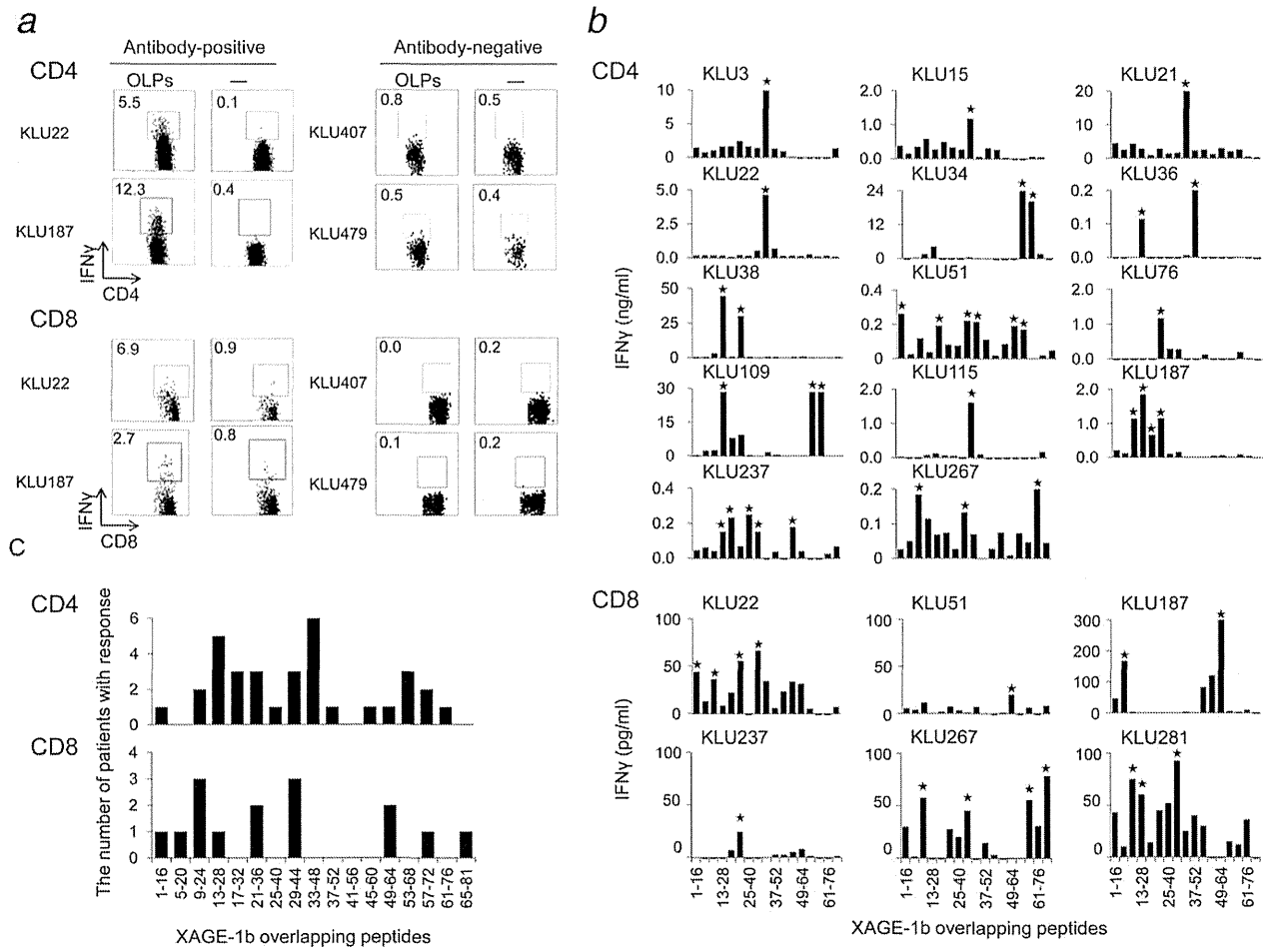

Figure 3. CD4 and CD8 T-cell responses against XAGE-1b (GAGED2a). (*a*) IFNγ capture assays. Purified CD4 and CD8 T-cells from PBMCs of antibody-positive (KLU22 and KLU187) or -negative (KLU407 and KLU479) patients were cultured with an equal number of irradiated (40 Gy), autologous CD4- and CD8-depleted PBMCs as APCs in the presence of 17 16-mer OLPs (1 μM) spanning the entire XAGE-1b (GAGED2a) protein using a micro-culture plate for CD4 T-cells (2×10^6 per well) and a 96-well culture plate for CD8 T-cells (1×10^4 per well) for 10–14 days. IFNγ secretion by CD4 and CD8 T-cells (5×10^4) was assayed against autologous EBV-B cells (5×10^4) pulsed with XAGE-1b (GAGED2a) OLPs after incubation for 4 hr in CD4 and for 8 hr in CD8. HLA types of the antibody-positive patients KLU22 and KLU187 are shown in Table 1. Those of the antibody-negative patients KLU407 and KLU479 are DRB1*15:02, -; DQB1*06:01, -; DPB1*09:01, -; A*24:02, -; B*52:01, -; C*12:02, - for KLU407: DRB1*08:03, *15:01; DQB1*06:01, *06:02; DPB1*02:01, 05:01; A*02:06, 02:07; B*39:01, *46:01; C*01:02, 07:02 for KLU479. *XAGE-1b* mRNA expression in a tumor specimen was positive in KLU407 and negative in KLU479. (*b*) The peptide regions recognized by CD4 and CD8 T-cells from antibody-positive patients. The culture supernatant of T-cells (1×10^4) stimulated with autologous EBV-B cells (1×10^4) in the presence of individual OLPs (5 μg/mL) for 24 hr was assayed by ELISA for IFNγ secretion. Predominant responses are marked by a star. HLA types of the patients are shown in Table 1. (*c*) The number of patients with CD4 and CD8 T-cell responses for individual 17 16-mer OLPs.

Effector cells were incubated with 5,000 target cells at various ratios in 96-well round-bottomed culture plates for 12 hr at 37°C in a 5% CO_2 atmosphere. The plate was read by a luminometer (multi-detection microplate reader, DS Pharma, Osaka, Japan).

Statistical analysis

All values are expressed as the mean ± S.D. of individual samples. Samples were analyzed using the Student's *t*-test for two groups and ANOVA for multiple groups using IBM SPSS Statistics 19 for Windows (IBM, New York, NY). Values of $p < 0.05$ were considered significant.

Results

XAGE-1b (GAGED2a) expression in lung cancer cell lines

We previously showed that XAGE-1b (GAGED2a) was expressed predominantly in lung adenocarcinoma and rarely observed in esophagus, stomach, liver, colon or breast cancer.[18,19,22] In normal adult tissues, XAGE-1b (GAGED2a) was expressed only in the testes. In this study, we investigated XAGE-1b (GAGED2a) expression in lung cancer cell

Table 1. HLA class II and I in patients analyzed for CD4 and CD8 T-cell responses, respectively, in Figure 3

	HLA class II						HLA class I					
Patient	DRB1		DQB1		DPB1		A		B		C	
KLU3	*08:03	*15:02	*06:01	–	*02:02	*09:01						
KLU15	*04:05	*15:02	*04:01	*06:01	*05:01	*09:01						
KLU21	*08:02	*08:03	*04:02	*06:01	*02:01	*05:01						
KLU22	*04:01	*09:01	*03:01	*03:03	*05:01	*41:01	*02:01	*24:02	*15:01	*15:11	*03:03	*04:01
KLU34	*12:01	*14:06	*03:01	–	*04:01	*05:01						
KLU36	*04:05	*15:02	*04:01	*06:01	*05:01	*13:01						
KLU38	*04:05	*15:02	*04:01	*06:01	*05:01	*09:01						
KLU51	*08:03	–	*06:01	–	*02:01	*02:02	*11:01	*24:02	*39:01	*46:01	*01:02	*07:02
KLU76	*11:01	*14:01	*03:01	*05:02	*02:01	*02:02						
KLU109	*04:05	*13:02	*04:01	*06:09	*03:01	*09:01	31:01	*33:03	*51:01	*58:01	*03:02	*1402
KLU115	*04:05	*15:02	*04:01	*06:01	*05:01	*09:01	*11:01	*24:02	*40:02	*52:01	*03:04	*12:02
KLU187	*04:05	*04:06	*03:02	*04:01	*02:01	*05:01	*02:06	*24:02	*35:01	*59:01	*01:02	*03:04
KLU192	Not determined						Not determined					
KLU237	*14:03	*15:01	*03:01	*06:02	*05:01	–	*24:02	*31:01	*40:02	*51:01	*03:04	*14:02
KLU267	*09:01	*14:06	*03:01	*03:03	*05:01	–	*26:02	*26:03	*15:01	–	*03:03	–
KLU281	*09:01	*15:02	*03:03	*06:01	*03:01	*09:01	*24:02	–	*07:02	*52:01	*07:02	*12:02

lines by Western blot and CLSM. In a conventional Western blot, we found that XAGE-1b (GAGED2a) mAb (clone USO 9-13) gave a 9 kDa band in cell lysates from XAGE-1b transfectants,[22] but not in *XAGE-1b*-mRNA expressing tumor cell lines. For this reason, we used a modified method in which cell lysates were first incubated with the mAb and the antigen/antibody complex was purified using protein G Sepharose beads. Then, the eluate of the XAGE-1b (GAGED2a)-enriched solution was analyzed by Western blot. As shown in Figure 1, *XAGE-1b*-mRNA-positive cell lines gave a 9 kDa band. CLSM showed nuclear localization of the protein with a granular or diffuse pattern in XAGE-1b (GAGED2a)-positive lung cancer cell lines, consistent with the immunohistochemistry (IHC) results with lung cancer tissues.[22]

Antibody response against XAGE-1b (GAGED2a) in NSCLC patients

The antibody response against XAGE-1b (GAGED2a) was investigated in NSCLC patients by ELISA using synthetic XAGE-1b (GAGED2a) protein. Figure 2*a* shows titration curves of sera from 200 NSCLC patients and 50 healthy control donors. The frequency of antibody-positive patients was 10% (20/200). The frequencies of antibody-positive patients with adenocarcinoma and squamous cell carcinoma were 14% (16/118) and 2% (1/44), respectively. The frequency of antibody-positive patients with Stage IIIB/IV lung adenocarcinoma was 19% (13/69). Our previous results showed that the frequencies of *XAGE-1b* mRNA and IHC-positives were 31% and 23% in NSCLC, and 45% and 33%, respectively, in lung adenocarcinoma. No correlation was observed between *XAGE-1b* expression and disease stage or histologic grade.[20,22] These findings indicate that the frequency of antibody positives was 32–43% in NSCLC patients and 42–57% in Stage IIIB/IV lung adenocarcinoma patients with *XAGE-1b*-mRNA and/or XAGE-1b (GAGED2a)-expressing tumors.

Epitope peptides recognized by the antibody were analyzed by ELISA using 17 16-mer XAGE-1b (GAGED2a) OLPs. As shown in Figures 2*b* and 2*c*, various regions were recognized. Within these, peptides 25–40, 29–44, 33–48 and 57–72 were relatively frequently recognized. The USO 9–13 mAb used for XAGE-1b (GAGED2a) expression analysis recognized peptide 65–81.[19]

CD4 and CD8 T-cell responses against XAGE-1b (GAGED2a) in NSCLC patients

Purified CD4 and CD8 T-cells from PBMCs were stimulated with a mixture of 17 16-mer XAGE-1b (GAGED2a) OLPs using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMCs as APCs for 10–14 days. CD4 T-cells were cultured in a micro-culture plate (2×10^6/well) and CD8 T-cells in a 96-well culture plate (1×10^4/well). After culture, responding CD4 and CD8 T-cells were collected and stimulated with a mixture of OLPs using autologous EBV-B cells as APCs for 4 and 8 hr, respectively, and examined for IFNγ secretion by FACS.

As shown in Figure 3, CD4 and CD8 T-cell responses were observed in 14 of 16 and 6 of 9, respectively, of the XAGE-1b (GAGED2a) antibody-positive patients examined. Neither response was observed in 7 antibody-negative patients or five healthy donors.

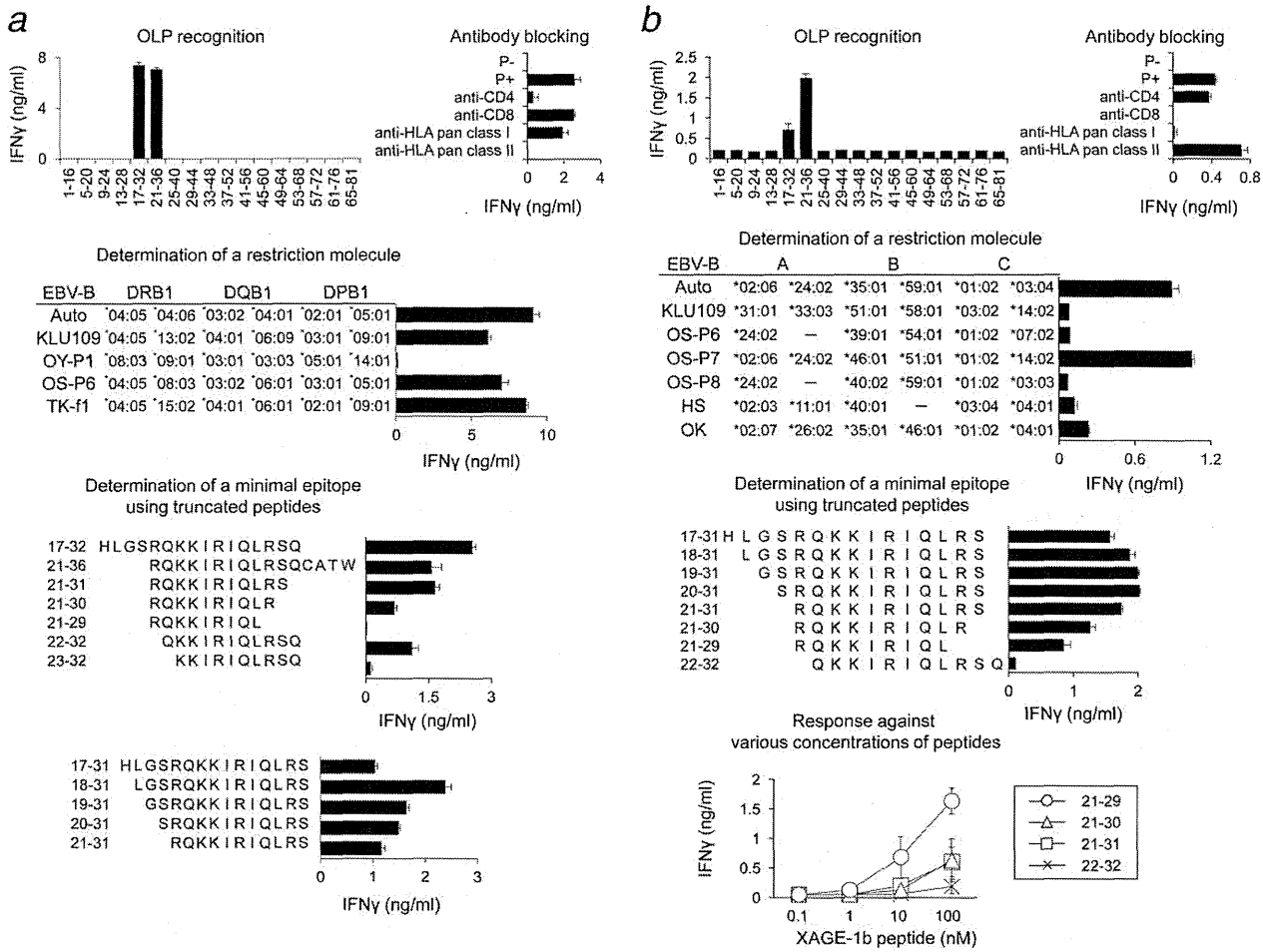

Figure 4. Determination of restriction molecules and minimal epitopes in recognition of XAGE-1b (GAGED2a) OLPs by CD4 (*a*) and CD8 (*b*) T-cell clones from patient KLU-187. For the method to establish CD4 and CD8 T-cell clones, see "Material and Methods" section. In *a*, recognition of XAGE-1b (GAGED2a) 17–32 and 21–36 OLPs by the CD4 T-cell clone (1×10^4) was determined by ELISA examining IFNγ in the culture supernatant after stimulation. In antibody blocking, the CD4 T-cell clone (5×10^3) was stimulated with the autologous EBV-B cells (5×10^3) in the presence of the peptide 17–32 (0.1 μM) and various antibodies (5 μg/mL). In determining a restriction molecule for peptide 17–32 recognition by the CD4 T-cell clone (5×10^3), EBV-B cells (5×10^3) with various HLA types were used for APCs. The recognition was restricted to DRB1*04:05. In determining a minimal epitope, various N- and C-terminal truncated peptides (0.01 μM in the upper panel and 0.001 μM in serum free medium in the lower panel) were examined for recognition by the CD4 T-cell clone (5×10^3). The peptide 18–31 (14-mer) was the epitope. In *b*, recognition of XAGE-1b (GAGED2a) 17–32 and 21–36 OLPs by a CD8 T-cell clone was similarly determined by ELISA. In antibody blocking, the CD8 T-cell clone (1×10^4) was stimulated with the autologous EBV-B cells (1×10^4) in the presence of the peptide 21–36 and various antibodies. In determining a restriction molecule for peptide 21–36 recognition by the CD8 T-cell clone, EBV-B cells with various HLA types were used for APCs. The recognition was restricted to A*02:06. In determining a minimal epitope, various N- and C-terminal truncated peptides were examined for recognition. The CD8 T-cell response to different concentrations of various peptides in serum free medium revealed peptide 21–29 (9-mer) to be the epitope. The values are the mean ± S.D. from the three independent experiments.

XAGE-1b (GAGED2a) peptide regions recognized by CD4 and CD8 T-cells

XAGE-1b (GAGED2a) peptide regions recognized by CD4 and CD8 T-cells were determined using 17 16-mer XAGE-1b (GAGED2a) OLPs. Purified CD4 and CD8 T-cells from PBMCs were stimulated once or twice with the OLPs using irradiated (40 Gy), autologous CD4- and CD8-depleted PBMCs as APCs as described above. The responding cells were collected and stimulated with individual OLPs using autologous EBV-B cells as APCs for 24 hr and assayed for IFNγ secretion in the culture supernatant by ELISA. As shown in Figures 3*b* and 3*c*, peptide regions recognized by CD4 and CD8 T-cells from 14 and 6 patients, respectively, who were XAGE-1b (GAGED2a) antibody-positive were quite diverse. Several peptide regions were relatively frequently recognized. CD4 T-cells recognized peptide 13–28 in 5 of 14, and peptide 33–48 in 6 of 14, XAGE-1b (GAGED2a) antibody-positive patients. On the other hand, CD8 T-cells recognized peptide 9–24 and 29–44 in three of six XAGE-1b (GAGED2a) antibody-positive patients. The patients' HLA

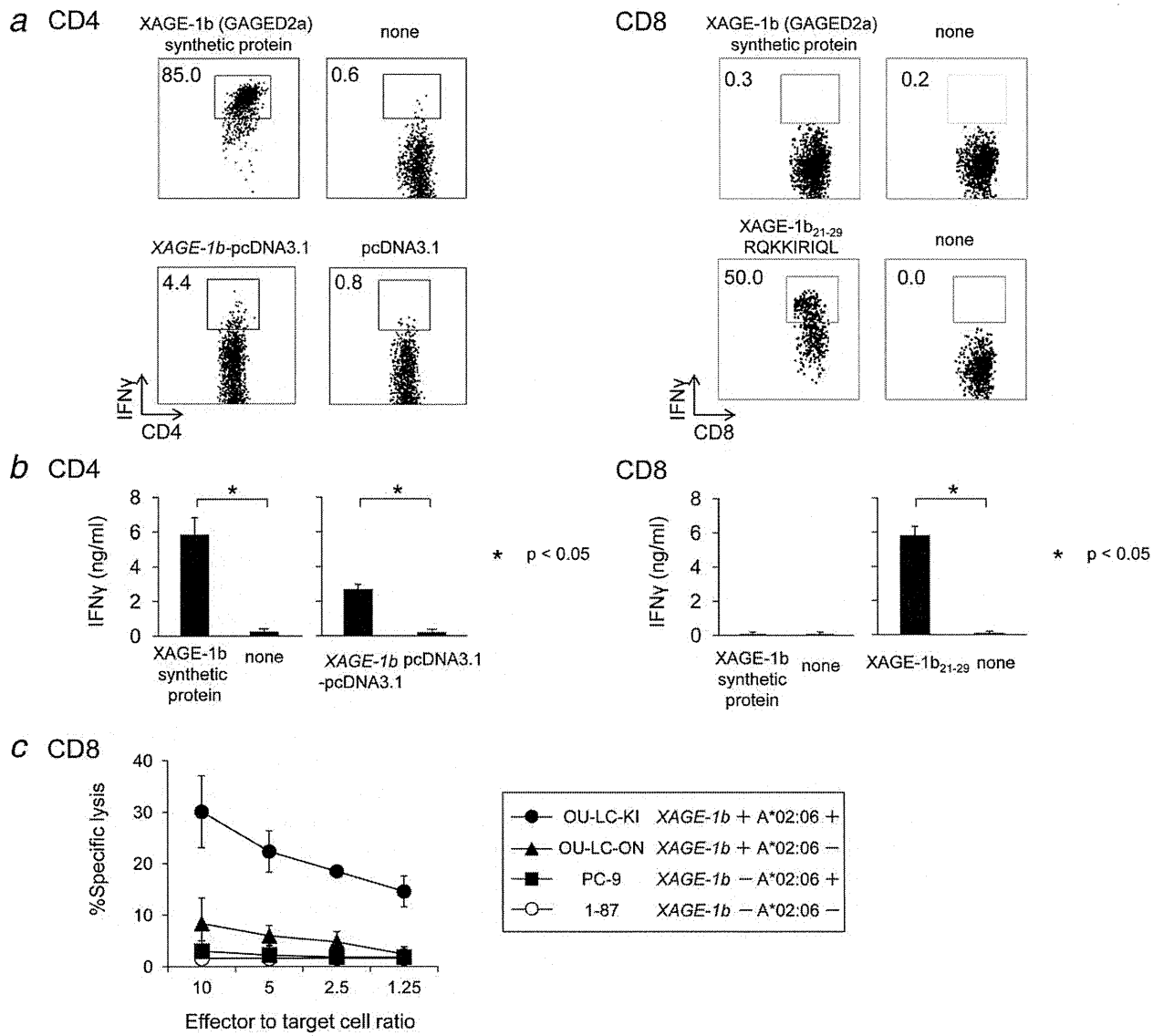

Figure 5. Recognition of a naturally processed XAGE-1b (GAGED2a) antigen by CD4 and CD8 T-cell clones from patient KLU187. In *a*, a CD4 T-cell clone (5×10^4) was stimulated for 4 hr with autologous DCs (5×10^4) pulsed with XAGE-1b (GAGED2a) synthetic protein (1 μM) or the lysate from *XAGE-1b*-transfected 293T cells (5×10^6). The CD8 T-cell clone (5×10^4) was stimulated for 8 hr with autologous DCs (5×10^4) pulsed with XAGE-1b (GAGED2a) synthetic protein (1 μM) or 9-mer epitope peptide (RQKKIRIQL) (1 μM). Then, the response was examined by an IFNγ capture assay. In *b*, the amount of IFNγ in the culture supernatant of the CD4 and CD8 T-cell clones (1×10^4) stimulated for 24 hr with autologous DCs (1×10^4) treated as above was determined by ELISA. In *c*, cytotoxicity of the CD8 T-cell clone for an XAGE-1b (GAGED2a)-positive and A*02:06-positive tumor cell line by a luminescent method using the aCella-Tox kit (see "Material and Methods" section) is shown. The values are the mean ± S.D. from the three independent experiments.

types are listed in Table 1. No specific correlation of the peptides recognized to HLA alleles was found.

Determination of restriction molecules and minimal epitopes in the recognition of XAGE-1b (GAGED2a) peptides by CD4 and CD8 T-cell clones

CD4 and CD8 T-cell clones were established from PBMCs of XAGE-1b (GAGED2a) antibody-positive patients. As shown in Figure 4*a*, the restriction molecule in recognition of peptide 17–32 by the KLU187 CD4 T-cell clone was determined by antibody blocking and by using various EBV-B cells as APCs to present the peptide, while the minimal epitope peptide was determined by using N- and C-termini truncated peptides. The recognition of peptide 17–32 was restricted by DRB1*04:05 and the minimal epitope was the 14-mer peptide 18–31.

The restriction molecule in the recognition of peptide 21–36 by the KLU187 CD8 T-cell clone and the minimal epitope

peptide were similarly determined. As shown in Figure 4*b*, the recognition was restricted to A*02:06 and the minimal epitope was the 9-mer peptide 21–29.

Recognition of naturally processed XAGE-1b (GAGED2a) antigen by CD4 and CD8 T-cell clones

We examined the recognition of the naturally processed XAGE-1b (GAGED2a) antigen by the DRB1*04:05-restricted CD4 T-cell clone, and the A*02:06-restricted CD8 T-cell clone shown in Figures 4*a* and 4*b*, respectively. As shown in Figures 5*a* and 5*b*, the DRB1*04:05-restricted CD4 T-cell clone recognized DCs pulsed with XAGE-1b (GAGED2a) synthetic protein or the lysate from *XAGE-1b*-transfected 293T cells. On the other hand, the A*02:06-restricted CD8 T-cell clone recognized DCs pulsed with the 9-mer epitope peptide, but not synthetic XAGE-1b (GAGED2a) protein. As shown in Figure 5*c*, the A*02:06-restricted CD8 T-cell clone showed cytotoxicity against a XAGE-1b (GAGED2a)-positive, A*02:06-positive lung cancer cell line OU-LC-KI, but not a XAGE-1b (GAGED2a)-positive, A*02:06 negative lung cancer cell line OU-LC-ON, a XAGE-1b (GAGED2a)-negative, A*02:06-positive lung cancer cell line PC-9, or a XAGE-1b (GAGED2a)-negative, A*02:06 negative lung cancer cell line 1-87.

Discussion

In this study, we showed that an antibody response against XAGE-1b (GAGED2a) was observed in 10% of NSCLC patients and in 19% of Stage IIIB/IV lung adenocarcinoma patients. We previously showed that the frequency of *XAGE-1b* mRNA and IHC-positives was 31% and 23% in NSCLC, and 45% and 33%, respectively, in lung adenocarcinoma. By calculation, this indicates that 32–43% of NSCLC patients and 42–57% of Stage IIIB/IV lung adenocarcinoma patients with *XAGE-1b*-mRNA and/or protein-expressing tumors elicited an antibody response. This high frequency of spontaneous antibody response against XAGE-1b (GAGED2a) in NSCLC patients was comparable to that against NY-ESO-1 in melanoma patients in a Caucasian population, and it has been shown to be one of the most immunogenic tumor antigens.[5,23] However, in lung cancer patients in the Japanese population, the frequency of the antibody response against NY-ESO-1 was approximately 5% (unpublished). On the other hand, no spontaneous antibody response was observed in lung cancer patients with MAGE-A3 or SSX2-expressing tumors.[7,23–27] The antibody response against p53 was shown to be high at around 7–27% in lung cancer patients.[27] These findings emphasize the strong immunogenicity of XAGE-1b (GAGED2a) in the antibody response in lung cancer patients.

In this report, we analyzed CD4 and CD8 T-cell responses in XAGE-1b (GAGED2a) antibody-positive patients. A CD4 T-cell response was detected in 14 of 16 (88%), and a CD8 T-cell response was detected in six of nine (67%) XAGE-1b (GAGED2a) antibody-positive patients examined. Occurrence of CD4 and CD8 T-cell responses in XAGE-1b (GAGED2a) antibody-positive patients showed the strong cellular immunogenicity of the XAGE-1b (GAGED2a) antigen. This is also similar to the findings with NY-ESO-1. Thus, an integrated immune response including antibody and CD4 and CD8 T-cell responses was repeatedly shown in patients with NY-ESO-1-expressing tumors.[28–30] With regard to both the XAGE-1b (GAGEG2a) and NY-ESO-1 antigens, CD4 and CD8 T-cell responses were elicited in PBMCs from antibody-positive patients after a single *in vitro* stimulation. *Ex vivo* detection of such responses was rarely possible due to the low frequencies of CD4 and CD8 T-cells responding to the antigens.[31] However, in XAGE-1b (GAGED2a)-antibody-positive patients, the CD8 T-cell response appeared to be somewhat weaker than the CD4 T-cell response.

In this study, we 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. We previously determined two XAGE-1b (GAGED2a) CD4 epitope peptides restricted to DRB1*04:10[32] and DRB1*09:01.[33] Moreover, we are currently determining other MHC I binding peptide epitopes. These CD4 and CD8 T-cell epitope peptides will be useful for designing vaccines and producing tetramers for immune monitoring. Tetramer production is now under investigation.

Recognition by CD4 and CD8 T-cell clones of a naturally processed XAGE-1b (GAGED2a) antigen was shown in this study. The CD8 T-cell clone showed cytotoxicity against an HLA-matched, XAGE-1b (GAGED2a)-positive tumor cell line.

XAGE-1b (GAGED2a) is 81 amino acids long and is expressed in most *XAGE-1b* mRNA expressing NSCLC. Thus, XAGE-1b (GAGED2a) was the predominant isoform in NSCLC. However, in hepatocellular carcinoma or prostate cancer, although *XAGE-1b* and *d* mRNA expression have been frequently observed, XAGE-1b (GAGED2a) protein expression has rarely been observed by IHC. It is possible that another isoform, XAGE-1d (GAGED2d), was expressed in these tumors. Production of mAb detecting XAGE-1d (GAGED2d) is now being studied.

Acknowledgements

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Heteroclitic serological response in esophageal and prostate cancer patients after NY-ESO-1 protein vaccination

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NY-ESO-1 is a prototypic cancer/testis antigen. In a recent phase I clinical trial, we vaccinated 13 patients bearing NY-ESO-1-expressing tumors with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein (CHP-NY-ESO-1) and showed efficient induction of NY-ESO-1 antibody, and CD4 and CD8 T cell responses using peripheral blood from the patients. In our study, we analyzed heteroclitic serological responses in those patients after vaccination. Serological response against 11 tumor antigens including MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SOX2, SSX2, XAGE1B and p53 was examined by enzyme-linked immunosorbent assay (ELISA) using sera from ten vaccinated patients. Expression of tumor antigens was determined by reverse transcription-polymerase chain reaction or immunohistochemistry. Eight of nine patients who showed antibody responses against NY-ESO-1 also showed an antibody response against at least 1 of these 11 tumor antigens after vaccination. In one patient, seven tumor antigens were recognized. Specificity analysis of the antibody response by ELISA using control recombinant proteins and synthetic peptides and by Western blot showed that the response was not against His6-tag and/or bacterial products included in a preparation of CHP-NY-ESO-1 used for vaccination. Thus, heteroclitic serological responses appear to be indicative of the overall immune response against the tumor, and their analysis could be useful for immune monitoring in cancer vaccine.

NY-ESO-1 antigen was originally identified in esophageal cancer by serological expression cloning (SEREX) using autologous patient serum[1,2] and has been shown to be strongly immunogenic. Patients bearing NY-ESO-1-expressing tumors often show antibody and CD4 and CD8 T cell responses spontaneously.[3–5] Clinical trials using NY-ESO-1 peptide,[6–9] protein[10,11] and viral constructs[12] as cancer vaccine have been conducted. Those studies have demonstrated efficient induction of antibody, and CD4 and CD8 T cell responses, and have also shown in some sporadic cases efficacy of immunotherapy.[2,6–12]

In a recent phase I clinical trial, we vaccinated 13 patients including eight Stage IV esophageal cancer patients, four

Key words: NY-ESO-1, cancer vaccine, cancer testis antigen, heteroclitic antibody response

Abbreviations: CHP: cholesterol-bearing hydrophobized pullulan; CMV: Cytomegalovirus; CT antigen: cancer/testis antigen; CTL: cytotoxic T lymphocytes; DHFR: dihydrofolate reductase; EBV: Epstein–Barr virus; OD: optical density; OLPs: overlapping peptides; RT-PCR: reverse transcription-polymerase chain reaction; SEREX: serological expression cloning

Additional Supporting Information may be found in the online version of this article

The authors declare that there is no conflict of interest

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Stage D3 prostate cancer patients and a Stage IV malignant melanoma patient with a complex of cholesterol-bearing hydrophobized pullulan (CHP) and NY-ESO-1 protein (CHP-NY-ESO-1).[13–16] We showed that the vaccine was well tolerated and had a potent capacity to induce NY-ESO-1 antibody and CD4 and CD8 T cell responses using peripheral blood from patients. Beneficial effects such as tumor regression or no tumor growth for a prolonged period were observed in six of seven disease-evaluable patients. However, all patients died eventually. Immunohistochemical analysis of the tumors that grew after vaccination suggested involvement of different mechanisms resulting in immune impairment.[15] NY-ESO-1-antigen loss was observed in a patient, disappearance of tumor-infiltrating CD4 and CD8 T cells was observed in two patients and an increase in the number of $CD68^+$ macrophages was observed in another patient. Further study will be necessary to elucidate cellular mechanisms including regulatory T cells causing immune suppression at the local tumor site.

It has been shown that cytotoxic T cell responses to tumor antigens other than the antigen used for immunization occur after vaccination.[17–26] This heteroclitic immune response following the initial response to a specific antigen was originally described in autoimmune disease and has been suggested to play a crucial role in clinical responses mediated by cancer vaccines.[27,28] Occurrence of CD8 T cell responses to unrelated tumor antigens was shown in studies of vaccination with MAGE-A1 and/or MAGE-A3,[19,20,25] HER-2/neu,[17,18,21,22] MART-1/Melan-A[23–25] and gp100.[26]

In our study, we analyzed the heteroclitic serological response against a panel of tumor antigens. Specificity analysis of antibody response was performed by enzyme-linked immunosorbent assay (ELISA) using control recombinant proteins and synthetic peptides, and by Western blot. The findings indicate frequent occurrence of heteroclitic serological responses in patients after CHP-NY-ESO-1 vaccination.

Material and Methods

Patients and sera

Eight advanced esophageal cancer patients (E-1, E-2, E-3, E-4, E-5, E-6, E-7 and E-8) and two prostate cancer patients (P-2 and P-3) were enrolled in the clinical trial (protocol LUD 2002-005 of the Ludwig Institute for Cancer Research, New York, NY) (http://clinicaltrials.gov/ct2/show/NCT00106158?term=CHP+NY-ESO-1&rank=2).[13–16] Peripheral blood was drawn from patients with written informed consent with the permission of the ethics committees of Osaka and Okayama Universities. Sera from MAGE-A4 seropositive esophageal cancer patients (EC-1, EC-2 and EC-3) and five healthy donors were drawn with written informed consent with the permission of the ethics committees of Osaka University. Sera were stored in −80°C freezer until use.

Preparation of a complex of cholesterol-bearing hydrophobized pullulan and NY-ESO-1 protein (CHP-NY-ESO-1)

Preparation of recombinant NY-ESO-1 protein for vaccine was described elsewhere.[29] A complex of CHP and NY-ESO-1 protein (CHP-NY-ESO-1) and the schedule of vaccine were described previously.[14] Briefly, patients with advanced cancers expressing NY-ESO-1 were injected 2–31 times subcutaneously at biweekly intervals with 100 μg of NY-ESO-1 recombinant protein formulated with 2 mg of CHP.

Recombinant protein and overlapping peptides

N-His6-tagged recombinant proteins, NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, p53, SOX2, SSX2, XAGE1B, dihydrofolate reductase (DHFR) and Akt,[30–32] were produced by using pQE30 vector (QIAGEN, Hilden, Germany) and expressed in M15 *E. coli* cells. N-His6-tagged p53 produced in a *Baculovirus* system was purchased (Enzo Life Sciences, New York, NY). N-His6-tagged CCDC-62 protein was produced in a *Baculovirus* system at Okayama University.[33]

A series of 22 25-mer MAGE-A4 overlapping peptides (OLPs) were synthesized using standard solid-phase methods based on *N*-(9-fluorenyl)-methoxycarbonyl chemistry on an ABIMED Multiple Peptide Synthesizer (AMS422, ABIMED, Langenfeld, Germany) at Okayama University. MAGE-A4 OLPs: 1 (1–25), 2 (15–39), 3 (29–53), 4 (43–67), 5 (57–81), 6 (71–95), 7 (85–109), 8 (99–123), 9 (113–137), 10 (127–151), 11 (141–165), 12 (155–179), 13 (169–193), 14 (183–207), 15 (197–221), 16 (221–235), 17 (225–249), 18 (239–263), 19 (253–277), 20 (267–291), 21 (281–305), 22 (295–317).

MAGE-A4 transfectants

CMS5a, a murine fibrosarcoma cell line from a strain of BALB/c origin, was stably transfected with pcDNA3.1 MAGE-A4 plasmid as described previously.[34] The plasmid was provided by Dr. A. Kuroda and Dr. M. Miyamoto (Hokkaido University, Sapporo, Japan) and purified using plasmid Mini kit.

ELISA

Serially diluted sera were added to 96-well plates coated overnight at 4°C with 1 μg/ml recombinant protein or 5 μg/ml peptide solution and blocked for 2 hr at room temperature. After overnight incubation, plates were extensively washed with PBS containing 0.2% Tween 20. For determining reciprocal titer by ELISA, shown in Figure 1, serum IgG bound to antigens was detected by alkaline phosphatase-conjugated specific monoclonal antibody (Southern Biotech, Birmingham, AL). After addition of ATTOPHOS substrate (Fisher Scientific, Waltham, MA), absorbance was measured using a Cytofluor Series 4000 fluorescence reader (PerSeptive Biosystems, Framingham, MA). A reciprocal titer was calculated for each sample as the maximal dilution still significantly reacting to a specific antigen. Specificity was determined by

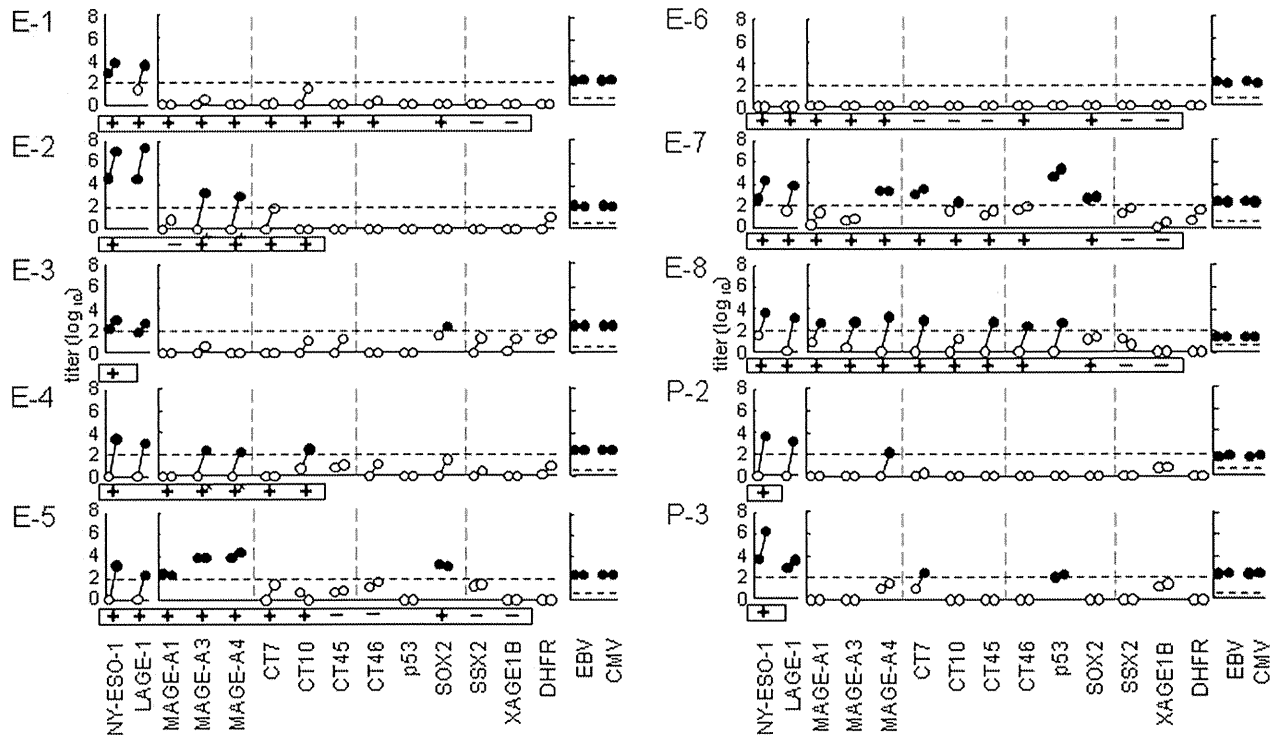

Figure 1. Reciprocal antibody titer against 13 tumor antigens in sera from patients vaccinated with CHP-NY-ESO-1 by ELISA. Serially diluted sera obtained before and after vaccination were assayed against N-His6-tagged recombinant proteins NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, p53, SOX2, SSX2, XAGE1B and DHFR. The reciprocal titer was the maximal dilution showing significant reaction (open and closed circles). Closed circles indicate reciprocal titers exceeding 100 (positive reaction). In each assay, antibody positive and negative sera were included as controls. Positive (+) and negative (–) expression of tumor antigens indicated in boxes under each panel was analyzed by RT-PCR and/or IHC when sample was available (see Supporting Information Table). Titer of EBV and CMV antibody in sera were measured by EBV and CMV kits, respectively. Values exceeding 4.0 were positive by manufacturer's indication. +*; 57B and M3H67 mAbs generated against MAGE-A3 and MAGE-A4 recombinant proteins, respectively, were both shown to recognize multiple MAGE-A family molecules.

comparing seroreactivity among the various antigens tested.[31] In each assay, positive and negative control sera were included. A positive result was defined as reciprocal titers >100. For conventional ELISA, peroxidase-conjugated goat anti-human IgG or IgM (Jackson Immuno Research Laboratory, West Grove, PA) was added to the wells for second antibody. After washing, signals were developed with *o*-phenylene diamine dihydrochloride, and absorbance at 490 nm was read using an ELISA reader (Benchmark Microplate Reader; Bio-Rad, Hercules, CA). Positivity was defined as sample optical density (OD) greater than three times that of the value for irrelevant control protein. Titers of Epstein–Barr virus (EBV) and Cytomegalovirus (CMV) antibody in sera were measured by EBV VCA kit (Denka Seiken, Tokyo, Japan) and CMV kit (Denka Seiken), respectively.

Western blot

Recombinant protein (20 ng) or cell lysate (20 μg) in sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS and 1 mM dithiothreitol) was boiled for 5 min and subjected to SDS-PAGE with 10–20% polyacrylamide BioRad Ready-Gels (Bio-Rad). After electrophoresis, the membrane (Hybond-P membrane, Amersham Pharmacia Biotech, Buckinghamshire, UK) was blocked with 5% FCS/PBS and then incubated with patients' sera diluted 1:1,000 for recombinant protein or 1:200 for cell lysate for 1 hr at room temperature. After washing, alkaline phosphatase-conjugated goat anti-human IgG (Jackson Immuno Research Laboratory) was added to the membrane. Signals were developed with a 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium chromogenic substrate kit (Bio-Rad). Polyclonal rabbit anti-MAGE-A1 serum (Abcam, Cambridge, UK) and monoclonal anti-MAGE-A4 (clone 3D12; Abnova, Taipei, Taiwan), anti-p53 (clone PAb421; Enzo) and anti-His6-tag (clone OGHis; MBL, Nagoya, Japan) antibodies were used for positive controls at 1:1,000 dilution.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using formalin-fixed paraffin-embedded specimens. Monoclonal antibodies

used were anti-MAGE-A1 (clone MA454), anti-MAGE-A3 (clone M3H67), anti-MAGE-A4 (clone 57B), anti-CT7/MAGEC1 (clone CT7-33) and anti-CT10/MAGEC2 (clone LX-CT10.5). For cancer-testis (CT) antigens, only strong nuclear and/or cytoplasmic staining as observed in testicular tissue (positive control) in at least 5% of cells was scored as positive. 57B and M3H67 mAbs generated against MAGE-A3 and MAGE-A4 recombinant proteins, respectively, were both shown to recognize multiple MAGE-A family molecules.[35,36]

Table 1. Heteroclitic antibody response and clinical response after CHP-NY-ESO-1 vaccination

ID	Heteroclitic response No. of antigens	Weeks (the No.)	Clinical response
E-1	0	89 (31)	Regression
E-2	2	14 (7)	Partial regression
E-3	1	28 (12)	Stable
E-4	3	12 (6)	Progressive
E-5	2	22 (11)	Partial regression
E-6	0	4 (3)	N.E.
E-7	4	2 (2)	N.E.
E-8	7	54 (27)	Stable
P-2	1	28 (10)	PSA stabilization
P-3	2	29 (13)	PSA stabilization

Abbreviations: Weeks (the No.): weeks after the start of vaccination and the number of vaccinations given; N.E.: not evaluable.

Reverse transcription-polymerase chain reaction

Total cellular RNA was extracted from frozen tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA). Conventional reverse transcription-polymerase chain reaction (RT-PCR) was performed against NY-ESO-1, LAGE-1, MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SOX2, SSX2 and XAGE1B.[30,31]

Results

Antibody response against 13 tumor antigens in CHP-NY-ESO-1-vaccinated patients

We analyzed antibody responses against NY-ESO-1, NY-ESO-1-related antigen LAGE-1, other CT antigens MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT10/MAGEC2, CT45, CT46/HORMAD1, SSX2 and XAGE1B, SOX2 and p53 in esophageal cancer patients E-1, E-2, E-3, E-4, E-5, E-6, E-7 and E-8 and prostate cancer patients P-2 and P-3 before and after a cycle of CHP-NY-ESO-1 vaccination (Fig. 1 and Table 1). Before vaccination, strong antibody responses against NY-ESO-1 and/or LAGE-1 were observed in E-2 and P-3 and defined as baseline seropositive. Additionally,

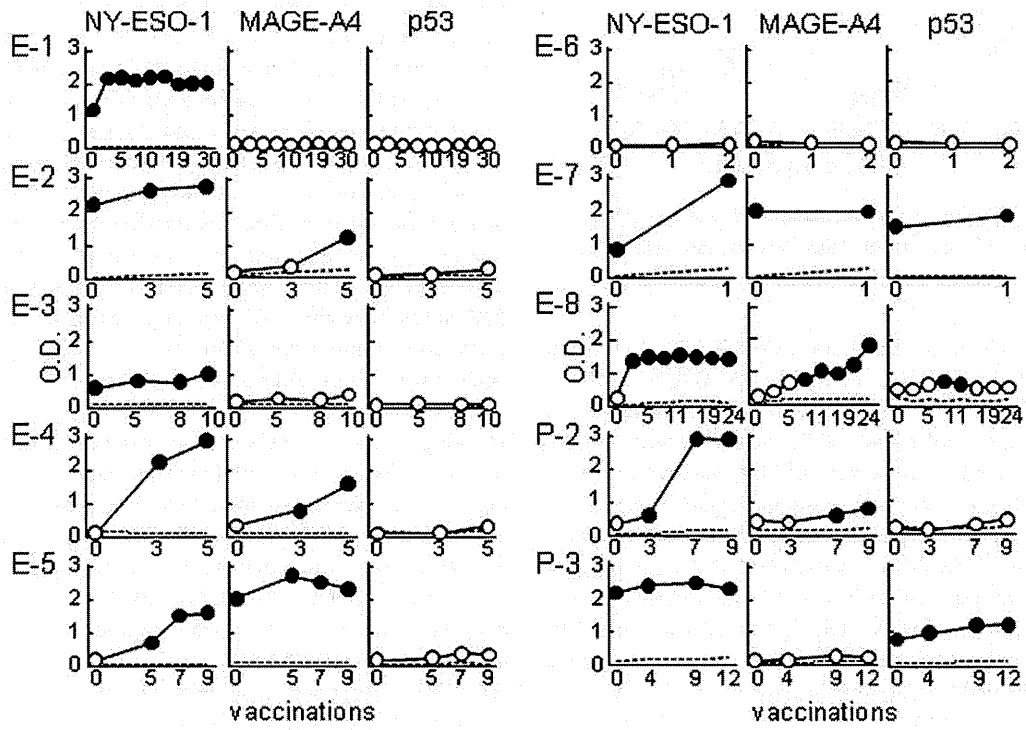

Figure 2. IgG antibody response against NY-ESO-1, MAGE-A4 and p53 in sera from patients before and after CHP-NY-ESO-1 vaccination by ELISA. Sera diluted at 1:100 were assayed against N-His6-tagged recombinant proteins NY-ESO-1, MAGE-A4 and Akt produced in *E. coli* and recombinant proteins p53 and CCDC-62 produced in *Baculovirus*. Akt and CCDC-62 were included as negative control (dotted line). Positive reaction (closed circles) represented the OD values exceeding three times the control OD value.

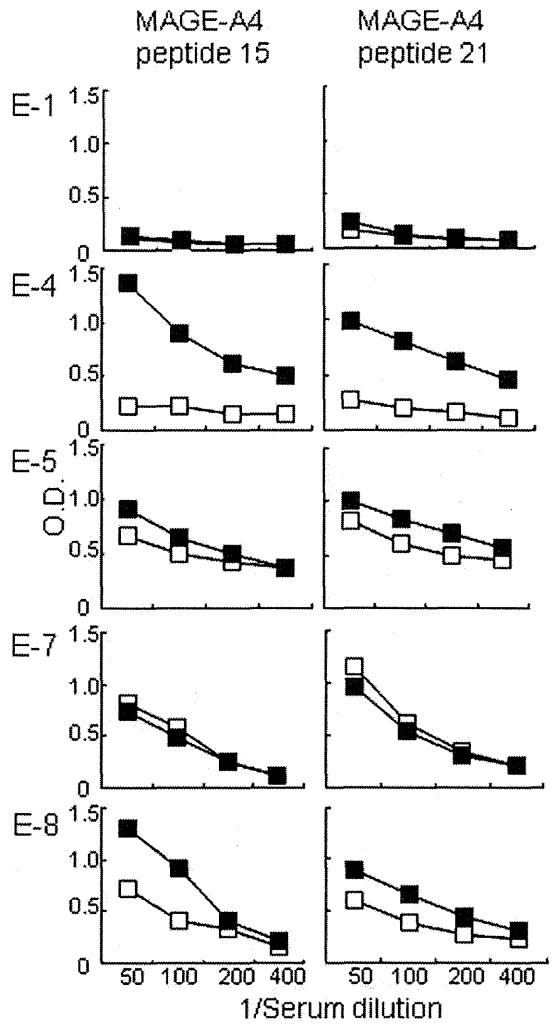

Figure 3. Serially diluted sera from patients before (open squares) and after (closed squares) CHP-NY-ESO-1 vaccination were assayed against MAGE-A4 peptide 15 and peptide 21 by IgG ELISA.

marginal antibody responses were observed in E-1, E-3 and E-7. In E-5, antibody against MAGE-A1, MAGE-A3, MAGE-A4 and SOX2 was observed. In E-7, antibody against MAGE-A4, CT7/MAGEC1, p53 and SOX2 was observed. In P-3, antibody against p53 was observed. After vaccination, in all patients except E-6, antibody response against NY-ESO-1 and LAGE-1 was increased or induced. In E-2, antibody responses against MAGE-A3 and MAGE-A4 were induced. In E-3, antibody response against SOX2 was induced. In E-4, antibody responses against MAGE-A3, MAGE-A4 and CT10/MAGEC2 were induced. In E-5, antibody responses against MAGE-A3 and MAGE-A4 were increased. In E-7, antibody responses against CT7/MAGEC1, p53 and SOX2 were increased and that against CT10/MAGEC2 was induced. In E-8, antibody responses against MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1, CT45, CT46/HORMAD1 and p53 were induced. In P-2, antibody response against MAGE-A4 was induced. In P-3, antibody response against CT7/MAGEC1 was induced and that against p53 was increased. No antibody against DHFR included as a control was detected in any patient. Furthermore, no increase of antibody response was observed against EBV and CMV after CHP-NY-ESO-1 vaccination.

Expression of 13 tumor antigens in tumor specimens

Expression of NY-ESO-1 was detected by RT-PCR and IHC in tumors from all patients before vaccination. Expression of other tumor antigens except p53 was analyzed by RT-PCR in E-1, E-5, E-6, E-7 and E-8, and expression of MAGE-A1, MAGE-A3, MAGE-A4, CT7/MAGEC1 and CT10/MAGEC2 was also analyzed by IHC in E-1, E-2, E-4, E-5, E-6, E-7 and E-8 (Fig. 1 and Supporting Information Table). Mutation of p53 was not determined in our study. Expression of corresponding antigen was confirmed with tumor specimens in patients who showed antibody against tumor antigens.

Antibody response against tumor antigens in CHP-NY-ESO-1-vaccinated patients: No involvement of antibody against His6-tag and the product of E. coli present in the vaccine

Antibody responses against selected tumor antigens were further confirmed in sera obtained at each time during multiple vaccinations. As shown in Figure 2, IgG antibody against MAGE-A4 was detected in sera from E-5 and E-7 before vaccination, and the response was increased or induced in E-2, E-4, E-5, E-8 and P-2 after vaccination. IgG antibody against p53 was detected in sera from E-7 and P-3 before vaccination, and the response was increased or induced in E-7, E-8 and P-3.

Induction of IgM antibody against MAGE-A4 was detected in sera from E-8 after vaccination (Supporting Information Fig. 1). IgM antibody against p53 was detected in sera from E-7 before vaccination. Increase or induction of IgM antibody against p53 was detected in E-7 and E-8 after vaccination. Interestingly, in E-8, transient IgM response against MAGE-A4 and p53 was followed by IgG response.

Recombinant NY-ESO-1 protein used for vaccination has His6-tag in the N-terminus and was produced in *E. coli* as the host cells. All antigens shown in Figure 1 also have His6-tag and were produced in *E. coli*. To exclude the possibility of detecting antibody against His6-tag and/or the product of *E. coli* in the assay that might be raised by vaccination, DHFR was tested as control. No antibody against DHFR was detected (see above). To further exclude the possibility, the antibody response against control antigens was examined by IgG ELISA using serum samples obtained in each time during multiple vaccinations. As shown in Figure 2, antibody against Akt protein with His6-tag and produced in *E. coli* was within a background level (<0.2 OD value). p53 used in the experiments shown in Figure 2 and Supporting Information Figure 1, but not in Figure 1, and CCDC-62 protein

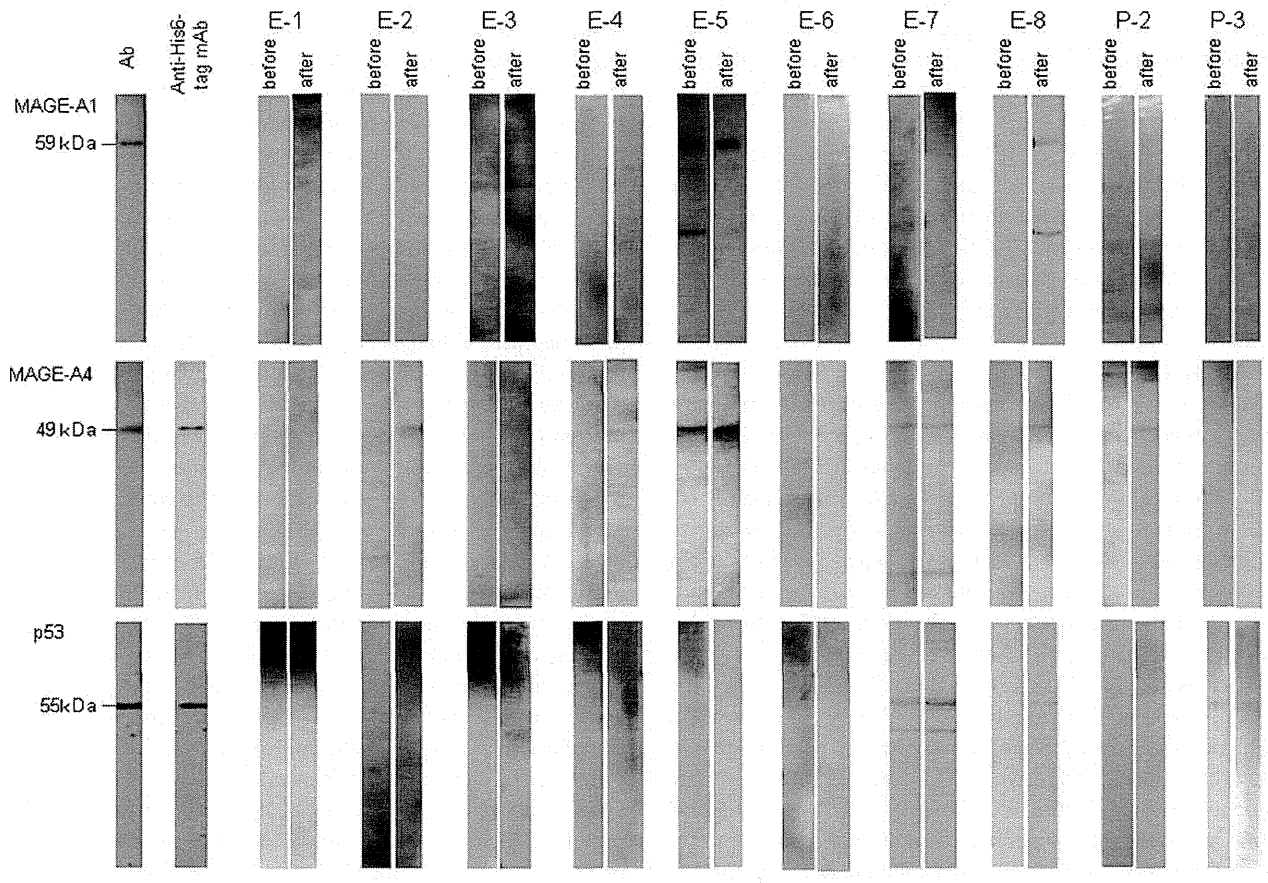

Figure 4. Western blot analysis. Reaction of sera against MAGE-A1, MAGE-A4 and p53 was investigated. Recombinant proteins (20 ng) were run by SDS-PAGE and transferred to a membrane by electrophoresis. Sera (1:1,000) from all patients obtained before and after vaccination were examined. Marker and control bands of each protein detected by monoclonal or polyclonal antibody (1:1,000) are also shown.

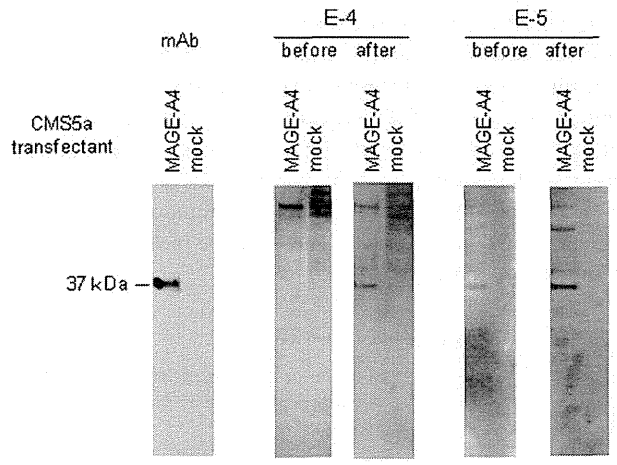

Figure 5. Western blot analysis of sera against MAGE-A4 in lysate of MAGE-A4-transfected CMS5a cells. Cell lysate (20 μg) was run by SDS-PAGE, transferred to a membrane by electrophoresis and sera (1:200) from E-4 and E-5 patients obtained before and after CHP-NY-ESO-1 vaccination were examined. Control band of the protein detected by monoclonal antibody (1:1,000) is shown.

share His6-tag and were produced by *Baculovirus*. Antibody against CCDC-62 was undetectable in sera from any patients.

Next, we synthesized MAGE-A4 OLPs and investigated antibody response by ELISA. Antibody response against MAGE-A4 peptides 15 and 21 was frequently observed in patients showing antibody response against MAGE-A4 protein (Supporting Information Fig. 2). Serially diluted sera from patients E-4, E-5, E-7 and E-8 obtained before and after CHP-NY-ESO-1 vaccination were examined against MAGE-A4 peptides 15 and 21 by IgG ELISA (Fig. 3). Increase or induction of antibody response was observed in E-4, E-5 and E-8, but not E-7 after vaccination. No antibody response was detected in E-1 included as negative control. These results were consistent with those by ELISA using recombinant MAGE-A4 protein in Figure 2.

Western blot analysis

The specificity of antibody against MAGE-A1, MAGE-A4 and p53 in sera from all patients vaccinated was further analyzed by Western blot (Fig. 4). Each antibody as positive control showed the representative band for MAGE-A1 protein at 59 kDa, for MAGE-A4 protein at 49 kDa and for p53 protein

at 55 kDa. Increase of reaction with the bands was observed with recombinant MAGE-A1 protein in sera from E-8, with recombinant MAGE-A4 protein in sera from E-2, E-4, E-5, E-8 and P-2 and with p53 in sera from E-7, E-8 and P-3 obtained after vaccination.

Specificity of the reaction was further confirmed using transfectants. As shown in Figure 5, sera from E-4 after vaccination and from E-5 before and after vaccination reacted to MAGE-A4 in lysate of MAGE-A4-transfected murine fibrosarcoma CMS5a cells. No reaction was observed with lysate of mock-transfected CMS5a cells.

Discussion

Efficient elicitation of host immune response is a prerequisite for successful immunotherapy using cancer vaccine, and immune monitoring of specific antibody, CD4 and CD8 T cell responses against tumor antigens after vaccination is crucial to evaluate the response. In our study, we investigated antibody response against 13 tumor antigens by ELISA using recombinant proteins to evaluate the immune response more precisely. Nine of ten patients analyzed except E-6 showed an increase or induction of antibody response against NY-ESO-1 and its related LAGE-1 antigen after CHP-NY-ESO-1 vaccination. Eight of these nine patients showed an increase or induction of antibody response to either of these antigens after vaccination. Previously, it was reported that sera from patients vaccinated with recombinant NY-ESO-1 protein and CpG in Montanide sometimes showed nonspecific production of antibody against other recombinant proteins used for control,[11,37] and some of these responses could be attributed to reactivity against bacterial components or His6-tag. To address this possibility, we performed specificity analysis of the antibody response using control recombinant proteins, synthetic peptides and by Western blot that showed heteroclitic responses were not against His6-tag and/or bacterial products included in a preparation of CHP-NY-ESO-1 used for vaccination.

We reported previously that those patients showed NY-ESO-1 specific antibody and CD4 and CD8 T cell responses during vaccination.[14,15] The findings suggest that increase or induction of antibody response against tumor antigens, *e.g.*, MAGE-A3 and MAGE-A4, as well as NY-ESO-1 after CHP-NY-ESO-1 vaccination may be caused by their release from tumor cells damaged by NY-ESO-1-specific immunity. Therefore, antibody response to multiple tumor antigens may suggest an intensity of the overall host immune response against the tumor, and detection of multiple heteroclitic serological responses using a panel of recombinant proteins would be a new tool of immunological monitoring for antitumor responses. A clear correlation between heteroclitic antibody responses and clinical outcomes could not be established in the limited number of patients analyzed in our study (Table 1). However, antibody response as well as CD4 and/or CD8 T cell responses to heteroclitic tumor antigens would be useful for evaluating overall immune response to tumor.

A number of studies have shown the relationship between heteroclitic immune response and clinical response. Germeau *et al.*[19] reported that the frequency of CTL precursor increased tenfold in some patients after vaccination using MAGE antigenic peptides, although they found no significant difference in the levels against immunizing antigens between the tumor-regressor and -progressor patients. They then analyzed CTL precursors against other tumor antigens than that utilized for vaccine and found that the immune responses elicited to those irrelevant antigens after vaccination might contribute to the whole immune response to a given tumor and was correlated to clinical responses. Similarly, Butterfield *et al.*[23,24] reported that peptide-specific T cell response was efficiently induced in most patients by immunization with MART-1/Melan-A peptide pulsed dendritic cells. However, cellular immune responses against not only MART-1/Melan-A but also gp100 and tyrosinase were detected only in a complete clinical responder. These findings suggest a relationship between heteroclitic CTL responses and clinical responses. Furthermore, Disis *et al.* reported induction of both cellular and humoral responses against other intramolecular determinants in patients immunized with HER-2/neu peptide vaccine, and of antibody response to p53 in patients immunized with HER-2/neu peptide vaccine.[17,22] They further studied the effect of HER-2/neu T-helper peptide-based vaccinated patients receiving trastuzumab therapy and observed prolonged immune responses against not only the vaccine antigen but also cryptic antigens.[38] Collectively, the presence of either humoral or cellular immune response to multiple tumor antigens appears to be indicative of the strength of overall response against the tumor and predictive of clinical response. In our study, we used a panel of 13 tumor antigens for the detection of the humoral response. Serological detection of responses to multiple tumor antigens that were shown to be highly immunogenic in cancer patients would be convenient and could be included in routine immune monitoring.

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Original Article

Effects of Ghrelin Administration During Chemotherapy With Advanced Esophageal Cancer Patients

A Prospective, Randomized, Placebo-Controlled Phase 2 Study

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BACKGROUND: Cisplatin reduces plasma ghrelin levels through the 5-hydroxytryptamine (5-HT) receptor. This may cause cisplatin-induced gastrointestinal disorders and hinders the continuation of chemotherapy. The authors of this report conducted a prospective, randomized phase 2 trial to evaluate the effects of exogenous ghrelin during cisplatin-based chemotherapy. **METHODS:** Forty-two patients with esophageal cancer who were receiving cisplatin-based neoadjuvant chemotherapy were assigned to either a ghrelin group (n = 21) or a placebo group (n = 21). They received either intravenous infusions of synthetic human ghrelin (3 μg/kg) or saline twice daily for 1 week with cisplatin administration. The primary endpoint was changes in oral calorie intake, and the secondary endpoints were chemotherapy-related adverse events; appetite visual analog scale (VAS) scores; changes in gastrointestinal hormones and nutritional status, including rapid turnover proteins, and quality of life (QoL) estimated with the European Organization for Research and Treatment of Cancer QoL core questionnaire (QLQ-C30). **RESULTS:** Two patients were excluded from the final analysis: One patient suspended ghrelin administration because of excessive diaphoresis, and another patient in the placebo group failed to monitor the self-questionnaire. Food intake and appetite VAS scores were significantly higher in the ghrelin group than in the placebo group (18.2 ± 5.2 kcal/kg/day vs 12.7 ± 3.4 kcal/kg/day [$P = .001$] and 6.2 ± 0.9 vs 4.1 ± 0.9 [$P < .0001$], respectively). Patients in the ghrelin group had fewer adverse events during chemotherapy related to anorexia and nausea than patients in the control group. Significant deterioration was noted after chemotherapy in the placebo group in QoL scores, appetite, nausea and vomiting, and global health status. **CONCLUSIONS:** Short-term administration of exogenous ghrelin at the start of cisplatin-based chemotherapy stimulated food intake and minimized adverse events. ***Cancer* 2012;118:4785-94.** © *2012 American Cancer Society.*

KEYWORDS: ghrelin, esophageal cancer, food intake, appetite, cisplatin-based chemotherapy.

INTRODUCTION

Neoadjuvant and/or adjuvant chemotherapy using multiple antitumor agents is an important component of any therapeutic regimen for advance-stage solid tumors.[1] Cisplatin plays a central role in the success of such multidrug chemotherapy regimens for various cancers[2]; however, it is also associated with an assortment of adverse effects, including nephrotoxicity, myelosuppression, and gastrointestinal disorders like nausea, vomiting, and appetite loss. These gastrointestinal symptoms generally are nonlethal and reversible; however, their high frequency and strength can strongly impair the patient's quality of life (QoL) and, in general, may preclude the completion of chemotherapy.

The acute phase of cisplatin-induced gastrointestinal disorders involve increased serotonin (5-hydroxytryptamine [5-HT]) secretion from enterochromaffin cells.[3] Consequently, a 5-HT3-receptor antagonist was developed and is widely used for patients with cancer who are receiving cisplatin-based chemotherapy.[4] Despite this advance, many patients still suffer from gastrointestinal disorders because of cisplatin, especially in the later phases of treatment.

Ghrelin is an endogenous ligand for the growth hormone (GH) secretagogue receptor and is secreted predominantly by gastric endocrine cells.[5] It induces dose-dependent, GH-releasing activity[5,6]; stimulates appetite and food intake; and triggers a positive energy balance through a central mechanism involving hypothalamic neuropeptides.[5-9] In rodents, ghrelin increases GH secretion, feeding, and body weight when administered centrally or peripherally.[7-9] We also reported

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previously that intravenous administration of ghrelin enhanced oral feeding and diminished weight loss in patients who underwent total gastrectomy[10] and esophagectomy.[11]

In rodents, cisplatin markedly decreased plasma ghrelin concentrations, whereas the administration of exogenous ghrelin improved cisplatin-induced decreases in food intake.[12,13] These observations suggested that ghrelin also may be effective in minimizing the gastrointestinal disorders induced by cisplatin in humans, although there are huge differences in feeding activity between the 2 species. Accordingly, we undertook a randomized clinical trial to elucidate the effect of exogenous ghrelin on patients with esophageal cancer who were receiving cisplatin-based neoadjuvant chemotherapy.

MATERIALS AND METHODS

Patients

This prospective, randomized, placebo-controlled phase 2 study enrolled 42 patients with advanced esophageal cancer who received cisplatin-based neoadjuvant chemotherapy. The Human Ethics Review Committee of Osaka University School of Medicine approved the study protocol, and a signed consent form was obtained from each enrolled patient before study entry in accordance with the Declaration of Helsinki. This study was registered on the University Hospital Medical Information Network (R000005924). It began in February 2010, and enrollment of patients ended in January 2011. The eligibility criteria for the study were as follows: 1) histopathologically confirmed squamous cell carcinoma of the esophagus; 2) stage II or III disease according to criteria of the International Union Against Cancer (UICC), sixth edition[14]; 3) ages 20 to 80 years; 4) no esophageal obstruction by tumor and capacity for oral intake of soft solid foods; 5) adequate function of major organs; 6) no other active malignancy; 7) an Eastern Cooperative Oncology Group performance status (PS) of 0 or 1; and, 8) provision of written informed consent. The exclusion criteria for the study were as follows: 1) pregnant or potentially (willingly) pregnant women; 2) a past history of other chemotherapy or radiotherapy; and, 3) patients judged to be ineligible by the investigator.

A coordinating center (a section of the Department of Gastroenterological Surgery, Osaka University Medical School) was responsible for creating the treatment allocation code using a computer-generated randomization table with a statistician. Patients were randomized at a 1:1 ratio to receive intravenous infusion of either synthetic human ghrelin (3 μg/kg) or placebo (saline). Treatment allocation was arranged before the beginning of chemotherapy. The study was performed in a single-blind manner, ie, without knowledge of allocation to the patients.

Calculation of Sample Size

We estimated that oral intake of food calories during the study period in the placebo group would be 1600 ± 300 kcal/day. The power calculation was based on a 20% improvement by ghrelin administration in oral food intake calories, with a power of 85% and an α value of 5%, requiring at least 17 patients per study group. Assuming that approximately 20% of patients in each group would not complete the study, the initial proposal aimed to recruit 20 patients in each group.

Neoadjuvant Chemotherapy Regimen

The enrolled patients received cisplatin-based chemotherapy. This was a regimen consisting of either 5-fluorouracil, cisplatin, and doxorubicin (ACF)[3,15] or 5-fluorouracil, cisplatin, and docetaxel (DCF).[16] Both regimens entailed 2 treatments every 4 weeks. Specifically, the ACF regimen comprised cisplatin (70 mg) and doxorubicin (35 mg) on day 1 and a continuous infusion of 5-fluorouracil (700 mg/day) for 7 days, whereas the DCF regimen comprised cisplatin (70 mg) and docetaxel (70 mg) on day 1 and then a continuous infusion of 5-fluorouracil (700 mg/day) for 5 days. Supportive therapy and prophylaxis against expected side effects was provided. All patients were premedicated with intravenous ramosetron hydrochloride (0.3 mg), a representative 5-HT3 receptor antagonist. This was infused 1 hour before the administration of cisplatin on day 1 and every morning thereafter on days 2 through 7 (ACF regimen) or days 2 through 5 (DCF regimen). Hypersensitivity reactions were treated prophylactically with intravenous dexamethasone (8 mg), which was infused 1 hour before the administration of cisplatin. Adequate hydration was ensured before and after cisplatin infusion. Additional antiemetics or steroid preparations were recommended in case of grade 3 or greater anorexia, nausea, and vomiting according to toxicity grading criteria from the Common Terminology Criteria for Adverse Events version 4.0 (CTCAE).[17] After completion of the second cycle of neoadjuvant chemotherapy, the patient underwent curative resection, ie, subtotal esophagectomy with reconstruction by gastric tube, together with 2-field or 3-field lymphadenectomy.[18]

Evaluation of Adverse Events and Criteria for Dose Modifications

Adverse events were evaluated each day of chemotherapy and were scored by the most severe event in the first cycle (days 1-28) based on the toxicity grading criteria from the

CTCAE by each primary physician. Before starting the second cycle of chemotherapy, patients were required to have grade <2 hematologic toxicity. When patients did not recover within a 2-week delay or had grade 4 nonhematologic toxicity in the first cycle, the chemotherapy was discontinued, and surgical resection was considered.

Dose modifications in the second cycle were based on treatment-related adverse events recorded in the first cycle. In the ACF regimen, the doses of cisplatin and doxorubicin were reduced by 20% for grade 4 neutropenia that lasted >5 days, febrile neutropenia grade ≥3, and thrombocytopenia grade ≥3. In the DCF regimen, the doses of cisplatin and docetaxel were reduced by 20% for the same hematogenic toxicity. The dose of cisplatin was reduced by 20% in the second cycle in both regimens after a rise in serum creatinine level above 1.5 mg/dL during the first cycle. The dose of 5-fluorouracil was reduced by 20% for grade ≥3 diarrhea and mucositis. After completing 2 cycles of neoadjuvant chemotherapy, all patients were restaged by endoscopy and computed tomography to evaluate the clinical response to chemotherapy 2 weeks after the completion of chemotherapy. Clinical responses were categorized according to criteria based on the World Health Organization response criteria for measurable disease and the Japanese Society for Esophageal Diseases.[19]

Study Protocol

The study protocol is summarized in Figure 1A. Patients who were assigned to the ghrelin group received ghrelin treatment at a dose of 3 μg/kg body weight diluted in 50 mL saline given over 30 minutes twice daily (before breakfast and before dinner) for 7 consecutive days (days 1-7), as in our previous studies.[10,11] Synthetic ghrelin was prepared and supplied as described previously.[10,11] Patients in the placebo group received a corresponding placebo (pure saline) infusion in the same fashion. All participants received the same protocol of intravenous infusion in both groups, ie, 3000 mL/day from days 1 to 3 and 2000 mL/day from days 4 to 7 of chemotherapy, including 43 g glucose, 35 millequivalents (mEq) sodium, 20 mEq potassium, 35 mEq chloride, and 20 mEq lactate in 1000 mL.

Endpoints

The primary endpoint of this study was alteration in oral calorie intake from day 1 to day 7 of chemotherapy. Patients in this study were served standard meals and were allowed to receive extra food if desired. All dietary intake calories were calculated by a national registered dietitian at Osaka University Hospital by measuring the weight of each dish diet before and after every meal.[10,11] The secondary endpoints included changes in appetite, adverse events, QoL, body weight, nutritional status, hormonal assays, and blood tests. Appetite profiles were measured using a 100-mm visual analog scale (VAS), with the questions "How hungry are you?" and "How full do you feel?," which were anchored with "0 not at all" and "100-extremely." Patients were instructed to rate themselves by selecting the scale before each meal that was most appropriate to their feeling at that time. The mean VAS score was calculated each day. Questionnaires included the European Organization for Research and Treatment of Cancer core QoL questionnaire (QLQ-C30) before and after chemotherapy (day 8).[20] The QLQ-C30 contains 5 functional scales (physical, role, cognitive, emotional, and social), 3 symptom scales (fatigue, pain, and nausea/vomiting), a global health/QoL scale, and 6 single items (dyspnea, insomnia, appetite loss, constipation, diarrhea, and financial difficulties). All scale scores and single items scores range from 0 to 100. A high score for a functional scale represents a higher ("better") level of functioning, whereas a high score for a symptom scale or item represents a higher ("worse") level of symptoms.

Blood samples were collected before breakfast after an overnight fast before chemotherapy and on Days 3 and 8 of chemotherapy. The samples were transferred immediately into chilled tubes containing disodium ethylenediamine tetra-acetic acid and aprotinin, centrifuged at 4°C, separated for serum sampling, and stored at −50°C. The plasma samples were mixed with a 10% volume of 1 M hydrochloric acid before storing at −50°C. Plasma acyl-ghrelin and desacyl-ghrelin concentrations were measured with a sandwich-type enzyme immunoassay kit according to the protocol supplied by the manufacturer (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan).[21] Total plasma ghrelin concentration was calculated as acyl-ghrelin plus desacyl-ghrelin concentration. Serum GH, insulin, and leptin concentrations were measured using a GH "Daiichi" kit (TFB, Inc., Tokyo, Japan), a chemiluminescent enzyme immunoassay (Fujirebio, Inc., Tokyo, Japan), and a human leptin radioimmunoassay (RIA) kit (Linco Research Inc., St. Charles, Mo), respectively. Serum insulin-like growth factor-1 (IGF-1) levels were measured by RIA (SRL Company Ltd., Tokyo, Japan).

Statistical Analysis

Continuous variables are expressed as the mean ± standard deviation unless stated otherwise. Statistical differences between groups were calculated by using the Student *t* test, the Fisher exact test, the Mann-Whitney test, or the chi-square test, as appropriate. Comparisons of the time

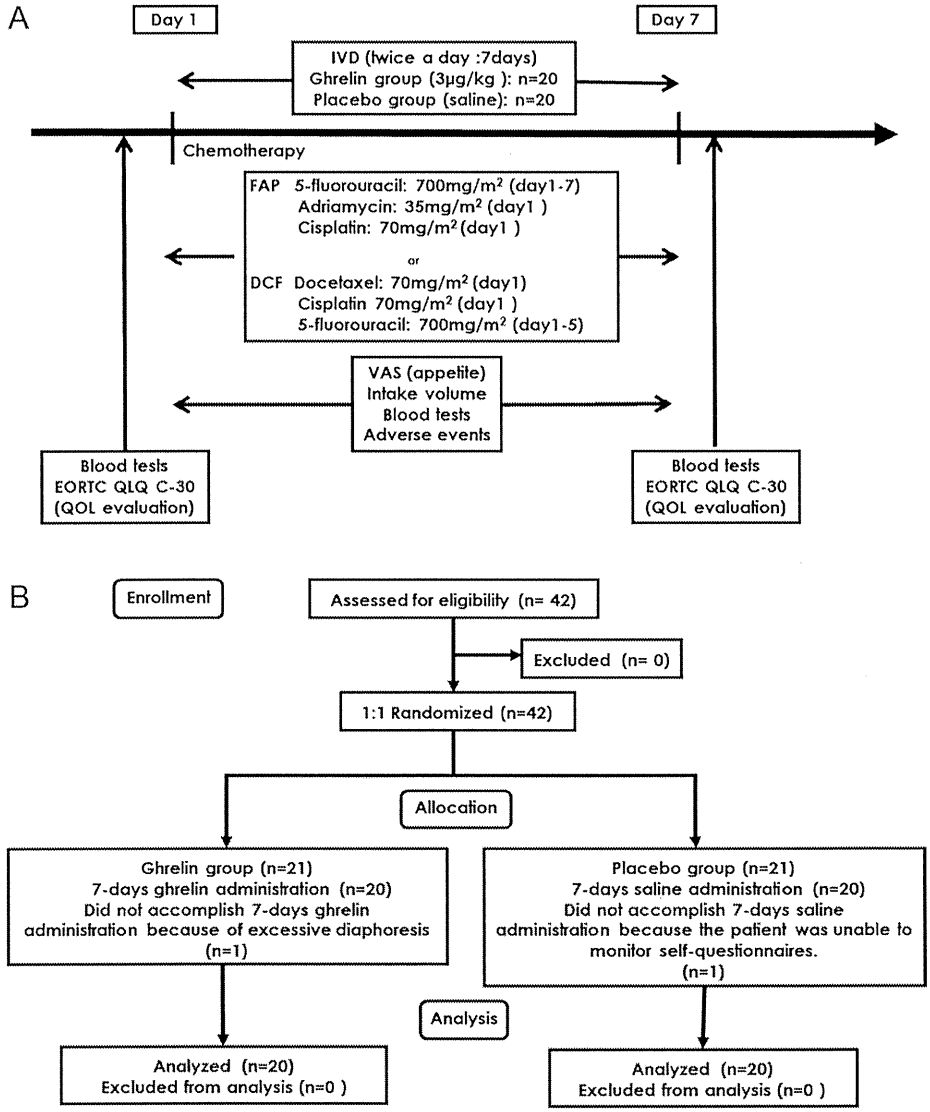

Figure 1. (A) This is a flow diagram of process through the trial. (B) The study protocol is illustrated. IVD indicates intravenous drip; FAP, combined 5-fluorouracil, doxorubicin (Adriamycin), and cisplatin; DCF, combined docetaxel, cisplatin, and 5-fluorouracil; VAS; visual analog scale; EORTC QLQ C-30, European Organization for Research and Treatment of Cancer Core-30 Quality-of-Life Questionnaire; QOL, quality of life.

course of food intake calories and appetite score were tested by using a 2-way repeated-measures analysis of variance (ANOVA). Statistical significance was set at $P < .05$. All calculations were performed using the JMP (version 9.0) software program (SAS Institute Inc, Cary, NC).

RESULTS

Patient Characteristics

In total, 42 enrolled patients were randomized into either the ghrelin group (21 patients) or the placebo group (21 patients). One patient (4.8%) in the ghrelin group who developed excessive diaphoresis during ghrelin infusion, equivalent to grade 2 according to CTCAE, and another patient (4.8%) in the placebo group who was unable to monitor the self-questionnaire because of general fatigue were excluded from the analysis (Fig. 1B). Table 1 lists the demographic and clinical characteristics of all patients. There were no significant differences in the background characteristics, including age, sex, body mass index, localization of cancer, clinical cancer staging, or chemotherapy regimen.

Table 1. Patient Characteristics

Parameter	No. of Patients		P
	Ghrelin Group	Placebo Group	
No. of patients	20	20	
Age: Mean±SD, y	65.8±5.2	61.8±10.9	.14
Sex			.28
Men	19	17	
Women	1	3	
BMI: Mean±SD, kg/m^2	21.6±.3	21.0±2.7	.44
Tumor localization			.27
Upper thoracic	4	1	
Middle thoracic	9	9	
Lower thoracic	7	10	
Clinical UICC TNM stage			
Tumor classification			.45
T1	0	0	
T2	6	4	
T3	8	12	
T4	6	4	
Lymph node status			.51
N0	8	6	
N1	12	14	
Metastasis classification			.43
M0	17	15	
M1	3	5	
Disease stage			.38
I	0	0	
II	9	7	
III	8	8	
IV	3	5	
Chemotherapy regimen			.74
ACF	13	12	
DCF	7	8	

Abbreviations: ACF: doxorubicin, cisplatin, and 5-fluorouracil; BMI, body mass index; DCF: docetaxel, cisplatin and 5-fluorouracil; SD, standard deviation; UICC, International Union Against Cancer.

Effect of Ghrelin on Dietary Intake and Appetite Scoring

The mean dietary intake gradually decreased after cisplatin administration to reach the lowest level on days 5 through 7. After completing chemotherapy, it took another 4 to 7 days for oral intake to recover and to allow hospital discharge. Although patients in the ghrelin and placebo groups reflected this trend, the decline in dietary intake with chemotherapy was significantly less in the ghrelin group compared with the placebo group (18.1 kcal/kg/day vs 12.7 kcal/kg/day overall), especially at day 1 (26.7 kcal/kg/day vs 23.1 kcal/kg/day) compared with day 7 (15.0 kcal/kg/day vs 8.5 kcal/kg/day) (Fig. 2A). In other words, the improved oral food intake because of ghrelin administration was more significant in the later phase of chemotherapy (repeated-measures ANOVA: ghrelin group vs placebo group, $P = .0027$). Changes in the VAS score reflected the changes in dietary intake between the 2 groups with a significant difference among them (repeated-measures ANOVA: ghrelin group vs placebo group, $P < .0001$, Fig. 2B). Notably, the appetite scores recovered more quickly after day 4 of chemotherapy in the ghrelin group than in the placebo group.

Effect of Ghrelin on Nutritional and Hormone Status

Table 2 details the blood test results before and after chemotherapy (day 8) in the ghrelin and placebo groups. There were no significant differences in nutritional parameters before chemotherapy, including hemoglobin, albumin, lymphocyte numbers, cholinesterase, total cholesterol, and the rapid turnover proteins (RTP) (prealbumin, retinol-binding protein, and transferrin). In the placebo group, significant declines after chemotherapy were observed for hemoglobin, prealbumin, and transferrin, but not for the other nutritional parameters tested. This RTP finding is consistent with ghrelin preventing nutritional deterioration because of chemotherapy compared with the placebo group (prealbumin: 26.4 ± 4.6 mg/dL vs 21.7 ± 2.8 mg/dL [$P = .042$]; transferrin: 205 ± 18 mg/dL vs 162 ± 32 mg/dL [$P = .037$]).

With respect to ghrelin and associated hormones, plasma total ghrelin levels (acyl-ghrelin plus desacyl-ghrelin) significantly decreased after chemotherapy, accounting for 61% of the baseline values (before chemotherapy) in the placebo group. GH, a target hormone for ghrelin, and IGF-1, a mediator of GH, consistently tended to decrease after chemotherapy. However, despite the poor dietary intake during chemotherapy, leptin tended to decrease rather than increase after chemotherapy. There were no significant differences in plasma ghrelin levels between the groups before and after chemotherapy because of its rapid turnover. Likewise, the levels of GH, IGF-1, insulin, and leptin did not differ between the ghrelin and placebo groups.

Adverse Events

Table 3 lists the hematologic and nonhematologic adverse events during the first cycle of chemotherapy. Diaphoresis is a known physiologic effect of ghrelin. One patient with grade 2 diaphoresis was excluded, whereas another with grade 1 diaphoresis completed the study protocol and was included in the analysis. Anorexia and nausea are the most common toxicities reported with cisplatin-based chemotherapy. In our study, grade ≥3 symptoms were noted in 55% (anorexia) and 60% (nausea) of patients in the placebo group. Ghrelin administration significantly reduced