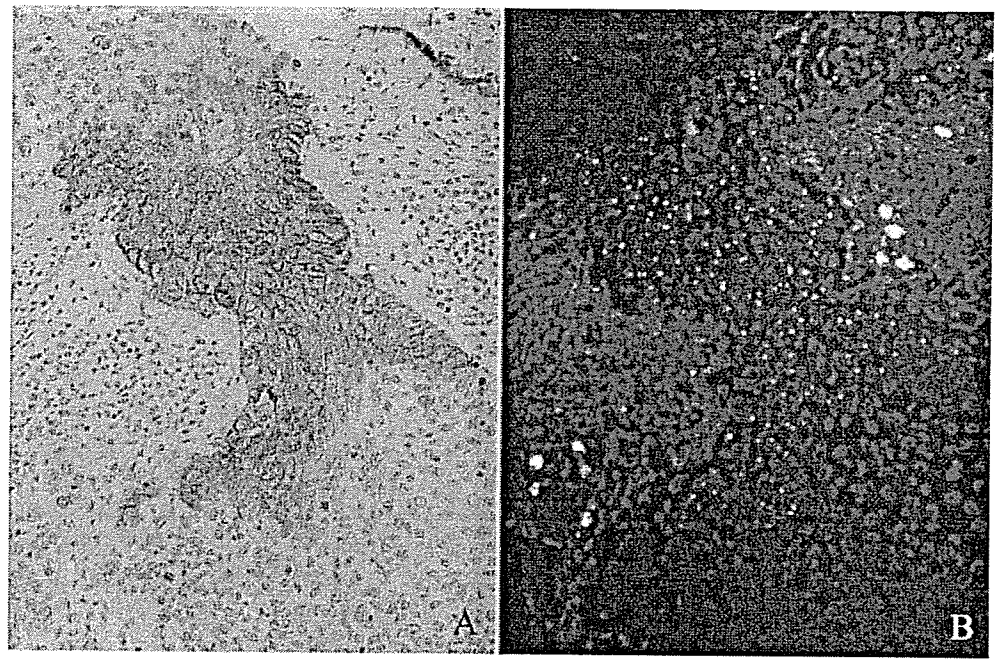


FIGURE 6 – (*a*) EGFR-positive cancer cells and negative cancer cells in nodal metastasis. (*b*) FISH analysis on the adjacent section shows amplification-positive cells and negative cells corresponding to protein overexpression (100×).

TABLE I – CORRELATION BETWEEN IHC AND FISH RESULTS

| IHC | FISH | | |
|---|---|---|---|
| | No. of cases with amplification | No. of cases with LA/poly | No. of cases with disomy |
| 3+ | 11 | 6 | 1 |
| 2+ | 4 | 26 | 5 |
| 1+[1] | 0 | 7 | 13 |
| 0[1] | 0 | 1 | 9 |
| *Total* | 15 | 40 | 28 |

Significant correlation between overexpression (3+ and 2+) and amplification (Fisher's exact test: $p < 0.0013$). High level amplification (3+) was associated with gene amplification compared to low level amplification (2+): $p < 0.0001$.

[1]Representative cases were used for FISH analysis.

TABLE II – CORRELATION BETWEEN EGFR OVEREXPRESSION AND DEPTH OF INVASION

| Depth of invasion | Overexpression | |
|---|---|---|
| | No. of positive cases | No. of negative cases |
| Tis and T1 | 7 | 28 |
| T2, T3 and T4 | 46 | 25 |
| *Total* | *53* | *53* |

Fisher's exact test: $p < 0.0001$.

prognostic significance. Univariate analysis revealed no significant difference in survival rates with respect to protein overexpression or gene amplification.

## Discussion

In the present study, overexpression of EGFR was found in 50% of the ESCC cases. Among those cases, *EGFR* amplification was found in 28% of the overexpressing tumors and in 14% of the overall study population. EGFR protein overexpression was associated with *EGFR* gene amplification, and in particular, high-level overexpression (3+ staining) was significantly associated with gene amplification. Although overexpression was associated with depth of invasion (T-factor), there was no significant differences in the incidence of gene amplification between Tis/T1 *vs.* T2–4 cancer. Furthermore, although several metastatic foci in lymph nodes contained amplified cells, there was no single colony composed exclusively of amplified cells. These results suggest that overexpression of EGFR may play a role during the process of tumor infiltration, but that in those tumors exhibiting *EGFR* gene amplification, this amplification occurs in the early stages of carcinogenesis. Later, in the process of cancer evolution, clones having gene amplification might be overtaken by amplification-negative clones because of some differences in growth advantages.

*EGFR* gene amplification, when observed in tumor samples, was always observed as clustered-type signals. It is generally accepted that clustered signals found by FISH correspond to amplified signals in homogeneously staining regions (HSR). The other type of amplification, multiple scattered signals, corresponds to double minute chromosomes (DM).[23] Our previous FISH studies demonstrated a clear organ-specific *EGFR* amplification pattern. In gastric[11] and colorectal carcinomas,[12] *EGFR* amplification was found to occur in a DM-type pattern. Another group has shown that *EGFR* amplification in gliomas is consistently of the DM type.[24] In contrast, in lung and esophageal carcinomas, *EGFR* amplification was found to be exclusively of the HSR type.[13] Although the clinical significance of these different amplification patterns remains to be elucidated, 1 of the *in vitro* studies demonstrated that DM pattern disappeared in response to chemotherapy,[25] suggesting that appropriate chemotherapy against tumors with *EGFR* amplification of the DM type may lead to downregulation of EGFR and consequent inhibition of EGFR-mediated intracellular signalling.[25]

Poly/LA was observed at a significant frequency. We considered the possibility that increased EGFR expression results from chromosome 7 polysomy, which is not uncommon in solid carcinomas, and in some cases, may account for moderate overexpression in the absence of specific gene amplification.[15,26] In our study, LA/Poly was statistically associated with protein overex-

pression; however, different from the gene amplification, in that, LA/Poly did not exactly correspond to protein overexpression on cell-by-cell basis.

We speculate that overexpression without gene amplification is probably caused by transcriptional or posttranscriptional activation, and various theories have been proposed to explain the mechanisms underlying this type of upregulation. Recent studies have demonstrated that *EGFR* transcription can be enhanced by polymorphic CA repeats[27] and by a 140 bp enhancer region,[28] both of which reside within intron 1 of *EGFR*. Another possibility is secondary upregulation of EGFR by local growth factor production. Accumulating data has shown that EGFR, when overexpressed in a variety of solid tumor types, can play a role in stromal invasion by stimulating the upregulation of substances such as laminin-5 gamma-2 chain and/or cyclooxygenase (COX)-2.[29] Conversely, there are studies showing that overexpression of COX-1 and COX-2 in human colon carcinoma cells stimulates the secretion of growth factors and subsequently induces EGFR expression.[30,31]

At the present time, 2 anti-EGFR therapies have been in clinical development: Small-molecule EGFR tyrosine kinase inhibitors such as gefitinib and humanized antibodies against EGFR represented by cetuximab. In preclinical studies, using cell lines or xenografts, both gefitinib and cetuximab were shown to be particularly effective in inhibiting proliferation of A431 cells,[32–37] although their effect was not necessarily correlated with the levels of EGFR expression in other cancer-derived cell lines.[33,34] On the basis of these experimental studies, several clinical trials have tested using gefitinib and cetuximab alone or in combination with cytotoxic therapy or radiotherapy, for the treatment of cancers of the head and neck, colon and lung.[38–43] In these latter studies, eligibility for treatment was determined by expression of EGFR as assayed by IHC, with low cut-off levels,[44] or without a prior determination of EGFR levels in individual tumors.[39,42,43]

Recently, several independent groups have published clinical studies showing that somatic mutations around the ATP-binding pocket of the tyrosine kinase domain in EGFR gene, but not overexpression of EGFR per se, predict sensitivity of pulmonary cancers to gefitinib. Lynch *et al.*[19] identified somatic mutations within exon 19 or 21 in 7 patients, and within exon 18 in 1 patient, out of a total of 9 patients exhibiting gefitinib-responsive lung cancer. No mutations were detected in 7 patients showing no response to gefitinib. Paez *et al.*[20] detected similar point mutations in either exon 19 or 21 in all of the 5 patients who responded to gefitinib compared to none of the 4 patients who progressed during gefitinib therapy. Interestingly, they also found that these mutations were frequent in Japanese patients with primary NSCLC compared to Caucasian patients (26% *vs.* 1.6%). These mutations of the tyrosine kinase domain were almost exclusively found in adenocarcinoma or bronchioalveolar carcinomas, and no case of squamous cell carcinomas was included in the previous reports, as in the present study.

In the current study, we did not detect any mutations in the tyrosine kinase domain, although the number of the cases examined may not be large enough. We are not aware of any clinical studies using gefitinib in the treatment of ESCCs or studies trying to relate other chemotherapeutic effects and EGFR gene mutations in ESCCs. However, if mutations in the tyrosine kinase domain decide the reactivity of ESCCs to gefitinib, as is the case for NSCLC, then most ESCCs would not be eligible for gefitinib treatment. This may suggest that, rather than using EGFR-protein inhibitor-based therapy such as gefitinib, 1 should use EGFR-targeting therapy to treat the patients with ESCC exhibiting overexpression of EGFR. The present study has demonstrated a high concordance between gene amplification and protein overexpression of EGFR in ESCC, similar to what is observed for c-*erb*B-2 in breast cancers. Unlike c-*erb*B-2 in breast cancers, detection of *EGFR* gene amplification by FISH has not been approved as a test for determining the applicability of these therapies.

In conclusion, anti-EGFR therapies, especially those using monoclonal antibodies, may be appropriate for patients with ESCC, since overexpression of EGFR was significantly correlated with the depth of invasion of the tumor. We assume that combined analyses by IHC, FISH and examination of gene mutations should clarify aberrations in protein and gene function, and could help to identify those patients who are going to benefit from anti-EGFR therapies. It may be important to keep in mind that there are at least 2 different mechanisms of EGFR overexpression: Those involving gene amplification and those that do not.

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# Frequencies of HER-2/neu expression and gene amplification in patients with oesophageal squamous cell carcinoma

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The utilisation of antitumour T cells induced by cancer vaccination with HER-2 peptides or antibodies (Herceptin) against HER-2, as immunotherapy for oesophageal cancer, is a novel and attractive approach. It is important to clarify the frequencies of HER-2 expression and gene amplification in patients with oesophageal squamous cell carcinoma (SCC) and to evaluate the relationship between HER-2 status and HLA haplotype, since the candidates for HER-2 peptide-based vaccination are restricted to a certain HLA haplotype. We determined the frequency of HER-2 expression using the HercepTest™ for immunohistochemistry and HER-2 gene amplification by fluorescence *in situ* hybridisation (FISH) assay in oesophageal SCC ($n = 66$). HER-2-positive tumours (1+/2+/3+) analysed by a HercepTest were observed in 30.3% of all the patients and HER-2 gene amplification evaluated by FISH was observed in 11.0% of all the patients, in which all HercepTest (3+) tumours were found to have gene amplification and three of six moderately positive (2+) tumours showed gene amplification. Furthermore, HER-2-positive cells were present more diffusely and were larger within each tumour in the patients who were HercepTest 3+ than those who were HercepTest 1+. Moreover, the survival rate in HER-2-positive group was significantly worse than that in HER-2-negative group. Also, the survival rate in the patients with HER-2 gene amplification was significantly worse than that without HER-2 gene amplification. In addition, oesophageal SCC patients with both HLA-A24-positive and HER-2-positive tumours (1+/2+/3+) accounted for 26% of these cases, and both HLA-A2- and HER-2-positive tumours accounted for 18% of them.

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While most patients with oesophageal cancer in Western countries have adenocarcinoma, most of those in Japan have squamous cell carcinoma (SCC). Despite various treatments such as surgical resection with extensive lymphadenectomy (Maillet *et al*, 1982; Altorki and Skinner, 1990; Kato *et al*, 1991; Akiyama *et al*, 1994) and the surgery combined with chemotherapy (Hayashi *et al*, 2001) and/or radiotherapy (Le Prise *et al*, 1995; Adham *et al*, 2000; Ishikura *et al*, 2003), the prognosis for advanced patients with oesophageal SCC remains poor. The utilisation of antitumour T cells or antibodies against tumour antigens, as immunoadjuvant therapy for oesophageal SCC, is therefore an attractive approach.

The HER-2/neu (designated as HER-2) proto-oncogene located on chromosome 17(17q12–q21.32) (Popescu *et al*, 1989) encodes a 185-kDa transmembrane glycoprotein with tyrosine-specific kinase activity (Coussens *et al*, 1985). The HER-2 proto-oncogene is amplified and overexpressed in approximately 30% of human ovarian and breast tumours (Slamon *et al*, 1989), and in 8.2% of gastric cancers (Takehana *et al*, 2002). The humanised monoclonal antibody (mAb) Herceptin, which specifically targets HER-2, exhibits potent growth inhibitory activity against HER-2-over expressing tumours (Sliwkowski *et al*, 1999). Herceptin has boosted the interest of clinicians in immunotherapy based on this molecule as it represents the first mAb approved for therapeutic use with proved survival benefit in patients with HER-2-positive breast cancer with metastasis (Baselga *et al*, 1996; Slamon *et al*, 2001). Moreover, abundant examples from experimental models and clinical trials suggest that HER-2 can be immunogenic and generate antibodies, CTL- and helper T-cell-specific responses in individuals with HER-2-overexpressing tumours (Fisk *et al*, 1995; Kono *et al*, 1998). Based on the above reports, anti-HER-2 immune targeting could be utilised as an attractive approach to treat oesophageal cancer. Thus, it is important to clarify the frequency of HER-2 expression and gene amplification relating to the HLA haplotype in order to determine possible candidates for HER-2-based immunotherapy, since the candidates for HER-2 peptide-based vaccination are restricted to a certain HLA haplotype.

With respect to oesophageal SCC, the frequencies of HER-2 overexpression analysed by immunohistochemistry (IHC) ranged from 0 to 55.9% (Mori *et al*, 1987; Chang *et al*, 1992; Suo *et al*, 1992; Shiga *et al*, 1993; Suwanagool *et al*, 1993; Suo *et al*, 1995; Hardwick *et al*, 1997; Lam *et al*, 1998; Akamatsu *et al*, 2003). Furthermore, reports describing HER-2 gene amplification ranged from 0 to 25%, and these studies were performed by Northern blot, slot blot or RT–PCR analysis (Shiga *et al*, 1993; Ikeda *et al*, 1996; Tanaka *et al*, 1997; Friess *et al*, 1999). The discrepancy in the HER-2 frequencies among the reports may be related to the methodology, including the different mAbs used in IHC or

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inaccurate analysis for gene amplification. There has been no previous report describing HER-2 gene amplification in oesophageal SCC analysed by fluorescence *in situ* hybridisation (FISH) analysis.

In the present study, we determined the exact frequency of HER-2 abnormalities using the HercepTest™ for IHC and the PathVysion test for FISH in oesophageal SCC, and analysed patient's data for the survival rate. Both the HercepTest and the PathVysion FISH assay are approved by the US Food and Drug Administration (FDA) for determining the eligibility for Herceptin treatment in breast carcinoma. Furthermore, we have evaluated a possible candidate for anti-HER-2 immune targeting therapy for oesophageal SCC.

## MATERIALS AND METHODS

### Patients and samples

In all, 66 consecutive patients with primary oesophageal SCC who were histologically diagnosed and treated in the First Department of Surgery, University of Yamanashi Hospital, between 1998 and 1999, were enrolled in the present study and all the patients were followed up for 5 years. None of the patients had received any treatment before surgery (preoperative radiotherapy or chemotherapy) and all patients had undergone oesophagectomy with two-field ($n=39$) or three-field ($n=27$) lymph node dissection. The patients were classified using the tumour node metastasis (TNM) classification. The characteristics of the patients are shown in Table 1. The study was approved by the ethical committee of University of Yamanashi and written informed consent was obtained from all individuals.

Formalin-fixed, paraffin-embedded tissue blocks were used for IHC and FISH analysis.

**Table 1** Clinical features of the patients ($n=66$)

| | |
|---|---|
| *Age (years old)* | |
| Mean | 65.3 |
| Range | 45–81 |
| *Gender* | |
| Male | 62 |
| Female | 4 |
| Primary tumour[a] | |
| pTis | 2 |
| pT1a | 8 |
| pT1b | 18 |
| pT2 | 5 |
| pT3 | 32 |
| *LNM* | |
| Negative | 29 |
| Positive | 37 |
| *SCC differentiation* | |
| Well differentiated | 15 |
| Moderate differentiated | 35 |
| Poorly differentiated | 14 |
| *Stage*[a] | |
| 0 | 9 |
| I | 5 |
| II | 25 |
| III | 19 |
| IVa | 6 |
| IVb | 1 |

LNM = lymph node metastasis; SCC = squamous cell carcinoma. [a]The grade of tumour and stages were defined according to the UICC (TMN) classification.

### HLA class I typing

Heparinised peripheral blood was obtained from patients prior to the operation. Peripheral blood lymphocytes (PBLs) were purified by centrifugation on a Ficoll gradient (Pharmacia, Uppsala, Sweden). For class I typing, PBLs were subjected to a complement-dependent microcytotoxicity assay using antisera to HLA-A loci. Peripheral blood lymphocytes were typed for A loci 1, 2, 3, 9, 10, 11, 19, 23, 24, 25, 26, 28, 29, 30, 31, 32, 33, 34, 36, 43, 66, 68, 69 and 74.

### IHC analysis

Immunohistochemical staining was performed using the HercepTest™ (DaKoCytomation, Denmark) according to the manufacturer's recommendations. Archival, formalin-fixed, paraffin-embedded material was used to obtain 4-$\mu$m-thick sections from the main tumour and the regional lymph nodes. Briefly, deparaffinised and rehydrated tissue sections were incubated with the Epitope Retrieval Solution in a heat water bath for 40 min at 95–99°C. Then, the sections were cooled at room temperature for 20 min and washed with TRIS buffer for 5 min. Next, endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min. The primary antibody was a rabbit polyclonal antibody to human HER-2, which recognises an intracytoplasmic part of HER-2, and the primary negative control antibody was an immunoglobulin fraction of normal rabbit serum at an equivalent protein concentration as the antibody to HER-2. The sections were washed with a TRIS buffer for 5 min and incubated with the primary antibody or the primary negative control antibody at room temperature for 30 min. After rewashing with a TRIS buffer for 5 min × 2 times, the primary antibody was detected using the Visualisation Reagents, which were a dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-rabbit immunoglobulins, for 30 min of incubation at room temperature. Subsequently, following rewashing with TRIS buffer for 5 min × 2 times, diaminobenzidine was added as a visualisation reagent for 10 min and the section was counterstained with haematoxylin. Control slides provided with the HercepTest™ kit, which contained three human breast cancer cell lines with staining intensity scores of 0, 1+ and 3+, were used in the present study. IHC analysis was performed by two observers (KM and KK) according to the staining intensity scores provided by the HercepTest™ kit. Each section was classified into four categories (0, 1+, 2+, 3+), in which tumour cells with complete absence of staining were scored as 0, those with incomplete membranous staining were classified as 1+, those with moderate, complete membranous staining were classified as 2+ and those with strong, complete membranous staining were classified as 3+ (Figure 1).

Furthermore, we evaluated the immunostaining pattern into two patterns, spot type or diffuse type (Figure 2), in which the diffuse type indicated tumour cells with membranous staining spread throughout the tumour tissue continuously. On the other hand, the spot type indicated tumour cells with membranous staining on one part or several parts separately in the section.

### FISH analysis

FISH analysis was performed using the PathVysion® HER-2 DNA Probe Kit (VYSIS, Downers Grove, IL, USA). The HER-2/neu-SpectrumOrange probe is specific for the HER-2 gene locus (17q11.2–q12). The CEP 17 (chromosome enumeration probe)/SpectrumGreen probe is specific for the alpha-satellite DNA sequence (centromere region of chromosome 17). To determine the copy number for chromosome 17, we used CEP 17 as the control. FISH procedures were conducted according to the manufacturer's guidelines, except the removal of the protein from

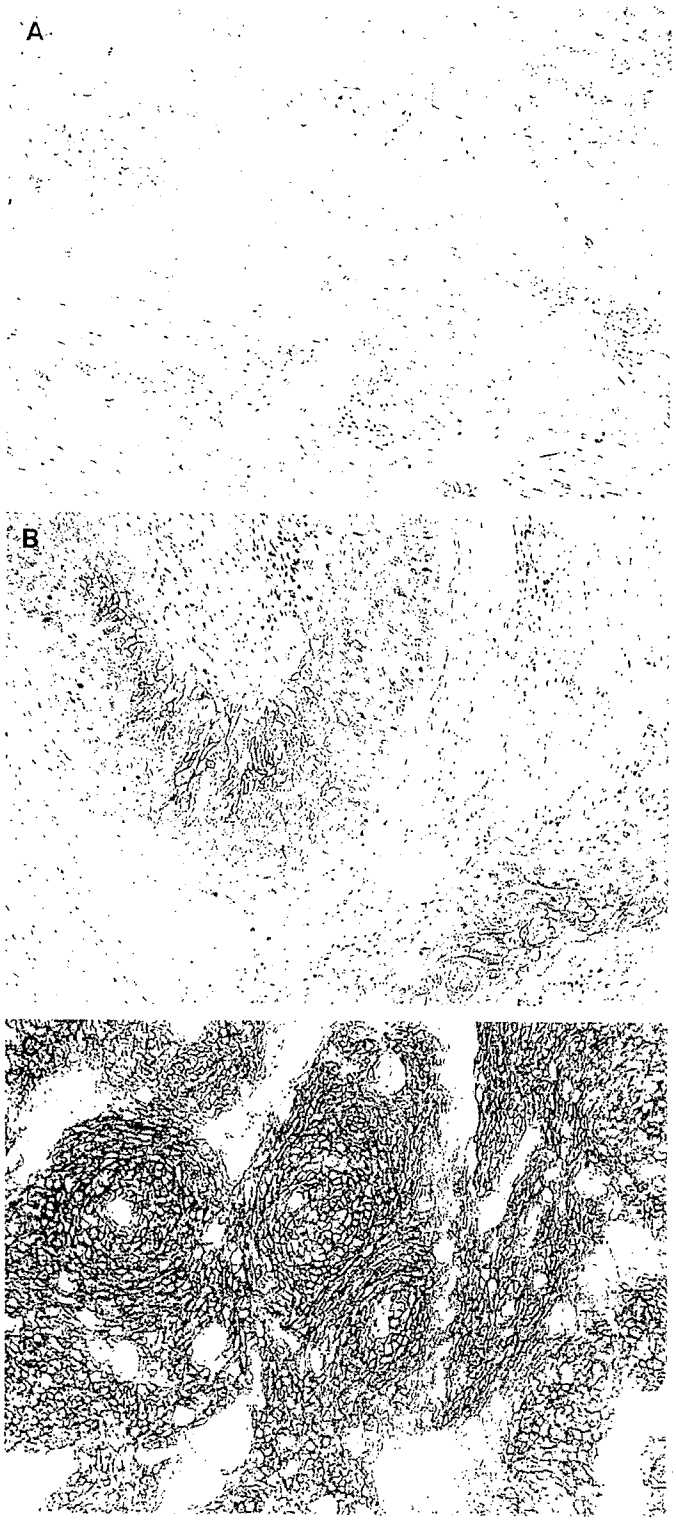


**Figure 1** Representative immunostaining of HER-2-positive cells. (**A**) 1+ staining cases, (**B**) 2+ staining cases and (**C**) 3+ staining cases. Original magnification × 200.

the section where we used our own protocol as described previously (Takehana *et al*, 2002). Briefly, sections were deparaffinised, dehydrated and incubated in 20% sodium bisulphate/2 × standard saline citrate at 43°C for 20 min. Sections were washed with SCC and treated with proteinase K (Boehringer-


**Figure 2** Heterogeneity in the pattern of HER-2 immunostaining. The staining pattern was categorised as spot type and diffuse type. (**A**) Spot type and (**B**) diffuse type. Original magnification × 100.

Mannheim, Mannheim, Germany) at 37°C for 25 min. Subsequently, denaturation, hybridisation and posthybridisation washing were performed according to the manufacturer's guidelines, and after hybridisation and posthybridisation washing, the sections were counterstained with DAPI (4′,6-diamidine-2′-phenylindole dihydrochloride). FISH analysis was performed using a fluorescence microscope (Olympus, Tokyo, Japan) equipped with Triple Bandpass Filter sets (Vysis). Signals were counted for at least 40 cancer nuclei per tumour. In accordance with earlier studies with FISH, a cell was considered to show amplification when a definite cluster or more than 10 orange signals of HER-2 was observed (Takehana *et al*, 2002). A positive control, which is breast tumour with previously identified HER-2 amplification and overexpression, was used as a positive control for HER-2 FISH.

## Statistical analysis

The $\chi^2$ test was applied to examine the differences in frequencies of the HLA-A haplotype and HER-2 expression in oesophageal SCC, the differences in HercepTest score and the rate of HER-2-positive cells in each tumour, and the differences in the lymph node metastasis of HER-2-positive patients and HER-2-negative patients. Actuarial overall survival rates were analysed by the Kaplan–Meier method and survival was measured in months

from operation to death or last review. The log-rank test was applied to compare with the two groups. Univariate and multivariate survival analysis were calculated according to Cox's proportional-hazards model. All statistical analyses were performed using Statview 5.0 for Windows software and statistically significant difference was considered as $P$-values $<0.05$.

## RESULTS

### Frequencies of HER-2 expression and gene amplification

We studied 66 oesophageal SCC tumours and their regional lymph nodes. In IHC, each section was classified into four categories (negative, 1+, 2+, 3+) according to the staining intensity scores provided by the HercepTest kit. Positive immunostaining (1+/2+/3+) of HER-2 expression was found in 20 (30.3%) of the 66 patients with oesophageal SCC (Table 2). The clinicopathological data and their FISH analysis are summarised in Table 3. Three patients (4.5%) showed strong positive staining (3+) and 6 (9.1%) showed moderate positive staining (2+).

With respect to FISH, a cell was considered to show amplification when a definite cluster or more than 10 orange signals of HER-2 was observed in accordance with earlier studies with FISH (Takehana *et al*, 2002). In FISH analysis for IHC-positive oesophageal SCC ($n=20$), HER-2 gene amplification (cluster, Figure 3) was found in seven tumours (Table 3). In the three tumours, cancer nuclei showed more than three HER-2 signals accompanied with the same number of centromere 17 signals. They were judged as polysomy 17 (Table 3).

With respect to the comparison of FISH and IHC analysis, all the strong positive (3+) tumours were found to have gene amplification, as shown in Table 3. Among six moderate positive (2+) tumours, three showed gene amplification (cluster) and one showed polysomy. In 11 weak positive (1+) tumours, one showed gene amplification (cluster) and two showed polysomy.

Taken together, positive immunostaining (1+/2+/3+) for HER-2 expression was found in 30.3%. Moreover, moderate and strong positive patients (2+/3+) had a high frequency of gene amplification, while weak positive (1+) patients showed a low frequency of gene amplification.

**Table 2** Frequencies of HER-2-positive patients detected by IHC in oesophageal SCC

| | **Patients (total = 66)** |
|---|---|
| *IHC scores* | |
| 3+ | 3 (4.5%) |
| 2+ | 6 (9.1%) |
| 1+ | 11 (16.7%) |

IHC = immunohistochemistry; SCC = squamous cell carcinoma.

### Immunostaining pattern and rate of HER-2-positive cells within each tumour

When the clinical application of anti-HER-2 immune targeting is considered, it is important to clarify the heterogeneity in the pattern of HER-2-positive tumour cells within each tumour. According to the DAKO HercepTest kit, the grading of the HercepTest depends on the intensity of membranous staining, indicating that the grading does not reflect how many cells are HER-2 positive. Thus, in the present study, we evaluated the immunostaining pattern and rate of HER-2-positive cells within the tumour.

We recognised that there was heterogeneity in the pattern of HER-2 immunostaining and categorised the staining pattern into a spot type and diffuse type (Figure 2). Also, HER-2-positive cells were semiquantified by counting the average number in each tumour. As a result, all three strong positive (3+) cases showed the diffuse-type pattern (Table 3) and had HER-2-positive cells in more than 30% of tumours (Table 4), while most of the weak positive (1+) patients showed that HER-2-positive cells were less than 30% (Table 4) ($P=0.057$, $\chi^2$ analysis). Thus, HER-2-positive

**Table 3** Patients with HER-2-positive oesophageal SCC detected by IHC and their FISH analyses

| | | | | | **IHC** | | | |
|---|---|---|---|---|---|---|---|---|
| **Case number** | **Age (years)** | **Sex** | **Stage[a]** | **Histological[b] classification** | **Score[c]** | **Pattern** | **Score[c] of LN** | **FISH** |
| 1 | 73 | M | III | Mod | 3+ | Diffuse | 3+ | Cluster |
| 2 | 54 | M | III | Por | 3+ | Diffuse | 3+ | Cluster |
| 3 | 62 | M | IVa | Mod | 3+ | Diffuse | 3+ | Cluster |
| 4 | 62 | M | IVa | Mod | 2+ | Spot | 0 | Cluster |
| 5 | 59 | M | III | Well | 2+ | Diffuse | 0 | Cluster |
| 6 | 57 | M | III | Mod | 2+ | Spot | 2+ | Polysomy |
| 7 | 55 | M | II | Por | 2+ | Diffuse | 2+ | No amplification |
| 8 | 69 | M | II | Well | 2+ | Spot | No | No amplification |
| 9 | 76 | M | I | Well | 2+ | Spot | No | Cluster |
| 10 | 56 | M | II | Mod | 1+ | Spot | 0 | Polysomy |
| 11 | 74 | M | III | Well | 1+ | Diffuse | 0 | Polysomy |
| 12 | 60 | M | III | Mod | 1+ | Spot | 0 | No amplification |
| 13 | 60 | M | III | Por | 1+ | Diffuse | 0 | No amplification |
| 14 | 64 | M | III | Mod | 1+ | Diffuse | 0 | No amplification |
| 15 | 67 | M | III | Mod | 1+ | Diffuse | 0 | No amplification |
| 16 | 71 | M | III | Mod | 1+ | Spot | 0 | No amplification |
| 17 | 47 | F | II | Por | 1+ | Diffuse | 1+ | No amplification |
| 18 | 74 | M | 0 | Por | 1+ | Spot | No | Cluster |
| 19 | 70 | M | II | Mod | 1+ | Diffuse | No | No amplification |
| 20 | 80 | M | II | Por | 1+ | Spot | No | No amplification |

SCC = squamous cell carcinoma; IHC = immunohistochemistry; FISH = fluorescence *in situ* hybridisation; LN = lymph node; No = no lymph node metastasis. [a]Stages were defined according to the TNM classification. [b]Well = well-differentiated SCC; mod = moderately differentiated SCC; por = poorly differentiated SCC. [c]IHC score was defined by the staining intensity of tumour cells (0, 1+, 2+, 3+).

cells were present more diffusely within each tumour in the HercepTest 3+ patients than those who were HercepTest 1+.

## Correlation of HER-2 expression in primary tumours and metastatic lymph nodes

Of all 66 cohorts, 37 (56.1%) had metastatic lymph nodes diagnosed by histopathological determination. While 15 (75.0%) in the 20 HER-2-positive patients had metastatic lymph nodes, 22 (47.8%) in the 46 HER-2-negative patients had metastatic lymph nodes (Table 3), indicating that there was a high frequency of lymph node metastasis in the HER-2-positive patients in comparison to HER-2-negative patients ($P<0.05$, $\chi^2$ analysis). All the strongly positive (3+) patients in the primary tumours were found to have HER-2-positive tumours (3+) in the metastatic lymph nodes, while there was only one patient with a HER-2-positive tumour in the metastatic lymph node in the weak positive (1+) patients with lymph node metastasis ($n=8$).

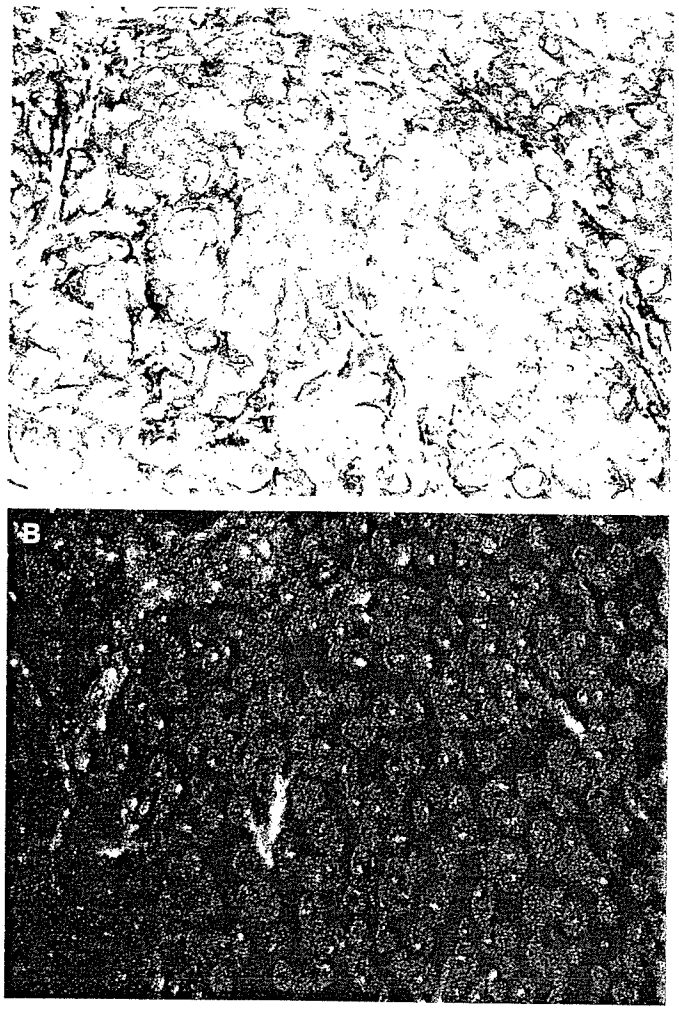


**Figure 3** Representative FISH analysis of HER-2 gene amplification (cluster formation). The serial sections in the representative case were analysed by IHC (**A**) (3+ staining cases, × 400) and FISH analysis ( × 400 in (**B**)).

**Table 4** Correlation between IHC scores and the rate of HER-2-positive tumour cells in each tumour

| | Rate of HER-2/neu-positive cells in each section | | |
|---|---|---|---|
| | ~30 (%) | 31–60 (%) | 61~ (%) |
| *IHC scores* | | | |
| 3+ ($n=3$) | 0 | 2 | 1 |
| 2+ ($n=6$) | 5 | 0 | 1 |
| 1+ ($n=11$) | 8 | 1 | 2 |
| | | $P=0.057$ by $\chi^2$ test | |

IHC = immunohistochemistry.

## Frequency of the HLA haplotype relating to HER-2 status

When anti-HER-2 immunotherapy such as cancer vaccination is considered for HER-2-positive patients, the candidates are restricted to a certain HLA haplotype (Kono *et al*, 2002a). It is important to clarify the HER-2 status relating to the HLA haplotype in patients with oesophageal SCC. In the patients tested for HLA haplotypes ($n=50$), the distribution of the HLA-A haplotype in patients with oesophageal SCC is shown in Table 5. The most frequent HLA-A haplotypes in oesophageal SCC are HLA-A24 (64%), HLA-A2 (52%) and HLA-A11 (28%). Also, oesophageal cancer patients with both HLA-A24-positive and HER-2-positive tumours accounted for 26% of these cases, and both HLA-A2-positive and HER-2-positive tumours accounted for 18% of them. There was no significant relation between the frequency of the HLA-A haplotype and the HER-2 status (Table 5).

## Analysis of the survival of patients with oesophageal SCC

The survival rate in HER-2-positive (1+/2+/3+) group was significantly worse than that in HER-2-negative group (Figure 4A). Moreover, there was a significant difference in the survival rate between HER-2 (2+/3+) and HER-2-negative group (Figure 4B). The survival rate in the patients with HER-2 gene amplification was significantly worse than that without HER-2 gene amplification (Figure 5).

In univariate analysis, the factors such as HER-2 gene amplification, pT2 and pT3, lymph node metastasis, stages 3–4 were significant prognostic factors for survival (Table 6), although only pT2 factor reached to the significant level as independent risk factors for survival in multivariate analysis (Table 6).

**Table 5** Frequencies of the HLA-A haplotype related to HER-2 expression in oesophageal SCC ($n=50$)

| | HER-2 status detected by IHC | |
|---|---|---|
| | **Positive ($n=18$)** | **Negative ($n=32$)** |
| *HLA-A2* | | |
| Positive ($n=26$) | 9 (18%)[a] | 17 (34%) |
| Negative ($n=24$) | 9 (18%) | 15 (30%) NS[b] |
| *HLA-A11* | | |
| Positive ($n=14$) | 7 (14%) | 7 (14%) |
| Negative ($n=36$) | 11 (22%) | 25 (50%) NS |
| *HLA-A24* | | |
| Positive ($n=32$) | 13 (26%) | 19 (38%) |
| Negative ($n=18$) | 5 (10%) | 13 (26%) NS |

SCC = squamous cell carcinoma; HLA = human leucocyte antigen. [a]Percentage indicates the number of patients out of all patients ($n=50$). [b]Not significant by $\chi^2$ test.

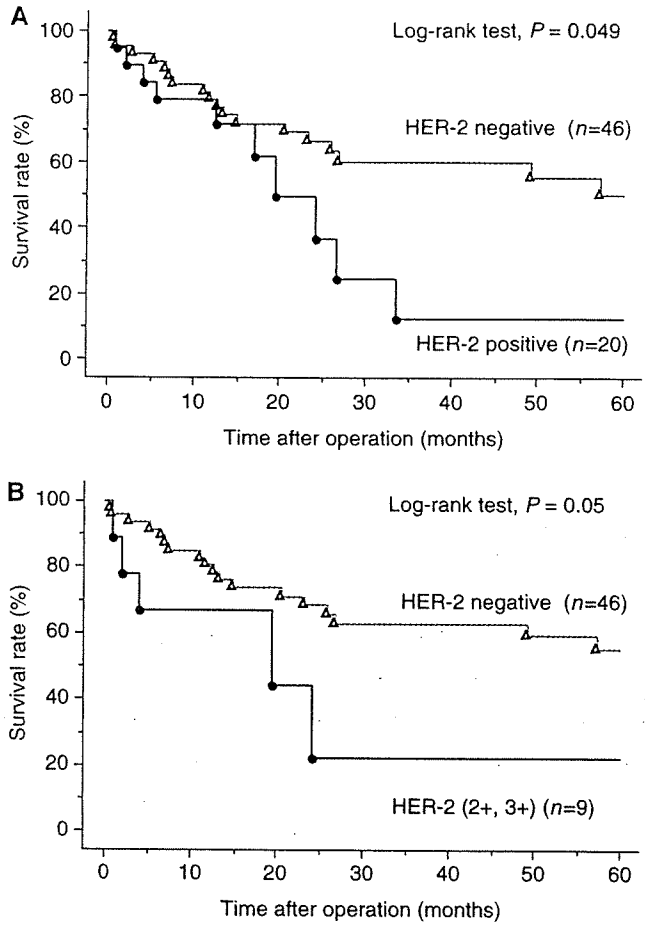


**Figure 4** (**A**) Survival curves of HER-2-negative and HER-2-positive (1+/2+/3+) groups. (**B**) Survival curves of HER-2-negative and HER-2 (2+/3+) groups.

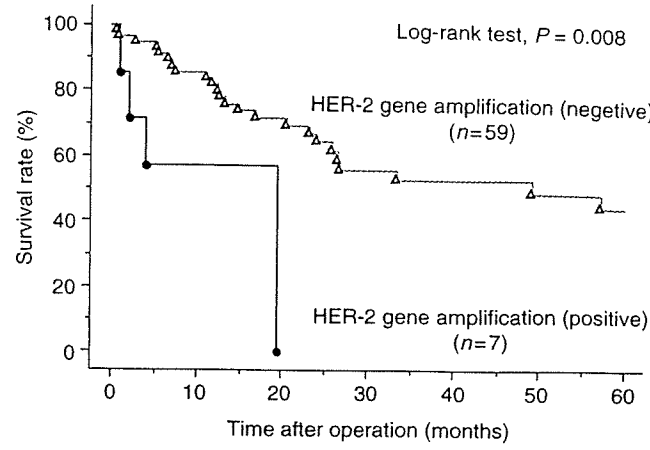


**Figure 5** Survival curves of HER-2 gene amplification-negative and HER-2 gene amplification-positive groups.

## DISCUSSION

The present study contains several important findings relevant to HER-2 status in oesophageal SCC. First, HER-2-positive tumours (1+/2+/3+) analysed by the HercepTest were observed in 30.3% of all patients and HER-2 gene amplification evaluated by FISH was observed in 11.0% of all patients, of which all IHC (3+) tumours were found to have gene amplification and three out of six tumours with moderate positive (2+) tumours showed gene amplification. Second, HER-2-positive cells existed more diffusely and were larger within each tumour in HercepTest 3+ patients than those who were HercepTest 1+. Thirdly, oesophageal SCC patients with both HLA-A24-and HER-2-positive tumours (1+/2+/3+) accounted for 26% of these cases, and both HLA-A2- and HER-2-positive tumours accounted for 18% of them.

The frequencies of HER-2 overexpression in oesophageal SCC analysed by IHC ranged from 0 to 55.9% (Mori *et al*, 1987; Chang

**Table 6** Significance of prognostic factors in univariate and multivariate survival analysis for patients with oesophageal SCC

| | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| | **Hazard ratio** | **95% CI** | ***P*-value** | **Hazard ratio** | **95% CI** | ***P*-value** |
| *FISH* | | | | | | |
| Negative | 1.0 | — | — | 1.0 | — | — |
| Positive | 4.05 | 1.31–12.53 | 0.015 | 2.94 | 0.78–11.09 | 0.111 |
| *HER-2 expression* | | | | | | |
| Negative | 1.00 | — | — | 1.0 | — | — |
| Positive | 2.06 | 0.93–4.57 | 0.074 | 0.92 | 0.35–2.41 | 0.861 |
| *Primary tumour*[a] | | | | | | |
| pTis-pT1b | 1.0 | — | — | 1.0 | — | — |
| pT2 | 5.85 | 1.56–21.91 | 0.009 | 5.00 | 1.06–23.52 | 0.042 |
| pT3 | 5.28 | 1.91–14.55 | 0.001 | 3.01 | 0.76–11.95 | 0.117 |
| *LNM* | | | | | | |
| Negative | 1.0 | — | — | 1.0 | — | — |
| Positive | 3.13 | 1.32–7.39 | 0.009 | 1.56 | 0.25–9.68 | 0.636 |
| *Stage*[a] | | | | | | |
| 0–II | 1.0 | — | — | 1.0 | — | — |
| III–IV | 5.21 | 2.33–11.64 | <0.0001 | 2.09 | 0.30–14.53 | 0.458 |

SCC = squamous cell carcinoma; CI = confidence interval; FISH = fluorescence *in situ* hybridisation; LNM = lymph node metastasis. [a]The grade of tumour and stages were defined according to the UICC (TMN) classification.

*et al*, 1992; Suo *et al*, 1992, 1995; Shiga *et al*, 1993; Suwanagool *et al*, 1993; Lam *et al*, 1998; Hardwick *et al*, 1997; Akamatsu *et al*, 2003). Furthermore, reports describing HER-2 gene amplification ranged from 0 to 25%, in which these studies were performed by Northern blot, slot blot or RT–PCR analysis (Shiga *et al*, 1993; Ikeda *et al*, 1996; Tanaka *et al*, 1997; Friess *et al*, 1999). This is the first report describing the HER-2 status in oesophageal SCC analysed by two FDA-approved tests, the HercepTest and FISH (PathVysion test). Moreover, there was no previous report describing HER-2 status evaluated by the HercepTest and FISH in relation to the survival rate in oesophageal SCC. As a result, HER-2-positive tumours analysed by the HercepTest were observed in 30.3% of all the patients and HER-2 gene amplification evaluated by FISH was observed in 11.0% of all the patients.

There is increasing evidence that there is a discrepancy in the detection of HER-2 status between the two FDA-approved test, the HercepTest and FISH (PathVysion test), in which the concordance rates ranged from 80 to 90% (Jacobs *et al*, 1999; Varshney *et al*, 2004). There have been several reports that cases with HER-2 overexpression without gene amplification mostly occurred in moderate positive cases (2+) (Perez *et al*, 2002; Varshney *et al*, 2004), in line with this study. Various explanations of this discrepancy have been proposed: transcriptional or post-translational activation (Slamon *et al*, 1989), artifactual high sensitivity of IHC (Varshney *et al*, 2004), the presence of chromosome 17 polysomy (Wang *et al*, 2002) or the low detection rate of FISH analysis (Jacobs *et al*, 1999). We found one case of polysomy in 2+ patients and two cases of polysomy in 1+ patients, suggesting that the presence of chromosome 17 polysomy might be one explanation for the discrepancy between the HercepTest and FISH in oesophageal SCC.

Interestingly, there was one case with HER-2 gene amplification in a HercepTest 1(+) tumour, indicating that the screening of HER-2 status by the HercepTest may underestimate HER-2 gene amplification. Since both the HercepTest and FISH assay have limitations in detecting HER-2 status, both methods should be applied when anti-HER-2 immune targeting, such as Herceptin or cancer vaccination, are considered in oesophageal SCC.

In this study, all the HercepTest 3+ patients had HER-2 gene amplification, and HER-2-positive cells in these cases were present diffusely and were larger within the tumours. Furthermore, the HER-2 expression in HercepTest 3+ patients was also preserved in the metastatic lymph nodes. The action of anti-HER-2-specific CTL correlated to the degree of HER-2 expression on the target tumour cells (Fisk *et al*, 1995; Kono *et al*, 1998). Also, the activity of ADCC induced by Herceptin correlated to the degree of HER-2 expression on the target tumour cells (Kono *et al*, 2002b). These results indicate that HercepTest 3+ patients in oesophageal SCC will be the best candidates for anti-HER-2 immune targeting. It has already been shown in breast cancer trials that there is a greater benefit from Herceptin therapy for 3+ patients compared to those who were 2+ (Cobleigh *et al*, 1999; Slamon *et al*, 2001).

The survival rate in patients with HER-2 expression or HER-2 gene amplification was significantly worse than that without HER-2 expression or HER-2 gene amplification. These results indicated that HER-2 status may be one of the prognostic factors to predict the clinical course of patients with oesophageal SCC, although the HER-2 status did not reach to the significant level as independent risk factors for survival in multivariate analysis in the present study. Since the sample size of the present study is limited, further studies with larger cohorts will be needed to draw valid conclusion.

When considering the cancer vaccination with HER-2-derived peptide epitopes, there is an HLA restriction. It has been shown that HLA-A2- or HLA-A24-restricted peptide epitopes were identified for immunodominant CTL epitopes derived from HER-2 (Fisk *et al*, 1995; Kono *et al*, 1998, 2002b). In the present study, oesophageal SCC patients with both HLA-A24- and HER-2-positive tumours (1+/2+/3+) accounted for 26% of these cases, and both HLA-A2- and HER-2-positive tumours accounted for 18% of them. These populations are considered for cancer vaccination with the HER-2 peptide in oesophageal SCC. In fact, we and others reported that clinical vaccination trials in gastric or breast cancer patients using DCs pulsed with HER-2 peptides confirmed the fact that vaccination with HER-2 peptides is immunogenic, and that HER-2 could be a good target for immunotherapy (Disis *et al*, 1999; Kono *et al*, 2002c). Furthermore, we have recently shown that Herceptin enhances MHC class I-restricted antigen presentation in HER-2-overexpressing tumours, resulting in a higher susceptibility of HER-2-overexpressing tumours to lysis by HER-2-specific CTL (Kono *et al*, 2004). These results suggested that the combination of Herceptin and anti-HER-2-specific CTLs may result in a synergic antitumour effect in oesophageal SCC. Anti-HER-2 immune targeting such as Herceptin or cancer vaccination with HER-2 peptides is novel and attractive approach for oesophageal SCC and the candidates for HER-2-based immunotherapy were limited, but significant populations of oesophageal SCC.

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# Trastuzumab-Mediated Antibody-Dependent Cellular Cytotoxicity against Esophageal Squamous Cell Carcinoma

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**Abstract** **Purpose:** In the present study, we investigated the degree of protein expression and gene amplification of HER-2 in esophageal squamous cell carcinoma (SCC) cell lines and freshly isolated tumors, and trastuzumab-mediated biological activity, in particular antibody-dependent cellular cytotoxicity (ADCC) against HER-2–expressing esophageal SCC cell lines.

**Experimental Design:** Ten different SCC cell lines with various levels of HER-2 status evaluated by flow cytometry, immunocytochemistry (HercepTest), and fluorescence *in situ* hybridization were evaluated for ADCC, growth inhibitory, or apoptosis-inducing activities mediated by trastuzumab.

**Results:** Trastuzumab induced ADCC against HER-2–expressing esophageal SCC and the activities reflected the degree of HER-2 expression analyzed by flow cytometric analysis, but not by HercepTest nor fluorescence *in situ* hybridization analysis. Furthermore, trastuzumab-mediated ADCC against transforming growth factor-β–producing SCC was enhanced by the treatment with SB-431542, which is a selective inhibitor of the phosphorylation induced by transforming growth factor-β. There were very marginal effects of anti-proliferative or apoptosis-inducing activities mediated by trastuzumab for HER-2–expressing esophageal SCC.

**Conclusion:** HER-2–expressing esophageal SCC cells could be killed by trastuzumab-mediated ADCC and the activity reflected the degree of HER-2 expression detected by flow cytometry.

Most patients with esophageal cancer in Japan have squamous cell carcinoma (SCC), whereas most of those in Western countries have adenocarcinoma. Despite aggressive treatment modalities such as surgical resection with extensive lymphadenectomy (1, 2) and surgery combined with chemotherapy (3) and/or radiotherapy (4, 5), the control of esophageal SCC at the advanced stage remains difficult. Therefore, for esophageal SCC patients, immunoadjuvant therapy such as the utilization of anti-tumor specific T cells or antibody against tumor antigens is very much needed.

The HER-2 proto-oncogene encodes a 185-kDa transmembrane glycoprotein with tyrosine-specific kinase activity (6). According to numerous examples from experimental models and clinical trials, HER-2 can be immunogenic and generate antibodies and cytotoxic T cell– and helper T cell–specific responses in individuals with HER-2–overexpressing tumors (7, 8). Moreover, the fully humanized monoclonal antibody (mAb) trastuzumab (Herceptin), which specifically targets the extracellular domain of the HER-2 protein, exhibits potent growth inhibitory activity against HER-2–overexpressing tumors (9). In fact, trastuzumab was clinically shown to have survival benefit in patients with HER-2–overexpressing breast cancer with metastasis (10, 11). Based on the above reports, anti–HER-2 immune targeting is as an attractive approach to treat esophageal SCC.

Many mechanisms are linked to the therapeutic effect of trastuzumab, including the blockade of signaling pathways (9), inhibition of tumor cell growth such as the down-regulation of the HER-2 receptor (9), activation of apoptotic signals of tumor cells (12), and enhancement of the immune system such as antibody-dependent cellular cytotoxicity (ADCC; refs. 9, 13). In addition, we recently reported that trastuzumab enhances class I–restricted antigen presentation recognized by HER-2–specific CTLs (14). These observations prompted us to use trastuzumab against esophageal SCC. However, there is little information about the possibility of applying trastuzumab to esophageal SCC patients.

In the present study, we investigated (*a*) the degree of protein expression and gene amplification of HER-2 in esophageal SCC cell lines and freshly isolated tumors evaluated by flow cytometry, immunocytochemistry, and fluorescence *in situ* hybridization (FISH); (*b*) the biological activity of trastuzumab against HER-2–expressing esophageal SCC cell lines; and (*c*) the synergic effect of trastuzumab and SB-431542, which is a selective inhibitor of the phosphorylation induced by transforming growth factor-β (TGF-β; ref. 15), in an SCC cell line producing TGF-β.

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## Materials and Methods

*Chemicals and antibodies.* Anti-HER-2 monoclonal antibody trastuzumab (Herceptin) and anti-CD20 mAb Rituxan, which is an isotype-matched control mAb for trastuzumab, were purchased from Roche (Basel, Switzerland). Phycoerythrin-labeled anti-HER-2 mAb (Becton Dickinson, San Jose, CA) and phycoerythrin-labeled mouse immunoglobulin G1 mAb (Beckman-Coulter, Miami, FL) as a negative control were used for immunostaining by flow cytometric analysis. Human recombinant TGF-β2 was purchased from AUSTRAL Biologicals (San Ramon, CA). Anti-TGF-β2 neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). SB-431542, which is a selective inhibitor of TGF-β/activin receptor-like kinase (ALK) type 1 activity, was purchased from TOCRIS (Ellisville, MO), and dissolved at concentration of 10 mmol/L in DMSO and stored at −20°C.

*Cell lines.* Esophageal SCC cell lines TE1, TE2, TE3, TE4, and TE5 were a gift from Dr. Nishihara (Institute of Development, Aging and Cancer, University of Tohoku, Sendai, Japan). Esophageal SCC cell lines KYSE30, KYSE50, KYSE70, KYSE110, and TT were purchased from Health Science Research Resources Bank (Osaka, Japan). Ovarian cancer cell line SKOV3 was obtained from American Type Culture Collection (Rockville, MD). All cell lines were kept in RPMI 1640 with 5% FCS, 50 units/mL penicillin, and 2 mmol/L L-glutamine.

*Cell preparation.* Primary solid tumor from esophageal SCC patients was isolated during surgery and was homogenized by mechanical mincing. Then, cell mixtures were passed through a cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) and suspended as a single-cell suspension. A single-cell suspension derived from solid tumors and malignant pleural effusion was purified by centrifugation with Ficoll-Paque (Pharmacia, Uppsala, Sweden) and subjected to flow cytometry or immunocytochemistry.

Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood obtained from healthy donors and esophageal SCC patients by centrifugation with Ficoll-Paque (Pharmacia).

*Immunocytochemistry analysis.* Immunocytochemical staining was done using the HercepTest (DakoCytomation, Glostrup, Denmark) according to the recommendations of the manufacturer. After each cell line was centrifuged into a cell pellet, pelleted cells were formalin fixed and paraffin-embedded. Archival, formalin-fixed, paraffin-embedded material was used to obtain 4-μm-thick sections. Briefly, deparaffinized and rehydrated tissue sections were incubated with the Epitope Retrieval Solution in hot water bath for 40 minutes at 95°C to 99°C. Then, the sections were cooled at room temperature for 20 minutes, and the sections were washed with TRIS-buffer for 5 minutes, and endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 minutes. The primary antibody was rabbit polyclonal antibody to human HER-2, which recognizes an intracytoplasmic part of HER-2, and the primary negative control antibody was an immunoglobulin fraction of normal rabbit serum at an equivalent protein concentration as the antibody to HER-2. The sections were washed with TRIS-buffer for 5 minutes and incubated with the primary antibody or the primary negative control antibody at room temperature for 30 minutes. After rewashing with TRIS-buffer for 5 minutes twice, the primary antibody was detected using visualization reagents, which were dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-rabbit immunoglobulins, for 30 minutes of incubation at room temperature. Subsequently, following the rewashing with TRIS-buffer for 5 minutes twice, diaminobenzidine was added as a visualization reagent for 10 minutes and the section was counterstained with Hematoxylin. The control slides provided with the HercepTest kit, which contained three human breast cancer cell lines with staining intensity scores of 0, 1+, and 3+, were used in the present study.

Immunocytochemistry analysis was done by two observers (K.M. and K.K.) according to the staining intensity scores provided by the HercepTest kit. Each section was classified into four categories (0, 1+, 2+, and 3+), in which tumor cells with complete absence of staining were scored as 0; those with incomplete membranous staining were classified as 1+; those with moderate, complete membranous staining were classified as 2+; and those with strong, complete membranous staining were classified as 3+.

*Fluorescence in situ hybridization analysis.* FISH analysis was done using the PathVysion HER-2 DNA Probe Kit (Vysis, Downers Grove, IL). The HER-2/neu-SpectrumOrange probe is specific for the *HER-2* gene locus (*17q11.2-q12*). The CEP 17 (chromosome enumeration probe)/SpectrumGreen probe is specific for the α-satellite DNA sequence (centromere region of chromosome 17). To determine the copy number for chromosome 17, we used CEP 17 as the control. FISH procedures were conducted according to the guidelines of the manufacturer, except the removal of the protein from the sections where we used our own protocol as previously described (16). The serial sections in each cell lines were analyzed by immunocytochemistry and FISH analysis. Briefly, sections were deparaffinized, dehydrated, and incubated in 20% sodium bisulfate/2× SSC at 43°C for 20 minutes. Sections were washed with SCC and treated with proteinase K (Boehringer-Mannheim, Mannheim, Germany) at 37°C for 25 minutes. Subsequently, denaturation, hybridization, and post-hybridization washing were done according to the guidelines of the manufacturer, and after hybridization and post-hybridization washing, the sections were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride. FISH analysis was done using a fluorescence microscope (Olympus, Tokyo, Japan) equipped with Triple Bandpass Filter sets (Vysis). Signals were countered for at least 40 cancer nuclei per tumor. In accordance with our previous studies with FISH, a cell was considered to show amplification when a definite cluster or more than 10 orange signals of HER-2 were observed (16).

A positive control, a breast tumor with previously identified HER-2 amplification and overexpression, was used as a positive control for HER-2 FISH.

*Antibody-dependent cell-mediated cytotoxicity assay.* After the target cells were labeled with 50 μCi of $^{51}Cr$ for 60 minutes, target cells ($5 \times 10^3$/well) and effector cells at various effector/target ratios were co-incubated in 200 μL of X-VIVO medium in a 96-well U-bottomed plate in triplicate for 8 hours at 37°C with trastuzumab (2 μg/well; Roche) or a control antibody, Rituxan (2 μg/well; Roche). After 8 hours of incubation, the radioactivity of the supernatant (100 μL) was measured with a γ counter. The percentage of specific lysis was calculated according to the following formula: % specific lysis = 100 × (experimental cpm − spontaneous cpm) / (maximum cpm − spontaneous cpm). Controls included the incubation of target cells alone with trastuzumab.

*Immunoblotting for phosphorylated Smad2.* PBMCs ($7 \times 10^6$) from healthy donors were pre-treated with SB-431542 or DMSO (vehicle) at indicated concentrations for 30 minutes in X-VIVO (1 mL) medium in a six-well plate and then pre-treated PBMCs were cultured in the presence of TGF-β2 (5 ng/mL) for 60 minutes. After incubation, each sample of PBMCs was washed twice with sterile PBS and then suspended in Laemmli sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromophenol blue; Bio-Rad Laboratories, Inc., Hercules, CA] supplemented with 5% β-mercaptoethanol. Extracts were cleared by centrifugation. Whole cell extracts (10 μg) were fractionated on 10% SDS-polyacrylamide gels and transferred onto Immobilon-P membrane (Millipore, Billerica, MA). Immunoblotting was done using antibodies against Smad2/3 (Upstate Biotechnology, Lake Placid, NY) and phosphorylated Smad2 (Upstate Biotechnology). Immunoreactive bands were detected by enhanced chemiluminescence (ECL-plus, Amersham Life Science, Piscataway, NJ) using horseradish peroxidase-linked anti-rabbit or anti-mouse immunoglobulin G (Amersham Life Science).

*Treatment with anti-transforming growth factor-β2 neutralizing monoclonal antibody or SB-431542 of trastuzumab-mediated antibody-dependent cellular cytotoxicity.* In experiment with anti-TGF-β2 neutralizing mAb, after the target cells were labeled with 50 μCi of $^{51}Cr$ for 60 minutes, target cells ($5 \times 10^3$/well) and PBMCs ($1 \times 10^5$/well) were co-incubated in 200 μL of X-VIVO medium in a 96-well U-bottomed plate in triplicate for 8 hours at 37°C with or without anti-TGF-β2 mAb (5 ng/mL; R&D Systems) in the presence of trastuzumab (2 μg/well; Roche).

In experiment with SB-431542, after PBMCs from healthy donors were pretreated with SB-431542 or DMSO (vehicle) at indicated concentrations for 30 minutes in X-VIVO medium, $^{51}Cr$-radiolabeled target cells ($5 \times 10^3$/well) and the pre-treated PBMCs ($1 \times 10^5$/well) were co-incubated in X-VIVO (200 μL) in a 96-well U-bottomed plate for 8 hours at 37°C with trastuzumab (2 μg/well) in the presence of SB-431542 or DMSO.

*Flow cytometric analysis.* To evaluate HER-2 expression, a phycoerythrin-labeled anti-HER-2/neu mAb (Becton Dickinson) and phycoerythrin-labeled mouse immunoglobulin G1 mAb (Beckman-Coulter, Miami, FL) as a negative control were used for immunostaining by flow cytometric analysis.

*3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay.* Each cell line (2,500/well) was cultured in 200 μL of X-VIVO with trastuzumab (2 μg/well) in 96-well flat-bottomed plates (Corning, Corning, NY) in triplicate. After 96 hours of incubation in a humidified incubator at 37°C, 5% $CO_2$, 50 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (2 mg/mL, Sigma, St. Louis, MO) were added to each well and incubation was done for 4 hours. Then, the supernatant was discarded and the crystal products were eluted with DMSO (50 μL/well, Sigma). The colorimetric evaluation was tested using a spectrophotometer at 570 nm. The inhibition of proliferation was shown as percent cell growth inhibition induced by trastuzumab in comparison with that induced by control mAb.

*Apoptosis.* Each cell line ($2 \times 10^5$ cells) was cultured in 2 mL of X-VIVO with or without trastuzumab (10 μg/mL) at 37°C for 24 hours in a six-well plate. After incubation, apoptosis in each cell line was measured by staining with FITC-conjugated Annexin-V and propidium iodide using a MEBCYTO Apoptosis kit (MBL, Nagoya, Japan) following the recommendations of the manufacturer.

*Quantitative determination of interleukin-10 and transforming growth factor-β.* Each cell line ($2 \times 10^5$ cells) was cultured in 2 mL of X-VIVO at 37°C for 24 hours in a six-well plate. After incubation, the culture supernatants in each cell line were collected and tested for cytokine using a human interleukin 10 (IL-10) Enzyme Immunometric Assay Kit (Assay Designs, Ann Arbor, MI), Human TGF-β1 Immunoassay Kit (R&D Systems), and Human TGF-β2 Immunoassay kit (R&D Systems) following the recommendations of the manufacturers.

*Statistics.* To evaluate statistical differences between groups, a non-paired Student's $t$ test was done. Statistically significant difference was considered at $P < 0.05$.

## Results

*The status of HER-2 expression and gene amplification of esophageal squamous cell carcinoma.* HER-2 expression in esophageal SCC cell lines ($n = 10$) was assessed by flow cytometric analysis, immunocytochemistry, and FISH. By flow cytometric analysis, there were variable levels of HER-2 expression (Table 1; Fig. 1). Furthermore, by immunocytochemistry analysis using the HercepTest, only the TE4 cell line was strong positive (3+) with the HercepTest, whereas the remaining nine cell lines showed no staining (Table 1). The HER-2 level of TE4 analyzed by flow cytometry was almost equal to that of SKOV3, which is a well-known HER-2-overexpressing ovarian cancer cell line (Fig. 1). In FISH analysis, *HER-2* gene amplification was only found in TE4 (Table 1), and polysomy, in which cancer nuclei showed more than three HER-2 signals accompanied with the same number of centromere 17 signals, was found in cell lines (Table 1). These results indicated that there was a difference in the detection of HER-2 status among flow cytometry, immunocytochemistry analysis, and FISH.

Next, we examined HER-2 expression in freshly isolated tumors (primary tumor and malignant pleural effusion) derived from two different esophageal SCC patients. Both samples revealed the moderate levels of HER-2 expression in comparison with esophageal SCC cell lines analyzed by flow cytometry (Fig. 1). Furthermore, we did immunocytochemistry (HercepTest) analysis for the same samples. In spite of the HER-2 expression in flow cytometric analysis, both samples showed no staining in HercepTest (data not shown). These results indicated that there was a difference in the detection of HER-2 status between flow cytometry and immunocytochemistry analysis, and flow cytometric analysis might be more sensitive in the detection of HER-2 status.

**Table 1.** Anti-proliferative activity and apoptosis-inducing activity of trastuzumab

| Cell lines | HER-2 status | | | % Inhibition of growth (MTT) | Apoptosis (%) | |
|---|---|---|---|---|---|---|
| | FACS (MFI) | HercepTest | FISH | | Medium | Medium + trastuzumab |
| TE4 | 666 | 3+ | Cluster | 16.3 ± 3.9 | 3.7 | 4.9 |
| KYSE30 | 123 | — | Polysomy | 0 | 5.9 | 6.6 |
| KYSE50 | 114 | — | Polysomy | 5.1 ± 2.0 | 11.7 | 10.7 |
| TE5 | 113 | — | Polysomy | 12.0 ± 4.8 | 3.4 | 2.3 |
| TE1 | 107 | — | Polysomy | 10.0 ± 2.2 | 3.9 | 3.7 |
| TE2 | 71 | — | Polysomy | N.D.* | N.D. | N.D. |
| TT | 56 | — | Polysomy | 5.3 ± 2.1 | 9.3 | 9.9 |
| TE3 | 47 | — | Polysomy | N.D. | N.D. | N.D. |
| KYSE70 | 34 | — | No amplification | N.D. | N.D. | N.D. |
| KYSE110 | 25 | — | Polysomy | 0 | 4.6 | 4.6 |

Abbreviations: MFI, mean fluorescence intensity; FACS, fluorescence-activated cell sorting; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
*N.D., not determined.

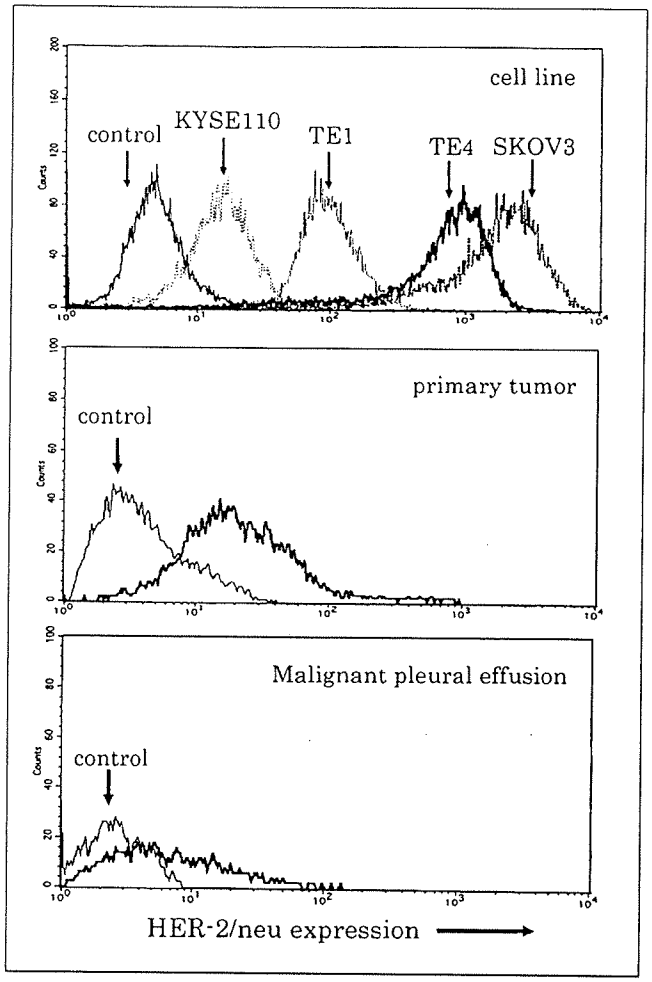


Fig. 1. The degree of HER-2 expression on esophageal SCC cell lines and freshly isolated SCC tumors. HER-2 expression was evaluated by flow cytometric analysis on esophageal SCC cell lines and freshly isolated SCC tumors derived from two different patients. Esophageal SCC cell lines KYSE110, TE1, and TE4 and ovarian cancer cell line SKOV3, which is a standard cell line with strong HER-2 expression, were used.

*The biological activity of trastuzumab against HER-2–expressing esophageal squamous cell carcinoma cell lines.* To evaluate the anti-proliferative activity and apoptosis-inducing activity of trastuzumab, we did 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and Annexin V and propidium iodide staining for esophageal SCC cell lines. Inhibition of tumor cell growth induced by trastuzumab was found in TE4, TE1, and TE5 with very marginal but significant levels (Table 1). In addition, significant levels of apoptosis induced by trastuzumab were not found in all tested cell lines (Table 1).

*Trastuzumab mediates antibody-dependent cellular cytotoxicity against HER-2–expressing esophageal squamous cell carcinoma.* To investigate whether trastuzumab induces ADCC against esophageal SCC, we did an ADCC assay of PBMCs from healthy donors and esophageal SCC patients against several different esophageal SCC cell lines with various levels of HER-2 (Table 1; Fig. 1). Summarized data from healthy donors ($n$ = 7) showed that trastuzumab could induce ADCC activities against esophageal SCC cells and the activities reflected the degree of HER-2 expression by flow cytometric analysis on the esophageal SCC cell lines (Fig. 2). However, even when the HER-2 levels were almost the same among TE5, TE1, KYSE30, and KYSE50, there were significant differences in the levels of trastuzumab-induced ADCC (Fig. 2). There was no direct cytotoxicity for the targets using trastuzumab alone (Fig. 2).

Furthermore, we did an ADCC assay of PBMCs from esophageal SCC patients ($n$ = 5) against KYSE110, TE1, and TE4 (Fig. 3). Trastuzumab could also induce ADCC activities against esophageal SCC cells and the activities reflected the degree of HER-2 expression (Fig. 3), although the levels of ADCC in esophageal SCC patients were weaker in comparison with those of healthy donors (Figs. 2 and 3).

*Modification of trastuzumab-mediated antibody-dependent cellular cytotoxicity by SB-431542 in transforming growth factor-β–producing esophageal squamous cell carcinoma.* As indicated in Fig. 2, the levels of trastuzumab-induced ADCCs were different among TE5, TE1, KYSY30, and KYSE50, although the HER-2 levels in these four cell lines were almost equal. Therefore, we next investigated the quantity of immunosuppressive cytokines, IL-10 and TGF-β, in the culture supernatants in KYSE30, KYSE50, TE5, and TE1, as it was well known that both IL-10 and TGF-β suppress natural killer (NK) activity (17–20). As a result, there were detectable TGF-β2 in the supernatants, in which TE1 produced much higher TGF-β2 (Fig. 4A), whereas no cell lines produced detectable levels of TGF-β1 or IL-10 (data not shown).

Next, we investigated whether anti–TGF-β2 neutralizing mAb enhanced trastuzumab-mediated ADCC against TGF-β2–producing TE1. Representative ADCC assays from three independent experiments from different healthy donors ($n$ = 3) were shown (Fig. 4B). We found that the treatment with anti–TGF-β2 mAb resulted in the enhancement of trastuzumab-mediated ADCC against TE1, whereas there were no effects for TE5 and KYSE30 (Fig. 4B). These results indicated that TGF-β2 produced by TE1 might inhibit the activity of trastuzumab-mediated ADCC against TE1.

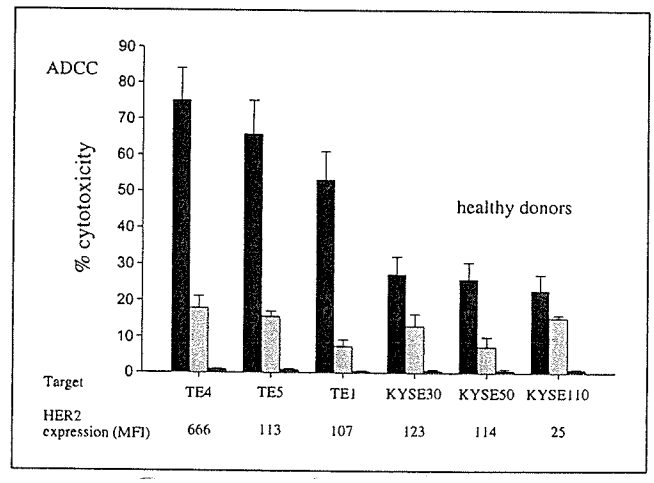


Fig. 2. Trastuzumab-mediated ADCC against HER-2–expressing esophageal SCC in healthy donors. Trastuzumab-mediated ADCC assays of PBMCs from healthy donors ($n$ = 7) against esophageal SCC cell lines. The HER-2 expression on esophageal SCC cell lines was evaluated by flow cytometric analysis. *MFI*, mean fluorescence intensity ■, Target + PBMC + Trastuzumab; ▨, Target + PBMC + Control mAb; ▩, Target + Trastuzumab (PBMC/target = 20:1).

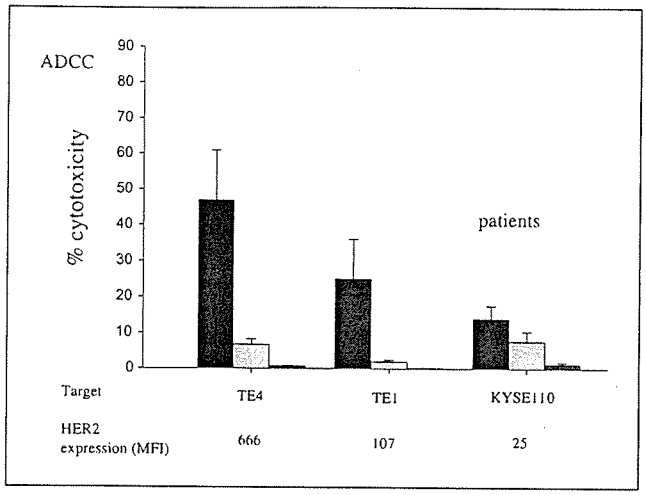


Fig. 3. Trastuzumab-mediated ADCC against HER-2–expressing esophageal SCC in esophageal SCC patients. Trastuzumab-mediated ADCC assays of PBMCs from esophageal SCC patients ($n$ = 5) against esophageal SCC cell lines. The HER-2 expression on esophageal SCC cell lines was evaluated by flow cytometric analysis ■, Target + PBMC + Trastuzumab; □, Target + PBMC + Control mAb; ▩, Target + Trastuzumab (PBMC/target = 20:1).

Furthermore, we investigated whether SB-431542 blocks TGF-β2–induced phosphorylation in PBMCs and whether inhibition of TGF-β signaling results in enhancement of trastuzumab-mediated ADCC against esophageal SCC. It has been shown that SB-431542 is a specific inhibitor of ALK-4, ALK-5, and ALK-7, the type I receptors for TGF-β, and inhibits the phosphorylation of Smad2/3 induced by TGF-β (15). A representative immunoblotting assay for phosphorylated Smad2 is shown in Fig. 5A, indicating that SB-431542 blocks TGF-β2–induced phosphorylation of Smad2 in PBMCs in a dose-dependent manner. Representative ADCC assays from six independent experiments from different healthy donors ($n$ = 6) were shown in Fig. 5B. We found that SB-431542 significantly enhanced trastuzumab-mediated ADCC for TGF-β2–producing TE1 in comparison with that in the DMSO control (Fig. 5B). However, SB-431542 did not enhance trastuzumab-mediated ADCC against TE5 and KYSE30 (Fig. 5B). Furthermore, these observations were confirmed in another five different healthy donors and the average synergic effect of SB-431542 was 12.2 ± 5.5% increase of trastuzumab-mediated ADCC, in which the maximal response was observed at around 0.1 μmol/L of SB-431542 (Fig. 5B).

## Discussion

The present study contains several important findings relevant to the action of trastuzumab against HER-2–expressing esophageal SCC. First, trastuzumab was able to induce ADCC against HER-2–expressing esophageal SCC and the activities reflected the degree of HER-2 expression analyzed by flow cytometric analysis, but not by HercepTest or FISH analysis. Second, trastuzumab-mediated ADCC against TGF-β–producing SCC was enhanced by treatment with SB-431542, which is a selective inhibitor of the phosphorylation induced by TGF-β.

There are many mechanisms linked to the therapeutic effect of trastuzumab, including the blockade of signaling pathways (9), inhibition of tumor cell growth (9), activation of apoptotic signals of tumor cells (12), and enhancement of ADCC (9, 13). In the present study, we clearly showed that HER-2–expressing esophageal SCC cells were killed by trastuzumab-mediated ADCC, as well as breast cancer cells and gastric cancer cells with HER-2 overexpression (9, 13). However, the anti-proliferative activity of trastuzumab for HER-2–expressing esophageal SCC was marginal and there was no effect on the activation of apoptosis.

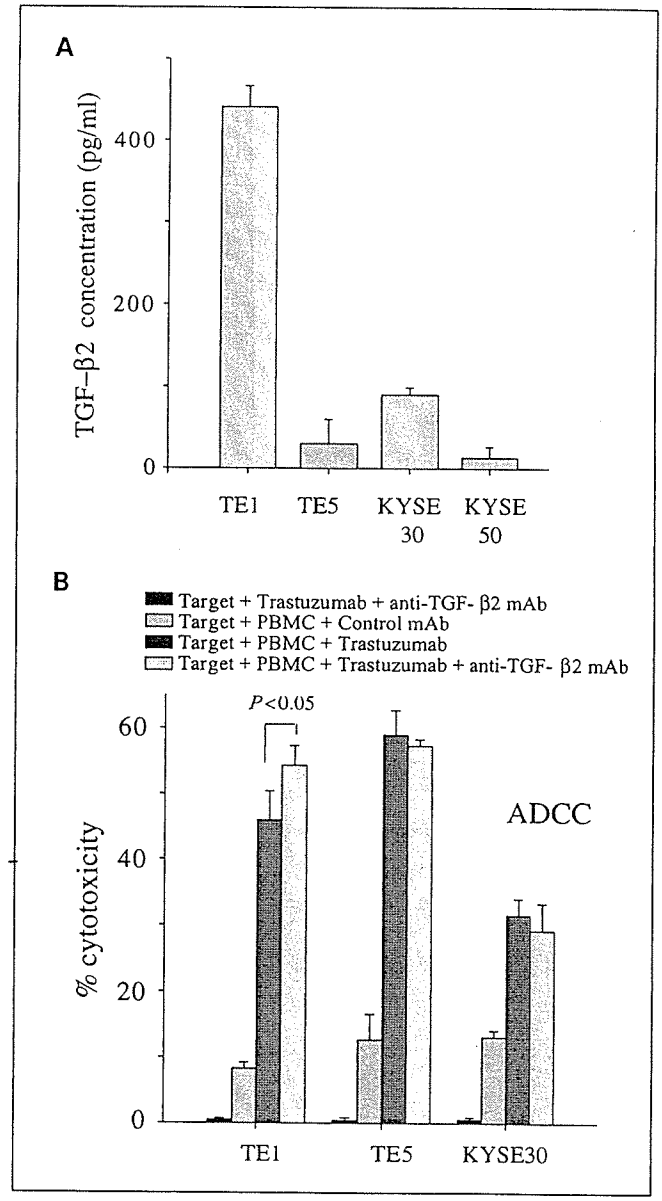


Fig. 4. Quantification of TGF-β2 in the culture supernatants in esophageal SCC ($A$) and treatment with anti-TGF-β2 neutralizing mAb in trastuzumab-mediated ADCC ($B$). $A$, each cell line ($2 \times 10^5$ cells) was cultured in 2 mL of X-VIVO at 37°C for 24 hours in a six-well plate. After incubation, the culture supernatants were measured with a Human TGF-β2 Immunoassay Kit. $B$, representative ADCC assays from three independent experiments from different healthy donors ($n$ = 3). After the target cells were labeled with 50 μCi of $^{51}Cr$ for 60 minutes, target cells ($5 \times 10^3$/well) and PBMCs ($1 \times 10^6$/well) were co-incubated in 200 μL of X-VIVO medium in a 96-well U-bottomed plate in triplicate for 8 hours at 37°C with or without anti-TGF-β2 mAb (5 ng/mL) in the presence of trastuzumab (10 μg/mL). Statistical analysis was done with a Student's $t$ test.

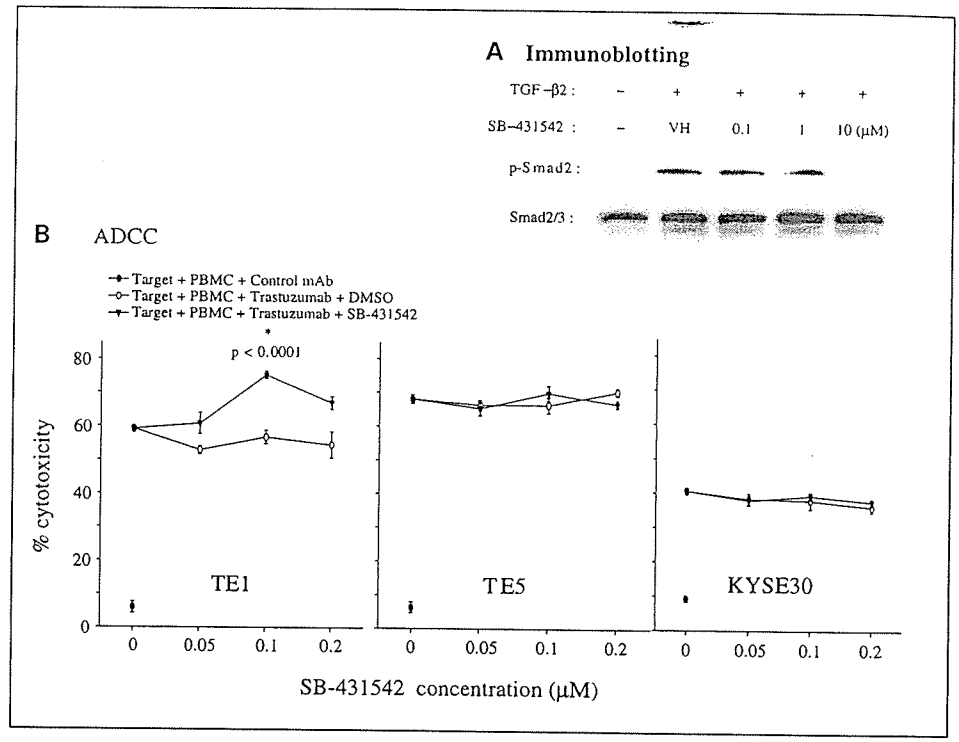


Fig. 5. SB-431542 significantly blocks TGF-β2–induced phosphorylation of Smad2 in PBMCs (*A*) and enhances trastuzumab-mediated ADCC for a TGF-β–producing esophageal SCC TE1 cell line (*B*). *A*, representative immunoblotting assay for phosphorylation of Smad2 in PBMCs. PBMCs ($7 \times 10^6$) were pre-treated with SB-431542 or DMSO (vehicle, *VH*) for 30 minutes in X-VIVO medium and then pre-treated PBMCs were cultured in the presence of TGF-β2 (5 ng/mL) for 60 minutes. After incubation, whole cell extracts (10 μg) were fractionated on 10% SDS-polyacrylamide gels and immunoblotting was done using antibodies against Smad2/3 and phosphorylated Smad2 (*p-Smad2*). *B*, representative ADCC assays from six independent experiments from different healthy donors ($n = 6$). The TE1 cell line produced much higher TGF-β2, whereas the TE5 and KYSE30 cell lines did not produce significant amounts of TGF-β2 (Fig. 4A). After PBMCs from healthy donors were pre-treated with SB-431542 or DMSO for 30 minutes, $^{51}$Cr-radiolabeled target cells ($5 \times 10^3$/well) and the pre-treated PBMCs ($1 \times 10^5$/well) were co-incubated for 8 hours with trastuzumab (10 μg/mL) in the presence of SB-431542 or DMSO. Statistical analysis was done with a Student's *t* test.

In esophageal SCC, TE4 was shown to have high HER-2 expression analyzed by both flow cytometric analysis and HercepTest, and to possess *HER-2* gene amplification detected by FISH in the present study. Thus, it is reasonable to assume that TE4 was efficiently killed by trastuzumab-mediated ADCC. However, another six cell lines which had moderate levels of HER-2 expression analyzed by flow cytometry, but negative staining with the HercepTest, were also killed by trastuzumab-mediated ADCC, and the activity reflected the degree of HER-2 expression by flow cytometric analysis. In a previous study, Bunn et al. (21) investigated the relationship among flow cytometric analysis, HercepTest, and FISH (PathVysion test) in human lung cancer cell lines and human breast cancer cell lines. They reported that there was a correlation between these three assays in the detection of HER-2 status. However, our present study indicated that the HercepTest may not be enough, or may underestimate, in the sensitivity of screening for trastuzumab-mediated ADCC. Furthermore, FISH analysis detected *HER-2* gene amplification or polysomy in the tested SCC cell lines. However, there was a discrepancy between the HER-2 status analyzed by FISH and the levels of trastuzumab-mediated ADCC. Also, freshly isolated tumor samples in the present study revealed moderate levels of HER-2 expression detected by flow cytometry, whereas these samples showed no staining in HercepTest. Thus, these results indicated that flow cytometric analysis may be a better method for screening the candidates when targeting the action of ADCC in trastuzumab therapy for esophageal SCC.

The trastuzumab-mediated ADCC of PBMCs from patients with esophageal SCC was impaired in comparison with that in healthy donors, in line with our previous report for gastric cancer patients (13). We showed that NK cell dysfunction contributed to the impaired trastuzumab-mediated ADCC in gastric cancer patients (13). It is likely that esophageal SCC patients might also be in the immunosuppressive state with NK cell dysfunction.

Clyens et al. and ourselves reported that NK cells and monocytes play a role in trastuzumab-mediated ADCC (13, 22). It is well known that immunosuppressive cytokines such as IL-10 and TGF-β are produced within the tumor microenvironment and suppressed the activity of NK cells, monocytes, or T cells (17–20). Therefore, to enhance the effect of trastuzumab-mediated ADCC, we next investigated the involvement of IL-10 and TGF-β in the trastuzumab-mediated ADCC for esophageal SCC. As a result, trastuzumab-mediated ADCC against TGF-β–producing TE1 cells was enhanced by treatment with anti–TGF-β2 neutralizing mAb and by blocking the TGF-β signaling with SB-431542. SB-431542 is a specific inhibitor of ALK-4, ALK-5, and ALK-7, the type I receptors for TGF-β, which affected the ALK-1– and ALK-5–mediated signaling induced by TGF-β and inhibited the phosphorylation of Smad2/3 induced by TGF-β (15). In the present study, we confirmed that SB-431542 could block TGF-β2–induced phosphorylation of Smad2 in PBMCs. It is likely that the inhibition of TGF-β signaling by SB-431542 on the effector cells (PBMCs) may result in the enhancement of trastuzumab-mediated ADCC. Considering these results, some modalities, such as the inhibition of TGF-β signaling aimed at enhancing trastuzumab-mediated ADCC, may be useful for successful trastuzumab treatment of TGF-β–producing esophageal SCC. Also, these observations suggest that if, in the setting of adoptive immunotherapy, *ex vivo* activated T cells and NK cells are pre-treated with SB-431542, adoptively transferred T cells and NK cells may overcome the immunosuppression induced by TGF-β *in vivo*. However, it is impossible to explain the inhibition of trastuzumab-mediated ADCC with immunosuppression by TGF-β alone. In fact,

ADCC activity for KYSE30 and KYSE50 cell lines was relatively low in comparison with that for TE5 or TE1 with almost the same amount of HER-2 levels, although the KYSE30 and KYSE50 cell lines did not produce significant amounts of TGF-β1 and TGF-β2. Further study will be needed to clarify the involvement of other immunosuppressive factors.

In conclusion, HER-2–expressing esophageal SCCs were killed by trastuzumab-mediated ADCC and the activity reflected the degree of HER-2 expression detected by flow cytometry. Furthermore, SB-431542 significantly enhanced the trastuzumab-mediated ADCC against a TGF-β–producing cell line.

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# Expansion of α-Galactosylceramide-Stimulated Vα24+ NKT Cells Cultured in the Absence of Animal Materials

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**Summary:** Vα24+ NKT is an innate lymphocyte with potential antitumor activity. Clinical applications of Vα24+ natural killer (NK) T cells, which are innate lymphocytes with potential antitumor activity, require their in vitro expansion. To avoid the potential dangers posed to patients by fetal bovine serum (FBS), the authors evaluated non-FBS culture conditions for the selective and efficient expansion of human Vα24+ NKT cells. Mononuclear cells (MNCs) and plasma from the peripheral blood of normal healthy donors were used before and after G-CSF mobilization. MNCs and plasma separated from apheresis products were also used. MNCs were cultured for 12 days in AIM-V medium containing α-galactosylceramide (α-GalCer) (100 ng/mL) and IL-2 (100 U/mL) supplemented with FBS, autologous plasma, or autologous serum. The cultured cells were collected and their surface markers, intracellular cytokines, and cytotoxicity were evaluated. The highest expansion ratio for Vα24+ NKT cells was obtained from G-CSF-mobilized MNCs cultured in medium containing 5% autologous plasma. Cultures containing MNCs and autologous plasma obtained before and after G-CSF mobilization had approximately 350-fold and 2,000-fold expansion ratios, respectively. These results suggest that G-CSF mobilization conferred a proliferative advantage to Vα24+ NKT cells by modifying the biology of cells and plasma factors. Expanded Vα24+ NKT cells retained their surface antigen expression and production of IFN-γ and exhibited CD1d-independent cytotoxicity against tumor cells. Vα24+ NKT cells can be efficiently expanded from G-CSF-mobilized peripheral blood MNCs in non-FBS culture conditions with α-GalCer and IL-2.

**Key Words:** NKT cells, G-CSF, α-galactosylceramide

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Murine Vα14+ natural killer (NK) T cells express an extremely restricted T-cell receptor (TCR) consisting of a Vα14-Jα281 α chain paired mainly with a Vβ8.2 β chain. Human Vα24+ NKT cells are similar to murine Vα14+ NKT cells, as Vα24+ NKT cells have an invariant Vα24-JαQ α chain preferentially paired with a Vβ11 chain.[1–3] α-Galactosylceramide (α-GalCer) is a specific ligand for human Vα24+ NKT cells and murine Vα14+ NKT cells.[4] Both types of NKT cells are activated by α-GalCer presented by CD1d. After stimulation with α-GalCer, Vα24+ NKT cells exhibit CD1d-dependent cytotoxicity against various types of tumor cells.[5–7] Because CD1d is probably a class I molecule expressed mainly on antigen-presenting cells (APCs) such as dendritic cells, macrophages, and B cells, it is speculated that NKT cells primarily interact with APCs.[6,8,9] NKT cells regulate innate tumor immunity by rapidly producing a large amount of IFN-γ and IL-4.[4,10]

The extremely low frequency of Vα24+ NKT cells in human peripheral blood[7,11,12] is an obstacle for their clinical application. To overcome this problem, the establishment of an effective in vitro expansion system for Vα24+ NKT cells by stimulation with α-GalCer has been explored. Significant expansion was reported in human Vα24+ Vβ11+ NKT cells cultured with a combination of IL-15, IL-7, IL-2, and α-GalCer.[13] Up to a 76-fold expansion of human Vα24+ Vβ11+ NKT cells was reported after culture with IL-7, IL-15, and α-GalCer-loaded monocyte-derived dendritic cells.[14] Alternative expansion methods use a combination of IL-2 and IL-15,[15] or α-GalCer and IL-2, with or without APCs.[16] Previously, we observed that Vα24+ NKT cells could be expanded 350-fold from human granulocyte-colony stimulating factor (G-CSF)–mobilized peripheral blood cells, upon stimulation with α-GalCer and IL-2 for 12 days.[17] However, in these culture systems, 10% fetal bovine serum (FBS) was used in the medium. To remove the potential risks related to FBS, we developed an efficient non-FBS expansion system for Vα24+ NKT cells.

## MATERIALS AND METHODS

### Cells, Plasma, and Serum Preparation

Peripheral blood and apheresis products were obtained from normal healthy individuals who were donating peripheral blood stem cells for allogeneic transplants. Written informed consent was obtained from the donors. This study was approved by our institution. Before and after G-CSF mobilization, samples were used immediately and cell fraction and

plasma were separated by centrifugation. The plasma and serum were obtained and cryopreserved at −80°C until use. Plasma and serum samples were heat-inactivated immediately before use. Mononuclear cells (MNCs) were isolated from peripheral blood and apheresis products by Ficoll-Hypaque (Immuno-Biologic Laboratories, Gunma, Japan) density gradient centrifugation. Apheresis plasma was also collected from the apheresis bags and used after heat inactivation.

## G-CSF Procedure for Apheresis Donor

The apheresis was indicated for a healthy donor whose relative needed peripheral blood stem cell transplantation. This indication was checked by the clinical team of stem cell transplantation unit in our hospital. G-CSF was administered subcutaneously at a dosage of 300 $\mu g/m^2$ divided twice daily for 3 days just before the apheresis procedure. On the day of apheresis, one more dose of G-CSF was administered in the morning before apheresis.

## Expansion of Vα24+ NKT Cells

MNCs were cultured in six-well culture plates or culture flasks (Costar, Corning, NY) at $1.0 \times 10^5$ cells/mL in medium supplemented with 100 ng/mL α-GalCer (Kirin Brewery Co, Tokyo, Japan) and 100 U/mL recombinant human (rh) IL-2 (R&D Systems, Minneapolis, MN) for 12 to 14 days. The environment for the incubation was under 20% $O_2$ and 5% $CO_2$. Cells were cultured in AIM-V (Life Technologies, Rockville, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 10% recombinant human serum albumin (rHSA), 5% or 10% autologous plasma, or 5% or 10% autologous serum. The rHSA was kindly provided by Mitsubishi Welpharma Corporation (Osaka, Japan). Fresh IL-2 was added every 3 days.

## Co-Culture and Expansion of Vα24+ NKT Cells

To determine whether G-CSF mobilization conferred any benefits to plasma or cells for the expansion of Vα24+ NKT cells, we tested the following culture conditions: (1) pre-G-CSF peripheral blood mononuclear cells (PBMCs) cultured in AIM-V with 5% pre-G-CSF plasma; (2) pre-G-CSF PBMCs cultured in AIM-V with 5% post-G-CSF plasma; (3) post-G-CSF PBMCs cultured in AIM-V with 5% pre-G-CSF plasma; and (4) post-G-CSF PBMCs cultured in AIM-V with 5% post-G-CSF plasma. After culturing cells with α-GalCer and IL-2 for 12 days, we quantified the expansion of Vα24+ NKT cells.

## Cell Surface Antigen Analysis

We used mouse anti-human mAbs conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), or peridinium chlorophyll (PerCP). CD3-PE, CD4-PerCP, CD8-PE, CD14-FITC, CD19-PE, CD25 (IL-2 receptor α chain)-FITC, and CD123 (IL-3 receptor)-PE mAbs were purchased from BD Biosciences (Mountain View, CA). Vα24-FITC, Vα24-PE, Vβ11-PE, CD124 (IL-4 receptor α chain)-PE, and CD127 (IL-7 receptor)-PE mAbs were purchased from Immunotech (Marseille, France). CD161-APC, CD114 (G-CSF receptor)-PE, and CD119 (IFN-γ recep-tor α chain)-PE mAbs were purchased from BD Pharmingen (San Diego, CA). PE-conjugated α-GalCer-CD1d tetramer was produced in our laboratory[18] and used to stain α-GalCer-loaded CD1d-reactive Vα24+ NKT cells. Biotinylated anti-CD1d-mAb, which was originally produced by Dr. Steven A. Porcelli (Albert Einstein College of Medicine, Bronx, NY), was a kind gift from Kirin Brewery Co. The biotinylated mAb was detected using streptavidin-PerCP (BD Biosciences). For cell surface antigen staining, cells were incubated with mAbs for 30 minutes on ice. After staining, cells were washed twice and resuspended in PBS. Propidium iodide (Sigma-Aldrich, St. Louis, MO) staining preceded all experiments to remove dead cells. Data were acquired by flow cytometry (FACSCalibur; BD Biosciences) and analyzed using CellQuest software (BD Biosciences).

## Vα24+ NKT Cell Separation

After expansion of Vα24+ NKT cells with α-GalCer and IL-2 in AIM-V with 5% autologous apheresis plasma for 12 days, cells were stained with Vα24-FITC for 20 minutes on ice and washed twice with 5 mM EDTA-PBS. After being incubated with anti-FITC microbeads (Miltenyi Biotec, Gladbach, Germany), Vα24+ NKT cells were sorted by a magnetic cell separation system (Super MACS; Miltenyi Biotec), according to the manufacturer's protocol. After separation, the purity of isolated Vα24+ NKT cells was determined to be more than 95% by flow cytometry. After the cells were re-cultured with 100 U/mL IL-2 for an additional 2 days, they were used for assays of cytotoxic activity against several tumor cell lines.

## Intracellular Cytokine Assay

The intracellular cytokine production of cultured cells was measured by flow cytometry. Cells were activated with 10 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich) for 4 hours at 37°C in 10 μg/mL Brefeldin A (Sigma-Aldrich) to prevent cytokine secretion. After activation, cells were stained with Vα24 antigens and permeabilized according to the manufacturer's protocol (BD Biosciences) for staining with IFN-γ-PE or IL-4-PE mAb (BD Biosciences). At least 30,000 gating events per sample were acquired by FACSCalibur, and the data were analyzed using CellQuest software.

## Cytotoxicity Assay

The cytotoxicity of isolated Vα24+ NKT cells against tumor cell lines was studied. The following cell lines were purchased from ATCC: Daudi (B-cell lymphoma), K562 (chronic myelogenous leukemia), and Jurkat (T-cell lymphoma).

Target cells were labeled with 50 μCi sodium [$^{51}$Cr] chromate (NEN Life Science Products, Inc, Boston, MA) per $5 \times 10^5$ cells for 1 hour, washed three times, and resuspended at $1 \times 10^5$ cells/mL in medium. Next, 100 mL of effector cells and 100 mL of $^{51}$Cr-labeled target cells ($1 \times 10^4$ cells/well) were added to 96-well round-bottomed plates (Nunc, Roskilde, Denmark) at effector-to-target (E/T) ratios of 10:1, 3:1, and 1:1. Plates were incubated for 4 hours at 37°C, and $^{51}$Cr release from lysed target cells was measured by a gamma counter. The percentage of specific $^{51}$Cr released in each well was analyzed using the following formula: specific lysis (%) = (test cpm − spontaneous cpm)/(total cpm − spontaneous cpm) × 100. "Test cpm" indicates the counts in experimental cultures of target cells and effector cells; "spontaneous cpm" indicates the counts in cultures containing only target cells and medium;