peripheral blood by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden) and monocytes were enriched by adherence to a plastic tissue culture flask (Corning, NY) for 90 min at 37°C. Adherent cells were cultured with 1,000 units/ml of granulocyte macrophage colony-stimulating factor (GM-CSF, Peprotech EC Ltd, London, UK) and 1,000 units/ml of IL-4 (Peprotech EC Ltd) in X-VIVO (Life Technologies, Inc., Gaithersburg, MD). On day 5, the DCs were matured with TNF-alpha (10 ng/ml, Peprotech EC Ltd), PGE_2 (1 μg/ml), IL-1β (10 ng/ml), IL-6 (1,000 U/ml). On day 7, the cytokine-treated cells were used as mature DC.

Generation of HER-2-specific CTL lines and CTL clone

After 7 days of culture as described above, mature DCs were pulsed with HER-2 peptide (20 μg/ml), which included the wild type peptide and the substitution analog peptide, in the presence of β_2-microglobulin (3 μg/ml) for 60 min at 37°C. Then, these peptide (wild type or substitution analog)-loaded mature DCs were co-incubated with autologous PBMCs, which were obtained from HLA-A24 healthy donors, at 1:10 in a 12-well plate in X-VIVO with 1% autologous serum, 100 IU/ml of IL-2 (Shionogi). Subsequent cultured cells were re-stimulated with these peptide-loaded, irradiated (25 Gy) mature DCs every 7 days. After four stimulations, the cultured CTL lines were tested for the reactivity with the enzyme linked immunospot (Elispot) analysis and cytotoxic assay. All CTL lines were generated from five different healthy donors.

CTL clone was then obtained from the CTL lines by limiting dilution. Briefly, the CTLs were isolated in 96-well U-bottom plates in X-VIVO with irradiated allogeneic PBMC (5×10^4 cells/well) from two different donors in the presence of HER-2 peptide (20 μg/ml) and 100 IU/ml of IL-2 (Shionogi). The CTL clones were expanded with irradiated allogeneic PBMC, HER-2 peptide and 100 IU/ml of IL-2.

Elispot analysis and cytotoxic assay

The HER-2-specific response was determined by the IFN-γ Elispot analysis and cytotoxic assay. Elispot analysis was performed with the Mabtech assay system (Nacka, Sweden). After 96-well plates with nitrocellulose membrane (Millipore) were pre-coated with a primary anti-IFN-γ antibody (1D1K) for 24 h, the plates were pre-treated with AIM-V containing 1% human serum albumin. Target cells (2×10^4 per well) and CTLs (2×10^3 per well) were incubated in 200 μl of AIM-V for 24 h in triplicate. Thereafter, a biotinylated secondary anti-IFN-γ antibody (7-B6-1) was added for 2 h and then the plates were incubated with the streptavidin-alkaline phosphatase reagent and stained with NBT and BCIP (Gibco). All Elispot analyses were performed in the same condition.

For the cytotoxic assay, a standard 4 h ^{51}Cr release assay was performed. To assess the peptide-specificity of CTL, TISI cells were pulsed with HER-2-derived, HLA-A24 restricted peptide for 16 h at 37°C. Thereafter, peptide-pulsed TISI cells were washed and subjected to the cytotoxic assay as a target. After the target cells were labeled with 100 μCi ^{51}Cr for 60 min, they (5×10^3 per well) and the effector cells at various effector/target ratios were co-incubated in 200 μl of X-VIVO medium in a 96-well U-bottom plate in triplicate for 4 h at 37°C. Subsequently, cold target inhibition was carried out using the non-radiolabeled TISI cells loaded with HER-2 peptide or with an irrelevant HIV peptide (as negative control) at various hot/cold target ratios. The supernatants were harvested and radioactivity was determined using a gamma counter. The percentage of ^{51}Cr release was calculated according to the following formula: % lysis = 100× (experimental release − spontaneous release)/(maximum release − spontaneous release).

Flow cytometric analysis and tetramer assay

For the evaluation of HER-2 expression, a PE-labeled anti-HER-2 mAb (Becton Dickinson, San Jose, CA) and PE-labeled mouse IgG1 mAb (Beckman-Coulter, Miami, FL) as a negative control were used for immunostaining by flow cytometric analysis.

To evaluate the specificity of the CTL905AA clone, FITC-labeled anti-CD8 (MBL, Nagoya, Japan) and a PE-labeled HLA-A*2402-HER-2(905AA) tetramer (NH_2–VYSYGVTVF–COOH:905AA peptide; MBL, Nagoya, Japan) were used for immunostaining, according to the manufacturer's recommendations.

Statistics

To evaluate statistical differences between the two groups, a non-paired Student's t test was performed. Statistically significant differences were considered to be P values < 0.05.

Results

Identification of HLA-A*2402 binding epitopes and generation of HER-2-derived, epitope-specific CTL lines

HER-2-derived epitopes were identified on the basis of the presence of an HLA-A2402 binding motif by scanning the HER-2 protein with a customized computer program which accounts for both primary and secondary HLA binding anchor residues contained within the HLA-A24 epitopes [16, 21]. In addition to the identified wild type peptides, sequences possessing suboptimal residues at anchor positions were modified to enhance the binding capacity for HLA-A*2402 molecules. Preferred anchor residues for A*2402 have been deter-

mined by Epimmune and others [21] to be tyrosine (Y) at position 2 and phenylalanine (F) at the carboxy terminus. In the present study, nineteen nonamers carrying the HLA-A24 binding motif were selected, including ten wild type peptides ($IC_{50} < 1{,}000$ nM) and nine substitution analog peptides ($IC_{50} < 50$ nM; Table 1). All of the substitution analog peptides showed a high binding affinity for HLA-A24 ($IC_{50} < 50$ nM), while wild type peptides showed a range of affinity from high to weakly intermediate.

Of these peptides, we excluded the already known peptide epitopes HER-2(8), HER-2(780) and HER-2(63) [9, 24, 31], and generated 16 different peptide-specific CTL lines from five different HLA-A24(+) healthy donors by using mature DC cells pulsed with each of the remaining peptides. Then, the CTL lines were tested for their specificity against the cognate peptide used for each CTL induction, in an Elispot analysis. The reactivities of the peptide-induced CTL lines are shown in Fig. 1. In the present study, each peptide was considered positive if the spot forming cells (SFC) against TISI targets pulsed with cognate peptide was more than twofold SFC against CTL only in an Elispot analysis. Three CTL lines (CTL780A, CTL905AA and CTL414AA induced by the HER-2(780A), HER-2(905AA) and HER-2(414AA) epitopes, respectively) out of the 16 T cell lines significantly recognized TISI targets pulsed with each inducing cognate peptide.

HER-2 peptide-specific CTL lines can specifically recognize HLA-A24 tumor cell lines overexpressing HER-2

The CTL780A, CTL905AA and CTL414AA lines, wherein each CTL line was generated from five different healthy donors, were tested against HER-2-expressing tumor cell lines in an Elispot analysis. In the present study, the response of the CTL line against the tumor cell line was positive if the SFC against HLA-A24 positive, HER-2 positive tumor cell line was more than threefold SFC against HLA-A24 negative, HER-2 positive tumor cell line in an Elispot analysis. Out of the three CTL lines, only the CTL 905AA line recognized HLA-A24 positive tumor cell lines overexpressing HER-2 (PC-9 and HCT-15), but not a HLA-A24 negative HER-2 positive MKN-7 tumor cell line (Fig. 2). To further confirm the reactivity, CTL 905AA lines, which were generated from five different healthy donors, were tested against several targets in a cytotoxicity assay. The CTL 905AA line lysed HER-2(+), HLA-A24(+) HCT-15 and KATOIII cells, but not MKN-7, SKOV-3 or K562 cells (Fig. 3). These results indicated that the HER-2(905AA) peptide-induced CTLs recognized and lysed HER-2-expressing and HLA-A24(+) tumors.

CTL905AA clones recognize HLA-A24 tumor cell lines overexpressing HER-2 and TISI target cells pulsed with the HER-2(905) wild type peptide

To further analyze the specificity of the HER-2(905AA) peptide, CTL clones were generated by limiting dilution methods from the CTL905AA line. Using the HER-2(905AA)–HLA-A24 tetramer, CTL 905AA clone, clone M2 was stained positive for both CD8 and the 905AA tetramer (Fig. 4), indicating that the T cell clone M2 was HER-2(905AA)-specific. In addition, clone M2 recognized the TISI cells pulsed with HER-2(905AA) peptide and also, to a lesser extent, TISI pulsed with the HER-2(905) wild type peptide (Fig. 5). These results revealed that the MHC anchor-substituted analog epitope HER-2(905AA) was more effective at breaking tolerance and inducing CTL which recognized the HER-2(905) wild type peptide, than the HER-2(905) wild type peptide

Table 1 Relative binding affinity of HER-2/neu-derived peptides to HLA-A24

Peptide	Sequence	A24 binding[2] IC_{50}
Wild type peptide		
HER-2(8)	RWGLLLALL	11.41
HER-2(780)	PYVSRLLGI	77.14
HER-2(951)	VYMIMVKCW	79.93
HER-2(440)	AYSLTLQGL	97.98
HER-2(907)	SYGVTVWEL	101.42
HER-2(905)	VWSYGVTVW	173.21
HER-2(63)	TYLPTNASL	323.50
HER-2(968)	RFRELVSEF	744.21
HER-2(342)	CYGLGMEHL	780.14
HER-2(887)	KWMALESIL	887.79
Substitution analog		
HER-2(8AA)	RYGLLLALF	1.47
HER-2(780A)	PYVSRLLGF	9.23
HER-2(905AA)	VYSYGVTVF	17.89
HER-2(887AA)	KYMALESIF	19.97
HER-2(951A)	VYMIMVKCF	19.98
HER-2(907A)	SYGVTVWEF	27.82
HER-2(968A)	RYRELVSEF	37.21
HER-2(414AA)	AYPDSLPDF	42.97
HER-2(63A)	TYLPTNASF	46.02

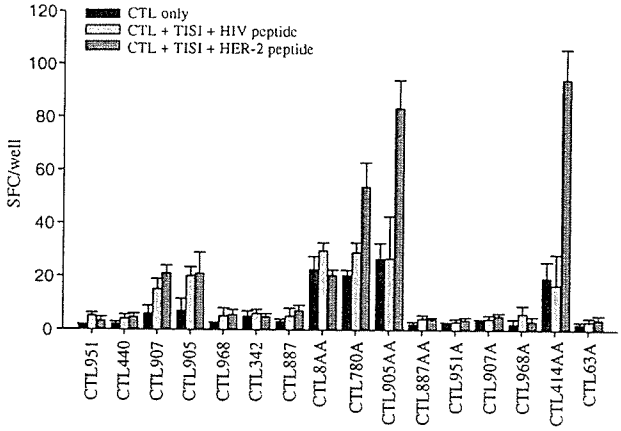

Fig. 1 The specificities of the HER-2 peptide-inducing CTL lines were evaluated with Elispot assay. Sixteen HER-2-derived peptide-specific CTL lines, designated by the inducing peptide epitope, were generated from five different HLA-A24(+) healthy donors by using mature DC cells pulsed with each peptide. Then, the CTL lines were tested for their specificity against cognate peptides, which were used for each CTL induction, in an Elispot assay described in Material and methods. Elispot assays were performed against all the CTL lines generated from five different healthy donors. Representative data from five independent experiments are shown. In the present study, each peptide was considered positive if the spot forming cells (SFC) of the CTL line against TISI targets pulsed with cognate peptide is more than the twofold SFC of CTL only. As a result, CTL780A, CTL905AA and CTL414AA significantly recognized TISI targets pulsed with each cognate peptide. *Error bars* indicate the standard error of the mean

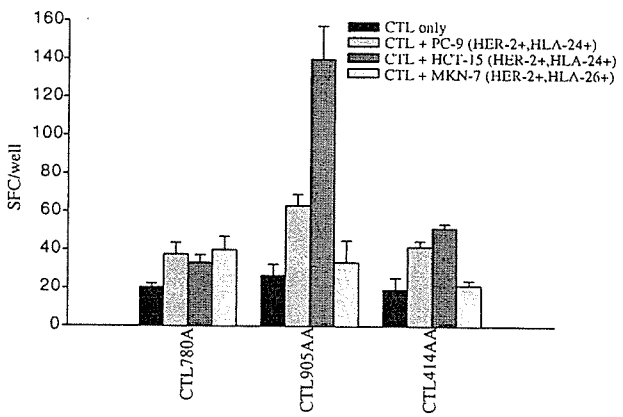

Fig. 2 Reactivities for the HER-2-expressing tumor by peptide-inducing CTL lines were evaluated with the Elispot assay. The CTL780A, CTL905AA and CTL414AA lines, which were generated from five different healthy donors, were tested against tumor cell lines in an Elispot analysis described in Material and methods. Representative data from five independent experiments are shown. In the present study, the response of the CTL line against the tumor cell line was positive if the SFC of the CTL line against a HLA-A24 positive HER-2 positive tumor cell line is more than threefold the SFC of a CTL line against a HLA-A24 negative HER-2 positive tumor cell line. Out of the three CTL lines, only the CTL 905AA line recognized HLA-A24 positive tumor cell lines overexpressing HER-2 (PC-9 and HCT-15), but not the HLA-A24 negative HER-2 positive MKN-7 tumor cell line. *Error bars* indicate the standard error of the mean

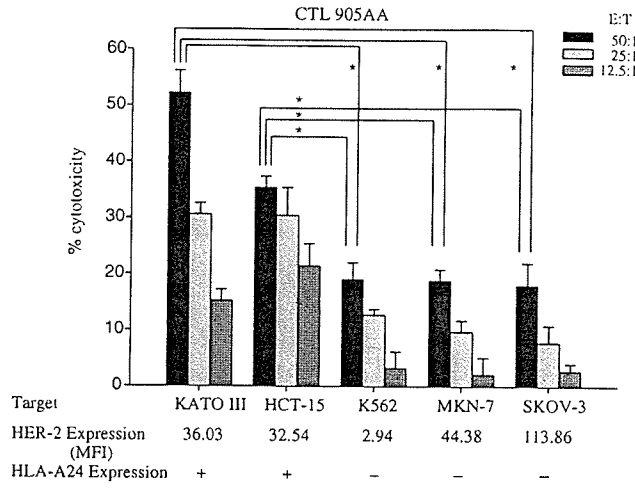

Fig. 3 Cytotoxic assay by CTL905AA line. CTL905AA lines generated from five different healthy donors were tested against several targets using 4 h ^{51}Cr-release assays at various effector/target ratios described in Material and methods. Representative data from five independent experiments are shown. CTL905AA line lysed HER-2(+), HLA-A24(+) HCT-15 and KATOIII, but not MKN-7, SKOV-3 or K562. HER-2 expression on the tumor cells was evaluated by flow cytometric analysis. Statistical analysis was performed with the Student's *t* test. $*P < 0.05$. *MFI* mean fluorescence intensity

which was less effective at inducing a primary in vitro CTL response (Fig. 1).

To further confirm the reactivity of the HER-2(905AA) peptide, various doses of the HER-2(905AA) peptides were tested for their capacity for sensitizing TISI by the clone M2. As expected, the reactivity of HER-2(905AA) peptide was dose-dependent (Fig. 6).

Moreover, another CTL clone derived from the CTL905AA line, M5, also demonstrated cytotoxicity against HER-2(+), HLA-A24(+) targets (HCT-15, MRKnu-1, KATOIII and PC-9) specifically (Fig. 7). When cold target inhibition assays were performed, a significant (84.3 or 75.2% inhibition at the 1:10 hot to cold ratio) inhibition of the killing for the HCT-15 was observed only with non-radiolabeled TISI loaded with HER-2(905AA) peptide or HER-2(905) wild type peptide but not with TISI loaded with an irrelevant control HIV peptide (Fig. 8). Collectively, these data indicated the HER-2(905AA) MHC anchor-substituted analog can efficiently induce HER-2-specific, HLA-A24 restricted CTLs.

Discussion

In the present study, we have screened seven wild type peptides and nine HLA anchor-substituted analogs derived from HLA-A24 binding, HER-2-derived peptides as possible CTL epitopes. Then, we showed that the analog HER-2(905AA) can efficiently induce HER-2-

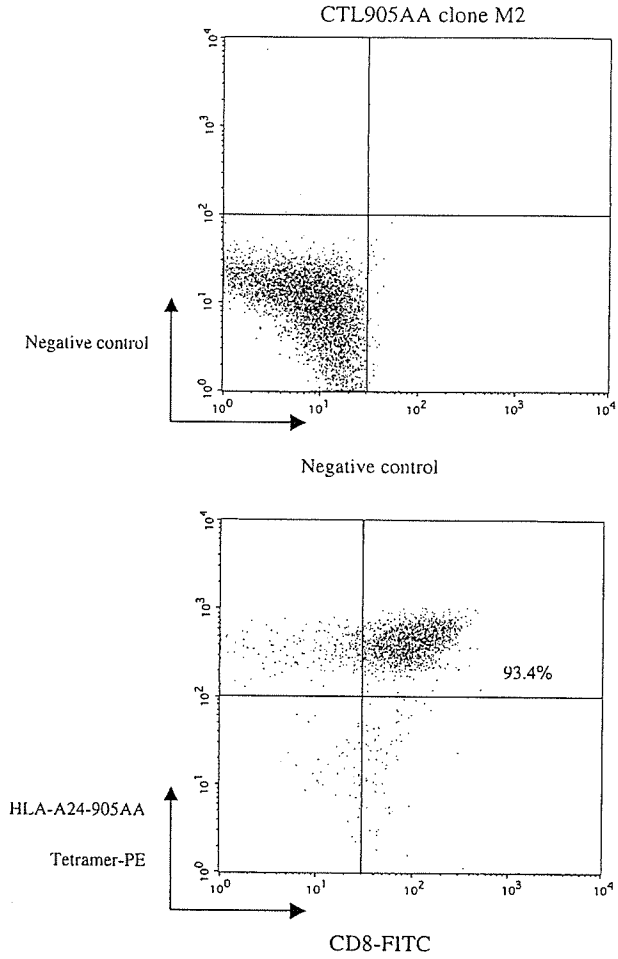

Fig. 4 Tetramer assay for the CTL905AA clone M2. HER-2(905AA)-reactive CTL clones were generated by limiting dilution methods from the CTL905AA line. HER-2(905AA) tetramer analysis showed that CTL905AA clone M2 was stained positive for both CD8 and the HLA-A24-HER-2(905AA) tetramer (93.4%)

specific, HLA-A24 restricted CTLs, which recognize and lyse tumor cells presenting the naturally processed wild type HER-2 epitope.

Deliberate substitutions of amino acids in peptide epitopes are generally thought to be effective in inducing peptide-specific CTLs by improving the binding affinity to HLA molecules. In previous studies, analogs substituted at MHC anchor residues have been tested in several tumor Ags, such as GP2, NY-ESO-1, gp100 as well as MART-1, and some of them successfully improved the immunogenicity of the CTL epitopes [3, 15, 25, 32, 35]. In the present study, to improve the immunogenicity of relatively low binding, HER-2-derived peptides, we generated anchor-substituted analogs (Table 1) and tested them for the immunogenicity. Although every substituted analog resulted in the enhancement of the binding affinity to HLA-A24 molecules, only analogs HER-2(780A), HER-2(905AA) and HER-2(414AA)

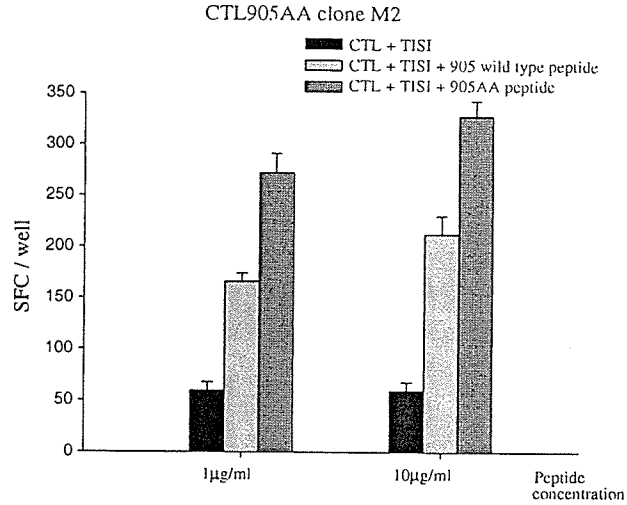

Fig. 5 The reactivity of the CTL905AA clone M2 in Elispot analysis. The CTL905AA clone M2 recognized TISI pulsed with HER-2(905AA) peptide and also, to a lesser extent, TISI pulsed with the HER-2(905) wild type peptide

were effective in inducing a peptide-specific CTL response (Fig. 1). Furthermore, out of three analogs, only the HER-2(905AA)-specific CTL resulted in the recognition and lysis of HLA-A24 tumor cell lines overexpressing HER-2 and the EBV-transformed cell line TISI pulsed with its wild type peptide. In addition, the cold target inhibition assay using the HER-2(905AA)-specific CTL clone further supported that a newly identified HER-2(905) peptide epitope is presented as the CTL epitope on HER-2 overexpressing tumor cell lines (Fig. 8).

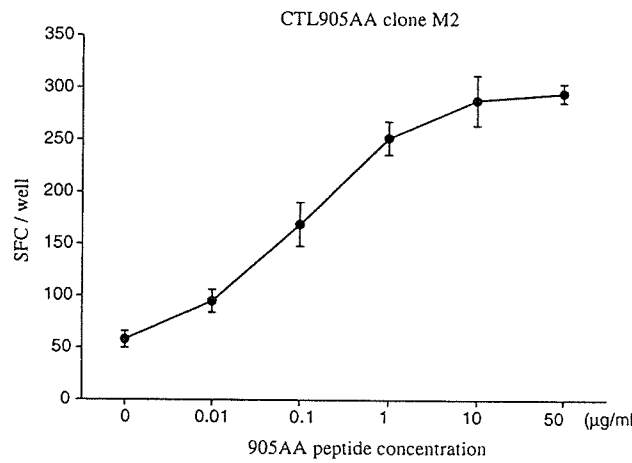

Fig. 6 Dose-dependent reactivity of the CTL905AA clone M2. To further confirm the reactivity of the CTL905AA clone M2, various doses of HER-2(905AA) peptide were tested for their capacity for sensitizing TISI by the CTL905AA clone M2 in an Elispot analysis. The reactivity of clone M2 for HER-2(905AA) peptide was dose-dependent. *Error bars* indicate the standard error of the mean

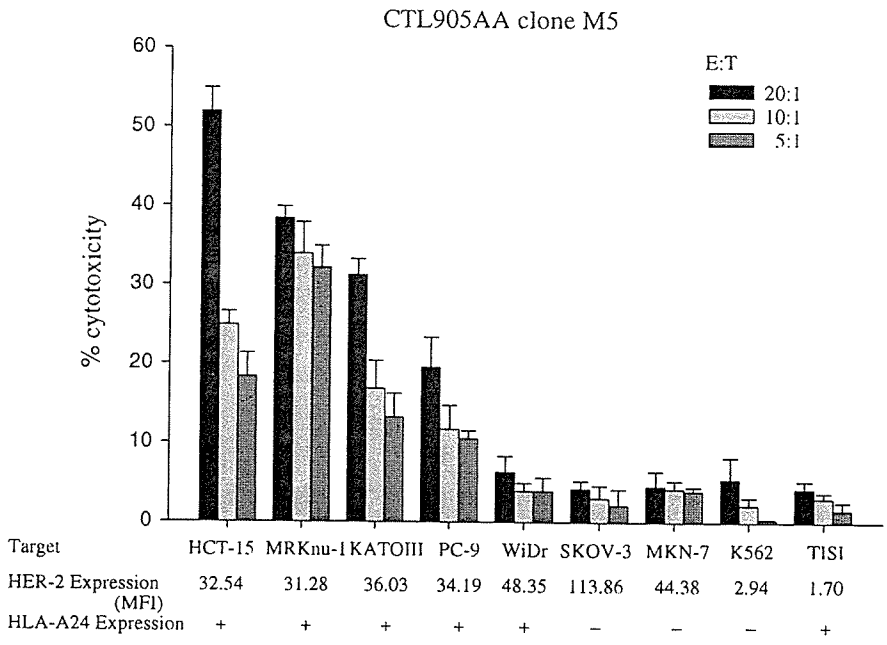

Fig. 7 The specificity of the CTL905AA clone M5 was evaluated with cytotoxic assay. The CTL905AA clone M5 was tested against several tumor cell lines using 4 h ^{51}Cr-release assays at various effector/target ratios. The clone M5 lysed the HER-2(+) and HLA-A24(+) tumor cell lines HCT-15, MRKnu-1, KATOIII and PC-9, while M5 did not react with WiDr, SKOV-3, MKN-7, K562 or TISI cells. HER-2 expression on the tumor cells was evaluated by the flow cytometric analysis. *MFI* mean fluorescence intensity

In general, low binding affinity for the MHC class I molecule makes it difficult to induce peptide-specific CTL as epitope peptides that have low binding affinity may permit T cells to escape from negative selection; however, these epitope peptides and T cells may be useful for tumor-specific immunity. In the present study, the HER-2(905AA) analog peptide, but not the HER-2(905) wild type peptide, was effective in inducing a peptide-specific CTL response, and the HER-2(905AA)-specific CTL specifically lysed TISI target cells pulsed with HER-2(905AA) compared to TISI targets pulsed with HER-2(905). It is possible that increased immunogenicity with the HER-2(905AA) peptide analog may be derived from a combination of efficient binding to HLA-A24 molecules and better interaction with T cell receptors of specific CTLs. It has been shown that MHC anchor-substituted analogs derived from gp100 or NY-ESO-1 can induce CTL responses more efficiently than their corresponding wild type peptide epitopes [3, 25].

Recently, we and a few others have suggested that tumor-specific immunotherapy based on HER-2-derived peptides may be a useful and novel approach to the treatment of cancer patients with HER-2 overexpressing tumors. In fact, we have shown that DCs pulsed with HER-2-derived, HLA-A2 restricted peptides can induce specific T cell responses in patients with gastric cancer [20]. HLA-A24 is one of the most common alleles in Japanese people and is shared by more than 60% of the Japanese gastric cancer patients [7]. Thus, it would be desirable to identify additional HLA-A24 restricted immunodominant epitope peptides derived from HER-2, in order to broaden tumor-specific immunotherapy based on HER-2. The HER-2(905AA) peptide analog could be used as cancer vaccine to induce potent anti-tumor CTL responses. We believe that HER-2-specific, HLA-A24 restricted CTLs generated by HER-2(905AA) may react with HER-2 overexpressing tumor in vivo. In conclusion, the substitution analog peptide, HER-2(905AA), can efficiently induce HER-2-specific, HLA-A24 restricted CTLs.

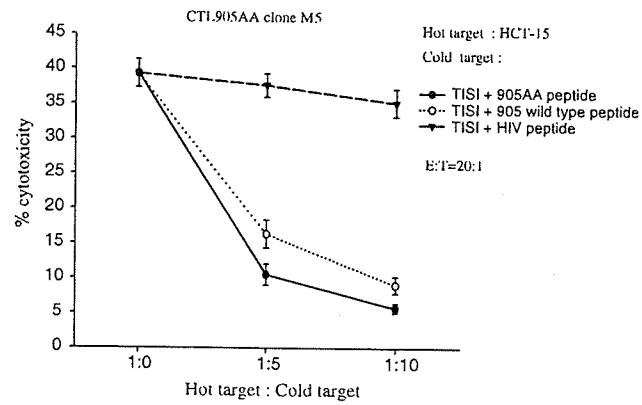

Fig. 8 Cold target inhibition assays with the CTL905AA clone M5. Cold target inhibition assays were performed using non-radiolabeled TISI cells loaded with the HER-2(905AA) peptide, the HER-2(905) wild type peptide or an irrelevant HIV peptide at various hot/cold target ratios. A significant (84.3 or 75.2% inhibition at the 1:10 hot to cold ratio) inhibition of the killing for the HER-2(+) and HLA-A24(+) HCT-15 mediated by clone M5 was observed when non-radiolabeled TISI cells loaded with HER-2(905AA) peptide or HER-2(905) peptide were added, but not when TISI cells were loaded with the control HIV peptide. *E:T* effector:hot target ratio

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ORIGINAL ARTICLE

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CD4(+)CD25^{high} regulatory T cells increase with tumor stage in patients with gastric and esophageal cancers

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Abstract *Purpose*: Regulatory T cells (T regs) can inhibit immune responses mediated by T cells. It has been shown that there is an increased proportion of T regs in several different human malignancies, although the actual mechanism remains unclear. In the present study, we evaluated the prevalence of CD4(+)CD25^{high} T regs in PBMCs from patients with gastric and esophageal cancers in relation to the clinical outcome. *Methods*: PBMCs in 72 patients with gastric cancer and 42 patients with esophageal cancer were evaluated for the proportion of CD4(+)CD25^{high} T cells, as a percentage of the total CD4(+) cells, by flow cytometric analysis with triple-color staining. Actuarial overall survival rates of the patients were analyzed by the Kaplan–Meier method. *Results*: The percentages of CD4(+)CD25^{high} T cells for cases of gastric cancer ($4.9 \pm 1.2\%$) and esophageal cancer ($5.2 \pm 2.1\%$) were significantly higher than those for healthy donors ($1.9 \pm 1.1\%$, $P < 0.01$). There were significant differences in the prevalence of CD4(+)CD25^{high} T cells between the early and advanced disease stages, both in gastric cancer (stage I vs. III, $P < 0.05$; stage I vs. IV, $P < 0.05$) and esophageal cancer (stage I vs. IV, $P < 0.05$). The patients with a high proportion of CD4(+)CD25^{high} T cells showed poorer survival rates in comparison to those with a low proportion, in both gastric and esophageal cancers. After patients received curative resections of gastric cancers ($n = 57$), the increased proportions of CD4(+)CD25^{high} T cells were significantly reduced, and the levels were almost equal to those in normal healthy donors. In addition, studies of gastric cancer patients with postoperative recurrent tumors ($n = 6$) revealed that the prevalence of CD4(+)CD25^{high} T cells individually increased compared to 2 months after the operations. CD4(+)CD25^{high} T cells expressed FOXP3 mRNA and had abundant CD45RO and intracellular CTLA-4 molecules. *Conclusions*: These results strongly suggest that tumor-related factors induce and expand CD4(+)CD25^{high} T regs.

Keywords Regulatory T cells · Gastric cancer · Esophageal cancer · CD4(+)CD25^{high} T cells

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Introduction

Regulatory T cells (T regs) are thought to be a functionally unique population of T cells, and function to maintain immune homeostasis [1–4]. T regs can inhibit immune responses mediated by CD4(+) and CD8(+) T cells, and it was reported that T regs play an important role in preventing allograft rejection, graft-versus-host disease, and autoimmune disease [5, 6].

Within CD4(+) T cells with a suppressive function, there are at least three different cell populations: CD25(+)CD4(+)-naturally occurring T regs, IL-10-producing Tr1 cells, and Th3 cells [7–14]. Furthermore, although CD25(+)CD4(+)-naturally occurring T regs were originally characterized by the coexpression of CD4 and CD25 in mice, it has recently been shown in humans that the CD4(+)CD25^{high} subset corresponded to naturally occurring T regs populations with a suppressive capacity, while CD4(+)CD25^{int} T cells contaminated effector or memory T cells with no suppressive capacity [15–17].

There is accumulating evidence that increased populations of T regs are present in patients with gastric cancer [18–20], colorectal cancer, gall bladder cancer, pancreatic cancer [20, 21], ovarian cancer [22], and lung cancer [23]. Moreover, we showed that the population of T regs in tumor-infiltrating lymphocytes (TILs) of patients with advanced gastric cancer was significantly larger than that of TILs in patients with early gastric cancer [18].

There is no clear evidence to suggest the mechanisms for the induction of T regs in cancer-bearing hosts. It has recently been shown that tumor cells and microenvironmental macrophages produce the chemokine CCL20, which mediates the trafficking of T regs to the tumor [24]. Also, we have shown that the levels of CD4(+)CD25^{high} T regs in tumor-draining lymph nodes adjacent to tumors were greater than those distant from tumors [25]. These data indicate that tumor-derived factors may induce and expand T regs pools.

In the present study, we evaluated the prevalence of CD4(+)CD25^{high} T regs in PBMCs from patients with gastric and esophageal cancers, and clarified the correlation between prevalence and clinical outcome for the patients.

Materials and methods

Patients

Seventy-two patients with gastric cancer and 42 patients with esophageal cancer, who were treated in the University of Yamanashi Hospital from 1999 to 2000, were enrolled in the present study, and their clinical features were evaluated according to the TNM classification for gastric and esophageal cancers. The patients with gastric cancer were 70.8 ± 18.3 years old (mean ± SD), and 40 patients were men and 32 were women. Thirty-six tumors belonged to stage I, 8 were stage II, 8 were stage III, and 20 tumors were stage IV.

The patients with esophageal cancer were 72.9 ± 11.3 years old, and 40 patients were men and 2 were women. Three tumors belonged to stage I, 16 were stage II, 15 were stage III, and 8 tumors were stage IV.

None of the patients received radiotherapy, chemotherapy, or other medical interventions before the study. This study was approved by the ethical committee of the University of Yamanashi, and written informed consent was obtained from all individuals.

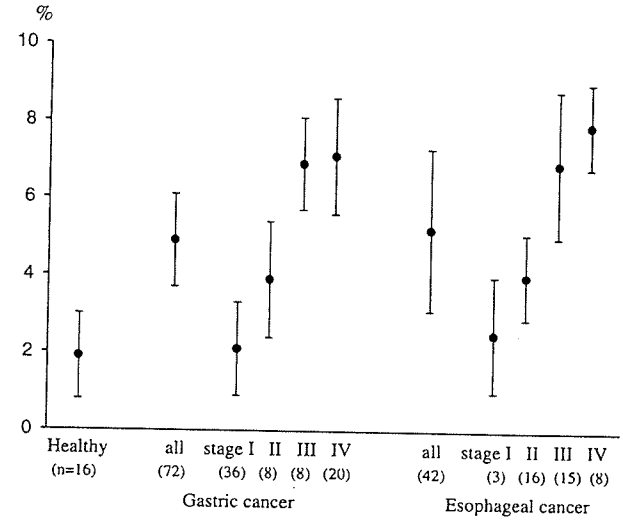

Fig. 2 Increased populations of CD4(+)CD25^{high} T cells in PBMCs from patients with gastric and esophageal cancers. The percentages of CD4(+)CD25^{high} T cells in cases of gastric ($n=72$, 4.9 ± 1.2%) and esophageal cancers ($n=42$, 5.2 ± 2.1%) were significantly higher than those of healthy donors ($n=16$, 1.9 ± 1.1%, $P<0.01$). There were significant differences in the prevalence of CD4(+)CD25^{high} T cells between stage I and III ($P<0.05$), stage I and IV ($P<0.05$) in gastric cancer, as well as stage I and IV ($P<0.05$) in esophageal cancer. Stage classification was defined according to the TNM classification

Cell preparations

PBMCs were isolated with a Ficoll (Amersham, Uppsala, Sweden) density gradient and routinely stored in liquid nitrogen in Cell Stock Media (IBL, Gumma, Japan).

Flow cytometric analysis

PBMCs were stained for molecules to determine their immunophenotype using anti-CD25-FITC, anti-CD4-PerCP, anti-CD3-APC, anti-CD152 (CTLA4)-PE, and

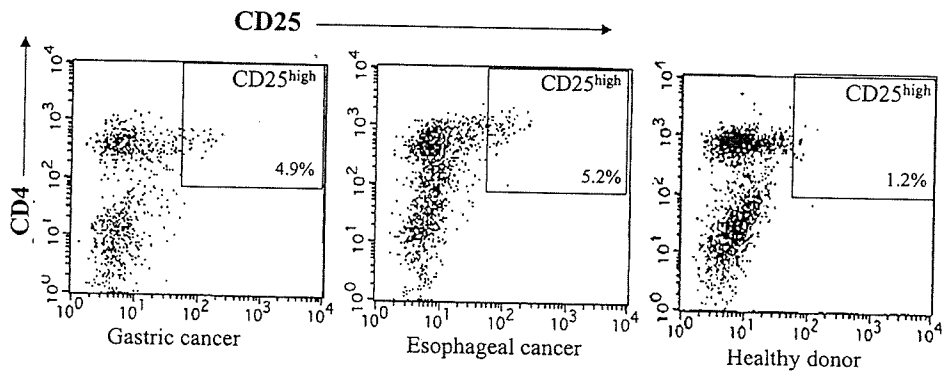

Fig. 1 CD4(+)CD25^{high} T cells in PBMCs from patients with gastric and esophageal cancers. The population of CD4(+)CD25^{high} T cells as a percentage of the total CD4(+) cells was evaluated by flow cytometric analysis with triple-color staining. Representative flow cytometric data from gastric (stage III) and esophageal cancer patients (stage III) and healthy donors are shown. *Rectangular gates* indicate CD4(+)CD25^{high} T cell populations

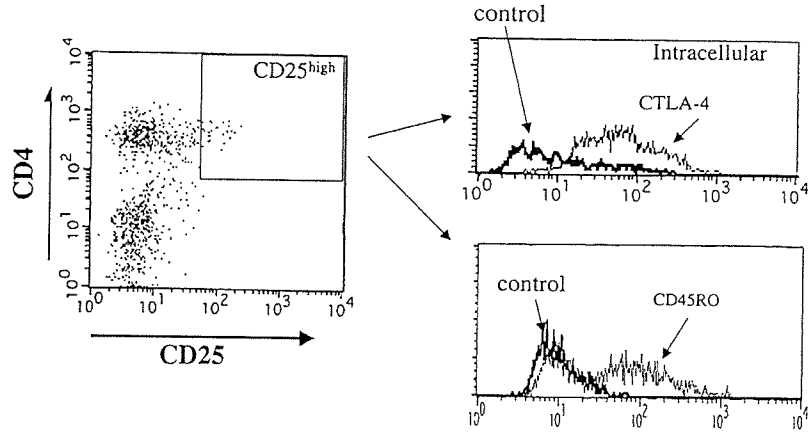

Fig. 3 The expression of CD45RO and intracellular CTLA-4(CD152) on CD4(+)CD25^{high} T cells. Representative flow cytometric data from gastric cancer patients (stage III) showed the expression of CD45RO and intracellular CTLA-4 after the gating of CD4(+)CD25^{high} T cells

anti-CD45RO-PE (Dako, Glosprup, Denmark) antibodies. Triple- or four-color flow cytometry was performed using FACSCalibur (Becton Dickinson, San Jose, CA, USA). Cells were analyzed using Cell Quest software.

To analyze the prevalence of T regs, CD4(+) CD25^{high} cells after gating on CD3(+) were evaluated and expressed as a percentage of the total CD4(+) cells.

Intracellular cytokine assay

Briefly, cells were incubated in RPMI 1640 (Sigma-Aldrich Cheme, Taufkirchen, Germany) with 5% FCS, 2 µl IC block (Biosource, Camarillo, CA, USA), and 2 µl phorbol myristate acetate (PMA, Sigma-Aldrich Cheme; final concentration of 25 ng/µl) for 4 h at 37°C. After staining with anti-CD25-FITC and anti-CD4-PerCP (Dako) for 30 min on ice and subsequent washing, cells were fixed in IC-Fix (Biosource) for 10 min on ice and washed twice with IC perm (Biosource). Thereafter, cells were stained with either the IgG negative control (Biosource), rat anti-human IL-10-PE (Biosource) or rat anti-human IFN-γ-PE (Biosource) and washed twice.

FOXP3 RT-PCR analysis

CD4(+)CD25^{high} T regs were separated by FACS sorting from PBMCs in gastric cancer patients ($n=6$). Total RNA was extracted from sorted CD4(+)CD25^{high} T cells according to the standard protocol with an RNeasy Minikit (Qiagen K.K., Tokyo, Japan). One microgram of total RNA was added to the reaction mixture using the OneStep RT-PCR Kit (Qiagen) and amplified in a GeneAmp PCR System 9700 (Applied Biosystems, CA, USA). Specific primers were designed as follows: *FOXP3* primers, CAG CTG CCC ACA CTG CCC CTA G (forward) and CAT TTG CCA GCA GTG GGT AG (reverse); *β-actin* primers, CTA CAA TGA GCT GCG TGT GC (forward) and CGG TGA GGA TCT TCA TGA GG (reverse). After the RT reaction with one cycle of 30 min at 50°C and 15 min at 95°C, for *FOXP3* PCR, the cycling conditions were as follows: 35 cycles of 45 s at 94°C for denaturation, 45 s at 59°C for annealing, and 1 min at 72°C for elongation. The amplified product (382 bp for *FOXP3* and 314 bp for *β-actin*) was electrophoresed on a 1.2% agarose gel (Ultra Pure, GIBCO BRL, New York, NY, USA) and equilibrated in TAE (40 mM Tris–acetate, 2 mM EDTA). Ethidium bromide (0.5 µg/ml) was added to the agarose–TAE gels with TAE electrophoresis buffers to visualize the amplified DNA fragments, and these were photographed using Polaroid film 667 under UV light.

Statistical analysis

Differences between the values were determined using the nonpaired or paired Student's t test. Actuarial overall survival rates were analyzed by the Kaplan–Meier method and survival was measured in days from diagnosis to death or the last review. The log-rank test was applied to compare the two groups. Multivariate analysis of prognostic factors for overall survival was made using Cox's proportional hazards model. All statistical analyses were performed using Statview 5.0 for Windows software and a significant difference was considered as $P<0.05$.

Results

Increased populations of CD4(+)CD25^{high} T cells in patients with gastric and esophageal cancers

PBMCs in patients with gastric cancer ($n=72$), esophageal cancer ($n=42$), and in healthy donors ($n=16$) were examined for the prevalence of CD4(+)CD25^{high} T cells as T regs, and the population of CD4(+)CD25^{high} T cells as a percentage of the total CD4(+) cells was evaluated. In representative flow cytometric data, the prevalence of CD4(+)CD25^{high} T cells in patients with gastric and esophageal cancers was higher than that in healthy donors (Fig. 1). Summarized data from all

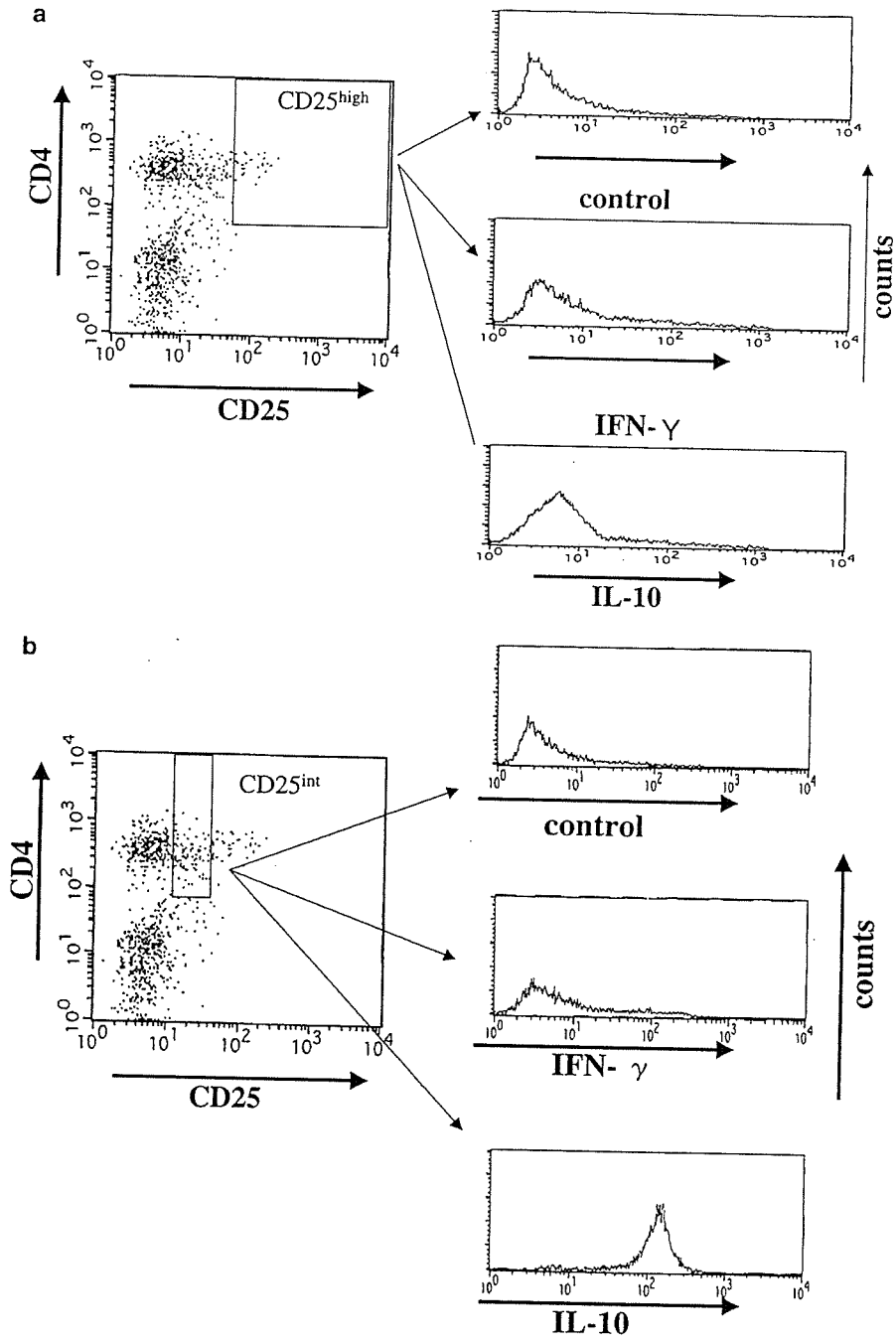

Fig. 4 Intracellular cytokine staining of CD4(+)CD25^{high} T cells. Intracellular cytokine staining (IFN-γ and IL-10) was performed gated on CD4(+)CD25^{high} T cells (**a**) or CD4(+)CD25^{int} T cells (**b**) derived from the PBMCs in the gastric cancer patient (stage III)

individuals indicated that the percentages of CD4(+)CD25^{high} T cells in gastric cancer patients ($4.9 \pm 1.2\%$) and esophageal cancer patients ($5.2 \pm 2.1\%$) were significantly higher than those of healthy donors ($1.9 \pm 1.1\%$, $P < 0.01$), as shown in Fig. 2. Moreover, there were significant differences in the prevalence of CD4(+)CD25^{high} T cells between the early and advanced disease stages, both in gastric cancer (stage I vs. III, $P < 0.05$; stage I vs. IV, $P < 0.05$) and esophageal cancer (stage I vs. IV, $P < 0.05$). These observations indicated that a tumor-bearing host with an advanced disease stage had an increased prevalence of CD4(+)CD25^{high} T cells in PBMCs in comparison to those with early disease stages or healthy donors.

Characterization of CD4(+)CD25^{high} T cells

In order to characterize the CD4(+)CD25^{high} T cells tested, we analyzed markers such as CD45RO and

Table 1 Significance of prognostic factors in multivariate survival analysis for patients with gastric and esophageal cancers

	Gastric cancer			Esophageal cancer		
	Hazard ratio	95% CI	*P* value	Hazard ratio	95% CI	*P* value
CD4(+)CD25^{high} T cells						
High	1.0	–	–	1.0	–	–
Low	0.81	0.93–4.57	0.71	0.72	0.30–1.41	0.56
Primary tumor[a]						
PTis–pT1b	1.0	–	–	1.0	–	–
PT2	2.85	0.92–5.18	0.74	4.21	1.06–19.52	0.04
PT3	2.28	0.61–11.55	0.63	2.93	0.66–13.95	0.11
Lymph node metastasis						
Negative	1.0	–	–	1.0	–	–
Positive	1.19	0.62–1.87	0.80	1.95	0.40–9.08	0.33
Stage[a]						
0–2	1.0	–	–	1.0	–	–
3–4	2.01	0.91–3.05	0.09	2.91	0.50–12.53	0.75

[a]The grade of tumor and stages were defined according to the UICC (TMN) classification

intracellular CTLA-4 (CD152). The expressions of CTLA-4 and CD45RO were analyzed in the gated CD4(+)CD25^{high} T cells. Representative flow cytometric data from gastric cancer patients with advanced disease stages showed that most of the CD4(+)CD25^{high} T cells expressed CD45RO and intracellular CTLA-4 (Fig. 3).

Furthermore, representative flow cytometric data with intracellular cytokine staining showed that CD4(+)CD25^{high} T cells derived from gastric cancer patients with advanced disease stages produced small amounts of IL-10 (Fig. 4a). In contrast, CD4(+)CD25^{int} T cells produced large amounts of IL-10 (Fig. 4b). Furthermore, CD4(+)CD25^{high} T cells as well as CD4(+)CD25^{int} T cells did not produce significant amount of IFN-γ (Fig. 4a, b), in line with previous reports [15–18, 25]. Thus, CD4(+)CD25^{high} T cells separated from PBMCs in the patients corresponded to CD25(+)CD4(+)-naturally occurring T regs [15–17].

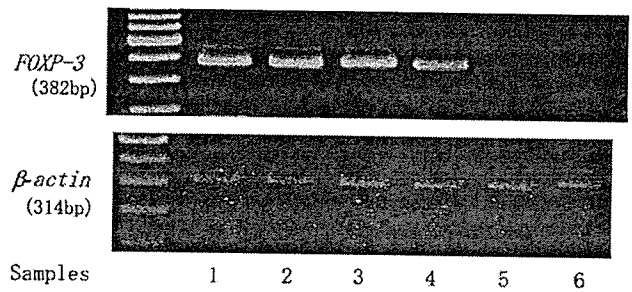

Fig. 5 FOXP3 mRNA analysis of CD4(+)CD25^{high} T cells. CD4(+)CD25^{high} T regs were separated by FACS sorting on CD4(+)CD25^{high} T cells in PBMCs from gastric cancer patients (stage III and IV). Total RNA was extracted from sorted CD4(+)CD25^{high} T cells (*samples 1–4*) or CD4(+)CD25int T cells (*samples 5, 6*) and RT-PCR analyze specific for FOXP3 mRNA was performed

FOXP3 analysis of CD4(+)CD25^{high} T cells

CD4(+)CD25^{high} T regs were separated by FACS sorting from the PBMCs of gastric cancer patients ($n=6$). Total RNA was extracted from sorted CD4(+)CD25^{high} T cells and RT-PCR analyze specific for *FOXP3 mRNA* was performed. Representative RT-PCR analysis is shown in Fig. 5 and sorted CD4(+)CD25^{high} T cells showed FOXP3 specific bands, indicating that CD4(+)CD25^{high} T cells corresponded to CD25(+)CD4(+)-naturally occurring T regs [15].

Survival rates of patients in relation to the prevalence of CD4(+)CD25^{high} T cells

When the patients were separated into high or low prevalence of T regs groups, classified by the mean values of CD4(+)CD25^{high} T cells (4.9% for gastric cancer and 5.2% for esophageal cancer), the gastric cancer patients with high numbers of CD4(+)CD25^{high} T cells (stage I, $n=5$; stage II, $n=6$; stage III, $n=7$; stage IV, $n=20$) showed significantly poorer survival rates in comparison to those with low numbers of CD4(+)CD25^{high} T cells (stage I, $n=31$; stage II, $n=2$; stage III, $n=1$) (Fig. 6a). Similarly, esophageal cancer patients with high numbers of CD4(+)CD25^{high} T cells (stage I, $n=1$; stage II, $n=2$; stage III, $n=11$; stage IV, $n=8$) showed significantly poorer survival rates in comparison to those with low numbers of CD4(+)CD25^{high} T cells (stage I, $n=2$; stage II, $n=14$; stage III, $n=4$) (Fig. 6b).

To assess whether prevalence of CD4(+)CD25^{high} T cells represented a prognostic parameter, we used Cox's proportional hazards model. The covariate parameters included stage of the disease, depth of tumor invasion, lymph-node metastasis, and prevalence of CD4(+) CD25^{high} T cells. Multivariate analysis revealed that the

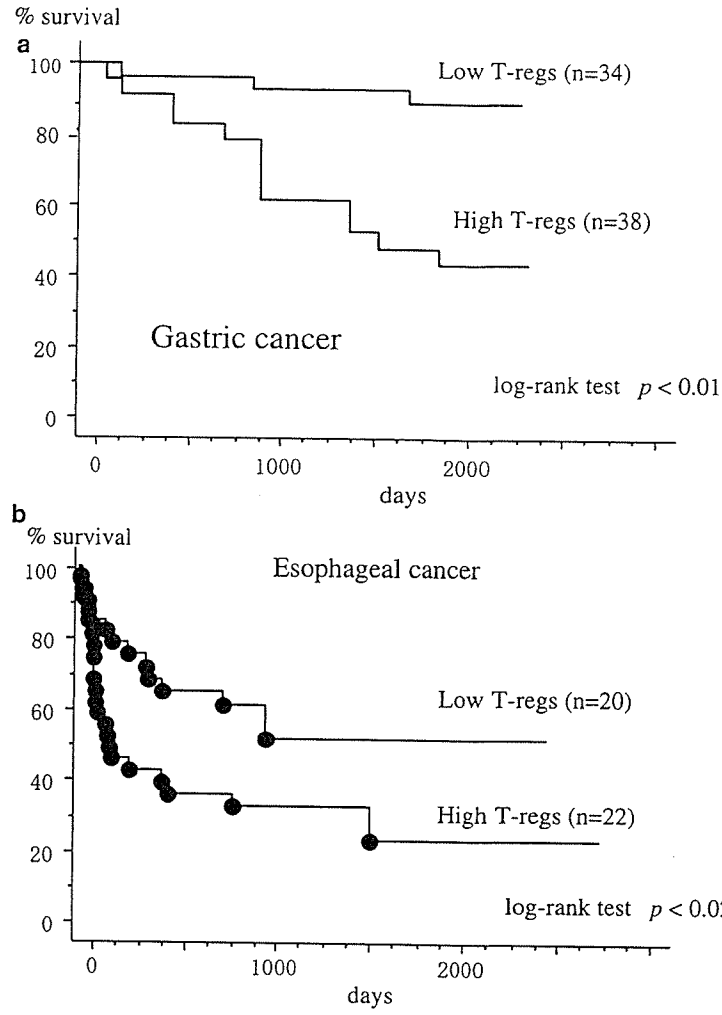

Fig. 6 Survival rates of patients in relation to the prevalence of CD4(+)CD25^{high} T cells. PBMCs were separated prior to the operation and analyzed for the prevalence of CD4(+)CD25^{high} T cells. When the patients were separated into high or low prevalence of T regs groups, classified by the mean values of CD4(+)CD25^{high} T cells, gastric cancer patients with high CD4(+)CD25^{high} T cell levels ($n=38$) showed poorer survival rates in comparison to those with low CD4(+)CD25^{high} T cell levels ($n=34$), analyzed by the log-rank test (a). Similarly, esophageal cancer patients with high CD4(+)CD25^{high} T cell levels ($n=22$) showed poorer survival rates in comparison to those with low CD4(+)CD25^{high} T cell levels ($n=20$) (b)

prevalence of CD4(+)CD25^{high} T cells was not an independent prognostic factor (Table 1).

Clinical significance of the prevalence of CD4(+)CD25^{high} T cells

In order to clarify the levels of CD4(+)CD25^{high} T cells in PBMCs from patients with or without tumor-bearing status, we compared preoperative and post-operative (2 months after the operation) levels. After patients received curative resections of gastric cancers ($n=57$), the increased proportions of CD4(+)CD25^{high} T cells were significantly reduced, and the levels were almost equal to those in healthy donors (Fig. 7a). In addition, studies of gastric cancer patients with postoperative recurrent tumors ($n=6$) revealed that the prevalence of CD4(+)CD25^{high} T cells individually increased compared to the periods without tumors (Fig. 7b). These results strongly suggest that tumor-related factors induce and expand T regs.

Discussion

The current report provides evidence for the relationship between the prevalence of CD4(+)CD25^{high} T regs in PBMCs and the clinical outcome in patients with gastric and esophageal cancers. We showed that patients with high levels of CD4(+)CD25^{high} T cells showed more advanced stages and poorer survival rates in comparison to those with low CD4(+)CD25^{high} T cell levels. Furthermore, after patients had received curative resections of tumors, the increased levels of CD4(+)CD25^{high} T cells significantly decreased to the levels of healthy donors.

Increased proportions of CD4(+)CD25(+) T cells in PBLs and TILs have been reported in several different human malignancies [18–23]. We recently reported about increased populations of CD4(+)CD25(+) T cells in PBL and TILs [18], and elevated CD4(+)CD25^{high} T cell levels in the tumor-draining lymph nodes [25] of patients with gastric cancer. In the

present study, we confirmed the increased prevalence of CD4(+)CD25^{high} T cells in PBMCs in larger cohorts in cases of gastric and esophageal cancers. Furthermore, we showed that patients with high levels of CD4(+) CD25^{high} T cells revealed poorer survival rates in comparison to those with low CD4(+)CD25^{high} T cell levels, in line with previous reports [20, 24]. However, multivariate analysis in the present study revealed that the prevalence of CD4(+)CD25^{high} T cells was not an independent prognostic factor. Since the sample size of the present study was limited and the groups were not sufficiently matched in composition, further studies will be needed to draw valid conclusion for the prognostic factor.

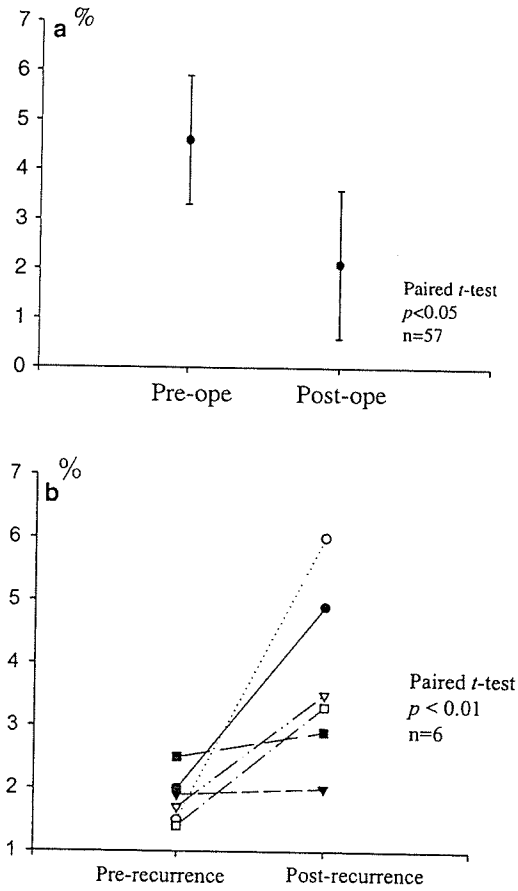

Fig. 7 Clinical course of patients in relation to the prevalence of CD4(+)CD25^{high} T cells. The prevalence of CD4(+)CD25^{high} T cells between preoperative and postoperative (2 months after the operation) periods are shown in **a**. After patients had received curative resections of gastric cancers ($n = 57$), the increased levels of CD4(+)CD25^{high} T cells were significantly reduced, analyzed by the paired t test (**a**). In addition, studies for gastric cancer patients with postoperative recurrent tumors ($n = 6$, postrecurrence) showed that the prevalence of CD4(+)CD25^{high} T cells individually increased in comparison to 2 months after the operation (prerecurrence) (**b**)

It has recently been shown that human CD4(+) CD25(+) T cells are not homogenous, and can be split into suppressive and nonsuppressive fractions by sorting CD25^{high} and CD25^{int} cells [15]. Furthermore, it was proposed that only a subset of high levels of CD25 and CTLA-4 molecules within CD4(+)CD25(+) T cell populations was capable of inducing a suppressive function, and that there was a difference in cytokine production profiles between CD25^{high} and CD25^{int} cells within CD4(+)CD25(+) T cells [15–17, 26, 27]. In the present study, we focused on CD4(+)CD25^{high} T cells as CD25(+)CD4(+)-naturally occurring T regs and evaluated the prevalence of CD4(+)CD25^{high} T cells in PBMCs. These subsets showed strong expressions of intracellular CTLA-4 and CD45RO and small amounts of IL-10 production, as indicated in a previous report which suggested that CD4(+)CD25^{high} T cells may correspond to human naturally occurring T regs [15]. In addition, we confirmed *FOXP3 mRNA* expression on CD4(+)CD25^{high} T cells in the present study. These results indicate that the population of CD4(+)CD25^{high} T cells in the present study correspond to human naturally occurring T regs. Since there is still a debate regarding the marker of T regs [15], further studies are required at a cloned T cell level or at a molecular level which targets more specific markers or functional profiles.

There is no clear evidence to suggest the mechanisms for the induction of T regs in cancer-bearing hosts. However, there are several possibilities, including the specific expansion of T regs induced by cancer-derived factors, or physiological defense phenomena against continuous inflammation induced by cancer. It has recently been shown that tumor cells and microenvironmental macrophages produce the chemokine CCL20, which mediates the trafficking of T regs to the tumor [24]. Also, we have shown that the levels of CD4(+)CD25^{high} T regs in tumor-draining lymph nodes adjacent to tumors were greater than those distant from tumors [25]. Furthermore, in the present study, we reported that after patients had received curative resections of tumors, the increased levels of CD4(+)CD25^{high} T cells were significantly reduced, and that the levels recovered to those of healthy donors. These results strongly suggest that tumor-derived factors may induce and expand T regs pools, although the precise mechanisms regulating CD4(+)CD25^{high} T regs remain unknown.

Recently, immunotherapy for cancer, including cancer vaccination or adoptive transfer of T cells, has been tested, but the results suggested it was limited in the effect on the regression of established tumors [28, 29]. The increased population of T regs, especially in the tumor environment, is one of the problems to be resolved in cancer immunotherapy. It was shown that the efficacy of therapeutic vaccination for cancer could be enhanced by removing T regs [30]. A better understanding of the underlying mechanism of T regs

regulation or a strategy for controlling T regs may lead to more effective immunotherapies against cancer.

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EGFR protein overexpression and gene amplification in squamous cell carcinomas of the esophagus

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Overexpression of epidermal growth factor receptor (EGFR) is observed in many cancers, sometimes accompanied by gene amplification. Recently, several clinical therapies targeting EGFR were developed, but the eligibility criteria for these therapies is not fully established. To develop such eligibility criteria for esophageal squamous cell carcinoma (ESCC), we sought to clarify: (*i*) the exact frequency of EGFR overexpression, (*ii*) the relationship between protein overexpression and gene amplification, (*iii*) the relationship between gene amplification and specific gene mutations and (*iv*) the correlation between the status of *EGFR* and clinical or pathological features. Immunohistochemistry revealed that EGFR protein is overexpressed in 53 (50%) of the 106 ESCC examined. Fluorescence *in situ* hybridization (FISH) indicated clear *EGFR* gene amplification in 15 of the 53 tumors, somewhat higher *EGFR* copy in 32 cases, and no increase in 6 cases. Gene amplification was significantly associated with high level overexpression. Direct sequencing of exons 19 and 21 of *EGFR* revealed no mutations in 15 tumors exhibiting gene amplification, and no mutations in 25 tumors not exhibiting gene amplification. Overexpression of EGFR was significantly correlated with depth of invasion of the tumor.In conclusion, anti-EGFR therapies may be appropriate for patients with ESCC. We assume that combined analyses by immunohistochemistry/FISH would clarify aberrations in protein and gene function, and could help to identify those patients who may benefit from anti-EGFR therapy.

Key words: *EGFR*; FISH; esophageal squamous cell carcinomas; immunohistochemistry; *EGFR*-targeted therapy

Esophageal cancer is the sixth most frequent cause of cancer death worldwide in 1990,[1] and squamous cell carcinoma is the predominant histologic type. The prognosis of esophageal squamous cell carcinoma (ESCC) is poor, with 5-year survival rates of about 10%, as reported by various cancer registries. Cure is expected only for superficial cancers.[2] In the future, we may be able to improve the prognoses of patients with these diseases and provide more appropriate therapies if we better understand the molecular genetic characteristics of each tumor. This may ultimately be as important as early diagnosis and effective treatment.

The epidermal growth factor receptor (*EGFR*) gene, located on chromosome 7p12, encodes a 170 kDa membrane glycoprotein. Upon activation by specific ligands such as EGF, its intrinsic kinase is activated and initiates a number of signaling pathways. In addition to being implicated in organ morphogenesis, maintenance and repair, upregulated EGFR signaling has been correlated in a wide variety of tumors with progression to invasion and metastasis.[3] *EGFR* was purified initially from the human squamous cell carcinoma cell line A431,[4] which overexpresses EGFR from 2- to 100-fold, resulting from a commensurate 3- to 110-fold increase in *EGFR* gene copy number.[5] Since then, many types of epithelial malignancies, including esophageal cancers, have been shown to express increased levels of EGFR expression on the cell membrane, with or without gene amplification.[6]

There have been several immunohistochemical (IHC) studies examining EGFR protein expression in ESCC; however, there is no consensus with regards to general frequencies of overexpression, distribution of overexpressing cells, or levels of expression.[7,8] Early studies, although small in scale, were able to quantify gene amplification and overexpression using combined Southern blot and ^{125}I-EGF binding assays: Hunts *et al.*[9] and Ozawa *et al.*[10] examined 10 and 32 ESCC tumors, respectively, and found a tumor exhibiting *EGFR* amplification as high as that in A431, with a 30-fold higher concentration of EGF binding sites than adjacent normal tissue.

Using a fluorescence *in situ* hybridization (FISH) technique combined with IHC, we have demonstrated that the major mechanism of EGFR overexpression, especially high level overexpression, in gastric,[11] colorectal,[12] pulmonary[13] and bile duct carcinomas,[14] as well as soft tissue sarcomas[15] is gene amplification. However, we also found another group in which low level overexpression occurred without gene amplification.[12–15] This led us to speculate that there may be 2 different mechanisms of EGFR overexpression: gene amplification and transcriptional/translational enhancement. We believe that detection of gene amplification by FISH may be the best method for elucidating the mechanisms of overexpression in cancers. However, there has been no study to date using this technique to detect *EGFR* aberrations in ESCC.

EGFR has recently attracted much attention because of the development of clinical therapies that target this receptor. IMC-C225 (cetuximab or Erbitux™, ImClone Systems, Branchburg, NJ), a monoclonal antibody against EGFR, is now approved for use in patients with colorectal cancers (FDA News, February 12, 2004). Among the various small molecule inhibitors of tyrosine kinases, gefitinib (ZD1839, Iressa™, AstraZeneca, Macclesfield, United Kingdom) has progressed to approval for clinical use in the USA for patients with non-small-cell lung cancer (NSCLC).

Our aim in the present study was to provide some rationale for the introduction of new adjuvant therapies for ESCC patients. First, we sought to determine the exact frequency of EGFR overexpression; second, to examine the relationship between protein overexpression and gene amplification; and third, to understand the relationship between gene amplification and gene mutation and, finally to clarify the correlation between the status of the *EGFR* gene and clinical or pathological features of the tumors.

Material and methods

Tissue samples

We examined 106 cases of ESCC and concurrently excised nodal metastases obtained from consecutive surgeries performed

Abbreviations: AJCC, American Joint Committee on Cancer; COX-2, cyclooxygenase-2; DM, double minute chromosomes; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ESCC, esophageal squamous cell carcinoma; FISH, fluorescence *in situ* hybridization; HSR, homogeneously staining regions; IHC, immunohistochemical; LA/Poly, low amplification/polysomy; NSCLC, non-small-cell lung cancer.

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at the Department of Surgery, University of Yamanashi between 1987 and 2004. The patients consisted of 97 men and 9 women, with a median age of 63 years (mean, 63.7; SD, 7.6; range, 43–80). Patients who had received irradiation and/or chemotherapy before surgery were excluded. The World Health Organization Classification of Tumors was used histologically to grade cases of ESCC.[2] The condition of the patients was assessed according to the system for staging primary tumor/regional lymph nodes/distant metastasis (TNM) described in the American Joint Committee on Cancer Staging Manual.[16] The 106 patients were classified into the TNM stages as follows: Stage 0, 2 patients; Stage I, 21 patients; Stage IIA, 33 patients; Stage IIB, 15 patients; Stage III, 32 patients and Stage IV, 3 patients. This laboratory study was approved by the Institutional Review Board at the University of Yamanashi, and written informed consent was obtained.

Immunohistochemistry (IHC)

Resected esophageal samples were immediately immersed in 20% buffered neutral formalin, fixed overnight and embedded in paraffin according to standard procedures. 1 or more representative blocks in which the carcinoma showed the deepest extent of invasion was used for histological analyses for each case. Serial sections (4 μm) were cut from representative formalin-fixed, paraffin-embedded cancer tissues, placed onto silanated glass slides and subjected to hematoxylin-eosin staining. EGFR IHC analysis and FISH analysis. IHC detection of EGFR was carried out on all primary tumors, as well as metastatic tumors of the lymph nodes, using a monoclonal antibody against the external domain of human EGFR (Novocastra Lab, Newcastle, UK; Working dilution, 1:20). The specificity and sensitivity of this antibody against EGFR were verified previously.[11,12] For IHC detection, a high-temperature antigen unmasking technique was used: *i.e.*, the section was autoclaved in 0.01 M citrate buffer (pH 7.0) at 121°C for 10 min. Antibody was visualized by avidin-biotin binding to peroxidase-conjugated secondary antibodies. In each analysis, a colon cancer section that had been previously confirmed to overexpress EGFR[12] was included as a positive control.

EGFR positivity in the IHC analyses was reviewed by 3 pathologists (MH, SS, YD), who were unaware of the gene amplification status of the sample. The intensity of reactivity was scored using a four-tier system, which we have established in previous studies on EGFR expression: negative, no discernible staining or background type staining; 1+, definite cytoplasmic staining and/or equivocal discontinuous membrane staining; 2+, unequivocal membrane staining with moderate intensity; 3+ strong and complete plasma membrane staining.[11–13] Samples exhibiting 2+ or 3+ immunostaining were classified as "overexpression", since we have previously demonstrated that only cases showing 2+ or 3+ positivity in IHC by our criteria were frequently associated with gene amplification of *EGFR*.[11–13,15] The extent (%) of positive staining cells was measured in a representative large section of each tumor.

FISH analysis

FISH analysis was undertaken for all cases of primary tumors exhibiting 3+ or 2+ staining (53 cases), and for another 20 representative tumors exhibiting 1+ staining. In addition, 10 tumors that scored negative for EGFR were selected at random as negative controls. Metastatic nodes from the primary tumors exhibiting gene amplification were also examined. Gene amplification of *EGFR* (7p12) was determined using a DNA probe set (LSI™ EGFR/CEP™ 7; Vysis, Downers Grove, IL) consisting of a SpectrumOrange™-labeled *EGFR* (locus)-specific probe and a SpectrumGreen™-labeled probe that hybridizes to the centromeric region of chromosome 7. The latter probe was used as the control to normalize copy number for chromosome 7. FISH was performed using standard methods, with a modification to incorporate an intermittent, short-term microwave treatment during the initial period of hybridization as described earlier.[12,17] The tissue sections were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride and p-phenylenediamine in phosphate-buffered saline and glycerol (DAPI II) (Vysis) and examined under a fluorescence microscope (Olympus, Tokyo, Japan). The microscope was equipped with a Triple Bandpass Filter set (Vysis) to discriminate DAPI II, SpectrumOrange™ and SpectrumGreen™, as well as filter sets specific for SpectrumOrange and SpectrumGreen. As positive controls, colon cancer tissues that had been previously confirmed to have *EGFR* gene amplification were used.[12] The number of *EGFR* signals and centromere 7 signals in the nucleus of cancer cells were counted and evaluated as follows. Basically, a cell in which the number of *EGFR* signals was greater than the number of centromere 7 signals was interpreted as positive for amplification. Among these, i) a cell with a definite cluster of *EGFR* signals or a total of more than 10 *EGFR* signals was scored as exhibiting high-level amplification[11–13,15] and ii) a cell with 3–10 *EGFR* signals was scored as low-level amplification. In addition, a cell in which both centromeric and *EGFR* signals were equally increased was scored as polysomic.[18] FISH images were taken using a photographic camera and recorded on film slides.

Direct sequencing of exons 19 and 21 of the EGFR *gene*

To clarify the possible relationship between gene amplification and specific mutations in *EGFR*, we performed direct sequencing. Given the apparent clustering of *EGFR* mutations in gefinitib-sensitive non-small-cell lung carcinoma,[19,20] we sequenced only exons 19 and 21 in 40 primary tumors, consisting of 15 tumors with gene amplification and 25 tumors without amplification. DNA was extracted from 40 paraffin-embedded tumors (15 tumors with amplification and 25 without amplification) using DEXPAT™ (TaKaRa, Kyoto, Japan). DNA was also extracted from 9 tumors (2 with amplification and 7 without amplification) by phenol and chloroform, in which fresh surgical tissues could be obtained. The polymerase chain reaction (PCR) was used to amplify exons 19 and 21 according to a previous report by Lynch *et al.*[19] Primer pairs used for the amplification were as follows: Exon 19, GCAATATCAGCCTTAGGTGCGGCTC (sense) and CATAGAAAGTGAACATTTAGGATGTG (antisense), and Exon 21, CTAACGTTCGCCAGCCATAAGTCC (sense) and GCTGCGAGCTCACCCAGAATGTCTGG (antisense). For the amplification of DNA extracted from paraffin-embedded tumor tissues, nested PCR amplification was performed as follows. An initial PCR product was generated using primers described earlier. Subsequently, 2 μl of this reaction was amplified in a secondary PCR, using the following internal primer pairs; Exon 19, CC TTAGGTGCGGCTCCACAGC (sense) and CATTTAGGATGTG GAGATGAGC (antisense); Exon 21, CAGCCATAAGTCCTCGA CGTGG (sense) and CATCCTCCCCTGCATGTGTTAAAC (antisense). In both reactions, the annealing temperature was 58°C. The PCR amplicon was purified using SUPREC™ (TaKaRa, Kyoto, Japan). Purified DNA was cycle-sequenced using the ABI BigDye Termination kit v1.1 (ABI, Foster City, CA) according to manufacturer's instructions. Sequence reactions were electrophoresed on an ABI 3730xl DNA analyzer. Electrophoregrams were analyzed in both the sense and antisense directions and compared with the *EGFR* sequence deposited in the GenBank data base, Access No. AY588246 for the presence of mutations.

Statistical analysis

Agreement among observers in their interpretation of IHC specimens was tested by kappa (κ) statistics.[21] In accordance with the criteria of Landis and Koch,[22] the κ values were divided into several scales to evaluate the strength of the agreement: $\kappa < 0.00$, poor; $0.00 < \kappa < 0.20$, slight; $0.21 < \kappa < 0.40$, fair; $0.41 < \kappa < 0.60$, moderate; $0.61 < \kappa < 0.80$, substantial; $0.81 < \kappa < 1.00$, nearly perfect. A χ^2 test for independence was used to examine the correlation among the status of EGFR protein, gene and the several clinicopathological factors. Patients' survival was analyzed by the Kaplan-Meier method, with Logrank test for univariate analysis.

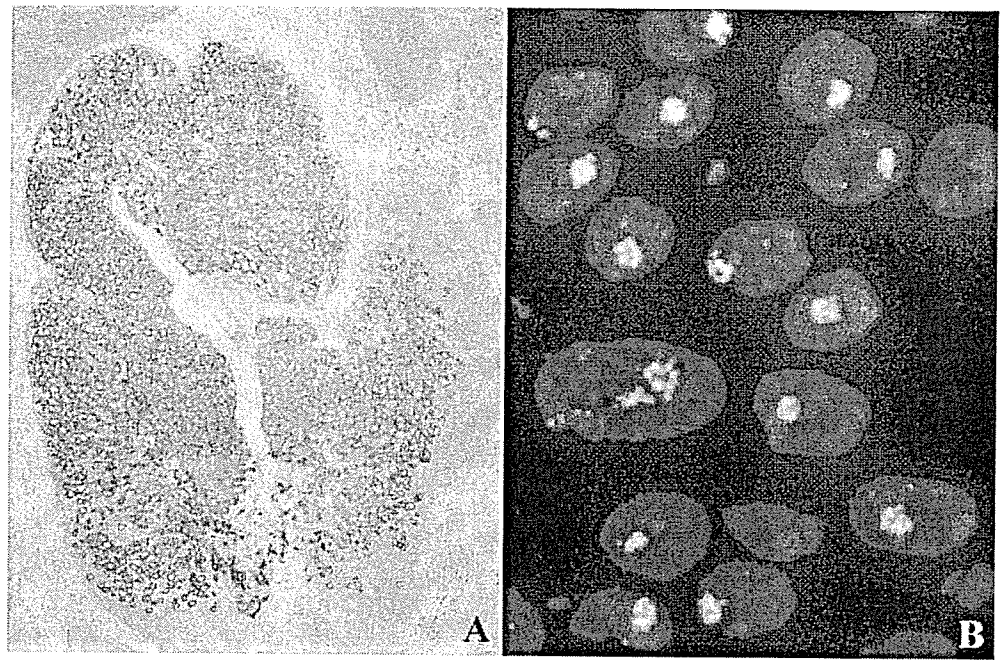

FIGURE 1 – Infiltrating squamous cell carcinoma of the esophagus. (*a*) 3+ immunostaining cells are localized to a tumor nest. (50×) (*b*) FISH analysis shows clusters of orange signals in cancer nuclei corresponding to the *EGFR* gene (320×).

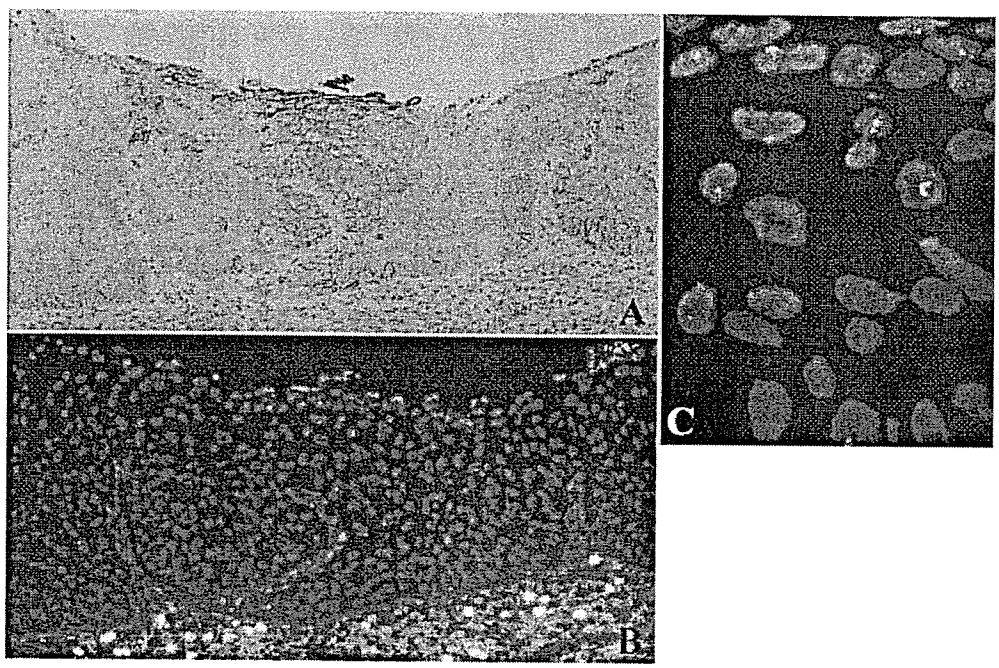

FIGURE 2 – T1 tumor. (*a*) 3+ immunostaining cells are localized to mucosal cancer (50×). (*b*) FISH analysis on the adjacent section shows 3+ immunostaining cells with clustered *EGFR* signals (50×). (*c*) Higher magnification of B (250×).

Results

EGFR *protein expression*

Overexpression of EGFR (2+ and 3+ staining) was exclusively confined to cancer cells. Some focal staining was present in basal cells and parabasal cells in normal esophageal epithelium; however, their intensities did not exceed 1+ staining.

Among the 106 ESCC cases analyzed, overexpression of EGFR was found in 53 cases (50%), of which 18 cases scored 3+ for EGFR staining and 35 cases scored 2+ staining. Low level (1+) immunoreactivity similar in intensity to the basal cell of normal esophageal mucosa was found in 41 tumors (39%), and these were scored as not overexpressing EGFR protein. No EGFR immunoreactivity was detected in 12 (11%) of the tumors. Although overall

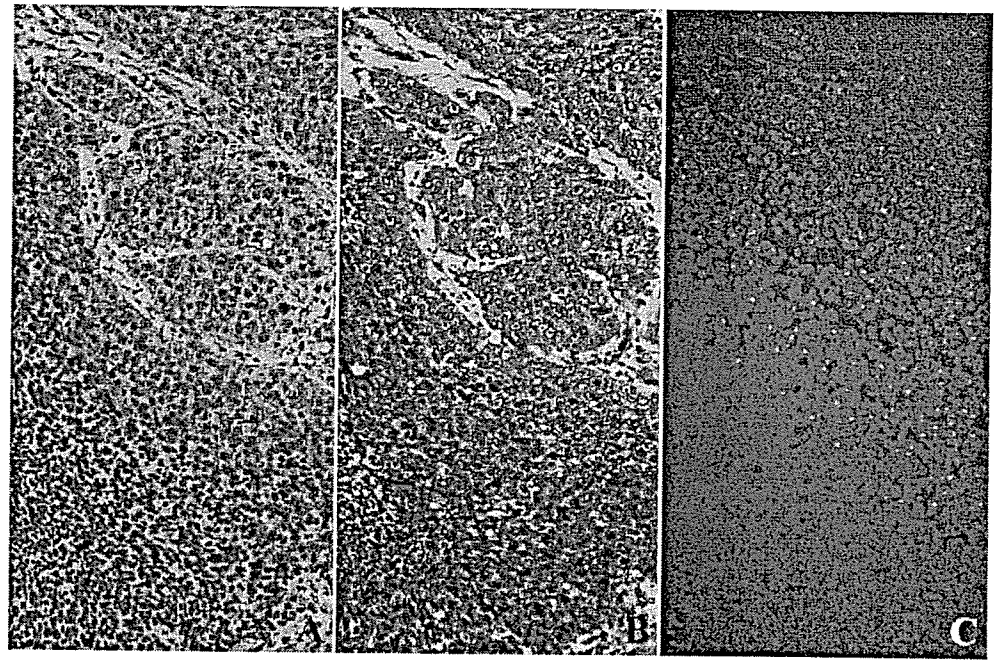

FIGURE 3 – Comparison of immunohistochemistry, histology and FISH on 3 serial sections (50×). (*a*) 2+ immunostaining cells and negative cells are found to be apposed. (*b*) overexpressing cell have abundant cytoplasm and clear nucleolus; however, negative cells show scant cytoplasm and dark nuclei. (*c*) Gene amplification is restricted to overexpressing cells.

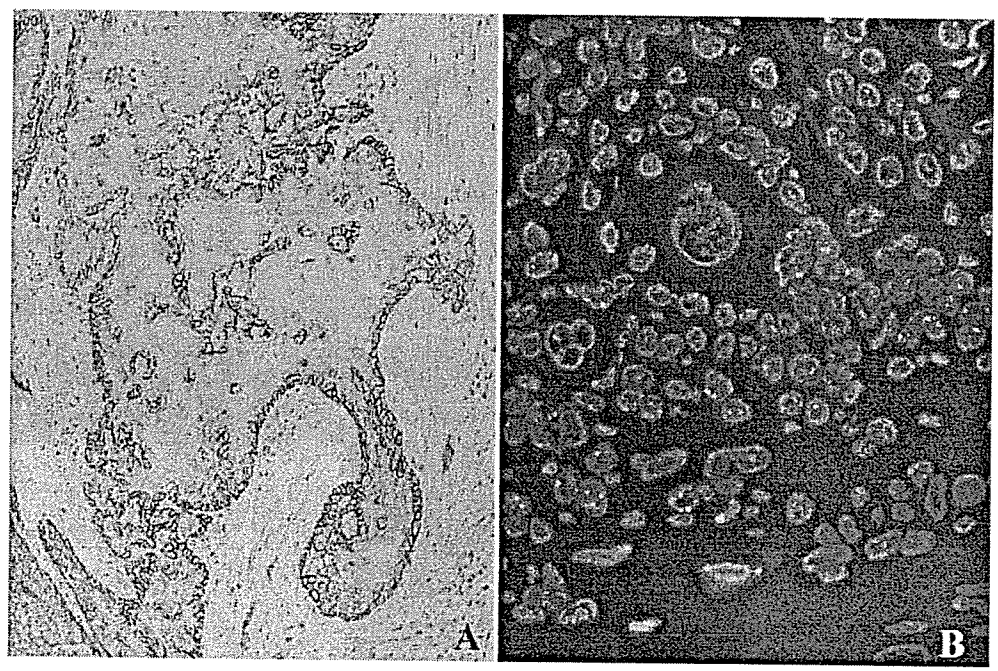

FIGURE 4 – (*a*) 2+ immunostaining cells distributed in the basal zone of cancer nests (50×). (*b*) No difference is found in the numbers of genes between cells in the central area (upper) and cells in the peripheral area (lower) (250×).

inter-observer agreement was moderate (κ = 0.75: 95% Confidence Interval, 0.69–0.81), it was nearly perfect for the negative and 1+ staining cases (κ = 0.98: CI, 0.88–0.99 and κ = 0.94: CI, 0.84–0.99, respectively).

EGFR-overexpressing cells were distributed heterogeneously and positive cells comprised different populations ranging from 10 to 90% (mean±SD, 54±24) of the total cells. In 23 of the tumors, which included 12 of the 3+ cases and 11 of the 2+ staining cases, positive cells were predominantly localized in separate zones or foci irrespective of infiltrative growth (Fig. 1*a*). This was found in 3 cases of superficial carcinoma (T1 tumors) (Fig. 2*a*). Advanced carcinomas sometimes displayed a distinctive distribu-

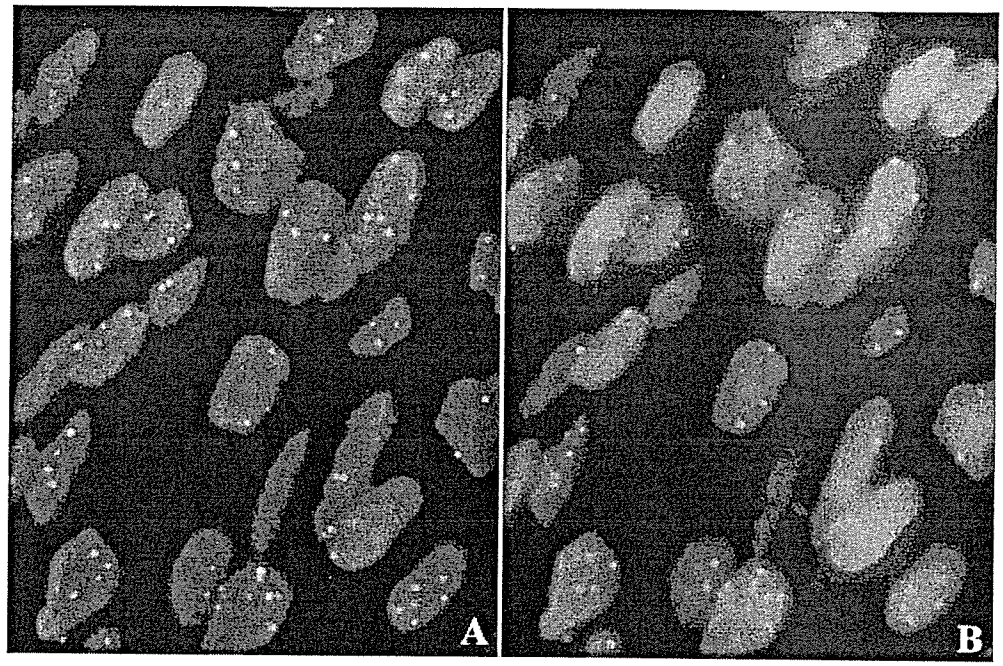

FIGURE 5 – Tumor of polysomy 7. The same field was observed by the triple band filter (*a*) (50×) and the SpectrumGreen™-specific filter (*b*). Cancer cells have 3–6 *EGFR* signals, accompanied with the same number of centromere 7 signals (250×).

tion of the overexpressing cells, consisting of areas of exclusively EGFR-positive cells and areas of mostly or all EGFR-negative cells. The demarcation between these positive and negative areas was clear and occasionally a morphological difference between those 2 areas was observed as well. EGFR-overexpressing cells generally had abundant cytoplasm and clear nucleolus, while negative cells had scant cytoplasm and dark nuclei. (Figs. 3*a* and 3*b*). Rest of the tumors contained areas where EGFR-positive cells, most of which were 2+ staining, were located in the basal zones of the tumor nests, and this staining gradually diminished in intensity towards the center of the cancer nests as shown in Figure 4*a*.

FISH analysis

FISH analysis revealed that 28% (15 of 53) of the tumors that were positive for EGFR protein overexpression were also positive for *EGFR* gene amplification, corresponding to 14% of the total cases. *EGFR* gene amplification was manifested as 1 or 2 distinct clusters of orange signals in each of these 15 cases (Figs. 1*b*, 2*c* and 3*c*). Each cluster consisted of approximately more than 10 signals, although precise enumeration was impossible in several cells because of the tight clustering and overlap of the signals.

In 40 tumors, the number of the orange *EGFR* spots ranged between 3 and 6. In 15 of these samples, there was a concomitant increase in the number of green spots, corresponding to centromere 7, as shown in Figure 5. These were thus scored as polysomy of chromosome 7. However, in the remaining 25 tumors, centromeric signals were too faint to count, even with using the filter set specific to SpectrumGreen™. As a result, clear identification of "polysomy of chromosome 7" or "low level amplification" according to the definition used in this study was not possible, and therefore, all 40 cases were arbitrarily designated as low level amplification/polysomy (LA/Poly). The remaining 28 tumors showed 2 orange signals, and thus, were classified as disomy 7. Normal epithelial cells and nonneoplastic stromal or inflammatory cells generally showed 2 faint signals for *EGFR*.

In the gene amplification-positive tumors, when tissue sections used for FISH were compared with serial sections used for immunostaining, the regions of cancer cells displaying gene amplification and protein overexpression overlapped completely. This colocalization could be confirmed on a cell-by-cell basis, as shown in Figures 2 and 3. However, EGFR-positive cells located in the basal zones of the tumor nests did not have gene amplification and demonstrate any differences in *EGFR* copy number from centrally located cells, showing no overexpression (Fig. 4).

Among the 15 cases exhibiting gene amplification of *EGFR* in the primary tumors, 8 had lymph nodes metastases. In 2 of those 8 cases, cancer cells with gene amplification were found in 1 of 1 metastatic node and 2 of 4 metastatic nodes in each case. The gene amplification was cluster-type and nonamplified cancer cells were colocalized, as shown in Figure 6. No single metastatic node composed exclusively of amplified cells.

Results of IHC and FISH were compared in Table I. Overall, overexpression of EGFR protein was associated with *EGFR* gene amplification ($p = 0.0013$), and in particular, high-level protein overexpression (3+) was significantly associated with gene amplification ($p = 0.0003$). In 30 cases of negative overexpression, gene amplification was not detected. Compared to disomy, LA/Poly was more frequently observed in tumors with protein overexpression (32 in 40 *vs.* 6 in 28, $p < 0.0001$).

DNA sequencing

Although all the PCR products, which had been amplified using genomic DNA extracted from paraffin-embedded tissues and fresh surgical tissues, were successfully sequenced, no mutations were detected in exons 19 and 21.

Comparison of EGFR *status and clinicopathological factors*

Protein overexpression and gene amplification were statistically evaluated for correlation with established clinicopathological factors. We found that neither protein overexpression nor gene amplification was correlated with the grade of squamous cell carcinoma (SCC) differentiation. Protein overexpression was significantly correlated with the depth of tumor invasion: the frequency of overexpression in T2, 3 and 4 tumors was significantly higher than that in Tis and T1 tumors ($p < 0.0001$) (Table II). EGFR overexpression and gene amplification were evaluated for their potential