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Bath-PUVA Therapy Decreases Infiltrating CCR4-Expressing Tumor Cells and Regulatory T Cells in Patients With Mycosis Fungoides

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Abstract

In this study, we analyzed the mechanism that bath-PUVA therapy to CCR4-expressing tumor cells and regulatory T cells (Treg) in patients with mycosis fungoides(MF). The CCR4 positive cell and Treg in patient blood and the skin were analyzed. Both type of cells decreased after bath-PUVA in the skin lesion, in contrast, bath-PUVA did not significantly change the percent circulating Treg. It suggested that bath-PUVA eliminated both pathogenetically relevant cells and Treg and systemic immunosuppression was not induced.

Background: Mycosis fungoides (MF) is a malignant lymphoma characterized by expansion of $CD4^+$ memory T-cell clones. Infiltrating cells express CCR4, which is attracted to CC chemokine ligands 17 and 22 (thymus and activation-regulated chemokine [TARC]/CCL17 and TARC/CCL22). Bath–psoralen plus ultraviolet A (PUVA) is effective against MF. In patients with psoriasis, bath-PUVA induces circulating regulatory T cells (Tregs), which suppress effector T cells. To understand the mechanisms in MF, we analyzed lesion-infiltrating cells before and after bath-PUVA therapy. **Patients and Methods:** Thirteen patients with MF (12 stage IB, 1 stage III; mean age 69.2 years, range 35-87 years; 6 men, 7 women) were recruited. **Results:** Immunohistochemical analysis revealed that lesion CCR4-positive ($CCR4^+$) cells and Tregs significantly decreased from 105.1 ± 164.8 cells/10^{-2} mm^2 to 31.4 ± 39.0 cells/10^{-2} mm^2 and from 78.1 ± 67.8 cells/10^{-2} mm^2 to 24.7 ± 25.0 cells/10^{-2} mm^2, respectively. Serum TARC levels significantly correlated with infiltrating $CD3^+$ (r = 0.997), $CCR4^+$ (r = 0.991), and forkhead box P3–positive ($Foxp3^{+)}$ cells (r = 0.843). Circulating Tregs before bath-PUVA therapy were not significantly different from those in healthy volunteers. Bath-PUVA did not significantly change the percentage of circulating Tregs. **Conclusions:** Bath-PUVA decreased $CCR4^+$ cells and Tregs in MF lesions but did not induce circulating Tregs, which might suppress effector T cells. Direct effects through skin lesions might eliminate both pathogenetically relevant cells and Tregs. Systemic immunosuppression was not induced.

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Introduction

Mycosis fungoides (MF) is a malignant cutaneous lymphoma with a chronic disease progression.[1] Because erythema and red plaques appear on the patient's whole body at an early stage, it is important to distinguish MF from other skin diseases. Various symptoms are associated with MF, including lymph node enlargement, skin tumors, and ulcer formation in the tumor stage. Symptom onset usually occurs in those older than 60 years of age, but the actual disease onset is earlier.[2] The histologic findings depend on the stage. In the erythema stage (stage I), the characteristic features include epidermal hyperplasia, lymphoid exocytosis, and band-like lymphoid infiltration in the

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AQ: 1 The Mechanism of Bath-PUVA Therapy for MF

Table 1 Patient Characteristics

Patient	Age	Sex	Disease Stage	Irradiation Frequency	Cumulative UV Doses (J/cm^2)
1	82	F	IB	46	170.4
2	68	F	III	42	146.4
3	35	F	IB	43	53.7
4	71	M	IB	20	45.0
5	87	M	IB	33	120.0
6	82	M	IB	5	8.0
7	77	M	IB	29	106.0
8	70	F	IB	37	138.0
9	83	M	IB	38	138.0
10	62	F	IB	14	29.5
11	56	M	IB	30	106.0
12	64	F	IB	42	150.0
13	62	F	IB	25	34.5
Mean ± SD	69.2 ± 14.1			31.1 ± 12.3	95.8 ± 54.5

Abbreviations: SD = standard deviation; UV = ultraviolet.

Figure 1 Immunohistochemical Analysis for Before Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy. Hematoxylin and Eosin Stain (A), Anti-CD3 (B), Anti-CCR4 (C), Anti-Foxp3 (D). A Pautrier Microabscess was Observed in the Epidermis. Many CCR4$^+$ Lymphocytes Were Observed. In Contrast, There Were a Few Foxp3$^+$ Cells. Yellow Triangles Were Some of Positive Cells

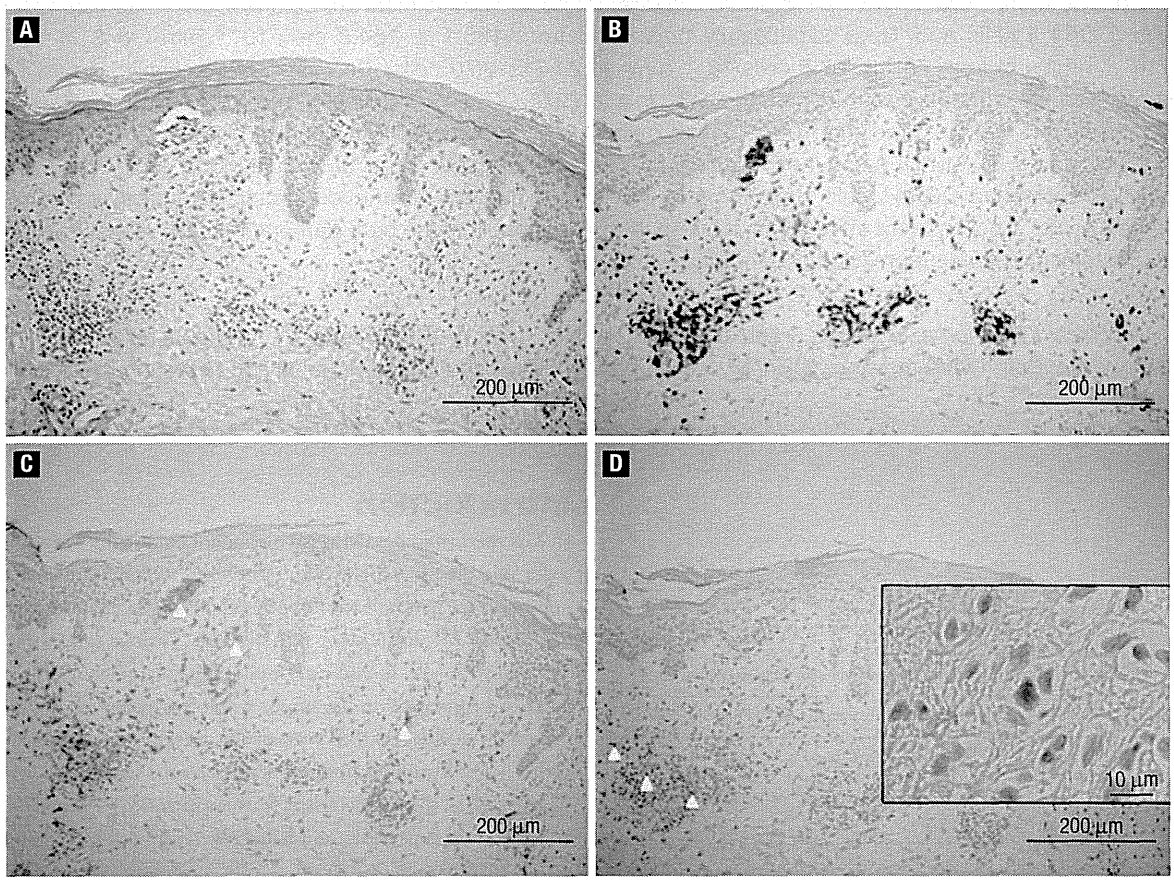
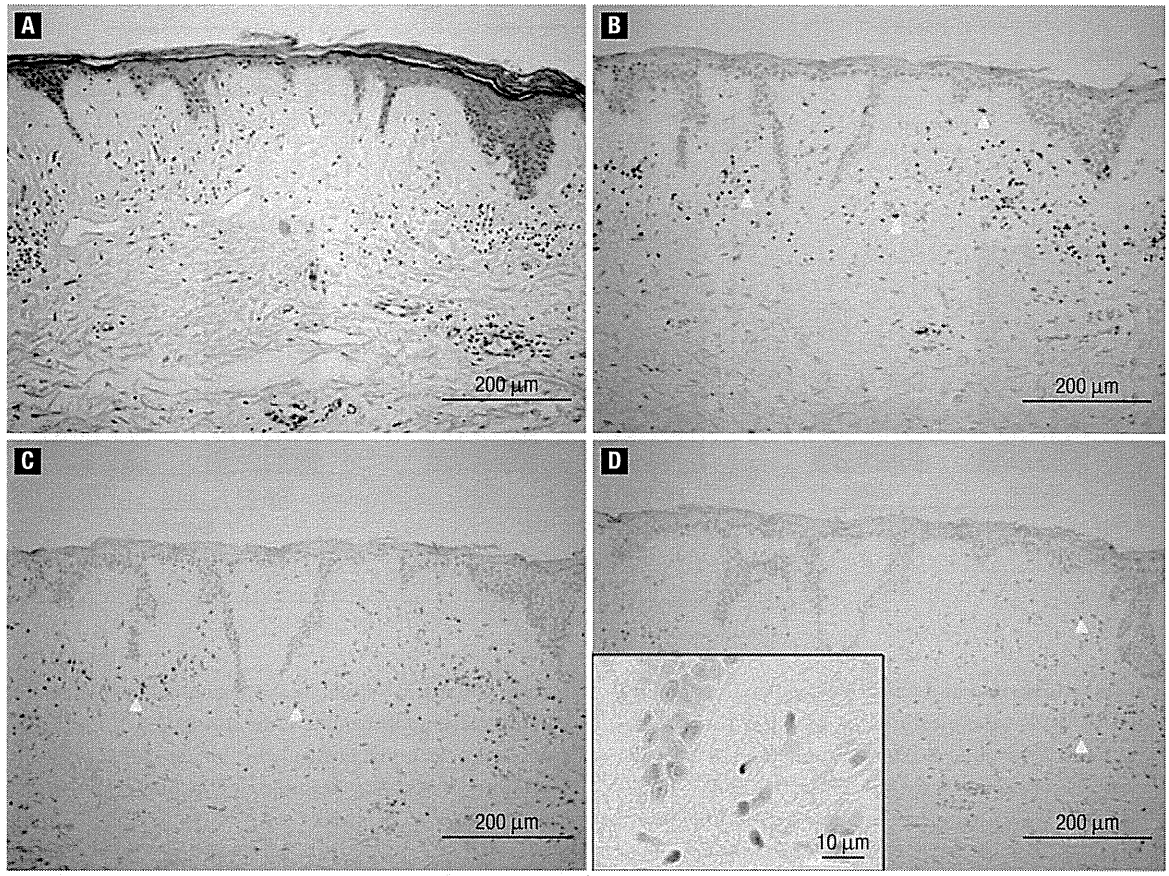


Figure 2 Immunohistochemical Analysis for After Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy. Hematoxylin and Eosin Stain (A), Anti-CD3 (B), Anti-CCR4 (C), and Anti-Foxp3 (D). The Number of Infiltrating Cells was Decreased. Yellow Triangles Were Some of Positive Cells



superficial dermis. In the plaque stage (stage II), Pautrier microabscesses are often observed. In the tumor stage (stage III), tumor cells infiltrate the nodular lesions and proliferate with necrosis, and then ulcers form in the tumorous lesions. In stages I and II, the 5-year survival rate is > 90%, but in stage III the rate drops to approximately 40%.[3]

In the initial stage, topical corticosteroids are used for red plaques.[4] Psoralen ultraviolet A (PUVA) or narrowband UVB is used mainly for stage I.[5,6] More severe cases require radiotherapy and chemotherapy. There are some clinical reports of bath-PUVA therapy for MF.[5] We recently reported that bath-PUVA therapy induces circulating regulatory T cells (Tregs), which suppress effector T cells such as Th17, in patients with psoriasis.[7] The underlying mechanisms of bath-PUVA therapy in MF, however, are unclear. Therefore, we analyzed cells infiltrating the lesions before and after bath-PUVA therapy. Circulating lymphocytes in the peripheral blood were also analyzed.

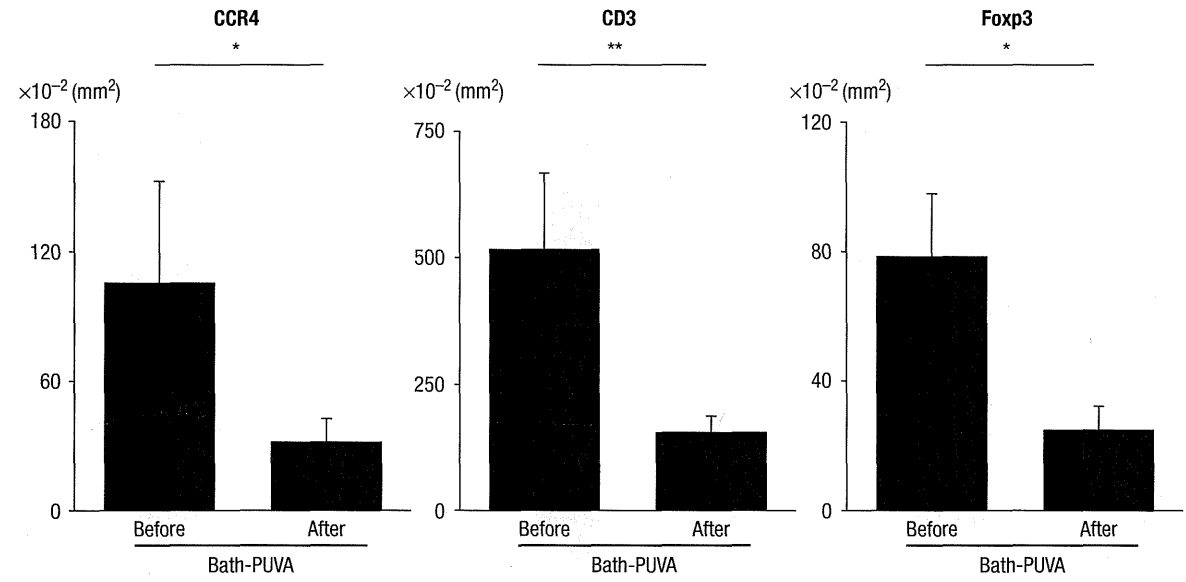
CCR4 is a chemokine receptor expressed on certain types of T-cell neoplasms, including MF and adult T-cell leukemia/lymphoma (ATLL).[8-10] Clinical development of the therapeutic humanized anti-CCR4 monoclonal antibody KW-0761 is in progress.[11] A phase I clinical trial of KW-0761 for patients with relapsed CCR4-positive ($CCR4^+$) T-cell neoplasms including MF was completed.[12] The subsequent phase II clinical trials of KW-0761 against relapsed ATLL (http://ClinicalTrials.gov Identifier: NCT00920790), untreated ATLL (NCT01173887), and relapsed peripheral T-cell lymphoma (NCT01192984) are currently being conducted in Japan. In the United States, a phase I/II clinical trial of KW-0761 against relapsed peripheral T-cell lymphoma has also been conducted (NCT00888927). In the present study, we analyzed the relationship between $CCR4^+$lymphocytes and some parameters from MF patients.

Patients and Methods

Patients

Thirteen patients of Japanese origin diagnosed with MF (mean age, 69.2 years; range, 35-87 years; 7 women and 6 men; 12 patients with stage IB and 1 patient with stage III disease) and 10 healthy controls (mean age, 31.7 years; range, 23-46 years; 6 women and 4 men) were recruited for the study. Serum-soluble interleukin-2

Figure 3 Number of Infiltrating Lymphocytes Before and After Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy in Stage I MF. The Number of Lymphocytes Decreased After Bath-PUVA Therapy (*$P < .05$ by Wilcoxon signed rank test)



receptor (sIL2R) and thymus and activation-regulated chemokine (TARC)/CCL17 levels were measured in 13 and 5 patients, respectively, as part of the clinical blood examination at SRL Inc (Tokyo, Japan). Fluorescence-activated cell sorting (FACS) analysis (FACSCalibur Flow Cytometry System, Becton Dickinson, Franklin Lakes, NJ,) was applied in 6 cases. Punch biopsies of 3 or 4 mm were performed in the same lesion before and after bath-PUVA therapy. In healthy controls, only the peripheral blood was examined. The analysis was conducted with the approval from the Ethics Committee of Nagoya City University. Patient profiles are summarized in Table 1.

Bath-PUVA Therapy

Patients were placed in a 37°C bath containing 0.0001% 8-methoxypsoralen before UVA radiation treatment 5 times per week. A whole-body UVA radiation unit, the Dermaray TS (Eisai-Toshiba, Tokyo, Japan) with FLR100HBL/A/DMR fluorescence tubes, was used for UVA irradiation. The initial dose was 0.5 J/cm^2 with subsequent doses increased by increments of 0.5 J/cm^2 to a maximum dose of 4 J/cm^2. The mean number of irradiation treatments was 25.1. Mean cumulative UVA dose was 79.1 J/cm^2. The patient treatment profiles are summarized in Table 1.

Immunohistochemical Analysis

Staining for CD3, anti-CCR4 antibodies, and forkhead box P3 (Foxp3) was performed as follows. The sections were fixed with 10% neutral-buffered formalin. Formalin-fixed paraffin sections were stained with anti-CCR4 antibody (KM2160, Kyowa Hakko Kirin, Tokyo, Japan), polyclonal rabbit antihuman CD3 antibody (A0452, Dako, Carpinteria, CA), and antimouse monoclonal antibody to Foxp3 (236 A/E7, Abcam, Tokyo, Japan) following standard protocols using diaminobenzidine as the chromogen.[13] Positive cells in all sections were counted manually by 2 independent researchers (HK, CS). The sections were measured and the number of positive cells in each section was calculated.

FACS Analysis of Peripheral Blood Mononuclear Cells

Peripheral blood was obtained from patients before and after bath-PUVA therapy. Peripheral blood mononuclear cells (PBMCs) were obtained by density gradient centrifugation and stained with fluorescence-conjugated antihuman CD4 (MT310; Dako A/S, Glostrup, Denmark), CD25 (ACT-1, Dako A/S), Foxp3 (PCH101, eBioscience, San Diego, CA), and the appropriate isotype control antibodies followed by FACS analysis.

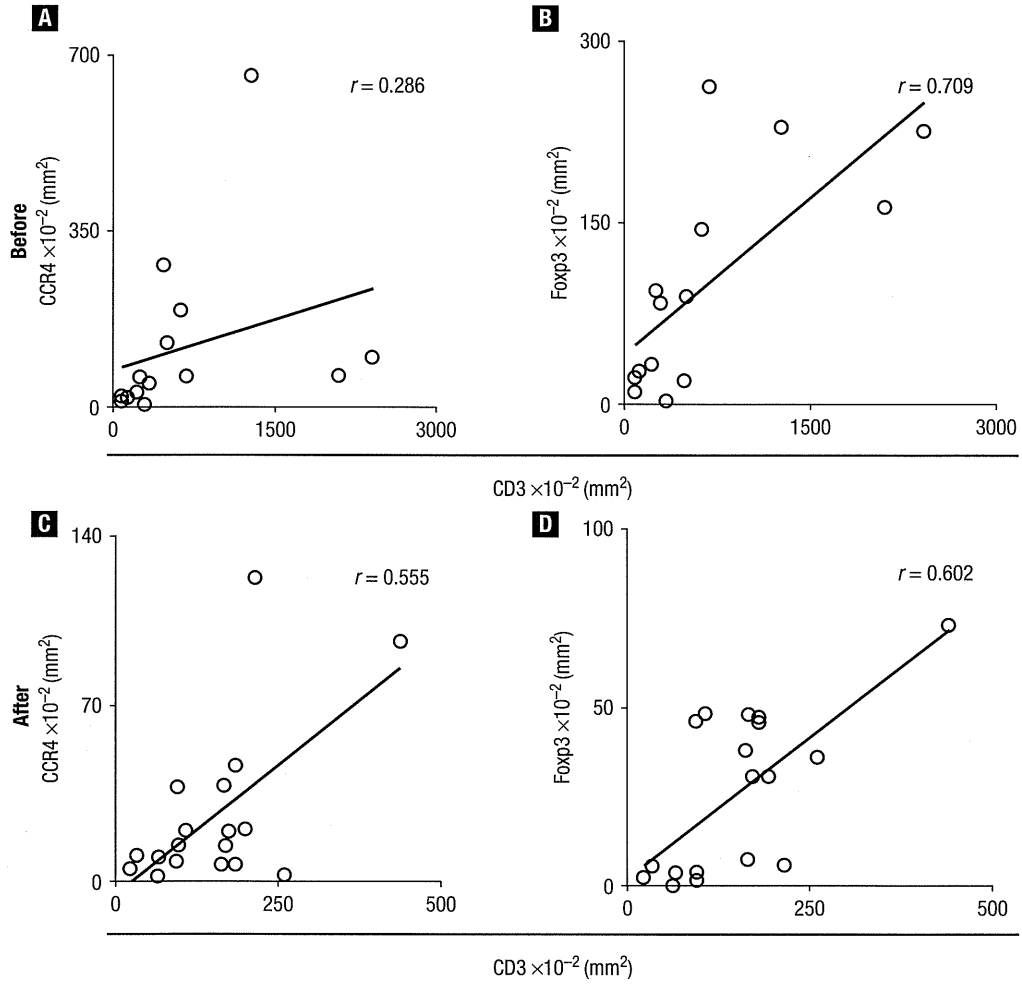
Statistical Analysis

The significance of changes in the variables before and after bath-PUVA therapy was examined using the Wilcoxon signed-rank test. Correlations between 2 variables obtained from patients with MF were assessed using the Spearman rank correlation coefficient. Differences in the variables between the 2 groups were examined with the Wilcoxon rank-sum test. Statistical analyses were performed using the Pharmaco Analyst II software (Human Life, Japan).

Results

Bath-PUVA therapy was well tolerated in all patients enrolled in the study. The red plaques in all patients improved clinically after bath-PUVA therapy. The number of atypical stage IB and stage III MF tumor cells are usually minimal, so it is difficult to clearly identify the tumor cells histologically from many reactive infiltrating

Figure 4 Correlation Between the Lymphocytes Before (A and B) and After (C and D) Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy. There was No Significant Correlation Between CD3 and CCR4 Before Bath-PUVA Therapy, But There was a Positive Correlation After Bath-PUVA Therapy. Correlation Coefficients Were Determined by Spearman Rank Correlation Analyses



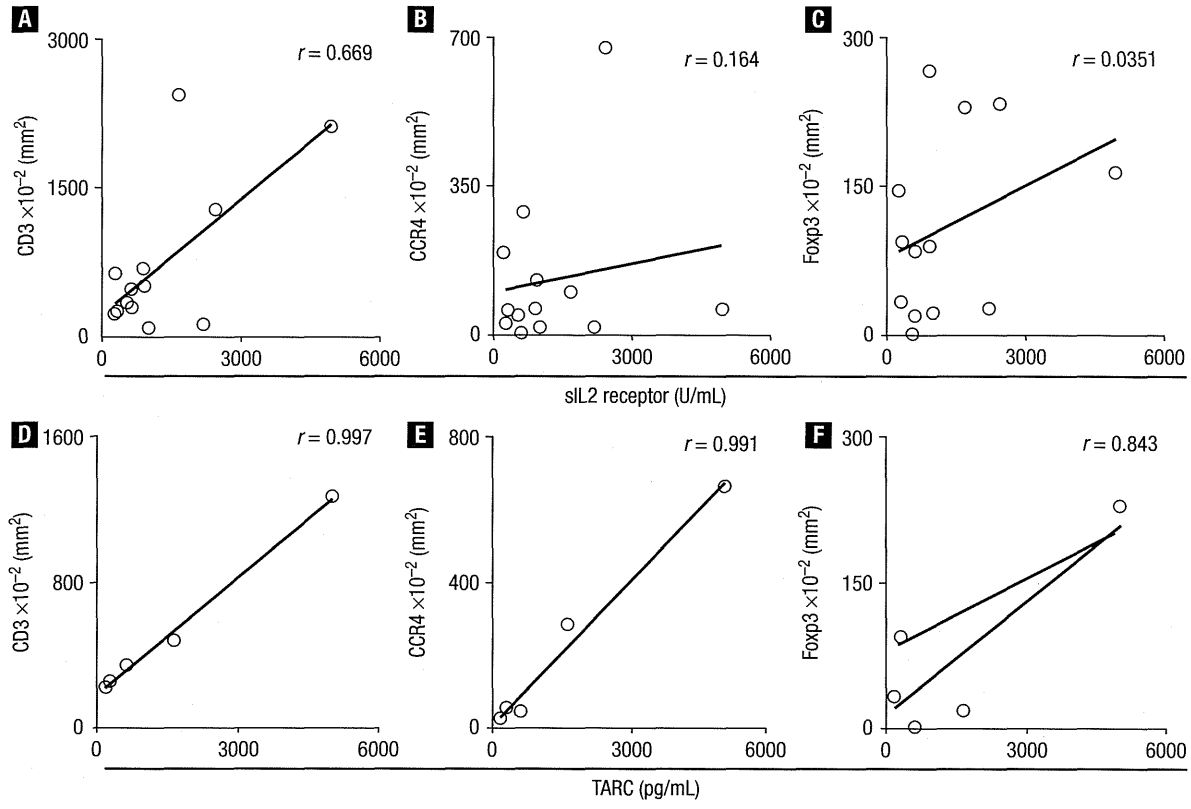
lymphocytes. Therefore we analyzed the numbers of whole stained lymphocytes, including both tumor and reactive cells (Figures 1 and 2). After bath-PUVA therapy, patients with stage I MF had a significant decrease in the number of infiltrating $CD3^+$, $CCR4^+$, and $Foxp3^+$ cells compared with the number of cells before therapy (Figure 3). The number of $CCR4^+$ cells in the lesion significantly decreased from 105.1 ± 164.8 cells/10^{-2} mm^2 to 31.4 ± 39.0 cells/10^{-2} mm^2. Similarly, Tregs in the lesion decreased from 78.1 ± 67.8 cells/10^{-2} mm^2 to 24.7 ± 25.0 cells/10^{-2} mm^2.

Before bath-PUVA, there was no significant correlation between the number of $CD3^+$ cells and $CCR4^+$ cells ($r = 0.286$) (Figure 4A), but after bath-PUVA, the levels of these cells were positively correlated ($r = 0.555$) (Figure 4C). The correlations between the number of $CD3^+$ and $Foxp3^+$ cells were significant before and after bath-PUVA therapy ($r = 0.709$; $r = 0.602$ (Figures 4B and D).

TARC/CCL17 is a CCR4 ligand used as a disease activity marker in atopic dermatitis,[14] and serum TARC/CCL17 levels are also correlated with MF disease activity.[15] Thus the correlation between the number of each type of infiltrating lymphocyte and sIL2R (n = 13) and TARC/CCL17 (n = 5) was evaluated (Figure 5). sIL2R levels were significantly correlated with the number of infiltrating $CD3^+$ cells ($r = 0.669$) but not with the number of infiltrating $CCR4^+$ and $Foxp3^+$ cells ($r = 0.164$ and $r = 0.351$, respectively). TARC/CCL17 levels were significantly correlated with the numbers of infiltrating $CD3^+$, $CCR4^+$, and $Foxp3^+$ cells ($r = 0.997$, $r = 0.991$, and $r = 0.843$, respectively) (Figure 5).

We then assessed the percentages of $CD4^+CD25^+Foxp3^+$ Tregs in $CD4^+$ T cells in PBMCs obtained from patients with MF and healthy volunteers. There was no significant difference between patients with MF and healthy volunteers. Bath-PUVA

Figure 5 Correlation Between Infiltrating Lymphocytes and Soluble IL-2 Receptor (sIL2) Levels (A-C) and Between Infiltrating Lymphocytes and Thymus and Activation-Regulated Chemokine (TARC) Levels (D-F). Soluble Interleukin-2 Receptor (sIL2) Levels Correlated Significantly With CD3, and TARC Levels Correlated Significantly With CD3, CDR4, and Foxp3. Correlation Coefficients Were Determined by Spearman Rank Correlation Analyses



therapy did not induce any significant change in the percentage of Tregs (Figure 6).

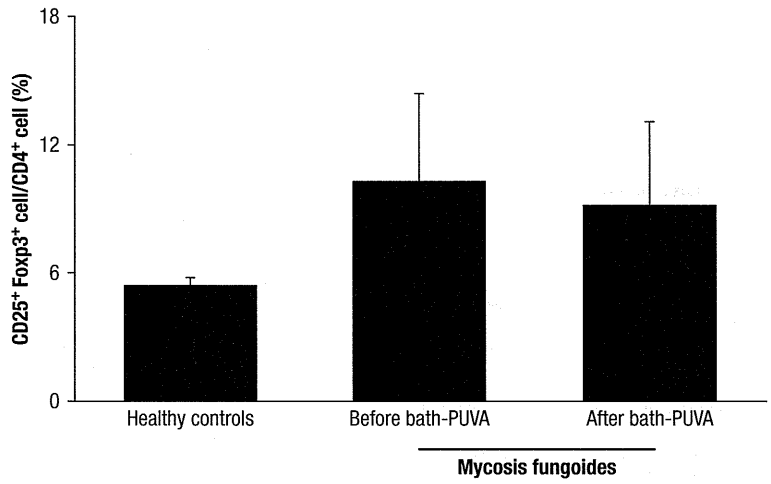
Discussion

The findings of the present study demonstrate that infiltration of both $CCR4^+$ and Tregs is eliminated by bath-PUVA therapy in patients with MF. The mechanism of the effectiveness of phototherapy for MF is not clear. For psoriasis treatment, there are 2 theories underlying the mechanisms of phototherapy: (1) induction of apoptosis in pathogenetically relevant cells[16] and (2) an immunosuppressive mechanism through the induction of Tregs.[7] The former is classified as an induction of apoptosis through oxygen radicals and caspase activation, leading to direct DNA damage. Photophoresis is a unique type of photochemotherapy using extracorporeal circulation and ultraviolet irradiation. In 1987, Edelson reported the efficacy of photophoresis treatment of cutaneous T-cell lymphoma.[17] A growing body of evidence indicates that photophoresis is also effective against other diseases.[18] Photophoresis induces monocytes to activate dendritic cells that express highly costimulatory molecules. The dendritic cells engulf the dying target cells,[19] leading to an immune response. Conversely, photophoresis induces antigen-specific Tregs. Therefore it is unclear how photophoresis regulates the immune responses toward both antiinflammatory and anticancer effects.

Leukocyte trafficking, which is critically regulated by chemokines and their receptors, shares many characteristics with tumor cell infiltration and metastasis. For example, CCR4 is a chemokine receptor selectively expressed on Tregs and Th2 cells[20-22] and also frequently expressed in ATLL cells, and its ligands TARC/CCL17 and macrophage-derived chemokine (MDC)/CCL22 are abundantly present in skin. We previously reported a significant association between the extent of CCR4 expression in ATLL cells and skin involvement.[9] With respect to MF, CCR4 expressed on the tumor cells also has a critical role in tumor formation in the skin.[23]

Generally, sIL2R is used as a tumor marker in cutaneous lymphoma such as in MF,[24] and in the present study sIL2R levels correlated positively with the number of infiltrating $CD3^+$ cells but not with that of infiltrating $CCR4^+$ and $Foxp3^+$ cells. TARC/CCL17 is also reported to be an MF tumor marker[15] and, as expected, serum TARC/CCL17 levels were significantly correlated with the numbers of infiltrating $CD3^+$, $CCR4^+$, and $Foxp3^+$ cells. These findings indicate that the number of the affected skin infiltrating $CD3^+$, $CCR4^+$, and $Foxp3^+$cells reflect the disease activity of MF.

Figure 6 Fluorescence-Activated Cell Sorting (FACS) Analysis With Peripheral Blood Mononuclear Cells (PBMCs) Obtained From Healthy Volunteers or Patients With MF Before and After Bath–Psoralen Plus Ultraviolet A (PUVA) Therapy. There was No Significant Difference Between Patients With Mycosis Fungoides (MF) Before Bath-PUVA and Healthy Volunteers (Wilcoxon rank-sum test). Bath-PUVA Therapy Did Not Induce Any Significant Change in the Percentage of T-Regulatory Cells (Tregs). The Significance of Changes in the Variables Before and After Bath-PUVA Therapy was Examined Using the Wilcoxon Signed-Rank Test



PUVA is widely used as an effective treatment for cutaneous T-cell lymphoma.[25] MF and Sézary syndrome are the most frequent forms of cutaneous T-cell lymphoma. We previously reported that bath-PUVA therapy induces circulating Tregs in patients with psoriasis.[7] Tregs is a T-cell subset with immune function[26] that is associated with some immune diseases.[27] *FOXP3* is a master regulator gene for the differentiation of Tregs, and Foxp3 is a molecular marker of Tregs.[28] *FOXP3* gene transfection in naive T cells transforms naive T cells into Tregs and, simultaneously, naive T cells acquire CCR4 on their surface.[29]

In the present study, bath-PUVA therapy eliminated $Treg^+$ and $CCR4^+$ cells. Considering that there are some $CCR4^+$ cells among Tregs, the efficacy of bath-PUVA is similar to anti-CCR4 antibody treatment, as previously reported, regarding the decrease in the number of $CCR4^+$ cells.[8,12] In contrast, it was unclear that anti-CCR4 antibody was effective against $Foxp3^+$ cells. Based on the positive correlation between $CCR4^+$ and $CD3^+$cells after bath-PUVA therapy, the other type of T cells may have been eliminated.

Recently, Tregs were divided into naturally occurring Tregs and inducible Tregs.[30] Inducible Tregs are derived from the peripheral blood after antigen stimulation. In MF, Tregs are present in the initial stage, but the number of Tregs decreases in the more advanced stages.[31] This progression suggests a correlation between the number of Tregs and the prognosis of MF. Theoretically, the number of Tregs must be reduced in patients with MF. In psoriasis, bath-PUVA suppresses immunity and concomitantly induces improvement of the lesions. Immunosuppression might lead to an increase of the tumor cells in MF. In the present study, Tregs in the skin decreased after bath-PUVA. Moreover, the number of circulating Tregs in peripheral blood was not changed after bath-PUVA.

Based on these results, systemic immunosuppression is not induced by bath-PUVA therapy in patients with MF. It is generally accepted that increased Tregs in the tumor microenvironment have an important role in tumor escape from host immunity in several different types of cancer. Therefore depletion of Tregs in the tumor vicinity is considered a potential strategy for boosting antitumor immunity. In this context, the bath-PUVA therapy–induced reduction of Tregs observed in the present study may induce antitumor immunity and subsequent tumor elimination in MF skin lesions.

Clinical Practice Points

- MF is a malignant cutaneous lymphoma with a chronic disease progression.
- There are some clinical skin forms in MF according to STAGE. The tumor cells were T lymph cells, especially, it reported that CCR4 was expressed highly in the tumor cells.
- CCR4 is a chemokine receptor expressed on certain types of T cell neoplasms. In ATLL, the subsequent phase II clinical trials targeted CCR4 was started. For MF, some treatments were used such as topical steroid, phototherapy, and chemotherapy. Especially, bath-PUVA therapy was effective for the early STAGE of MF. However, the mechanism of the bath-PUVA therapy for MF was unclear.
- In the present study, bath-PUVA therapy decreased CCR4 positive cells and Treg in MF lesions, but did not induce circulating Treg, which might suppress effector T cells. Direct effects through skin lesions might eliminate both pathogenetically relevant cells and Treg.

- Considering that there are some CCR4-positive cells among Treg, the efficacy of bath-PUVA is similar to anti-CCR4 antibody treatment. Systemic immunosuppression was not induced by bath-PUVA therapy. It is generally accepted that increased Treg in the tumor microenvironment has an important role in tumor escape from host immunity in several different types of cancer.
- Based on these results, bath-PUVA therapy had possibility that widely applied for other disease.

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Disclosure

The authors have stated that they have no conflicts of interest.

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FULL PAPER

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NY-ESO-1 antibody as a novel tumour marker of gastric cancer

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Background: NY-ESO-1 antibodies are specifically observed in patients with NY-ESO-1-expressing tumours. We analysed whether the NY-ESO-1 humoral immune response is a useful tumour marker of gastric cancer.

Methods: Sera from 363 gastric cancer patients were screened by enzyme-linked immunosorbent assay (ELISA) to detect NY-ESO-1 antibodies. Serial serum samples were obtained from 25 NY-ESO-1 antibody-positive patients, including 16 patients with curative resection and 9 patients who received chemotherapy alone.

Results: NY-ESO-1 antibodies were detected in 3.4% of stage I, 4.4% of stage II, 25.3% of stage III, and 20.0% of stage IV patients. The frequency of antibody positivity increased with disease progression. When the NY-ESO-1 antibody was used in combination with carcinoembryonic antigen and CA19-9 to detect gastric cancer, information gains of 11.2% in stages III and IV, and 5.8% in all patients were observed. The NY-ESO-1 immune response levels of the patients without recurrence fell below the cutoff level after surgery. Two of the patients with recurrence displayed incomplete decreases. The nine patients who received chemotherapy alone continued to display NY-ESO-1 immune responses.

Conclusion: When combined with conventional tumour markers, the NY-ESO-1 humoral immune response could be a useful tumour marker for detecting advanced gastric cancer and inferring the post-treatment tumour load in seropositive patients.

Gastric cancer is the second most common cause of cancer-related death worldwide (Health and Welfare Statistics Association: Tokyo, 2006; Katanoda and Yako-Suketomo, 2009). Although complete removal of the tumour by surgical resection is an ideal treatment option for patients with gastric cancer, many patients with advanced-stage gastric cancer need to be treated with intensive chemotherapy. Gastric cancer patients exhibit high relapse rates even after curative surgery and unresponsiveness to chemotherapy, resulting in dismal survival rates (Sasako *et al*, 2011). Several methods for the prediction and early detection of subclinical 'minimal residual cancer' after surgery (Austrup *et al*, 2000; Klein *et al*, 2002) or relapse have been developed, for example, peritoneal lavage, positron emission tomography, gene profiling, and so on. (Motoori *et al*, 2006; Makino *et al*, 2010; Graziosi *et al*, 2011), reliable markers that can specifically reflect gastric cancer disease status have not been determined.

Analysing serum level of tumour markers is employed for cancer detection, monitoring patients' disease status, and prognosis prediction. Several organ-specific tumour markers are used in the clinic, for example, prostate-specific antigen and prostatic acid

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phosphatase for prostate cancer (Seamonds *et al*, 1986; Ferro *et al*, 1987) and protein induced by vitamin K absence-II for liver cancer (Fujiyama *et al*, 1986). As no gastric cancer-specific markers have been determined, a combination of several nonspecific tumour markers, for example, carcinoembryonic antigen (CEA), CA19-9,and so on, is merely applicable for monitoring treatment efficacy, but not the diagnosis of gastric cancer (Takahashi *et al*, 1995, 2003). Carcinoembryonic antigen and CA19-9 are found in the sera of 20–60% of gastric cancer patients, and their expression levels in gastric cancer are related to clinical events, such as relapse (Kodera *et al*, 1996). Carcinoembryonic antigen value, in particular, is indicative of the formation of a large tumour, liver or peritoneal metastasis, and/or a high risk of relapse and poor prognosis (Ikeda *et al*, 1993; Yamamoto *et al*, 2004). However, as CEA, a cell surface-anchored glycoprotein, is expressed in normal cell membranes, 5% of CEA-positive cases are pseudopositives, that is, caused by heavy smoking, endometriosis, and ageing, and so on. (Alexander *et al*, 1976), suggesting the importance of novel markers for gastric cancer.

NY-ESO-1 antigen, a cancer/testis (CT) antigen, was originally identified in oesophageal cancer by serological expression cloning using autologous patient serum and has been shown to be strongly immunogenic. Spontaneous NY-ESO-1 antibody production is often observed in patients with NY-ESO-1-expressing tumours, for example, 9.4% of melanoma patients, 12.5% of ovarian cancer patients, 7.7–26.5% of breast cancer patients, 4.2–20.0% of lung cancer patients, and 52% of prostate cancer patients, but has not been detected in non-cancerous donors (Stockert *et al*, 1998; Nakada *et al*, 2003; Türeci *et al*, 2006; Chapman *et al*, 2007; Isobe *et al*, 2009; Gati *et al*, 2011). Thus, it is possible that the NY-ESO-1 humoral immune response could be used as a serological marker for detecting these cancers and to facilitate the clinical management of some patients with particular types of cancer (Gnjatic *et al*, 2006). Jäger *et al* (1999) found that the change in the NY-ESO-1 humoral immune response reflected the overall tumour load in 10 out of 12 patients with various cancers. However, there is ongoing controversy regarding the association between the NY-ESO-1 immune response and prognostic criteria (Yuan *et al*, 2011). To address these issues in gastric cancer, we investigated the clinical usefulness of the NY-ESO-1 humoral immune response for diagnosis, monitoring, and relapse prediction in gastric cancer patients.

MATERIALS AND METHODS

Serum sample and tissue specimen collection from gastric cancer patients. In all, 363 patients with histologically confirmed gastric cancer, who underwent surgical resection or chemotherapy at one of four institutions between 2004 and 2011, were included in this study after providing written informed consent. Serum samples were obtained from the 363 patients during their admission to hospital for surgical treatment and/or chemotherapy, and afterwards, serial serum samples were obtained at each follow-up visit from 25 patients who displayed NY-ESO-1 humoral immune responses. All serum samples were collected as surplus samples after routine blood tests and stored. Fixed and frozen gastric cancer tissue samples were obtained from 60 out of 363 patients during surgery and stored. The samples were subsequently subjected to expression analysis. Information regarding blood test results, tumour stage, histological type, depth of invasion, lymph node metastasis, and distant metastasis, which were obtained from pathological examinations and CT scans, were collected from the relevant patient databases. Serum samples obtained from 50 healthy donors were used as controls. This study was approved by the institutional review boards of Osaka University Hospital, Toyonaka Municipal Hospital, Ikeda City Hospital, and Minoh City Hospital.

Reverse transcription–polymerase chain reaction. Total cellular RNA was extracted from the frozen tissue using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). The total RNA (1 μg) was subjected to the reverse transcription (RT) in 20 μl buffer with oligo-$(dT)_{15}$ primer using a RT system (Promega, Madison, WI, USA). Conventional polymerase chain reaction (PCR) was performed in a 25-μl reaction mixture containing 1 μl of cDNA template, 500 nM of each primer, and 1 U of *Taq* DNA polymerase (AmpliTaq Gold, Roche Molecular Systems, Pleasanton, CA, USA) in the following conditions: one cycle of 95 °C for 12 min; followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min; and then a final step of 72 °C for 10 min. The sequences of the primers for *NY-ESO-1* were as follows: ESO1-1, 5′-AGTTC TACCTCGCCATGCCT-3′; and ESO1-2, 5′-TCCTCCTCCAGC GACAAACAA-3′. The integrity of each RNA sample was verified by performing RT–PCR for *porphobilinogen deaminase* (*PBGD*). The PCR products were subjected to electrophoresis on a 2% agarose gel and visualised with ethidium bromide.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissues were used for the immunohistochemistry (IHC) analyses. Slides were incubated with the primary antibody overnight at 4 °C. The monoclonal antibody E978, which was previously generated by our group, was used to detect NY-ESO-1. The slides were then subjected to a heat-based antigen retrieval technique by immersing them in a preheated buffer solution (hipH solution; Dako, Carpinteria, CA, USA). A polymer-based antibody detection system (PowerVision; Leica Microsystems, Buffalo Grove, IL, USA) was used as the secondary reagent, and 3,3-diaminobenzidine tetrahydrochloride (Liquid DAB; Biogenex, San Ramon, CA, USA) was used as the chromogen. Normal adult testis tissue as a positive control and appropriate negative controls were included for each case.

Enzyme-linked immunosorbent assay. A measure of 100 μl of 1 μg ml^{-1} recombinant protein in coating buffer (pH 9.6) were added to each well of 96-well PolySorp immunoplates (Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. The plates were then washed with PBS and blocked with 200 μl per well of 5% FCS/PBS for 1 h at room temperature. After being washed again, 100 μl of serially diluted serum were added to each well and incubated for 2 h at room temperature. Then, after extensive washing, goat anti-human IgG (Medical & Biological Laboratories, Nagoya, Japan) was added to the wells as a secondary antibody, and the plates were incubated for 1 h at room temperature. The plates were washed again, and the signals were developed with 100 μl per well of 0.03% *o*-phenylene diamine dihydrochloride, 0.02% hydrogen peroxide, and 0.15 M citrate buffer, and absorbance was read at 490 nm using an enzyme-linked immunosorbent assay (ELISA) reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA, USA). Ovalbumin (OVA; Sigma, St Louis, MO, USA) was used as the control protein. Levels of NY-ESO-1 humoral response were assessed using optical density (OD) values.

CEA and CA19-9. Serum CEA and CA19-9 levels were measured at each hospital's clinical laboratory department. Carcinoembryonic antigen and CA19-9 positivity were defined as serum levels of CEA and CA19-9 of >5.0 ng ml^{-1} and >37 U ml^{-1}, respectively.

Statistical analysis. Fisher's exact test was used to assess the associations between NY-ESO-1 antibody expression and clinicopathological parameters. Kaplan–Meier curves were plotted to assess the effect of the NY-ESO-1 antibody on overall survival. Survival curves were compared using the log-rank test.

Table 1. Frequencies of NY-ESO-1 antibody, CEA, and CA19-9 in gastric cancer patients

Stage	NY-ESO-1 Ab	CEA	CA19-9	CEA and/or CA19-9	CEA and/or CA19-9 and/or NY-ESO-1 Ab
I	6/176 (3.4)	24/176 (13.6)	6/176 (3.4)	27/176 (15.3)	31/176 (17.6)
II	2/45 (4.4)	8/45 (17.8)	7/45 (15.6)	11/45 (24.4)	12/45 (26.6)
III	17/67 (25.3)	22/67 (32.9)	11/67 (16.4)	25/67 (37.3)	35/67 (52.2)
IV	16/75 (20.0)	23/75 (30.7)	30/75 (40.0)	40/75 (53.3)	46/75 (61.3)
I + II	8/221 (3.6)	32/221 (14.5)	13/221 (5.9)	38/221 (17.2)	43/221 (19.5)
III + IV	33/142 (23.2)	45/142 (31.7)	41/142 (28.9)	65/142 (45.8)	81/142 (57.0)
Total	41/363 (11.1)	77/363 (21.2)	54/363 (14.9)	103/363 (28.4)	124/363 (34.2)

Abbreviations: Ab = antibody; CA = carbohydrate antigen; CEA = carcinoembryonic antigen. Values within parentheses are percentages.

RESULTS

Determination of NY-ESO-1 humoral immune response positivity. We first determined the OD cutoff value for NY-ESO-1 humoral immune response positivity. When the serum samples from the 50 healthy donors were examined for reactivity to the NY-ESO-1 recombinant protein by ELISA, their OD values ranged from 0.08 to 0.20, and their mean and standard deviation values were 0.15 and 0.05, respectively, at a dilution of 1 : 200. Thus, NY-ESO-1 humoral immune response positivity was defined as an OD value of >0.25 at a dilution of 1 : 200 (95% accuracy level) and >3 times of the OD value against control protein (OVA).

NY-ESO-1 humoral immune responses of gastric cancer patients. Serum samples were obtained from 363 gastric cancer patients, including 176 stage I, 45 stage II, 67 stage III, and 75 stage IV patients at admission (Table 1). The NY-ESO-1 antibody was detected in 3.4% (6 of 176) of stage I, 4.4% (2 of 45) of stage II, 25.3% (17 of 67) of stage III, and 20.0% (16 of 75) of stage IV gastric cancer patients, resulting in an overall detection rate of 11.1% (41 of 363). An analysis of the gastric cancer patients' characteristics found that NY-ESO-1 antibody positivity was significantly correlated with gender (male > female) and tumour progression (Table 2). In particular, the patients with progressive gastric cancer involving deeper tumour invasion, positive lymph node metastasis, positive distant metastasis, or a higher clinical stage tended to produce the NY-ESO-1 antibody.

Analysis of NY-ESO-1 antigen expression. *NY-ESO-1* mRNA and NY-ESO-1 protein expression were analysed by RT–PCR and IHC, respectively, in gastric cancer tissues obtained from 60 patients for whom both frozen and formalin-fixed specimens were available, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients (Table 3). *NY-ESO-1* mRNA was detected in six specimens. NY-ESO-1 was immunohistochemically detected in 19 specimens, including 6 and 13 that were positive and negative for *NY-ESO-1* mRNA, respectively. Most of the specimens displayed a heterogeneous staining pattern (data not shown).

NY-ESO-1 antibody and antigen expression. We analysed the frequency of NY-ESO-1 antibody positivity in gastric cancer patients in whom NY-ESO-1 antigen expression was or was not detected by RT–PCR or IHC. As shown in Table 3, 9 out of the 60 gastric cancer patients whose specimens were available for expression analysis possessed the NY-ESO-1 antibody in their sera. The NY-ESO-1 antibody was detected in 8 of 19 (42.1%) patients with IHC-positive gastric cancer and 5 of 6 (83.3%) patients with RT–PCR (and IHC)-positive gastric cancer, whereas only 1 of 41 patients in whom both RT–PCR and IHC analysis produced negative results displayed an NY-ESO-1 humoral immune responses.

Table 2. Relationship between NY-ESO-1 antibody positivity and clinicopathological features in gastric cancer patients

Variable	NY-ESO-1 Ab		*P*-value*
	Negative	Positive	
Gender			
Male	223 (86.4)	35 (13.6)	0.04307
Female	99 (94.3)	6 (5.7)	
Age (years)			
>65	178 (88.6)	23 (11.4)	0.9209
<65	144 (88.9)	18 (11.1)	
Histological type			
Differentiated	143 (89.4)	17 (10.6)	0.5605
Undifferentiated	132 (87.4)	19 (12.6)	
Depth of tumour invasion			
cT1–T2	193 (92.8)	15 (7.2)	0.0044
cT3–T4	129 (83.2)	26 (16.8)	
Lymph node metastasis			
Negative	196 (97.0)	6 (3.0)	<0.001
Positive	126 (78.3)	35 (21.7)	
Distant metastasis			
Negative	277 (91.1)	27 (8.9)	<0.001
Positive	45 (76.3)	14 (23.7)	
Stage			
I–II	213 (96.4)	8 (3.6)	<0.001
III–IV	109 (76.8)	33 (23.2)	

Abbreviations: Ab = antibody. Fisher's exact test was used for the statistical analysis. Values within parentheses are percentages.

Frequencies of NY-ESO-1 humoral immune responses and conventional tumour markers in gastric cancer patients. The frequency of the NY-ESO-1 humoral immune response was compared with those of conventional tumour markers in gastric

Table 3. Frequency of NY-ESO-1 antibody positives in gastric cancer patients in whom the NY-ESO-1 antigen was or was not detected by IHC or RT–PCR

	IHC		
	Positive	**Negative**	**Total**
mRNA			
Positive	5/6 (83.3)	0/0 (0.0)	5/6 (83.3)
Negative	3/13 (23.1)	1/41 (2.4)	4/54 (7.4)
Total	8/19 (42.1)	1/41 (2.4)	9/60 (15.0)

Abbreviations: IHC = immunohistochemistry; RT–PCR = reverse transcription–polymerase chain reaction. Frozen and formalin-fixed tissue specimens from 60 patients, including 12 stage I, 12 stage II, 20 stage III, and 16 stage IV patients, were analysed. All stage IV patients had previously undergone surgical treatment. Values within parentheses are percentages.

cancer patients. The serum CEA and CA19-9 levels of 363 gastric cancer patients were measured at admission (Table 1). Carcinoembryonic antigen and CA19-9 positivity were observed in 21.2% (77 of 363) and 14.9% (54 of 363) of the gastric cancer patients, respectively, and, except for CA19-9 in the stage III patients, they displayed higher frequencies than the NY-ESO-1 humoral immune response in all stages of the disease. We then analysed whether the addition of the NY-ESO-1 humoral immune response to CEA and CA19-9 increased the diagnostic frequency of gastric cancer. The combined use of CEA and CA19-9 tests produced positivity rates of 15.3% (27 of 176) in stage I, 24.4% (11 of 45) in stage II, 37.3% (25 of 67) in stage III, and 53.3% (40 of 75) in stage IV gastric cancer patients, resulting in an overall positivity rate of 28.4% (103 of 363). When the NY-ESO-1 humoral immune response was added to these two conventional tumour markers, the positivity rates of all stages increased, resulting in information gains of 14.9% (from 25 to 35 patients; 10 of 67) in stage III and 11.2% (from 65 to 81 patients; 16 of 142) in stage III and IV gastric cancer patients.

Changes in the NY-ESO-1 humoral immune responses of the patients during their clinical courses. Serial serum samples were obtained from 25 gastric cancer patients who displayed positive NY-ESO-1 antibody at admission, and the changes in their NY-ESO-1 humoral immune responses were examined throughout their clinical courses. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgical treatment, and 14 did not suffer recurrence. The NY-ESO-1 immune response levels of the patients who did not suffer recurrence decreased after treatment and had fallen below the cutoff level by 9 months after surgery in most cases and did not subsequently increase (Figure 1). The half-lives of their NY-ESO-1 humoral immune response levels were 1.5, 1.6, 2.1, 3.2, and 6.6 months in the stage I patients; 3.0 and 4.0 months in the stage II patients; and 1.6, 1.9, 2.3, 3.0, 3.2, 4.1, and 6.7 months in the stage III patients (mean: 3.0 months). On the other hand, the two patients who underwent curative surgery but subsequently suffered recurrence, M-2 (stage I) and M-11 (stage III), displayed not only incomplete decreases in their NY-ESO-1 humoral immune response levels but also their subsequent restoration to pretreatment levels (Figure 1 and Figure 2A and B). In a comparison between the patients' conventional tumour marker levels and their NY-ESO-1humoral immune response levels, we found that the changes in their CEA and CA19-9 levels were consistent with their NY-ESO-1 immune response levels in patient M-2, whereas patient M-11 was negative for both CEA and CA19-9 throughout their clinical course. Nine stage IV patients who received chemotherapy alone maintained high NY-ESO-1 humoral immune response levels throughout their clinical courses,

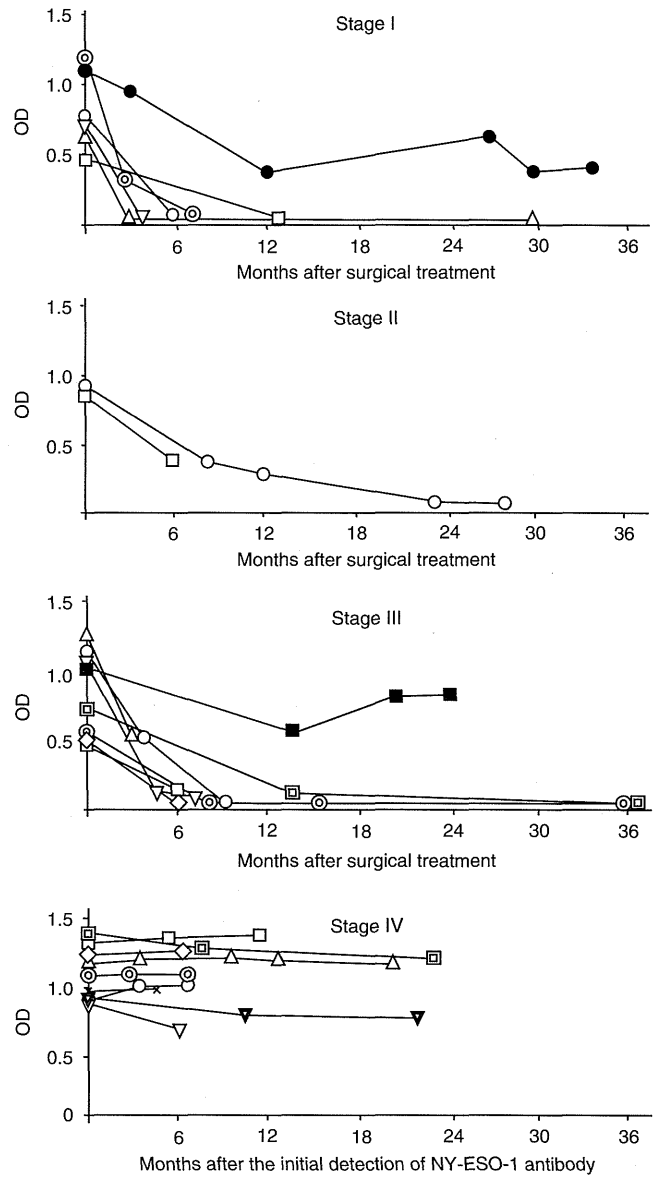

Figure 1. Change in the NY-ESO-1 humoral immune responses of gastric cancer patients after treatment. The serum NY-ESO-1 humoral immune responses of patients with stage I, II, III, or IV gastric cancer in whom NY-ESO-1 antibody production was detected before surgical treatment or chemotherapy were serially analysed. In all, 6 stage I, 2 stage II, and 8 stage III patients received curative surgery, and only 2 patients (●, ■) suffered recurrence. Other 14 patients did not suffer recurrence. Nine patients with stage IV gastric cancer received chemotherapy alone after the initial detection of NY-ESO-1 antibody. Each mark represents a patient. Optical density (OD) values were measured at a serum dilution of 1 : 200.

including some patients who achieved partial tumour responses after chemotherapy (Figure 1).

Prognostic value of the NY-ESO-1 humoral immune response in gastric cancer. The prognostic value of the NY-ESO-1 immune response was evaluated in gastric cancer patients. An analysis of the cumulative overall survival of the gastric cancer patients indicated that there was no difference in the survival rates of the patients who did and did not display positive NY-ESO-1 humoral immune responses (Figure 3A). However, among the patients with higher stage gastric cancer, overall survival was better in the patients in whom NY-ESO-1humoral immune responses were

detected, although the difference was not significant (Figure 3B). NY-ESO-1 protein expression, as detected by IHC, did not affect the overall survival rate (data not shown).

DISCUSSION

NY-ESO-1 antibody was detected in 23.2% of stage III and IV gastric cancer patients, and the combinatorial use of the NY-ESO-1 antibody with CEA and CA19-9 as tumour markers increase the percentage of tumour detection from 45.8 to 57.0%. As the frequency of NY-ESO-1 humoral immune response was relatively low in the patients with early-stage gastric cancer, analysing serum NY-ESO-1 antibody levels alone might not be useful for screening for early-stage gastric cancer. Nevertheless, the expression of NY-ESO-1, a CT antigen, is restricted to tumour tissues and NY-ESO-1 antibody is only detectable in patients with NY-ESO-1-expressing tumours (Stockert *et al*, 1998), indicating the highly specific nature of NY-ESO-1 humoral immune responses in cancer patients. Given that NY-ESO-1 expression by malignant cells is required for antibody induction (Stockert *et al*, 1998), the detection of NY-ESO-1 antibody would be helpful for diagnosing malignancy, although extensive analysis of serum samples from patients with non-cancerous disease, for example, liver or renal disorders, autoimmune diseases, and so on, would be necessary to confirm. In our expression analysis, more NY-ESO-1-positive cases were detected by IHC (19 of 60) than by RT–PCR (6 of 60). This was probably due to the heterogeneous expression of NY-ESO-1 in gastric cancer and the fact that a limited number of biopsy samples were used for the RT–PCR, whereas multiple slices from whole tumour specimens were used for the IHC. Extensive IHC analysis should be used for NY-ESO-1 expression studies of gastric cancer.

We detected a correlation between the NY-ESO-1 humoral immune response levels and the clinical outcome after therapy in gastric cancer patients. The patients who underwent surgery and did not suffer a subsequent relapse displayed consistent decreases in their NY-ESO-1 humoral immune response levels or even the complete disappearance of the NY-ESO-1 antibody from their sera. It is generally accepted that constant immunological stimulation is necessary to maintain a strong humoral immune response (Jager *et al*, 1999). Thus, reduction of antigen doses by the removal of NY-ESO-1-expressing tumour is one possible reason for the observed decreases in these patients' NY-ESO-1 humoral immune response levels after surgery. Patients M-2 and M-11, in whom NY-ESO-1 humoral immune responses remained high for 1 year after surgery and increased thereafter, may have a subclinical residual disease of the so-called 'minimal residual cancer' (Austrup *et al*, 2000; Klein *et al*, 2002) after curative surgery. Local recurrent tumours of 23 and 25 mm in diameter subsequently developed in M-2 and M-11, respectively, suggesting that even a small tumour burden is sufficient to stimulate antibody production. Patient M-2 showed a partial decrease in their NY-ESO-1 humoral immune response levels after the resection of the relapsed tumour, and we are carefully observing the progression of this tumour.

Nine patients with stage IV gastric cancer received chemotherapy alone. Among them, six patients displayed stable disease, two

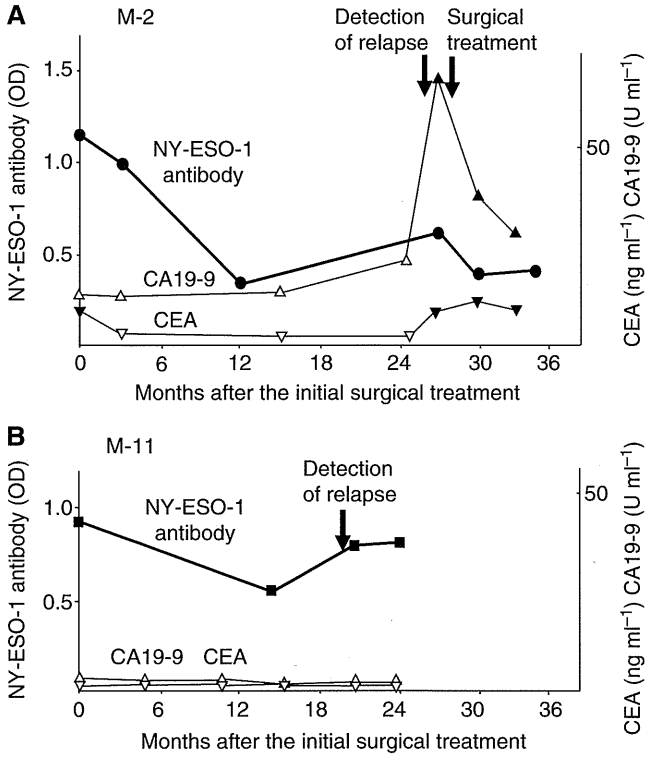

Figure 2. NY-ESO-1 humoral immune response, CEA, and carbohydrate antigen (CA)19-9 levels of patients who relapsed after curative surgery. The NY-ESO-1 humoral immune response (●, ■; Figure 1), CEA (▽), and CA19-9 (△) levels of two patients, M-2 (stage I) (**A**) and M-11 (stage III) (**B**), who underwent curative surgery but subsequently suffered recurrence, were serially analysed. OD values were measured at a serum dilution of 1 : 200. The closed marks indicate CEA or CA19-9 positivity.

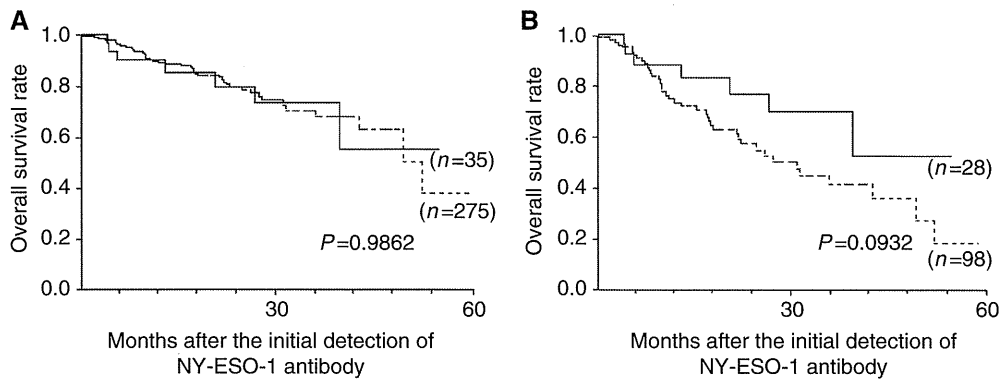

Figure 3. Prognostic role of NY-ESO-1 antibody in gastric cancer patients. The cumulative overall survival rate was analysed in all patients ($n=310$; **A**) and stage III and IV ($n=126$; **B**) gastric cancer patients in whom NY-ESO-1 antibodies were (continuous line) and were not detected (dotted line). The detection of NY-ESO-1 protein by IHC analysis did not affect the overall survival rate (data not shown). Survival curves were plotted using the Kaplan–Meier method. The log-rank test was used for comparisons between groups. *P*-values <0.05 were considered significant.

patients displayed progressive disease, and one patient (M-19) achieved a partial response. Serial analysis of the NY-ESO-1 humoral immune responses of these nine patients including M-19 showed that they barely changed throughout their clinical courses, suggesting that even small tumours are enough to provoke strong NY-ESO-1 humoral immune responses. In this regard, the NY-ESO-1 humoral immune response might not be suitable as a clinical marker for palliative therapy.

We have performed serial cancer vaccine clinical trials with NY-ESO-1 because of its strong immunogenicity and high specificity (Uenaka *et al*, 2007; Wada *et al*, 2008; Kakimi *et al*, 2011). The NY-ESO-1humoral immune response could be a reliable marker of the induction of immune response, as well as for predicting clinical responses in these trials. Furthermore, antibody-based examinations detected both intra- and intermolecular antigen spreading in the sera of patients who had been vaccinated with NY-ESO-1 protein (Kawada *et al*, 2012), suggesting the possible correlation of NY-ESO-1 humoral immne responses and clinical status. In addition, we have started a phase I study of vaccination with NY-ESO-1 protein mixed with Hiltonol (Poly ICLC), Picibanil (OK-432), and Montanide (ISA-51) in patients with NY-ESO-1-expressing cancers (UMIN000007954). Furthermore, NY-ESO-1 vaccine involving modulators of immune checkpoints, for example, anti-CTLA4 antibody and anti-PD-1 antibody, and reagents that are antagonistic to regulatory T cells, for example, anti-CCR4 antibody (Pardoll, 2012) should be considered.

Recently, the antibody against p53, another tumour antigen, has been recognised as a useful tumour marker (Lubin *et al*, 1995). Shimada *et al* (2000)) reported that p53 antibody was detected in 35% of serum samples from patients with *in situ* oesophageal cancer and that it disappeared after endoscopic mucosal resection, proposing that p53 antibody is useful for the early detection and subsequent monitoring of oesophageal cancer. In addition, Müller *et al* (2006) reported that p53 antibody was found in 23.4% of serum samples from cancer patients with 100% accuracy and was correlated with poor prognosis in hepatocellular carcinoma and breast cancer.

Here, we have demonstrated that the NY-ESO-1 humoral immune response could also be valuable as a marker for detecting advanced gastric cancer and inferring whether residual tumour cells remain after treatment, although its frequency in gastric cancer is not very high. We have started a prospective multi-institutional clinical study of NY-ESO-1 humoral immune responses in higher stage gastric cancer patients. In this new study, the NY-ESO-1 humoral immune responses of approximately 100 patients who relapsed after curative surgery will be serially analysed and then followed up. This trial has been registered as UMIN000007925 in Japan.

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Spontaneous antibody, and CD4 and CD8 T-cell responses against XAGE-1b (GAGED2a) in non-small cell lung cancer patients

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The spontaneous immune responses against XAGE-1b (GAGED2a) were analyzed in non-small cell lung cancer (NSCLC) patients. An antibody response against XAGE-1b (GAGED2a) was observed in 10% (20/200) of NSCLC patients and in 19% (13/69) of stage IIIB/IV lung adenocarcinoma patients. A CD4 T-cell response was detected in 88% (14/16) and a CD8 T-cell response in 67% (6/9) in the XAGE-1b (GAGED2a) antibody-positive patients examined. Frequent antibody responses and CD4 and CD8 T-cell responses in XAGE-1b (GAGED2a) antibody-positive patients indicate the strong immunogenicity of the XAGE-1b (GAGED2a) antigen in NSCLC patients. We established T-cell clones from PBMCs of antibody-positive patients and determined the DRB1*04:05-restricted XAGE-1b (GAGED2a) 18–31 peptide (14-mer) as a CD4 T cell epitope and the A*02:06-restricted XAGE-1b (GAGED2a) 21-29 peptide (9-mer) as a CD8 T cell epitope. As for peptide recognition, CD4 and CD8 T-cell clones responded to naturally processed antigen. The CD4 T-cell clone recognized DCs pulsed with the synthetic protein or a lysate from XAGE-1b-transfected 293T cells. The CD8 T-cell clone showed cytotoxicity against a tumor expressing XAGE-1b (GAGED2a) and the appropriate HLA class I allele. These findings establish XAGE-1b (GAGED2a) as a promising target for a lung cancer vaccine.

More than 70 cancer/testis (CT) antigen gene families have been identified by immunological or genetic approaches.[1–3] Several CT antigens such as the NY-ESO-1 antigen *etc.* have been shown to elicit humoral and cellular immune responses in cancer patients.[4,5] Because of their restricted expression in normal tissues and high immunogenicity, CT antigens are considered attractive targets for cancer vaccines.[6–9]

XAGE-1 was originally identified by the search for *PAGE/GAGE*-related genes using an expression sequence tag database[10] and was shown to exhibit CT antigen characteristics.[11,12] Five identical genes *XAGE1A to E* have now been identified, located in dispersed fashion in different orientations in a region of approximately 350 kilobases on chromosome Xp11.22.[13] They belong to X antigen family genes. The associated protein is designated as G antigen family D member 2 (GAGED2), and GAGED2a and d isoforms have been identified.[10,13] Four transcript variants *XAGE-1a, b, c* and *d* have been extensively studied and were shown to be expressed in metastatic melanoma, Ewing sarcoma, and various epithelial tumors such as breast, lung and prostate cancers.[14–17] In a serologic search for antigens using recombinant expression cloning (SEREX), we identified XAGE-1b as a dominant antigen recognized by serum from a lung adenocarcinoma patient using an autologous tumor cell line established from malignant pleural effusion as a source of the cDNA library.[18] From the analysis with transfected 293T cells using a USO 9–13 mAb specific for XAGE-1b (GAGED2a) protein, we showed that the *XAGE-1a* and *b* transcripts code for the 81 amino acid XAGE-1b (GAGED2a) protein.[19] The *XAGE-1c* transcript codes for 9- and 17-a.a. peptides from an alternative reading frame. The *XAGE-1d* transcript codes for a protein consisting of 69 amino acids (GAGED2d).

Key words: cancer/testis antigen, XAGE-1b (GAGED2a), non-small cell lung cancer, antibody and T-cell responses, cancer vaccine
Abbreviations: CLSM: confocal laser scanning microscopy; CT: cancer/testis; DAPI: 4′,6-diamidino-2-phenylindole; HRP: horseradish peroxidase; IHC: immunohistochemistry; NSCLC: non-small cell lung cancer; OLP: overlapping peptides; PHA: phytohemagglutinin
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In relation to clinical relevance, we showed that XAGE-1b (GAGED2a) expression by itself had no correlation with overall survival in non-small cell lung cancer (NSCLC) patients.[20] However, both XAGE-1b (GAGED2a) and HLA class I expression correlated with prolonged survival. Moreover, expression of XAGE-1b combined with down-regulated HLA class I expression correlated with even worse survival. These findings suggested that XAGE-1b (GAGED2a) and HLA expression elicited a T-cell response against tumors and resulted in prolonged survival.

In this study, we investigated spontaneous antibody response, and CD4 and CD8 T-cell responses, against XAGE-1b (GAGED2a) in NSCLC patients. We showed a high frequency of antibody responses in this patient population. CD4 and CD8 T-cell responses were detected in most of the antibody-positive patients. Furthermore, we determined CD4 and CD8 T-cell epitopes using cloned T-cell lines. A CD4 T-cell clone recognized naturally processed antigen on DCs pulsed with the synthetic protein or a lysate from *XAGE-1b*-transfected 293T cells, and a CD8 T-cell clone showed cytotoxicity against a tumor cell line expressing XAGE-1b (GAGED2a) and the appropriate HLA class I allele.

Material and Methods

Blood samples

Peripheral blood was drawn from lung cancer patients and healthy donors after obtaining written informed consent at Kawasaki Medical School Hospital from 2005 to 2010. Sera were obtained from 200 patients with NSCLC including 118 adenocarcinomas, 44 squamous cell carcinomas, six pleiomorphic carcinomas, four adenosquamous carcinomas, one large cell carcinoma and 27 unclassified. Sera were also obtained from 50 healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation using a Histo-paque 1077 (Sigma–Aldrich, St. Louis, MO). CD4-, CD8- and CD19-positive cells were purified by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). The residual cells were kept for use as antigen presenting cells (APCs). The cells were stored in liquid N_2 until use. HLA typing was done with PBMCs by a sequence-specific oligo-nucleotide probe and sequence-specific priming of genomic cDNA using a standard procedure.

Cell lines

OU-LC-KI, OU-LC-SK, OU-LC-ON, PC-9 and 1-87 were lung adenocarcinoma cell lines.[21] RERF-LC-AI was a lung squamous cell carcinoma cell line. HEK293T was a human embryonic kidney cell line. OU-LC-KI, OU-LC-SK and OU-LC-ON were established in our laboratory. RERF-LC-AI, 1-87 and HEK293T were obtained from RIKEN (Riken Bioresource Center, Ibaragi, Japan). These cell lines were kept in tissue culture by serial passage. Epstein-Barr virus (EBV)-B cells were generated from CD19-positive peripheral blood B cells using a culture supernatant from EBV-producing B95-8 cells. The medium used to maintain these cell lines was RPMI1640 supplemented with 2 mmol/L Glutamax, antibiotics, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Invitrogen, Carlsbad, CA) and 10% fetal calf serum (FCS) (JRM, Bioscience, Lenexa, KA). An HEK293T transfectant was generated by introducing pcDNA 3.1/Zeo (+) containing *XAGE-1b* cDNA using LipofectAMINE 2000 (Invitrogen).

Antibodies

Anti-human CD4, anti-human CD8, anti-HLA pan class I and anti-HLA pan class II mAbs were purchased from BD Bioscience (San Jose, CA).

Overlapping peptides

The following series of 17 16-mer overlapping XAGE-1b (GAGED2a) peptides spanning the entire protein 1-16, 5-20, 9-24, 13-28, 17-32, 21-36, 25-40, 29-44, 33-48, 37-52, 41-56, 45-60, 49-64, 53-68, 57-72, 61-76 and 65-81 were synthesized using Fmoc chemistry on a Multiple Peptide Synthesizer (AMS422, ABIMED, Langenfeld, Germany) at Okayama University.

Synthetic XAGE-1b (GAGED2a) protein

XAGE-1b (GAGED2a) protein (81 amino acids) was synthesized using a peptide synthesizer by GL Biochemistry (Shanghai, China).

Reverse transcription (RT)-PCR

Total RNA was obtained from cells using an RNeasy Mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Two micrograms of each sample was subjected to cDNA synthesis using Ready-To-Go first strand beads kit (GE Healthcare, Tokyo, Japan). Sequences of primer pairs for *XAGE-1b* were X-1, 5′-TTTCTCCGCTACTGAGACAC-3′ and X-2, 5′-CAGCTTGCGTTGTTTCAGCT-3′, and sequences for *G3PDH* were G3PDH-S, 5′-ACCACAGTCCATGC CATCAC-3′, G3PDH-AS, 5′-TCCACCACCCTGTTGCTG TA-3′. The amplification was performed using 30 cycles as described.[22]

Western blot analysis

The XAGE-1b (GAGED2a) antigen in the cell lysate was immunoprecipitated using USO 9-13 mAb and the antigen/antibody complex was purified using protein G Sepharose beads. The beads were washed with a lysis buffer [500 nM HEPES (pH7.9), 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid, 1% Triton X-100, 100 μM 4-(2-aminoethyl)-benzensulfonyl fluoride, 1 μg/mL leupeptin and pepstatin A], and the immune complex was dissolved in a sample loading buffer [250 mM Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate (SDS), 20% glycerol and 5% β-mercaptoethanol]. The sample was heated at 95°C for 5 min and then separated in a 10–20% gradient gel by SDS-PAGE. The materials in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was treated with a membrane-blocking reagent (GE Healthcare) and then incubated with USO 9-13 mAb at room temperature for 2 hr. After washing, the

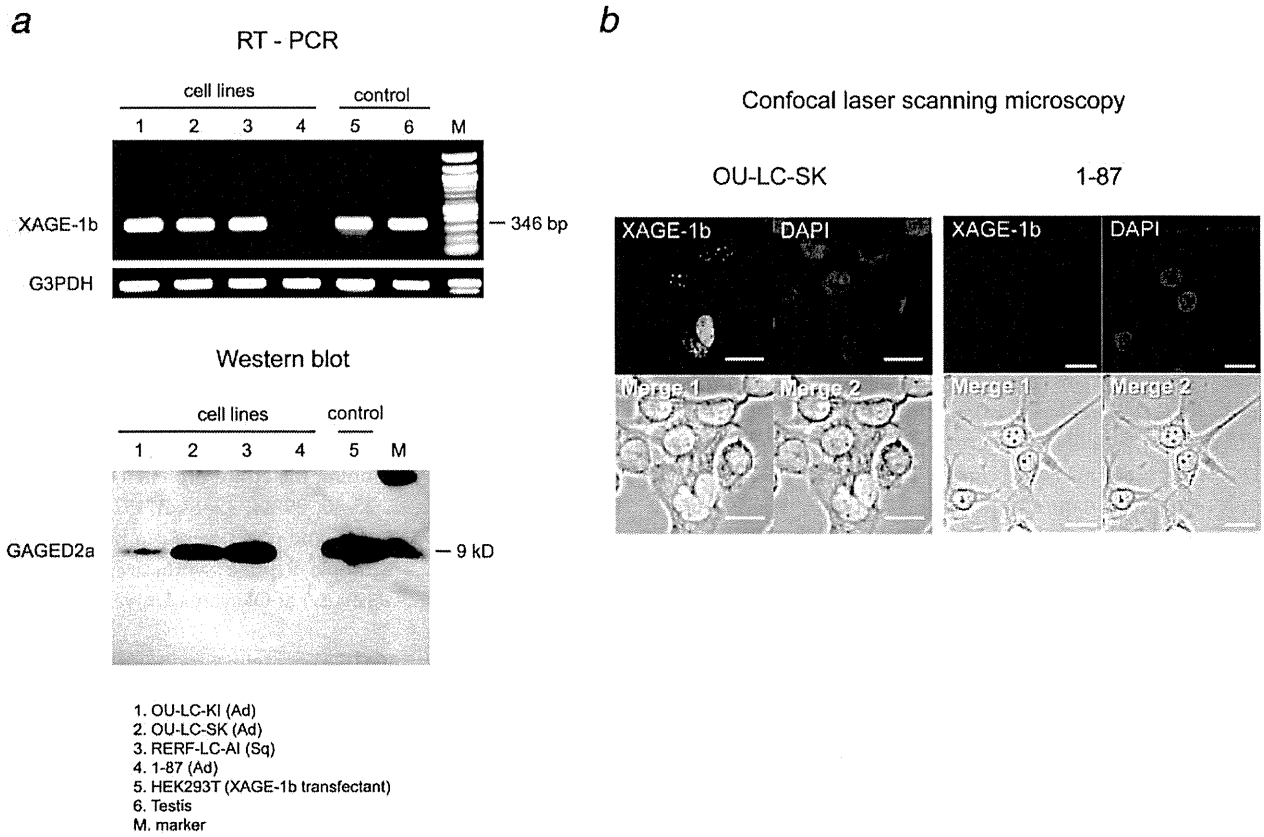

Figure 1. RT-PCR and Western blot analyses (*a*) and CLSM (*b*) for XAGE-1b (GAGED2a) expression in lung cancer cell lines. OU–LC–KI, OU–LC–SK and 1–87 are lung adenocarcinoma (Ad) cell lines. RERF-LC-AI is a lung squamous cell carcinoma (Sq) cell line. In RT-PCR, G3PDH was used as the internal control. In Western blot, the cell lysate was first incubated with USO 9-13 mAb and the antigen/antibody complex was purified using protein G Sepharose beads. The eluate was run on the gel. The pcDNA3.1/XAGE-1b-transfected HEK293T cell lysate was used as a control. In CLSM, the cells were stained with USO 9–13 mAb and FITC-conjugated goat anti-mouse IgG (green). Nuclei are stained with DAPI (blue). XAGE-1b (GAGED2a) derived fluorescence is merged with differential interference contrast (Merge 1) and DAPI (Merge 2). Scale bar indicates 20 μm. HLA expression of the cell lines was A*02:01, *02:06; B*39:01, *51:01; C*03:04, *14:02 for OU–LC–KI: A*24:02, -; B*51:01, -; C*14:02, - for OU–LC–SK: A*24:02, -; B*52:01, -; C*12:02, - for RERF-LC-AI: A*02:07, *11:01; B*46:01, *54:01; C*01:02, - for 1–87. No expression of HLA class II antigens was observed in these cell lines.

membrane was incubated with a peroxidase-conjugated second antibody (MBL, Nagoya, Japan) and the bands were visualized using an ECL plus Western Blotting Detection System (GE Healthcare).

Confocal laser scanning microscopy

Cells were fixed in ethanol (Wako Pure Chemical Industries, Osaka, Japan) and permeabilized with a 0.1% Tween 20/5% FCS/phosphate buffered saline (PBS) buffer. The cells were then stained with USO 9-13 mAb and FITC-conjugated goat anti-mouse IgG (Sigma–Aldrich). For intracellular localization, 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) was used. The stained cells were visualized under a confocal laser scanning microscope (CLSM) (Model Fluoview FV10i for the magnification of 60×, Olympus, Tokyo, Japan).

ELISA to detect the XAGE-1b (GAGED2a) antibody

Synthetic XAGE-1b (GAGED2a) protein (1 μg/mL) in a coating buffer was adsorbed onto a 96-well ELISA plate (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. Plates were washed with PBS and blocked with 5% FCS/PBS (200 μL/well) for 1 hr at 37°C. After washing, 100 μL of serially diluted serum was added to each well and incubated for 2 hr at 4°C. After washing, horseradish peroxidase (HRP)-conjugated goat anti-human IgG (MBL) was added to the wells, and the plates were incubated for 1 hr at 37°C. After washing and development, absorbance was read at 490 nm.

In vitro stimulation of CD4 and CD8 T-cells

CD4 (2×10^6/well) and CD8 (1×10^4/well) T-cells were cultured on a micro-culture plate and a 96-well culture plate (BD Bioscience), respectively, with an equal number of irradiated (40 Gy), autologous CD4- and CD8-depleted cells as APCs in

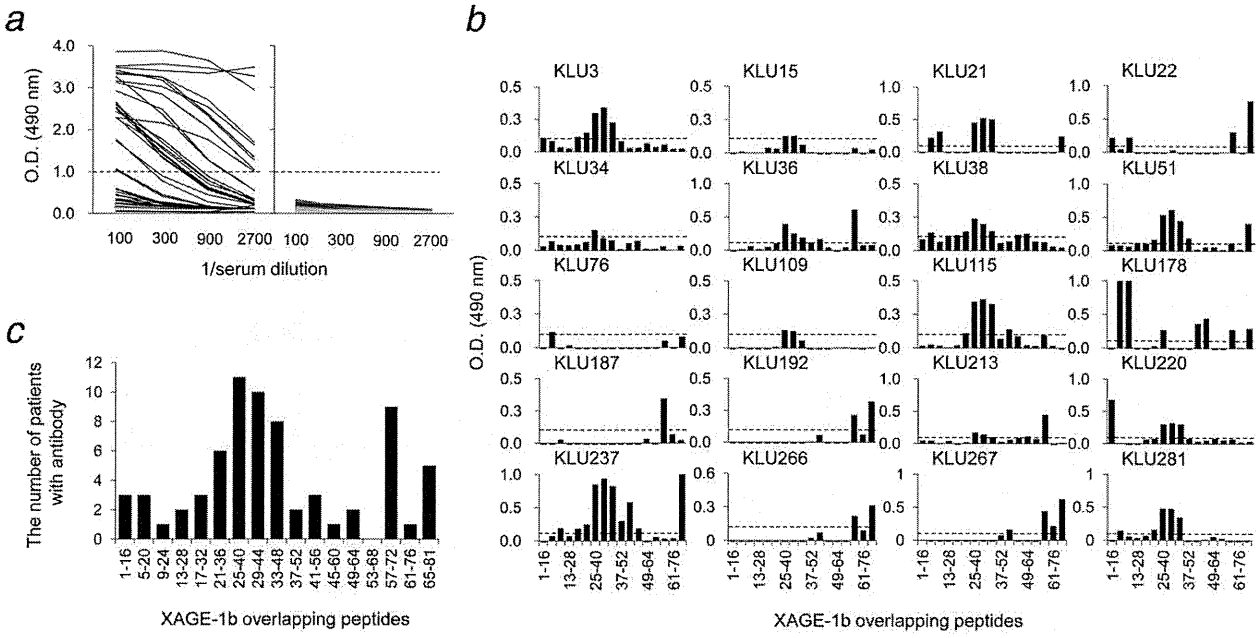

Figure 2. Antibody response against XAGE-1b (GAGED2a) in non-small cell lung cancer patients. (*a*) ELISA against synthetic XAGE-1b (GAGED2a) protein of serially diluted sera from 200 non-small cell lung cancer patients (left) and 50 healthy donors (right). O.D. values exceeding 1.0 were considered positive. (*b*) ELISA against 17 16-mer XAGE-1b (GAGED2a) overlapping peptides (OLPs) (5 μg/mL) of sera from 20 antibody-positive patients in *a* at 1:100 serum dilution. O.D. values exceeding 0.1 were considered positive. (*c*) The number of antibody-positive patients for individual 17 16-mer XAGE-1b overlapping peptides.

the presence of a mixture of 17 16-mer overlapping peptides (10^{-6} M) for 10–14 days at 37°C in a 5% CO_2 atmosphere. The medium was AIM-V (Invitrogen) supplemented with 5% heat-inactivated pooled human serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10 units/mL recombinant IL-2 (Takeda Chemical Industries, Osaka, Japan) and 10 ng/mL recombinant IL-7 (Peprotech, London, UK).

Establishment of CD4 and CD8 T-cell clones

CD4 and CD8 T-cells were cloned by limiting dilution after one or two *in vitro* stimulations in round-bottomed 96-well plates in the presence of irradiated (40 Gy), allogeneic PBMCs as feeder cells. The medium used was AIM-V (Invitrogen) supplemented with 5% heat-inactivated pooled human serum, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10 units/mL recombinant IL-2 (Takeda Chemical Industries), 10 ng/mL recombinant IL-7 (Peprotech) and 1 μg/mL phytohemagglutinin-L (PHA) (Sigma–Aldrich).

Preparation of dendritic cells (DCs)

Monocytes were isolated from PBMCs using anti-CD14 mAb-coated magnetic beads (Miltenyi Biotec) and cultured in AIM-V medium supplemented with 5% heat-inactivated pooled human serum, 10 ng/mL recombinant human (rh) granulocyte-macrophage colony-stimulating factor (GM-CSF) (Kyowa Hakko Kirin, Tokyo, Japan) and 10 ng/mL rhIL-4 (PeproTech) for 10 days at 37°C in a 5% CO_2 atmosphere.

Interferon (IFN) γ ELISA

Responder CD4 or CD8 T-cells (1×10^4) from a stimulation culture were cultured with EBV-B cells (1×10^4) pulsed with overlapping peptide (OLPs) in a 96-well round-bottomed culture plate for 24 hr at 37°C in a 5% CO_2 atmosphere. Culture supernatants were then collected and the amount of INFγ was measured by sandwich ELISA. For antibody blocking experiments, each mAb (5 μg/mL) was added to the assay culture.

IFNγ capture assay

Responder CD4 or CD8 T-cells (5×10^4) from the stimulation culture were cultured with autologous EBV-B cells (5×10^4) pulsed with OLPs for 4 or 8 hr, respectively. The cells were then treated with a bi-specific CD45 and IFNγ antibody (IFNγ catch reagent) (2 μL) for 5 min on ice. The cells were diluted in AIM-V medium (3 mL) and placed on a slow rotating device (Miltenyi Biotec) to allow IFNγ secretion at 37°C in a 5% CO_2 atmosphere. After incubation for 1 hr, the cells were washed with cold buffer and treated with PE-conjugated anti-IFNγ (detection reagent), and FITC-conjugated anti-CD4 or anti-CD8 mAb. After incubation for 10 min at 4°C, the cells were washed and analyzed by FACS Calibur or Canto II (BD Bioscience).

Cytotoxicity assay

Cytotoxicity was assayed by a luminescent method using a aCella-Tox kit (Cell Technology, Mountain View, CA).