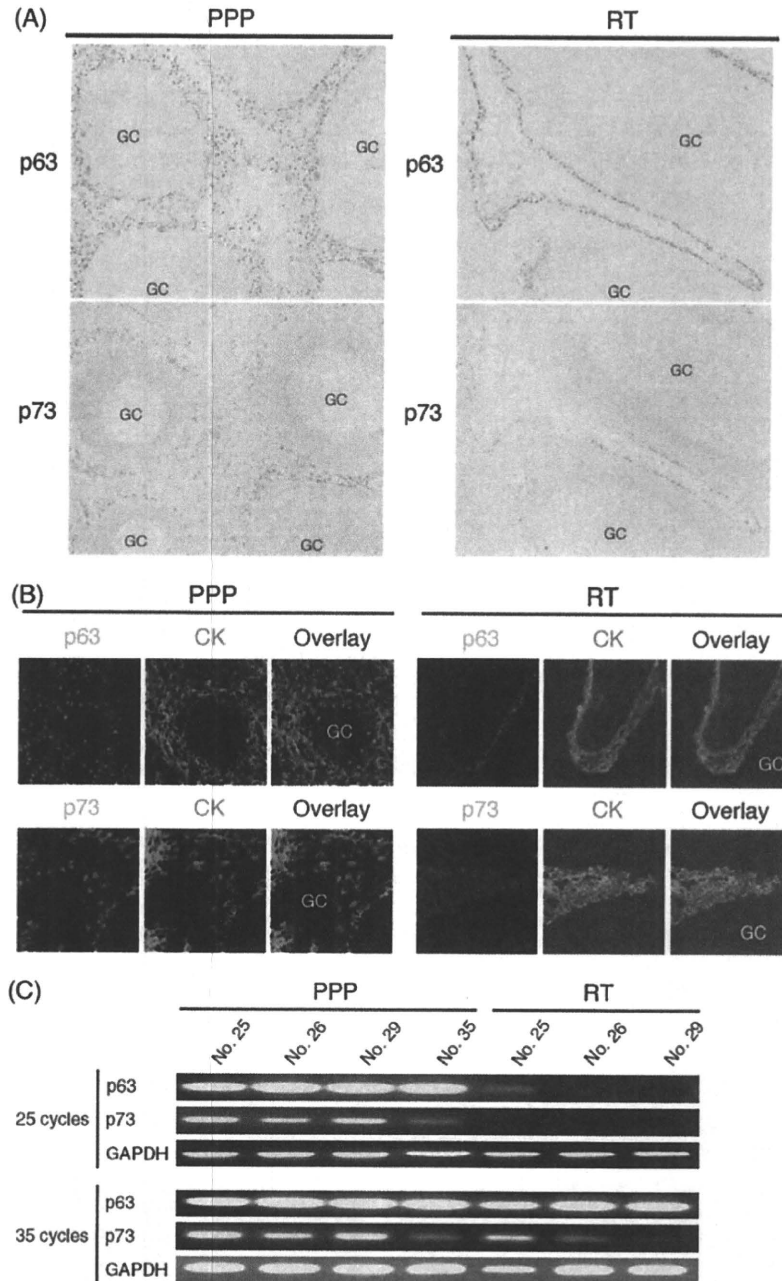


Figure 5. Expression of p63 and p73 of p53-related factors in TCECs of PPP tonsils. (A) p63 and p73, shown in the upper and lower panels, respectively, were extensively expressed in cells around lymphoid follicles with germinal centres (GC) in PPP tonsil (case 6), whereas there seemed to be much less expression of p63 and p73 in RT tonsil (case 19). Formalin-fixed, paraffin-embedded tissue sections of tonsils were immunostained and signals were detected by using DAB. Original magnification: $\times 400$. (B) Confocal laser microscopy after double immunostaining of frozen sections of PPP (case 30) and RT (case 33) tonsils shown in the left and right panels, respectively. TCECs of PPP tonsil showed high expression of p63 and p73 in the nuclei of PPP-TCECs, while low expression of p63 and p73 in the nuclei of TCECs of RT tonsil was observed. CK = cytokeratin. Original magnification: $\times 400$. (C) After a low number of PCR cycles (25 cycles), p63 and p73 were preferentially detected in PPP-TCEC rather than in RT-TCEC primary cultures as assessed by RT-PCR analysis. When the number of PCR cycles was increased to 35, p63 and p73 were detectable in primary RT-TCECs. GAPDH was used as a control. (D) Quantification of IL-6 at the transcriptional level in HSC-3 and HaCaT cells transfected by plasmid DNAs encoding p63 or p73 as assessed by real time-PCR analysis. VC = vector control. (E) Reporter analysis of the promoter region of the *IL-6* gene using HSC-3 cells. Promoter activity of the parental pIL6-luc651 construct (containing a full-length promoter, $-651/+1$), which contains 651 bp directly upstream of the transcription initiation site of the *IL-6* gene, was enhanced by the introduction of p63. While promoter activity of the deletion construct of the AP-1 site (pIL6-luc65 Δ AP-1) was still enhanced by the introduction of p63, promoter activity of the deletion construct of the NF- κ B site (pIL6-luc65 Δ NF- κ B) by p63 was clearly abrogated. Data shown are from three independent experiments. RLU = relative luciferase unit; VC = vector control