

ORIGINAL ARTICLE

Novel cancer-specific epidermal growth factor receptor antibody obtained from the serum of esophageal cancer patients with long-term survival

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Abstract

Although esophageal cancer has a poor prognosis after recurrence, some patients have shown long-term survival despite recurrence. We hypothesized that induction of either antitumor Abs or antitumor-specific CTLs could play a role in long-term survival (5 years or longer) in patients with recurrence and/or distant metastases. Therefore, we aimed to obtain Abs that specifically bind to cancer cells by using serum samples from patients with a good prognosis. A phage library was prepared using PBMC mRNA of the patients, and cell panning was carried out using an esophageal cancer cell line. Results showed the presence of an epidermal growth factor receptor (EGFR) Ab, KT112, that specifically bound to the cancer cell line. Notably, KT112 bound to only EGFR-positive cancer cells but failed to bind to normal esophageal cells. Furthermore, KT112 was characterized by responses to EGFR expressed on cancer cells but not to the recombinant extracellular domain of EGFR. Immunohistochemical analysis showed that KT112 reacted with 17.4% of esophageal squamous cell carcinoma tissue but not with any other cancer or normal tissue, suggesting that the Ab recognizes cancer-specific forms of EGFR and might have contributed to tumor suppression in patients with esophageal cancer. Furthermore, because of its high cancer specificity, KT112 could be a promising therapeutic option (e.g., in Ab-drug conjugates) for esophageal cancer.

KEYWORDS

antibody, cancer specificity, EGFR, esophageal squamous cell carcinoma, phage display

Abbreviations: ADC, antibody-drug conjugate; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; DNP, 2,4-Dinitrophenol; EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; PBST, PBS containing 0.1% Tween 20; SCC, squamous cell carcinoma; scFv, single-chain Fv; V_H, variable heavy; V_L, variant III; V_L, variable light.

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1 | INTRODUCTION

Esophageal cancer accounts for approximately 590,000 deaths per year worldwide, making it the sixth leading cause of cancer-related mortality. Furthermore, the prevalence of the disease is increasing worldwide, with 570,000 new cases diagnosed annually.¹ Most patients with esophageal cancer are diagnosed in the advanced stages of the disease, and the prognosis for such patients is poor despite the availability of multimodal therapies such as surgery, chemotherapy, and radiation therapy, with a 5-year survival rate of less than 20%.²⁻⁴ Due to its malignant potential, only a few patients with esophageal cancer have long-term survival after recurrence. In recent years, cancer immunotherapies, such as effector cell therapies, Ab therapies, and immune checkpoint inhibitor therapies, have been shown to be highly effective,⁵⁻⁷ and some cancer patients may have had a good prognosis due to suppression of disease progression by a tumor immune response. In this study, we explored esophageal cancer-specific target molecules, such as EGFR, vascular endothelial growth factor, HER2, MET, and PD-L1, for the development of molecular targeted drugs.⁸

Epidermal growth factor receptor, a tyrosine kinase receptor, is known to be involved in various cancer progression events, including signal cell proliferation, migration, and metastasis. Epidermal growth factor receptor has been shown to be upregulated in 30%–90% of esophageal cancers. Furthermore, 70% of esophageal cancers overexpress EGFR.⁹⁻¹¹ Currently, four major types of EGFR mAbs—cetuximab, panitumumab, nimotuzumab, and necitumumab—are clinically used for several types of cancers, including lung, head and neck, colon, and pancreatic cancer. In addition to these four approved drugs, various EGFR Abs have been tested in clinical trials, including monotherapy, combination therapy with small molecules, and ADCs, such as mAb A13, AMG595, depatuzumab (ABT-806), duligotuzumab (MEHD7945A, RG7597), futuximab (Sym004), GC1118, imgatuzumab (GA201), matuzumab (EMD72000), panitumumab (ABX-EGF), zalutumumab, humMR1, and tomuzotuximab.¹² Despite their effectiveness in preclinical studies, the clinical utility of EGFR-targeted therapies has been limited because of toxic side effects and poor clinical responses due to the broad expression profiles of the antigens that can cross various normal tissues. The most common toxicities were skin rashes, diarrhea, constipation, stomatitis, fatigue, and electrolyte abnormalities. Therefore, EGFR Abs with a higher tumor selectivity are desired.

We hypothesized that induction of either antitumor Abs or antitumor-specific CTLs could play a role in the long-term survival (5 years or longer) in esophageal cancer patients with recurrence. Using a phage library prepared from patient PBMC mRNA, we attempted to obtain Abs that specifically bind to cancer cells by cell panning using a cancer cell line. Subsequently, using antigen identification, antigen reactivity, and immunohistochemical analyses, we elucidated that the isolated Ab was a novel EGFR Ab.

2 | MATERIALS AND METHODS

2.1 | Ethics approval

All study participants gave consent for future analyses of their blood samples for research purposes. The protocol for this prospective study was approved by the ethics committee of Kyowa Kirin Co., Ltd. (IRB no. 2013_028) and Toho University (Ethical Committee no. 25-6). Patients provided written informed consent before enrollment.

2.2 | Patients

Serum samples were obtained from 17 patients with advanced esophageal cancer who had survived for 5 years or longer after initial treatment. Of these, four patients had developed recurrent tumors at the time of sampling. Of these four patients, two died 6 months and 5 years after recurrence, while the remaining two survived for 5 years or longer after recurrence. Serum Abs were analyzed using samples from one of the two patients with long-term survival. This patient had stage III (T3N1M0) disease with a high p53 Ab titer.¹³ Three years after surgical treatment, the patient had developed cervical lymph node recurrence, but he survived for 5 years or longer after recurrence.

2.3 | Evaluation of cell reactivity using serum-derived polyclonal Abs

Serum samples were collected using a blood collection tube containing a serum separator (# 367988; BD), allowed to stand for 30 min, and then centrifuged at 1000 g for 10 min. Antibodies were purified from serum samples using Protein G (#17-0618-02; GE Healthcare). The cells were suspended in D-PBS supplemented with 5% FBS, 1 mM EDTA, and 0.1% Na₂S₂O₃ (staining buffer) and dispensed into a 96-well plate. After centrifugation, the supernatant was removed and patient Ab and human Ig (#867279694, for blocking purpose; Japan Blood Products Organization) diluted to 100 µg/ml were added to the pellet and incubated for 30 min on ice. After washing, Alexa Fluor 647-labeled goat anti-human Ab (#A21445; Molecular Probes) was added and incubated for 30 min. After washing, the cells were suspended in staining buffer containing 7-AAD staining solution, and the fluorescence intensity of each cell was measured using a flow cytometer.

2.4 | Phage display

The scFv library was generated using modified phage display protocols.¹⁴⁻¹⁶ Briefly, RNA from blood collected in PAXgene tubes was extracted using the PAXgene Blood RNA Kit (PreAnalytiX) according

to the manufacturer's instructions. cDNA was synthesized using the SMARTer RACE cDNA Amplification Kit (#634859; Clontech). The V_H and V_L chain genes were amplified from the cDNA using PCR. The V_H and V_L PCR products were inserted into the phagemid vector pCANTAB 5E (Amersham Pharmacia) and transformed into *Escherichia coli* TG1 cells by electroporation. Thereafter, the cells were cultured in 2YTGA (2YT media, glucose, and ampicillin) and M13KO7 helper phages were added and incubated for 1 h. Subsequently, kanamycin was added and incubated at 37°C for 16 h and the phages were purified using a PEG/NaCl solution and resuspended in 1 ml staining buffer. Phages were added to MRC5 cells or HEK293F cells (negative control cells) and incubated at 4°C for 1 h. Unbound phages were transferred to OE21 cells and incubated at 4°C for 1 h. Phages that bound to OE21 cells were eluted, neutralized, and infected into TG1 cells. A total of five selection cycles were carried out.

2.5 | Construction and expression of Abs

The scFv regions of KT112 (phage clone) were cloned in-frame into an expression vector with a signal peptide and the human IgG4 Fc region (S228P/S235E/R409K).¹⁷ Immunoglobulin G4 (S228P/S235E/R409K) is a low-effector-activity Ab format that shows increased stability. The V_H and V_L regions of KT112, AM1 (AbbVie), and DNP (negative control Ab) were each cloned in-frame into an expression vector with a signal peptide and human IgG4 constant region (S228P/S235E/R409K). Similarly, cetuximab (DB00002; DrugBank) was cloned into an expression vector with a signal peptide and human IgG1 constant region. The plasmids were transfected into Expi293F cells using the Expi293 Expression System (Thermo Fisher Scientific). Antibodies and V_H -linker- V_L -hinge-CH2-CH3 (scFv-Fc) were purified using Protein A (GE Healthcare). After elution, the buffer in which the sample was dissolved was replaced with PBS.

2.6 | Flow cytometry

The cells were suspended in staining buffer and dispensed into a 96-well plate. After centrifugation, the supernatant was removed and each Ab was added to the pellet and incubated for 30 min on ice. After washing, R-phycoerythrin-labeled goat anti-human Ab was added and incubated for 30 min. After washing, the cells were suspended in staining buffer containing 7-AAD staining solution, and the fluorescence intensity of each cell was measured using a flow cytometer. The results were analyzed, and the mean fluorescence intensity was calculated using the geometric mean.

2.7 | Enzyme-linked immunosorbent assay

Human EGFR/HER1/ErbB1 protein (His Tag, #10001-H08H-20; Sino Biological) and recombinant human EGFR isoform VIII protein CF (#9565-ER-050; R&D Systems) were immobilized in a 96-well

immunoplate (MaxiSorp; Nunc). The plate was blocked with Super Block Blocking Buffer in PBS (Thermo Fisher Scientific). DNP IgG4PE (R409K), KT112scFv-Fc, cetuximab, 100 mg panitumumab (Takeda Pharmaceutical Co., Ltd.), and AM1 IgG4PE (R409K) diluted to required concentration using PBST were added and allowed to react at room temperature for 1 h. After washing with PBST, goat anti-human IgG (γ chain specific) HRP conjugated (#2040-05; SouthernBiotech) diluted in PBST was added as a secondary Ab at room temperature for 1 h. After washing with PBST, 3,3',5,5'-tetramethylbenzidine solution (Dako) was added and incubated. Hydrochloric acid was added to stop the reaction, and the absorbance was measured at a wavelength of 450 nm/570 nm.

2.8 | Antigen identification

Membrane proteins were purified using the Mem-PER Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific) from OE21 cells as samples and MRC5 cells as negative controls. Immunoprecipitation was then carried out using KT112 and plasma membrane proteins prepared using the Dynabeads ProteinA Immunoprecipitation Kit (Thermo Fisher Scientific). Proteins recovered by immunoprecipitation were subjected to SDS-PAGE. Bands specifically purified using the KT112 Ab were excised, and in-gel differentiation was performed using Trypsin/Lys-C Mix, Mass Spec Grade (Promega). Peptides extracted from the gels were used for protein identification by liquid chromatography with tandem mass spectrometry analysis. The Swiss-Prot database was used for analysis.

2.9 | siRNA silencing of EGFR expression

siRNA silencing of EGFR expression was carried out by using ON-TARGET plus Human EGFR siRNA-SMARTpool (Dharmacon) for EGFR and ON-TARGET plus Non-targeting Pool (Dharmacon) for the negative control according to the manufacturer's instructions. Briefly, OE21 and T.Tn¹⁸ cells were seeded in 6-well culture plates at 5×10^5 cells/well. siRNA in Opti-MEM I Reduced Serum Medium (Gibco) was mixed with Lipofectamine RNAiMAX Reagent (Invitrogen) in Opti-MEM and allowed to stand for 5 min at room temperature. A 9-pM siRNA solution was added to the OE21 cells and incubated for 72 h at 37°C in a CO₂ incubator. A 3-pM siRNA solution was added to the T.Tn cells and incubated for 48 h at 37°C in a CO₂ incubator.

2.10 | Antibody-dependent cellular cytotoxicity assay and CDC assay

Antibody-dependent cellular cytotoxicity was determined by the lactate dehydrogenase release assay as described previously,¹⁹ using cryopreserved human PBMCs (HemaCare) as effector cells and OE21 cells as target cells at an effector / target ratio of 25:1. Complement-dependent cytotoxicity was measured by nonradioactive methods of CCK-8 (Dojindo) as described previously.²⁰

2.11 | Internalization assay

Cells were seeded in 96-well plates and cultured overnight at 37°C. Antibodies were added at dilutions per the required concentration in the medium. Hum-ZAP (Advanced Targeting Systems) was added at 100 ng/well and incubated at 37°C. OE21 cells were cultured for 2 days and T. Tn and HEEpiC cells for 3 days. Viable cells after culture were detected using the Cell Proliferation Kit II (XTT assay; Roche Diagnostics). The XTT-labeled reagent and electron coupling reagent were mixed and added to a 96-well plate and incubated at 37°C for 4 h. The absorbance was measured at a sample wavelength of 490 nm and a reference wavelength of 630 nm.

2.12 | Immunohistochemical analyses

Antibodies were labeled with biotin using EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) according to the manufacturer's instructions. The slides were stained with biotinylated Abs and an avidin-biotin complex, developed with diaminobenzidine, and assessed using microscopy. Immunoexpression was evaluated based on the staining intensity (–, negative; +, weak; 2+, moderate; and 3+, strong).

3 | RESULTS

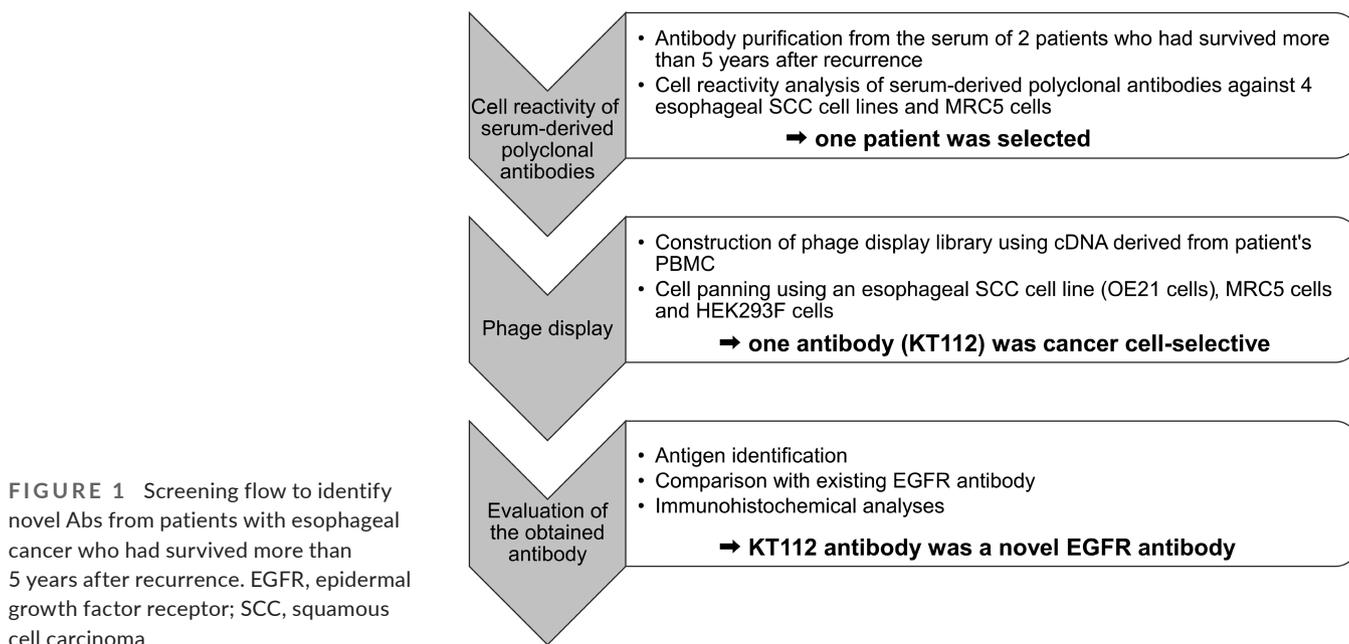
3.1 | Determination of esophageal SCC cell line-binding Abs in patients with a good prognosis

For identification of esophageal SCC cell line-binding Abs, IgG Abs were purified from the serum of a patient who had survived for 5 years or longer after recurrence (Figure 1). The purified IgG Abs

showed binding to four esophageal SCC cell lines as well as normal esophageal cells (Figure 2A). Notably, the purified IgG Abs also showed binding to a fibroblast cell line (MRC5 cells). Because IgG Abs obtained from serum are polyclonal in nature, an absorption assay was carried out to determine whether the Abs that bound to the esophageal SCC cell lines were the same as the Abs that bound to the MRC5 cells. Flow cytometry following absorption of MRC5 cells incubated with the purified Abs showed no decrease in reactivity of the esophageal SCC cell line (OE21), suggesting that the OE21 cell-binding Abs were different from the MRC5 cell-binding Abs (Figure 2B). Thus, the presence of esophageal SCC cell-binding Abs was confirmed, which formed the basis for isolation of these Abs using a phage library as described below.

3.2 | Isolation of esophageal SCC cell line-binding Abs from a patient phage library

In order to isolate cancer-specific mAbs, we prepared a phage display library using cDNA derived from PBMCs of the patient, wherein the variable regions of the Abs were designed to be displayed on the surface of the M13 phage coat protein as scFv molecules. To identify phages that specifically bound to esophageal SCC cells, we performed cell panning using an esophageal SCC cell line (OE21 cells). To eliminate phages with nonspecific binding, MRC5 cells and HEK293F cells were used for negative selection. After five cycles of affinity selection, there was an increase in the titer of phages binding to OE21 cells, following which clones were evaluated. Analysis of the reactivity of each clone to OE21 and HEK293F cells showed that 63 clones out of 91 did not react with HEK293F cells but reacted with OE21 cells, 19 of 91 reacted with both cell types, and 9 of 91 showed poor detection. Clones showing selective binding to OE21 cells were sequenced, and each Ab sequence was identified. Based



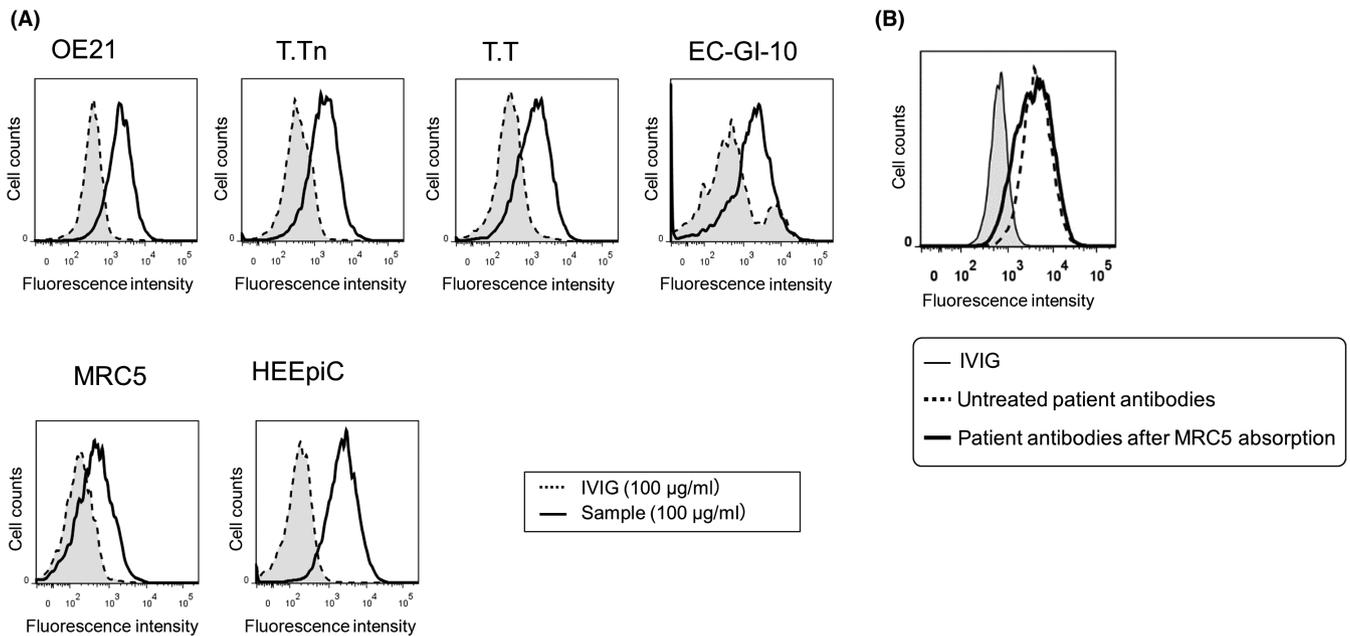


FIGURE 2 Reactivity of patient serum to esophageal cancer cell lines by flow cytometry analysis. (A) Top row shows the reactivity to esophageal cancer cell lines, and the bottom row shows the reactivity to fibroblast cell lines (MRC5) and normal esophageal cells (HEEpiC). Histograms for Abs derived from the patient's serum (solid line) and those for i.v. immunoglobulin (IVIG) used as a negative control (dotted line) are shown. The concentration of the Abs is 100 µg/ml. (B) Reactivity of patient Abs to OE21 cells after MRC5 absorption. Thin solid line shows IVIG, the dotted line shows untreated patient Abs, and the thick solid line shows histograms for patient Abs after MRC5 absorption

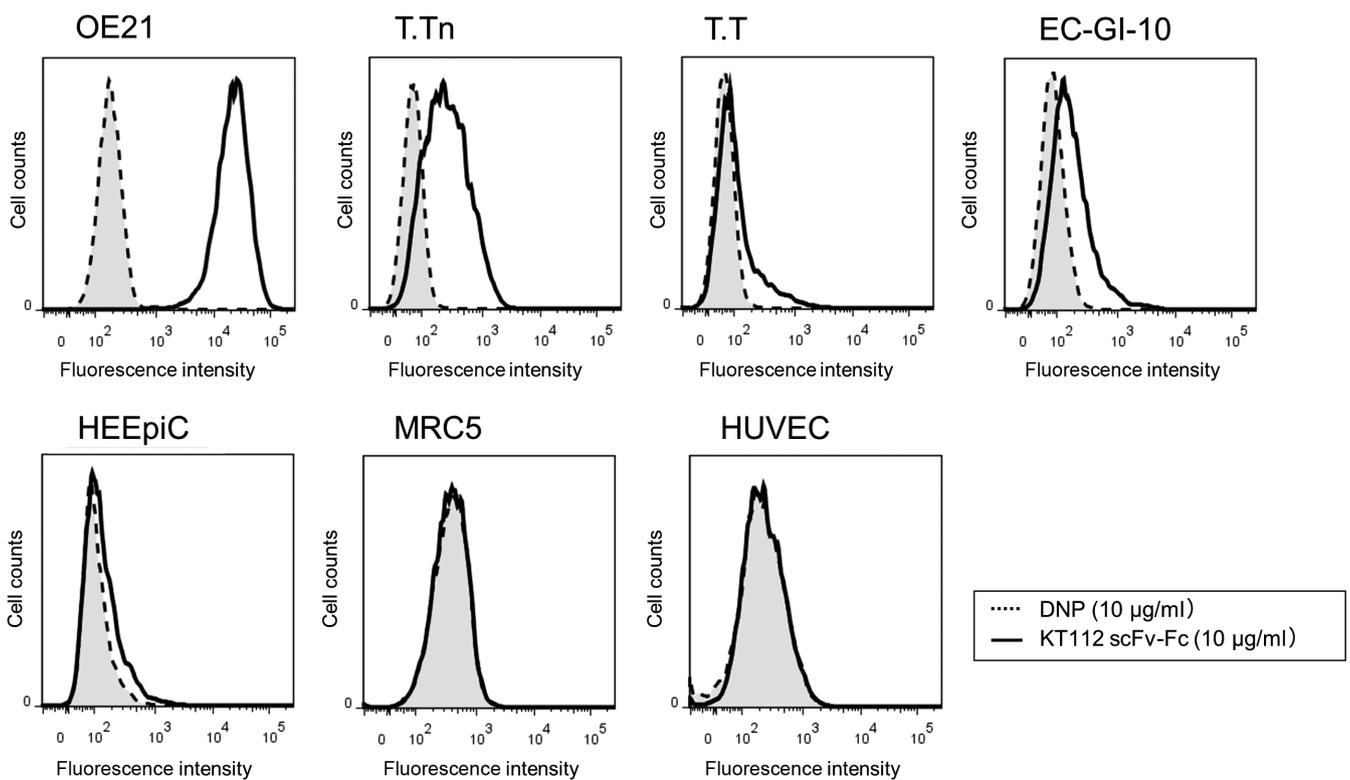


FIGURE 3 Reactivity of the KT112 Ab to esophageal cancer cell lines by flow cytometry analysis. Top row shows the reactivity to esophageal cancer cell lines, and the bottom row shows the reactivity to MRC5, HEEpiC, and HUVEC cell lines. Histograms for KT112 single chain Fv-Fc (solid line) and those for DNP Abs used as negative controls (dotted line) are shown. Antibody concentration is 10 µg/ml

on the complementarity-determining region sequence classification, eight independent sequences were obtained. However, as three of the eight clones identified did not express scFv-Fc, we decided to evaluate the cellular reactivity of only the five available scFv-Fc clones. Of the five Abs, four reacted with esophageal SCC cell lines. Of these four Abs, three were noncancer cell-selective and also reacted strongly with normal esophageal cells, whereas the remaining Ab (KT112) was cancer cell-selective. The KT112 Ab reacted with all four esophageal SCC cell lines, albeit the reaction with one of the strains was very weak, and only slightly with normal esophageal cells (Figure 3). Of note, KT112 did not bind to MRC5 cells or HUVECs (Figure 3).

3.3 | Recognition of EGFR by KT112

The membrane fractions of each cell were immunoprecipitated with each Ab and compared using SDS-PAGE (Figure 4A), showing a band that was specifically detected only in the eluted fraction of OE21 cells but not in the eluted fraction of MRC5 cells, the negative control. Immunoprecipitation analysis of the band showed that the KT112 Ab recognized EGFR as an antigen. Subsequently, OE12 cells knocked down for EGFR expression by siRNA were used to determine the specificity of the KT112 Ab for EGFR. The results of the cellular reactivity after siRNA treatment are shown in Figure 4B. Compared with the reactivity of the negative control siRNA (thick solid line), the reactivity of KT112 was decreased when EGFR siRNA was treated in both OE21 and T.Tn cells (dotted line), indicating that EGFR was indeed recognized by KT112.

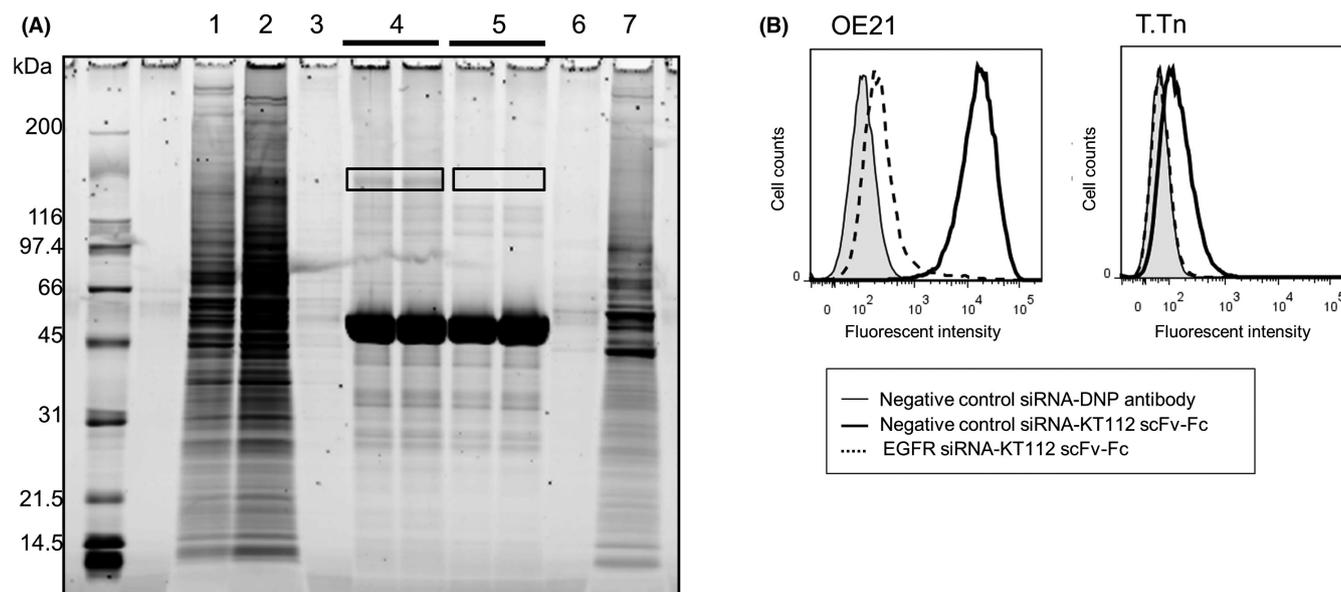


FIGURE 4 Identification of antigens recognized by KT112 Abs. (A) Results of SDS-PAGE after immunoprecipitation. Lane 1, OE21 membrane fraction; lane 2, OE21 supernatant fraction; lane 3, OE21 wash fraction; lane 4, OE21 elute fraction; lane 5, MRC5 elute fraction; lane 6, MRC5 wash fraction; and lane 7, MRC5 supernatant fraction. (B) Reactivity of the cell after siRNA treatment by flow cytometry analysis. Thin solid line shows the reactivity of the DNP Ab to cells treated with negative control siRNA, the thick solid line shows the reactivity of the KT112 Ab to cells treated with negative control siRNA, and the dotted line shows the reactivity of KT112 Abs to cells treated with epidermal growth factor receptor (EGFR) siRNA. vIII, variant III; scFv, single-chain Fv

3.4 | KT112 does not bind to recombinant EGFR extracellular domain

The reactivity of KT112 with the recombinant EGFR extracellular domain was analyzed. Panitumumab, AM1, and cetuximab, which are available as EGFR Abs, were used for comparison. AM1, a cancer-specific EGFR Ab,²¹ mainly recognizes EGFR vIII, but also binds to EGFR WT at higher expression levels. The responses to the recombinant EGFR extracellular domain and recombinant EGFR vIII extracellular domain were evaluated using an ELISA (Figure 5). Unlike cetuximab, panitumumab, and AM1, KT112 did not bind to the recombinant proteins of EGFR and EGFR vIII. The lack of response to recombinant EGFR suggests the possibility of a selective response to EGFR expressed on cancer cells.

3.5 | Comparison with known EGFR Abs

As KT112 was thought to have a different mechanism for EGFR reactivity compared with the known EGFR Abs, ADCC and CDC assays between KT112 and cetuximab were carried out. Figure 6 shows ADCC (Figure 6A) and CDC against OE21 cells (Figure 6B). KT112 had potent ADCC activity to OE21, the extent of which was lower than that of cetuximab. KT112 also showed no CDC activity. Subsequently, a competitive inhibition assay between KT112 and the known EGFR Abs was carried out. Figure 7 shows the reactivity of KT112 in the presence of the competitive Abs (Figure 7A) and the reactivity of each Ab in the presence of KT112 (Figure 7B). KT112 was competitively inhibited by cetuximab and panitumumab but not

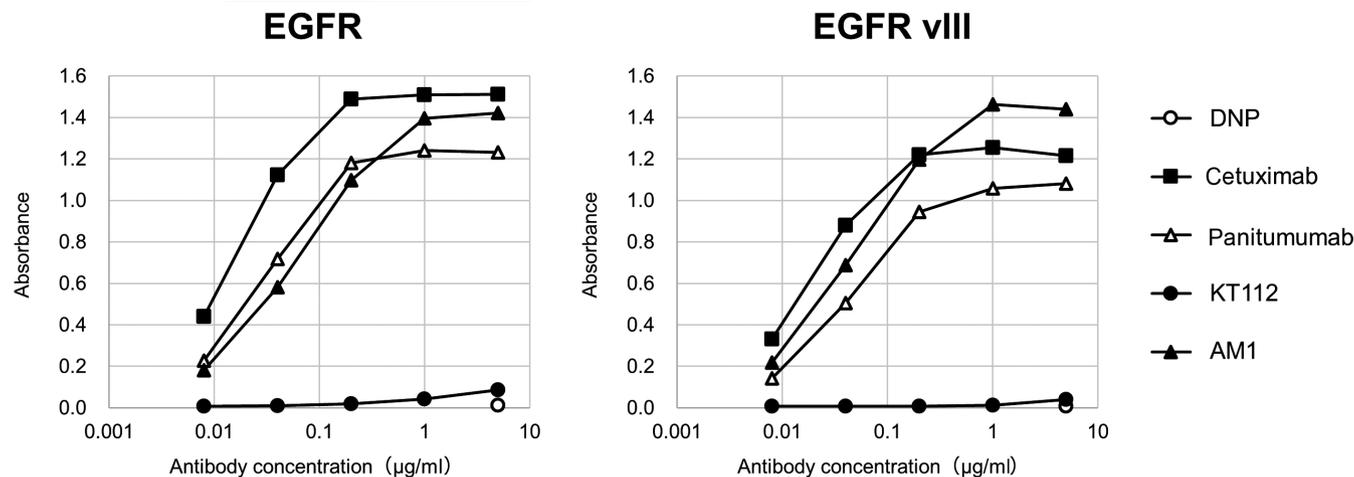


FIGURE 5 Reactivity to recombinant epidermal growth factor receptor (EGFR; left panel) and EGFR variant III (vIII; right panel) was analyzed by ELISA. Black circles, KT112 Ab; black squares, cetuximab; black triangles, AM1; white circles, DNP Ab (5 µg/ml only); white triangles, panitumumab

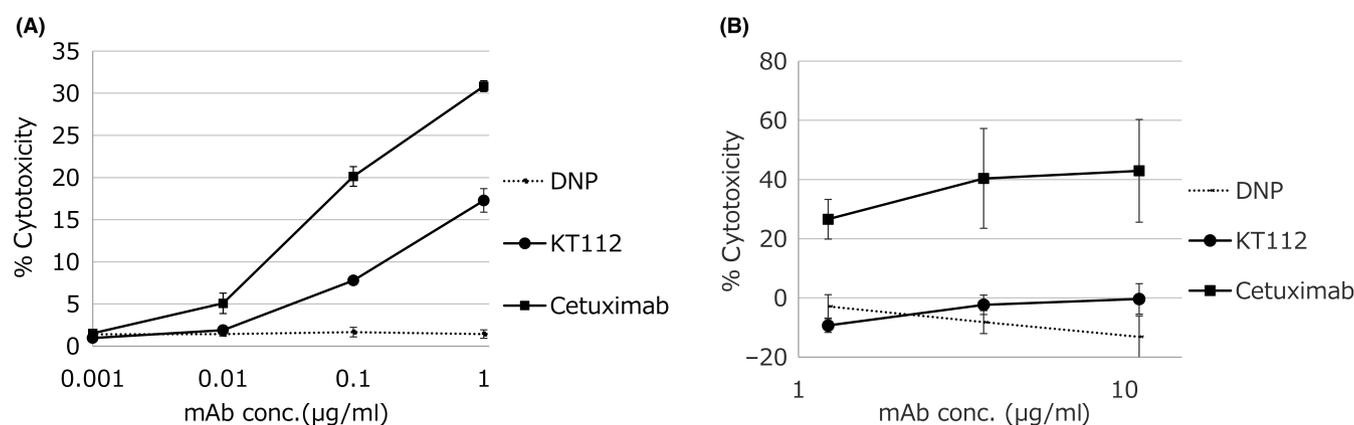


FIGURE 6 Effector function of KT112 Abs. (A) Ab-dependent cellular cytotoxicity and (B) complement-dependent cytotoxicity activity of KT112 against esophageal cancer cell-line OE21. Black circles, KT112 Ab; black squares, cetuximab; dashed line, 2,4-Dinitrophenol (DNP) Ab as a negative control

by AM1 (Figure 7A), whereas cetuximab, panitumumab, and AM1 were not competitively inhibited by KT112 (Figure 7B). This discrepancy might be attributable to the lower affinity of KT112 compared with cetuximab or panitumumab. Alternatively, binding of cetuximab or panitumumab might have altered the conformation of EGFR and thus prevented binding of KT112.

3.6 | KT112 has a similar internalization capacity as cetuximab

The internalization capacity of KT112 was confirmed using a saporin-labeled anti-human IgG Ab as a secondary Ab. Following internalization of the primary Ab, the saporin added to the secondary Ab was also taken up into the cell, resulting in inhibition of protein synthesis with inactivation of the ribosome, leading to cell death. Results of the detection of cell death in normal esophageal cells (HEEpiC) and in the esophageal SCC cell line (OE21 cells) are shown

in Figure 8A,B, respectively. Cetuximab, which is a known EGFR Ab, was also evaluated. Interestingly, KT112 was shown to be internalized to a similar level as cetuximab. Notably, cetuximab also bound to HEEpiC, normal esophageal cells that internalize and induce cell death, whereas KT112 did not bind to normal esophageal cells and did not induce cell death.

3.7 | KT112 specifically bound to esophageal cancer tissues

The KT112 Ab did not bind to formalin-fixed paraffin-embedded tissue samples but bound to frozen sections. Therefore, frozen array samples were prepared from the esophageal SCC and normal esophageal mucosa for immunohistochemical analyses. Frozen array samples were also used for the various types of cancer and normal tissues, since KT112 was obtained as an Ab that binds to esophageal SCC and lung SCC tissue. A summary of the immunohistochemical

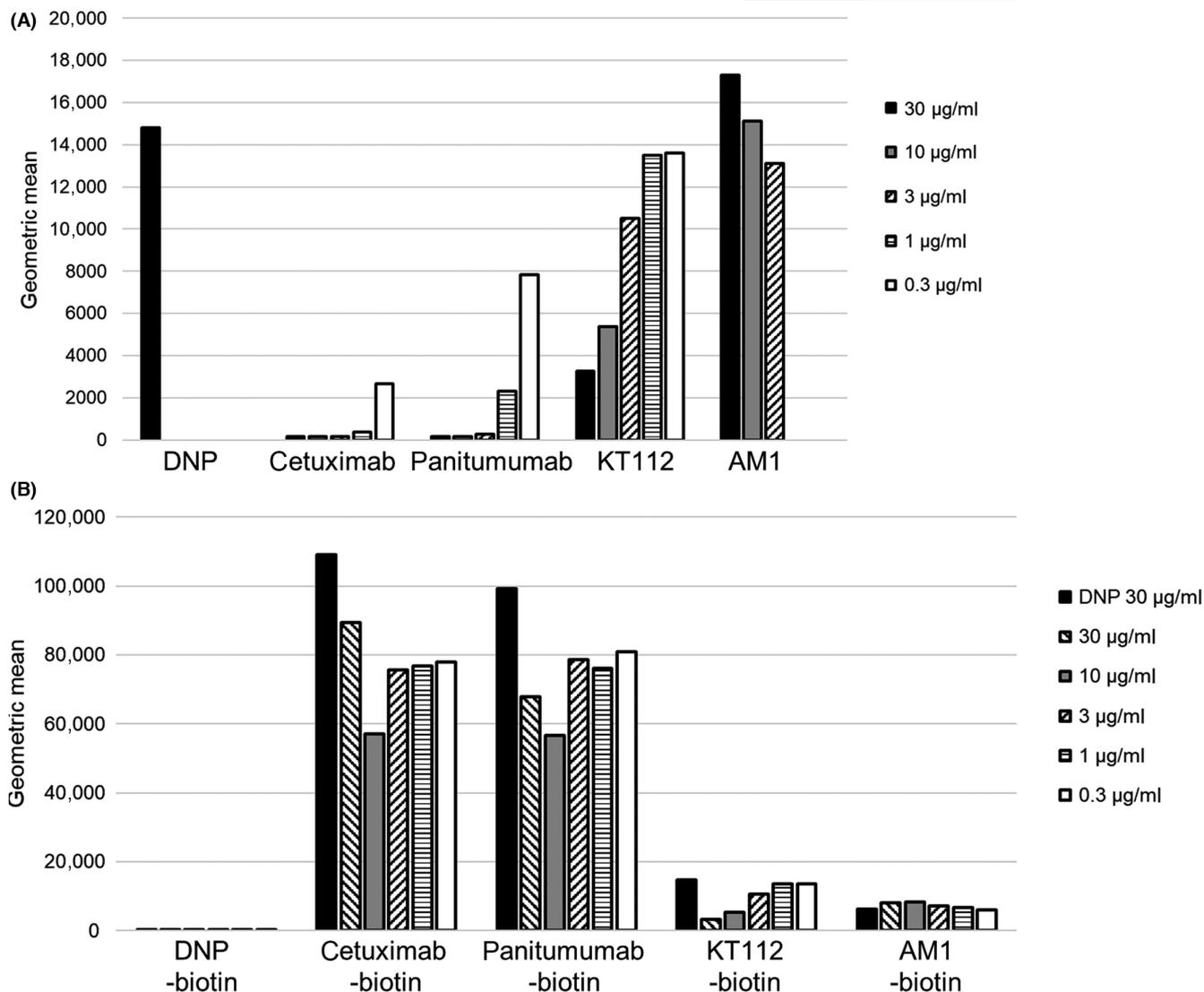


FIGURE 7 Competitive inhibition of each epidermal growth factor receptor (EGFR) Ab. The reactivity of each EGFR Ab to OE21 cells was analyzed by flow cytometry. The horizontal axis indicates the Ab name, and the vertical axis indicates mean fluorescence intensity. (A) Reactivity of the KT112 Ab in the presence of a competitive Ab is shown. Concentrations of the competitive Abs are 0.3, 1, 3, 10, and 30 µg/ml. 2,4-Dinitrophenol (DNP) Ab was analyzed only at 30 µg/ml. AM1 Ab was analyzed at 3, 10, and 30 µg/ml. The concentration of the biotinylated KT112 Ab for detection is 0.8 µg/ml. (B) Reactivity of each Ab in the presence of the KT112 Ab. Concentrations of the competitive Abs are 0.3, 1, 3, 10, and 30 µg/ml. Concentrations of the Abs for detection are 0.8 µg/ml for the biotinylated KT112 Ab, 0.1 µg/ml each for biotinylated cetuximab and panitumumab, and 2 µg/ml for the biotinylated AM1 Ab

analyses using KT112 is presented in Table 1 and Figure 9. Esophageal SCC tissue was stained with KT112 at a positive rate of 17.4% (4/23), while other cancer species and normal tissues were all negative, indicating that KT112 specifically bound to esophageal SCC tissue.

4 | DISCUSSION

A novel EGFR Ab, KT112, was obtained from serum samples of esophageal SCC patients with a good prognosis despite recurrence. This Ab specifically bound to esophageal cells but not to normal cells. In addition, this Ab selectively bound to EGFR expressed on cancer cells but not to recombinant EGFR protein. Immunohistochemical

staining with the KT112 Ab showed that 17.4% of esophageal SCC tissue was positive but other cancer or normal tissues were negative.

Possible mechanisms by which EGFR Abs exert antitumor activity include^{22–25}: (a) inhibition of EGFR downstream signaling by competitively binding to EGFR ligand binding sites such as EGF; (b) structural changes and steric inhibition of ligand binding upon binding of the Ab to EGFR; (c) internalization and degradation of EGFR by binding of the Ab, thereby inhibiting signal transduction downstream of EGFR; and (d) antitumor activity by the effector function of Abs, such as ADCC and CDC. In the cell growth inhibitory assay using cetuximab or KT112, cell growth inhibition was observed only with cetuximab, but not with KT112 (Document S1, Figure S1). Moreover, it was found that IgG1 was the predominant IgG subclass

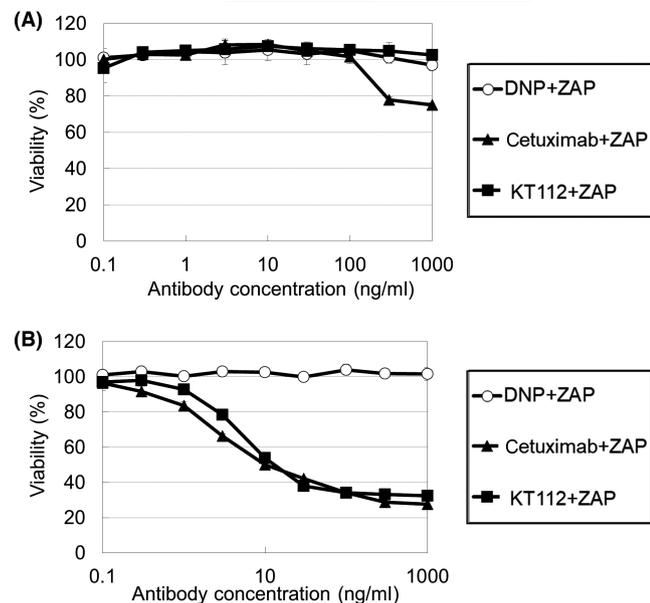


FIGURE 8 Analysis of the internalization ability of each Ab by cell death. The horizontal axis shows the Ab concentration, and the vertical axis shows survival. (A) Normal esophageal cells (HEEpiC) and (B) OE21 cells. Black triangles, cetuximab; black squares, KT112 Ab; white circles, 2,4-Dinitrophenol (DNP) Ab. ZAP

among cancer cell line-binding Abs isolated from the patient's serum (Document S1, Figure S2). As the IgG1 subclass is known to have an effector function, it is possible that KT112 contributed to antitumor activity in the patient through exertion of effector functions, especially ADCC, as shown in Figure 6.

Indeed, ADCC might be an important therapeutic mechanism of EGFR Abs, as suggested by the significant correlation between CD16 polymorphism, an individual factor that determines ADCC intensity,²⁶ and the clinical outcome in some clinical trials of cetuximab.²⁷ Therefore, enhancing ADCC by engineering Ab molecules might be a promising approach to improve anti-EGFR therapies. An ADCC-enhancing EGFR Ab, imgatuzumab (GA201), showed promising results in head and neck SCC based on the therapeutic kinetics of the peripheral immune cells and cytokines; however, it failed to show effectiveness in subsequent phase II trials.²⁸ Alternatively, using KT112 as a vehicle for ADCs could be a good approach for the treatment of esophageal cancer, considering its high internalizing activity. Several clinical trials have assessed ADCs using EGFR Abs.^{21,29,30} As EGFR is expressed systemically, it is considered important to enhance tumor selectivity in order to achieve strong efficacy with low toxicity. Our results indicated that KT112 was highly selective for esophageal SCC. Considering that KT112 was obtained

TABLE 1 Summary of the immunohistochemical analyses of various tumors and healthy tissues using KT112

Tumor		-	+	2+	3+	Positive/All samples (%)
Esophagus	Squamous cell carcinoma	19	4	0	0	4/23 (17.4)
Lung	Squamous cell carcinoma	11	0	0	0	0/11 (0)
	Adenocarcinoma	7	0	0	0	0/7 (0)
	Small-cell carcinoma	1	0	0	0	0/1 (0)
Kidney	Clear cell carcinoma	8	0	0	0	0/8 (0)
Suprarenal gland	Eosinophilic adenoma	1	0	0	0	0/1 (0)
Bladder	Transitional cell carcinoma	1	0	0	0	0/1 (0)
Liver	Hepatocellular carcinoma	6	0	0	0	0/6 (0)
Normal tissue	Score			Normal tissue	Score	
Cerebrum	-			Suprarenal gland	NE	
Medulla oblongata	-			Kidney	-	
Esophagus	-			Mammary gland	-	
Stomach	-			Ovary	-	
Small intestine	-			Prostate	-	
Colon	-			Testis	-	
Spleen	-			Skin	-	
Thymus	-			Smooth muscle	-	
Lung	-			Skeletal muscle	-	
Pancreas	-			Placenta	-	

Note: -, negative; +, weak; 2+, moderate; 3+, strong.

Abbreviation: NE, not evaluated.

Frozen tissue section of esophageal cancer

Esophageal cancer frozen tissue array

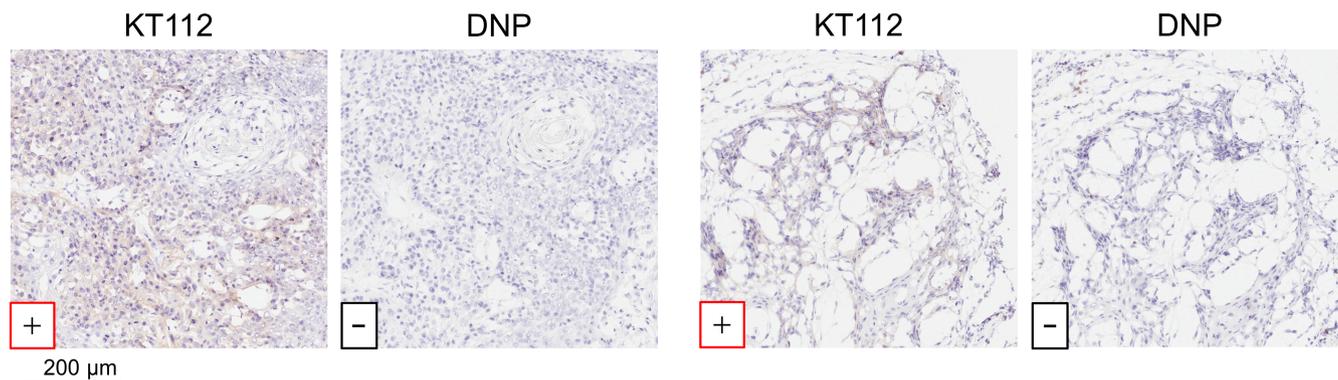


FIGURE 9 Analysis of esophageal cancer tissue reactivity with KT112 Ab. Left panels show the result of staining with frozen sections of esophageal cancer cells. Right panels show the result of staining with frozen tissue arrays of esophageal cancer cells. DNP, 2,4-Dinitrophenol

from the serum of a human participant, the possibility of unexpected off-target toxicity in humans may be low. Thus, KT112 could be a good candidate as an ADC Ab for esophageal SCC.

Nonetheless, two major aspects remain to be elucidated. First, the mechanisms by which KT112 exhibits cancer selectivity remain unclear. A possible explanation might be that KT112 recognizes EGFR mutations expressed in esophageal cancer cells, posttranslational modifications, or conformational changes. The fact that KT112 did not recognize recombinant EGFR protein produced by HEK293 cells and NSO suggests that KT112 recognizes some structural epitope or modifications characteristic of tumor cells; however, more detailed analyses are required to fully understand the mechanism of action. Next, although KT112 might be effective as an ADC that retains good antitumor activity without damaging normal tissues, further *in vivo* experiments using drug-conjugated KT112 are required to evaluate the therapeutic potential and safety profile of KT112-based ADCs.

In conclusion, we obtained a novel EGFR Ab, KT112, with high cancer selectivity for esophageal SCC. Development of this Ab could have contributed to cancer cell growth suppression in the patient who showed long-term survival despite recurrence. Further research is needed to determine whether this cancer-specific Ab can surpass existing anti-EGFR therapies by expanding its therapeutic indications in patients with esophageal SCC.

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DISCLOSURE

Hideaki Shimada is an editorial board member of *Cancer Science*. Sayaka Takagi-Maeda, Katsuaki Usami, Nobuaki Takahashi, and Rinpei Niwa are employees of Kyowa Kirin Co., Ltd. The other

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SUPPORTING INFORMATION

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