cancer who underwent upper gastrointestinal endoscopy.

Gastroduodenal invasion complicated with pancreatic cancer has been reported [1, 2, 3, 4, 6]. In a previous study, gastric invasion was observed in 20 (27%) and duodenal invasion was observed in 30 (40%) of 75 patients with pancreatic cancer at autopsy [1]. In another autopsy report, gastric invasion was observed in 9 (7%) and duodenal invasion in 51 (37%) of 138 patients [4]. However, there have been few endoscopic reports regarding gastroduodenal invasion as a complication of pancreatic cancer [2, 6]. In the present study, gastroduodenal invasion was observed in 11 (21%) of 53 patients at diagnosis, and 20 (27%) of 75 patients during the clinical course of pancreatic cancer. The high incidence of gastroduodenal invasion at autopsy is assumed to be correlated with the progressive state of pancreatic cancer in most cases. In the present study, gastroduodenal invasion developed in patients with pancreatic cancer located in the pancreatic head at a significantly higher rate than in those with lesions in the pancreatic body and/or tail. It is assumed that pancreatic head cancer is prone to invade the duodenum, because the pancreas adjoins the duodenum and the duodenal wall is thinner than the gastric wall. It has been reported that patients with pancreatic cancer often develop gastrointestinal bleeding due to direct tumor invasion into the gastrointestinal tract [2, 3, 6]. In the present study, upper gastrointestinal bleeding developed in 4 patients with duodenal invasion. Gastrointestinal bleeding was uncontrollable in 1 of these 4 patients and resulted in death. Gastrointestinal bleeding due to direct tumor invasion is thought to be a critical complication in the patients with pancreatic cancer.

Esophagogastric varices as a complication of pancreatic diseases have been reported previously [5, 7, 8, 9]. The mechanism underlying the development of esophagogastric varices is assumed to entail involvement of the splenic vein by pancreatic disease, a manifestation called left-sided portal hypertension [12]. In endoscopic studies, it was reported that esophagogastric varices were observed by endoscopic ultrasonography in 16 patients (21%) with pancreatic diseases (pancreatic tumors in 12 and pancreatitis in 4) of 76 patients in whom gastroesophageal varices were detected endoscopically [5]. In another endoscopic study, esophagogastric varices were found in 25 (26%) patients with pancreatic cancer among 96 patients who underwent upper gastrointestinal endoscopy at diagnosis [9]. This previous study indicated that esophagogastric varices tended to be observed in patients with pancreatic body and/or tail cancer more frequently in those with pancreatic head cancer. In the present study, esophagogastric varices were observed in patients with pancreatic body and/or tail cancer at significantly higher rates than in those with pancreatic head cancer. It is assumed that the splenic vein is more susceptible to invasion by pancreatic body and/or tail cancer than pancreatic head cancer. In the present study, although the red color sign was observed in about one third of the patients with esophagogastric varices, no F3 varices were identified. In addition, upper gastrointestinal bleeding developed in only 2 (14.3%) of 14 patients, although 1 patient died of repeated GI bleeding. We speculated that segmental portal hypertension in patients with pancreatic cancer is reduced by flow from the short gastric or gastroepiploic vein to the portal vein (hepatopetal route) in contrast to liver cirrhosis (hepatofugal route). In this study, portal hypertensive gastropathy developed in 3 patients with pancreatic cancer. Portal hypertensive gastropathy related to pancreatic cancer has not been reported previously. Upper gastrointestinal bleeding developed in 1 patient with portal hypertensive gastropathy, resulting in hemostasis by percutaneous portal vein stent.

Although radiation-induced gastroduodenal mucosal lesions with pancreatic cancer have been reported, the incidence is relatively low [13, 14]. However, a total of 9 (50%) of the 18 patients who were treated with radiotherapy suffered from radiation-induced mucosal lesions in this study. It was assumed that long-term follow-up and frequent upper gastrointestinal endoscopy at digestive symptoms may have influenced the high prevalence in this study. Although all 9 patients with radiation-induced gastroduodenal mucosal lesions have been treated with PPI or H_2 blockers since radiotherapy, upper gastrointestinal bleeding developed in 2 patients with radiation-induced gastritis 5 or 7 months after radiotherapy, resulting in hemostasis by argon plasma coagulation therapy. Therefore, attention should be paid to late-onset upper gastrointestinal bleeding in patients treated with radiotherapy. It is assumed that PPI or H_2 blockers would be insufficient to prevent radiation-induced mucosal lesions.

There have been few previous reports of studies regarding gastroduodenal ulcers complicated with pancreatic cancer. In the present study, a total of 8 cases with gastroduodenal ulcers (except radiation-induced ulcers) were observed. In addition to 5 patients with radiation-induced ulcers, a total of 13 cases of gastroduodenal ulcers were identified. Although 2 patients developed NSAID-induced gastroduodenal ulcers, they were not treated with PPI (1 patient was treated with H_2 blocker). PPI may have prevented gastroduodenal ulcers in these patients. In this study, although gastroduodenal ulcers were observed in only 3 patients at diagnosis, 10 lesions were newly identified especially in patients treated with radiotherapy, biliary drainage, or NSAIDs. Therefore, gastroduodenal ulcers should be considered in patients with pancreatic cancer receiving these therapies during the clinical course.

In the present study, about half of the upper gastrointestinal lesions presented with symptoms related to upper gastrointestinal lesions especially in patients with gastroduodenal invasion, radiation-induced gastroduodenal mucosal lesions, and

gastroduodenal ulcers (except radiation-induced ulcers). Upper gastrointestinal bleeding as a complication of pancreatic cancer has been reported previously [2, 3, 6, 7, 8]. In this study, 15 (27%) of 56 lesions were accompanied by upper gastrointestinal bleeding. Most of upper gastrointestinal bleeding was controlled by endoscopic or conservative treatment. However, 3 of 15 patients in whom bleeding was confirmed endoscopically died of upper gastrointestinal bleeding. In addition to 2 patients in whom upper gastrointestinal endoscopy was not performed at hematemesis, a total of 5 (7%) of 75 patients died of upper gastrointestinal bleeding. Therefore, attention must be paid regarding upper gastrointestinal bleeding in patients with pancreatic cancer. When upper gastrointestinal bleeding occurs or is suspected during follow-up of pancreatic cancer, upper gastrointestinal endoscopy should be performed promptly. Although progression of anemia often develops in patients with pancreatic cancer due to bone marrow suppression by chemotherapy or secondary anemia, upper gastrointestinal endoscopy is recommended for evaluation of upper gastrointestinal bleeding in cases in which anemia has developed.

In this study, 14 (25%) of 56 upper gastrointestinal lesions were observed on the second or later examination. In particular, gastroduodenal invasion and radiation-induced gastroduodenal mucosal lesions were predominant in these cases. Therefore, attention should be paid to patients in whom pancreatic tumors have enlarged or radiotherapy has been performed.

The survival period of patients with pancreatic cancer has been prolonged due to the development of gemcitabine [15] and S-1 [16]. Therefore, the appearance of upper gastrointestinal lesions during the clinical course of pancreatic cancer is predicted to increase. In addition, clinical symptoms related to upper gastrointestinal lesions would be often masked by NSAIDs or opioid in patients with pancreatic cancer. It is proposed that upper gastrointestinal endoscopy may be recommended in patients with pancreatic cancer at diagnosis and in whom pancreatic tumors have enlarged or radiotherapy has been performed, and should be performed in patients in whom upper gastrointestinal bleeding is suspected of.

In conclusion, the incidence of upper gastrointestinal lesions in patients with pancreatic cancer was relatively high. In addition, the incidence increased with the clinical course of pancreatic cancer. We should pay attention to upper gastrointestinal lesions in patients with pancreatic cancer. In particular, when the symptoms associated with upper gastrointestinal bleeding are observed, upper gastrointestinal endoscopy should be performed promptly.

Conflict of interest The authors have no conflict of interest

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Therapeutic antitumor efficacy of anti-epidermal growth factor receptor antibody, cetuximab, against malignant pleural mesothelioma

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Abstract. Epidermal growth factor receptor (EGFR) is commonly overexpressed in malignant pleural mesothelioma (MPM). Cetuximab is a chimeric mouse-human antibody targeted against EGFR and induces potent antibody-dependent cellular cytotoxicity (ADCC). The action of cetuximab against MPM cells has not been well studied. Therefore, in this study, we investigated the antitumor activity of cetuximab against MPM cell lines, particularly with respect to ADCC activity *in vitro* and *in vivo*. EGFR expression of MPM cells was measured by a quantitative flow cytometric analysis and immunohistochemistry. The effect of cetuximab on growth inhibition was assessed using a modified MTT assay. The ADCC activity was measured by a 4-h ^{51}Cr release assay using fresh or IL-2-activated peripheral blood mononuclear cells. *In vivo* antitumor activity of cetuximab was evaluated using an orthotopic implantation mouse model. Cetuximab-mediated ADCC activity against MPM cells was observed at low concentration (0.25 mg/ml) and was enhanced by IL-2, whereas no direct effect on growth inhibition was detected. A logarithmic correlation was observed between the number of EGFRs on MPM cells and ADCC activity. Low EGFR expression on the MPM cells, which was weakly detectable by immunohistochemistry, was sufficient for maximum ADCC activity. In the mouse model, cetuximab treatment with or without IL-2 significantly inhibited intrathoracic tumor growth and prolonged their survival. Our study shows that cetuximab has potent anti-MPM activity both *in vitro* and *in vivo*, mainly through the immunologic mechanism of ADCC. Cetuximab has the potential to be used as a novel therapy for MPM patients.

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Key words: malignant pleural mesothelioma, cetuximab, antibody-dependent cellular cytotoxicity, intrathoracic therapy

Introduction

Malignant pleural mesothelioma (MPM) is a rare and highly aggressive neoplasm, which arises from the pleural, pericardial, or peritoneal lining. Most patients with MPM have a history of exposure to carcinogenic asbestos fibers (1,2), particularly those of the amphibole type, or to naturally occurring erionite in some regions of Turkey (3,4). Although surgery, chemotherapy, radiotherapy, and combinations thereof play an important role in the treatment of MPM patients, the median survival of patients treated for MPM is dismal, at only 6-18 months (5-7). Despite advances in modern systemic chemotherapy using the combination of pemetrexed and cisplatin, long-term survival in patients with MPM remains limited (8). Therefore, more specific, effective, and less toxic therapies are needed. Research into the molecular pathways of MPM has led to novel targeted strategies that inhibit specific key molecules in tumor growth and progression.

Epidermal growth factor receptor (EGFR) is a tyrosine-kinase (TK) receptor involved in cell death and proliferation, cell motility, angiogenesis, and extracellular matrix composition (9). EGFR is overexpressed in many human malignancies, including lung, head and neck, colorectal, and breast cancers, where it is variably associated with patient prognosis (10,11). EGFR is reported to be overexpressed in 44-97% of MPM patients, as determined by various immunohistochemical studies with variability in outcomes (12-15). In a recent study, Destro *et al* demonstrated that both the immunohistochemical expression and corresponding mRNA levels of EGFR were higher in tumor specimens than in normal pleural samples (12). These data confirmed those of a previous study suggesting that EGFR could

play an important role in the oncogenic phenotype of MPM disease (9).

Two types of EGFR inhibitors have been developed: small molecule EGFR tyrosine kinase inhibitors (TKIs) (16,17) and monoclonal antibodies directed against the extracellular domain of EGFR (18-20). Gefitinib, a quinazoline derivative, is the first TKI developed that specifically inhibits the activation of EGFR TK through competitive binding to the ATP-binding domain of the receptor. Gefitinib has been shown to be effective in preclinical studies and clinical trials, and it received approval for use in Japan in patients with advanced non-small cell lung cancer refractory to chemotherapy in July 2002. Subsequently, it has gained approval in over 30 countries, including the United States. Gefitinib reduced the proliferation of MPM cells by inhibiting the EGFR signaling pathway *in vitro* (9); however, the clinical study revealed that gefitinib was not active in MPM patients (21). The same is true of erlotinib (14). These disappointing results for EGFR TK inhibitors have led to increased interest in monoclonal antibodies directed against EGFR, because these 2 classes of agents may have substantially different mechanisms of action.

Cetuximab is a chimeric mouse-human antibody directed against the extracellular domain of EGFR (22), thereby inhibiting the binding of activating ligands to the receptor. Consequently, cetuximab inhibits ligand-dependent activation of the EGFR and inhibits the downstream pathways that cause cell cycle progression, cell growth, and angiogenesis. In addition, the binding of cetuximab initiates EGFR internalization and degradation that leads to signal termination (23-25). In addition to these direct inhibitory effects to EGFR signaling, cetuximab potentially provokes immunologic antitumor effects called antibody-dependent cellular cytotoxicity (ADCC). This effect takes place in the presence of the host effector system, such as natural killer (NK) cells, because cetuximab has a human IgG1 backbone. Recently, we and others showed that this ADCC activity is crucial for the antitumor effects of cetuximab (26-28). Because this immunological mechanism is not activated by TKIs, cetuximab is expected to have more potent antitumor activities against MPM than TKIs, especially *in vivo*. However, no published *in vitro* or *in vivo* studies have focused on the effect of cetuximab against MPM cells, particularly with respect to ADCC activity.

In the present study, we investigated the biologic activity of cetuximab against a panel of MPM cells with respect to ADCC activity and the survival effects of intrathoracic treatment using an orthotopic implantation mouse model that reproduces the clinical behavior and therapeutic responsiveness of MPM in humans.

Materials and methods

Cell lines and cell culture. Five MPM cell lines (EHMES-1, MSTO-211H, H2052, EHMES-10 and H28) and an epidermoid carcinoma cell line (A431) were used in this study. MSTO-211H, H2052, H28 and A431 were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The other lines (EHMES-1, EHMES-10) were established from the pleural effusion of a patient with MPM at Ehime University (Ehime, Japan). All cell lines were maintained in RPMI-1640 supplemented with 10% FCS, 50 U/ml penicillin, 50 U/ml streptomycin and 2.05 mmol/l glutamine. The cells were incubated at 37°C in 5% CO_2.

Monoclonal antibody. Cetuximab was obtained from Bristol-Myers Squibb (New York, NY, USA). Rituximab, used as a control antibody, was obtained from Chugai Pharmaceutical (Tokyo, Japan). Anti-EGF receptor antibody (clone 528) for flow cytometry was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-EGF receptor antibody (clone 31G7) for immunohistochemical analysis was obtained from Zymed (South San Francisco, CA, USA).

Flow cytometric analysis. Cell surface EGFR expression of MPM cell lines was examined by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA) using a monoclonal antibody (clone 528). To determine the absolute number of antibody-binding sites per cell, we carried out a quantitative flow cytometric analysis using Dako QIFIKIT (DakoCytomation, Copenhagen, Denmark). Briefly, $1x10^4$ cells were incubated for 1 h at 4°C with 0.4 μg of the primary antibody or the isotype-control IgG2a antibody (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.01% sodium azide. After washing thrice with PBS, cells were incubated for 1 h with FITC-conjugated anti-mouse IgG (DakoCytomation) at 4°C. Similar to samples labeled with FITC-conjugated anti-mouse IgG from this kit, standard beads coated with a known amount of mouse IgG molecules were labeled with this secondary antibody. The labeled samples were washed thrice with PBS and analyzed using FACScan flow cytometer (Becton Dickinson). The number of antibody binding sites per cell was calculated by comparing the mean fluorescent intensity (MFI) value of the labeled cells with a calibration curve obtained by regression analysis of the MFI values of the standard beads.

Growth inhibition assay. Cell viability was assessed using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)2H-tetrazolium monosodium salt (WST-8) assay (Dojindo, Kumamoto, Japan). Cells were plated at $3x10^4$ cells/well in triplicate in 96-well plates in complete medium. Following an overnight incubation, cetuximab (0-1,000 μg/ml) was added in varying concentrations and incubated. After 72 h, WST-8 solution (Dojindo) was added to each well, followed by incubation for 4 h at 37°C, and absorbance was measured using a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at test and reference wavelengths of 450 and 655 nm, respectively. Cell viability was calculated by dividing the mean absorbance of wells containing treated cells by those of control wells with untreated cells. The concentration of cetuximab resulting in 50% growth inhibition (IC_{50}) was calculated. All experiments were done at least in triplicate and repeated at least 3 times.

Isolation of peripheral blood mononuclear cells (PBMCs) and interleukin-2 (IL-2) treatment. PBMCs were isolated from heparinized peripheral blood by lymphocyte-separation-medium (MP Biomedicals, Irvine, CA, USA) density gradient centrifugation. To investigate the effect of IL-2 (Sigma-Aldrich) on ADCC activity, PBMCs (10^6 cells/ml) were pre-incubated at 37°C for up to 18 h before cytotoxic assay in the presence of IL-2 (30 IU/ml) (29-31). Blood samples were collected at Tottori University in accordance with the Tottori University Review Board, and the healthy individuals provided written informed consent.

Table I. EGFR expression analysis by quantitative flow cytometry and IHC in malignant pleural mesothelioma cell lines.

Cell lines	EGFR expression (nos. of EGFR/cells)	Immunohistochemical score
EHMES-1	$6.54x10^3$	1+
MSTO-211H	$1.42x10^4$	1+
H2052	$2.73x10^4$	2+
EHMES-10	$3.16x10^4$	2+
H28	$4.51x10^4$	2+

Test for ADCC and NK activity. After the target MPM cells were labeled with 100 μCi ^{51}Cr (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) for 60 min, target cells (10^4/well) and effector cells at various effector:target (E/T) ratios were co-incubated in 200 μl of DMEM or RPMI-1640 in a 96-well U-bottomed plate in triplicate for 4 h at 37°C with 0.5 μg/ml of cetuximab (Bristol-Myers Squibb) or control antibody, rituximab (Chugai Pharmaceutical). Next, the amount of radioactivity in the supernatant liquid was measured by a gamma counter. The percentage of specific cytolysis was calculated as previously described (27). ADCC activity was calculated as the percentage of lysis in the presence of cetuximab minus the percentage of lysis in the presence of control antibody that is attributed to NK activity.

Immunohistochemical analysis. Paraffin-embedded cell blocks were prepared from each MPM cell lines, which were fixed in 4% paraformaldehyde. Tissue sections (3 μm) were de-waxed in xylene, rehydrated through a graded series of ethanol solutions, rinsed in distilled water for 5 min, and then immersed in 0.6% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase. For antigen retrieval, the sections then were microwaved in 0.01 mol/l of sodium citrate-buffered saline, pH 6.0, for 20 min at 92°C using a Microwave Processor model MI-77 (Azumaya, Tokyo, Japan). After rinsing in PBS for 5 min, the slides were pre-blocked with 10% normal rabbit serum at room temperature for 20 min and incubated at 4°C overnight with the primary antibody, anti-EGF receptor antibody (clone 31G7) (Zymed). The immunoreaction was visualized with 3.3'-diaminobenzidine and 100 μl of hydrogen peroxidase in 0.05 M Tris-HCl buffer, pH 7.6. Finally, the slides were counterstained with a 0.1% hematoxylin solution. The staining results were measured semiquantitatively on a scale of 0, 1+, 2+ and 3+ as follows: 0, no membranous staining in any of the cells; 1+, weak intensity membranous and cytoplasmic staining of nearly equal intensity; 2+, moderate to strong intensity staining predominantly in the membranes; and 3+, strong intensity staining clearly localized to the cell membranes. Representative examples of 0, 1+, 2+ and 3+ IHC staining for EGFR are demonstrated in Fig. 1. We performed the staining for the each cell line 3 times, and the intensity was evaluated by 2 independent pathologists.

Animals. Male C.B-17 SCID mice (5 weeks) were obtained from CLEA Japan (Osaka, Japan) and maintained under specific pathogen-free conditions throughout the study. Experiments were carried out in accordance with the guidelines established by the Tottori University Committee on Animal Care and Use.

Orthotopic implantation model. The cultured MSTO-211H cells were harvested by pipetting. The cells were washed 3 times and resuspended in Ca^{2+}- and Mg^{2+}-free PBS. For orthotopic implantation, SCID mice were anesthetized with ether and had their right chest wall shaved. After sterilization of the chest wall with 70% ethanol, the right chest skin and subcutaneous tissue was cut, and the parietal pleura was exposed. Thereafter, the tumor cells (10^6/100 μl PBS) were injected into the thoracic cavity of SCID mice using a 27G needle as described previously (32). Finally, the incisions were sutured to close the wound. The mice were treated with cetuximab (0.05 mg/mouse i.t.) or in combination with IL-2 (30 IU/ml i.t.) using the same methods on day 7, and sacrificed on day 21 to evaluate tumor development. The pleura-disseminated tumors were inspected macroscopically.

Area measurements. The intrathoracic tumor area was manually defined on intrathoracic pictures, and was measured with the image analysis software program Scion Image for Windows (PC version of NIH Image).

Statistics. The statistical comparison between the 2 groups was analyzed using Student's t-test. The survival times of SCID mice bearing MSTO-211H cells was determined using the Kaplan-Meier estimation (PRISM for Windows; GraphPad Software, La Jolla, CA, USA).

Results

Analysis of EGFR expression in MPM cell lines using flow cytometry and IHC. We first examined the expression of EGFR in 5 MPM cell lines. A431, an epidermoid carcinoma cell line, was used as a positive control for EGFR expression in most studies, since it has been reported to express high levels of EGFR (33,34). We measured the number of EGFRs on each MPM cell line by quantitative flow cytometric analysis (Dako QIFIKIT) (35) and compared them to the evaluation by immunohistochemistry (IHC) (scored from 0 to 3+). As shown in Table I, the level of EGFR expression in each MPM cell line, in ascending order, is as follows: EHMES-1, MSTO-211H, H2052, EHMES-10 and H28. As assessed using IHC, 2 cell lines (EHMES-1 and MSTO-211H), which express a low number of EGFRs (ranging from $6.54x10^3$ to $1.42x10^4$/cell), were stained and scored as 1+. The other 3 cell lines of MPM, which expressed moderate numbers of EGFR (ranging from $2.73x10^4$ to $4.51x10^4$/cell), were scored as 2+. The positive control cell line A431, expressing a large number of EGFRs ($3.51x10^6$/cell) scored 3+ (data not shown) (Fig. 1). These results indicated a good correlation between the number of EGFR molecules on the cells and their EGFR status as estimated by IHC.

Direct effects of cetuximab on growth inhibition in MPM cells. We next examined the effect of cetuximab against the proliferation of MPM cells using the WST-8 assay, which is a modified MTT assay. We found that all MPM cell lines were completely resistant to cetuximab treatment irrespective of the surface amount of EGFR (Fig. 2). These data suggest that direct growth

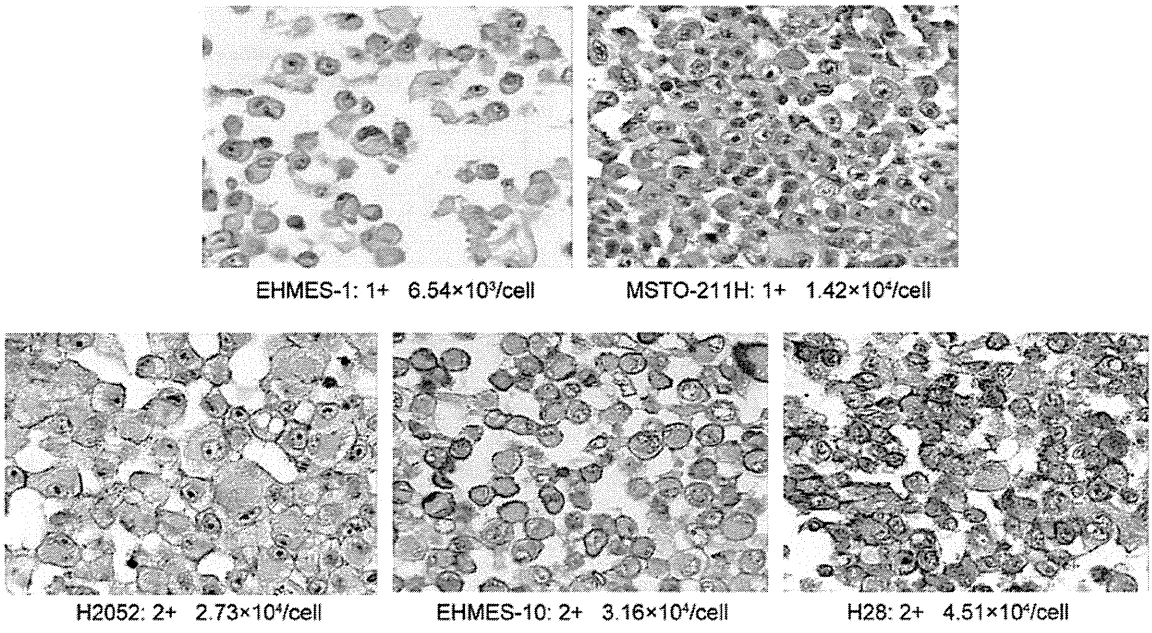

Figure 1. Immunohistochemical staining for EGFR expression. Representative EGFR immunohistochemistry scoring in 5 malignant pleural mesothelioma cell lines. The immunohistochemical score and the number of EGFR molecules/cell are also indicated (x400 magnification).

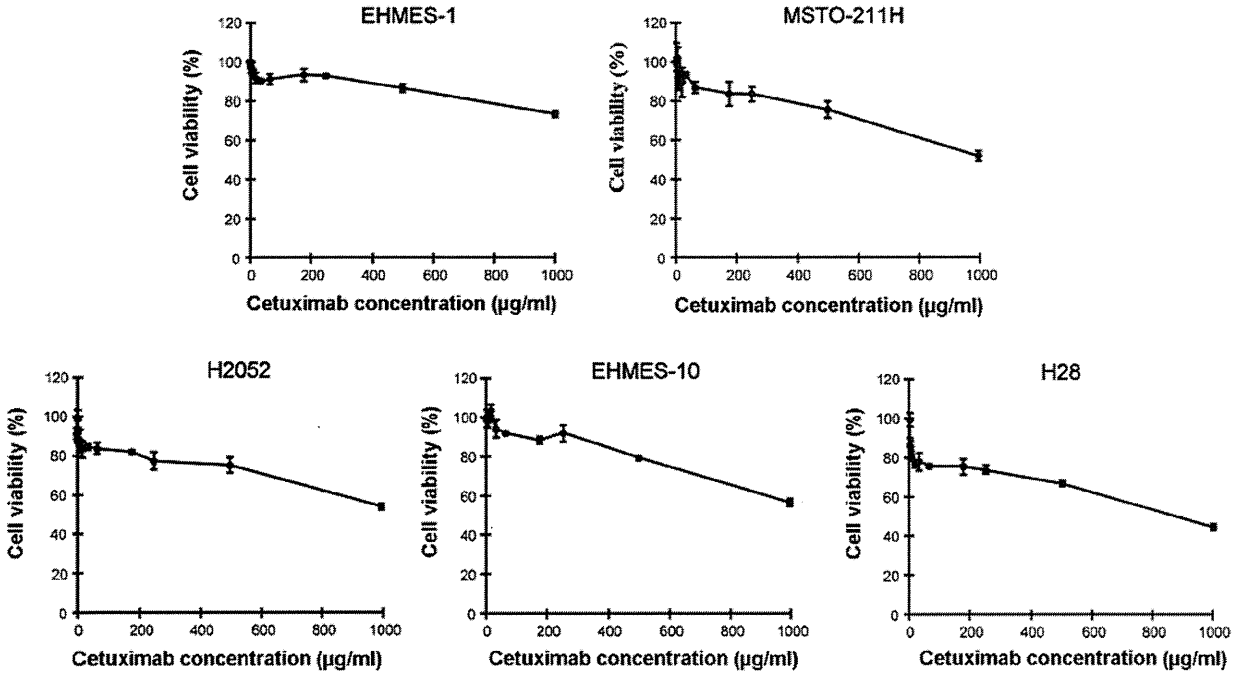

Figure 2. Direct effects of cetuximab on growth inhibition. Malignant pleural mesothelioma cell lines were treated with indicated concentration of cetuximab (0-1,000 μg/ml). Cell proliferation was measured by WST-8 assay after 72 h of continuous drug exposure.

inhibitory effects would not be expected in the anti-MPM action of cetuximab.

Cetuximab-mediated cytotoxicity against MSTO-211H cells by healthy human PBMCs. To test whether cetuximab induces ADCC activity against MPM cell lines, we performed a 4-h ^{51}Cr release assay of MSTO-211H cells that weakly express EGFR using human PBMCs at various E/T ratios (Fig. 3A). While low levels of cytolysis of MSTO-211H cells were induced by PBMCs at the higher E/T ratios of 80:1 and 40:1 in the absence of cetuximab (known as NK activity), the lytic activity of PBMCs increased significantly in the presence of cetuximab at both E/T ratios. There was no significant increase in lytic activity in the presence of the control antibody, rituximab (data not shown). These data suggest that cetuximab was capable of inducing ADCC activity efficiently, even against MPM cells that weakly express EGFR.

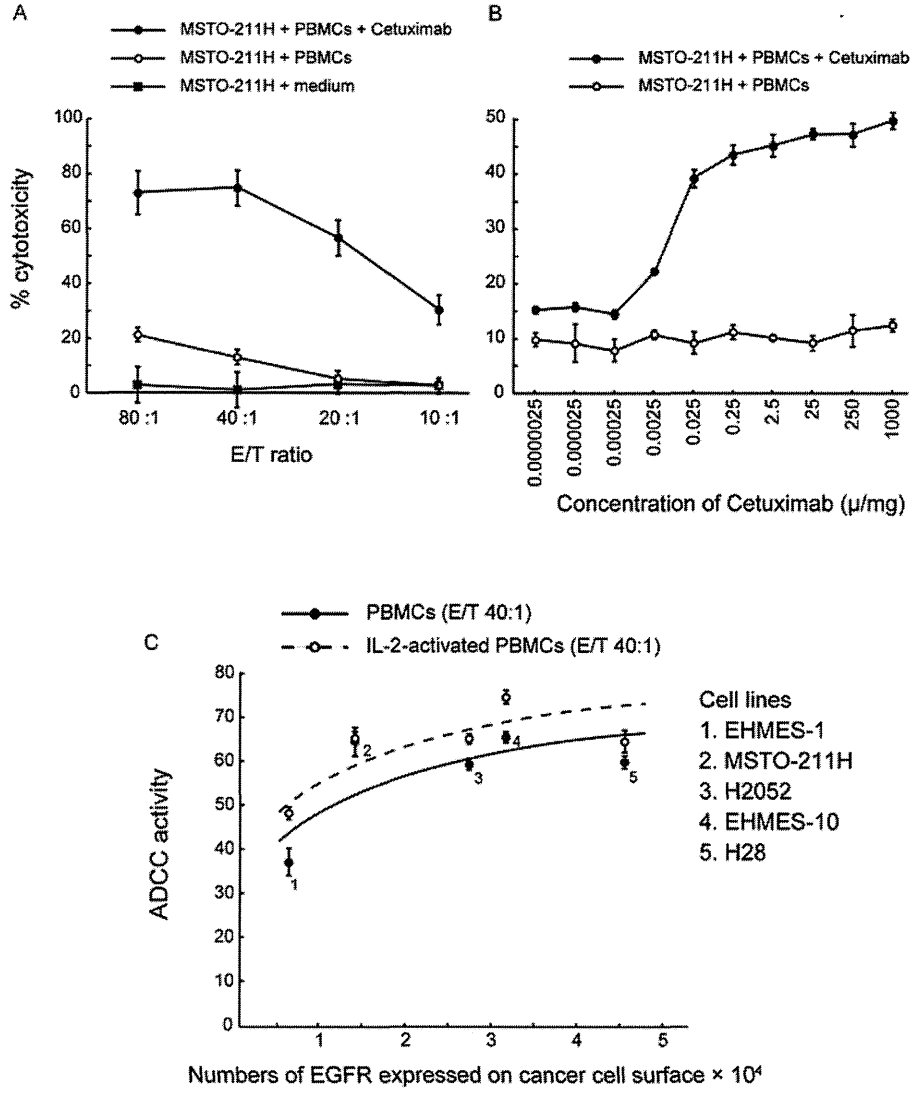

Figure 3. Cytotoxicity against a malignant pleural mesothelioma cell line mediated by cetuximab. (A) Cetuximab mediates cytotoxicity against the EGFR-expressing MSTO-211H mesothelioma cell line. Healthy human PBMCs, using 4 different E/T ratios, were tested for cytotoxicity in the presence or absence of cetuximab (2.5 μg/ml). The y-axis reveals cytotoxicity as determined by a 4-h ^{51}Cr release assays. (B) Concentration-dependent curve of cetuximab-dependent ADCC activity and NK activity against MSTO-211H cells by healthy human PBMCs. MSTO-211H cells were incubated with PBMCs at an E/T ratio of 20:1 along with or without indicated concentrations of cetuximab (0.0000025-1,000 μg/ml). Data are representative of 3 independent experiments. Points, mean of a triplicate experiment; bars, SD. (C) Correlation between EGFR expression levels of target malignant mesothelioma cell lines and cetuximab-mediated ADCC activity. The x-axis indicates the number of EGFR molecules expressed on the surface of the cancer cells. The y-axis represents the ADCC activity of cetuximab (0.25 mg/ml) as determined by a 4-h ^{51}Cr release assay. Healthy human PBMCs were incubated with or without IL-2 (30 IU/ml) at an E/T ratio of 40:1 for 18 h and tested for cetuximab-mediated ADCC activity against various ^{51}Cr-labeled cell lines. Data are representative of 5 independent experiments. The malignant mesothelioma cell lines used are indicated. Points, mean of a triplicate experiment; bars, SD.

Next, to identify the optimal cetuximab concentration for ADCC activity, we determined the ADCC activity with increasing concentrations of cetuximab, ranging from 2.5×10^{-6} to 1,000 mg/ml at an E/T ratio of 20:1. As shown in Fig. 3B, cetuximab-mediated ADCC activity against MSTO-211H cells was already detectable at a concentration of 2.5×10^{-3} mg/ml and was saturated at 0.25 mg/ml. These data indicate that a cetuximab concentration in excess of 0.25 mg/ml was sufficient for maximum ADCC activity. We used this concentration of cetuximab for the subsequent assays.

Cetuximab-mediated ADCC activity against MPM cell lines with various EGFR expression levels. To evaluate the correlation between the ADCC activity induced by cetuximab and EGFR expression levels on target MPM cells, we determined the ADCC activity in MPM cell lines with various EGFR expression levels at an E/T ratio of 40:1 in the presence of the optimal dose of cetuximab (0.25 mg/ml). As shown in Fig. 3C, the ADCC activity correlated logarithmically with the number of EGFR molecules expressed on the MPM cell surface. Near-maximum ADCC activity was observed in MSTO-211H cells, which have small numbers of EGFRs and scored 1+ by IHC. ADCC activity did not increase in cells with higher EGFR expression.

In addition, as IL-2 is known to activate PBMCs, we tested the effects of overnight treatment of PBMCs with IL-2 on cetuximab-mediated ADCC activity. Low doses of IL-2 increased ADCC activity in all cell lines, regardless of EGFR expression level (Fig. 3C). These data suggest that the very weak EGFR

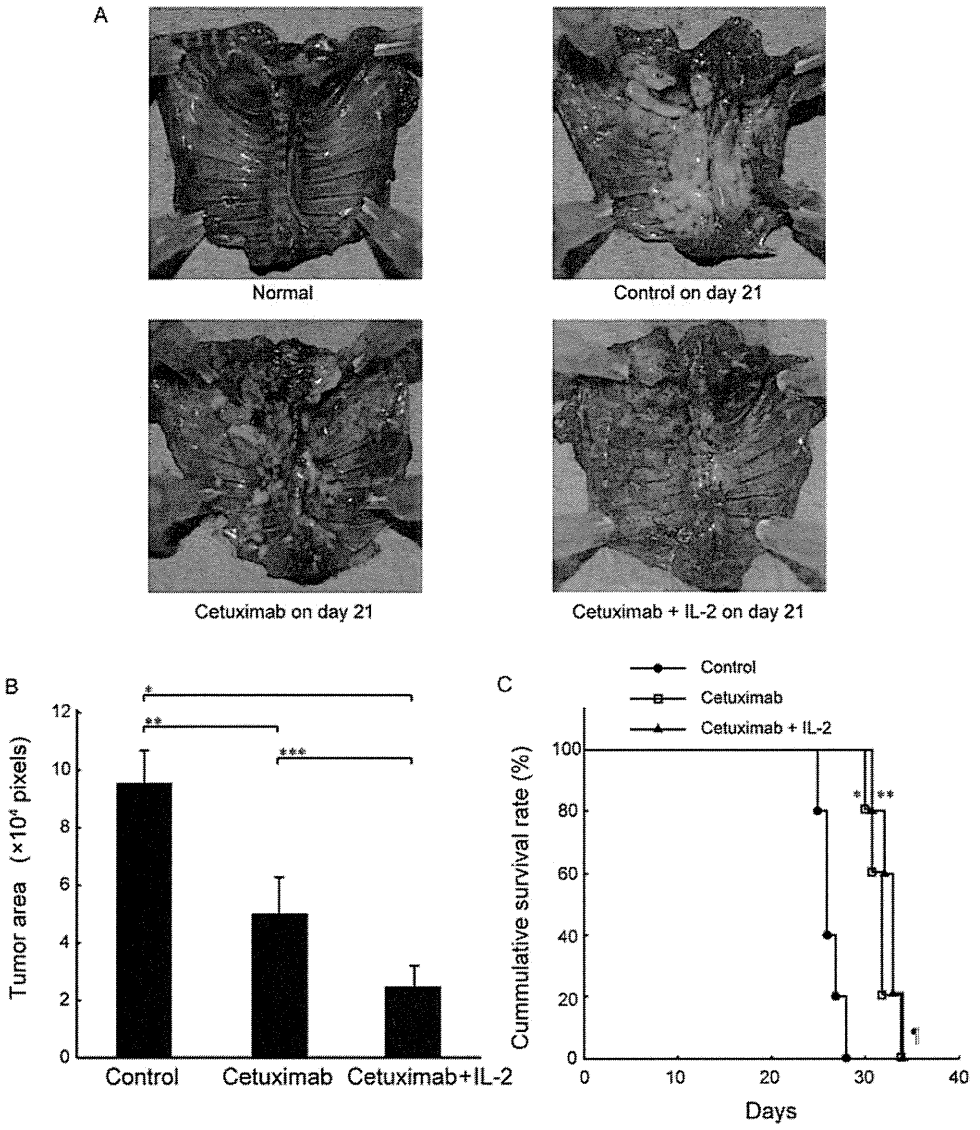

Figure 4. Effect of cetuximab alone and in combination with IL-2 on SCID mice bearing MSTO-211H cells. Mice were treated with cetuximab (0.05 mg/mouse i.t. on day 7) or in combination with IL-2 (30 IU/ml i.t. on day 7). (A) Representative intrathoracic pictures of SCID mice bearing MSTO-211H cells on day 21. The MSTO-211H cells produced small nodular tumors intrathoracic cavity. (B) Tumor area of the mice treated with cetuximab or in combination with IL-2 on day 21. Columns, mean pixels from 5 independent animals; bars, SD. *P<0.001 compared to the control group; **P<0.001 compared to the control group; ***P<0.01 compared to the cetuximab monotherapy group. (C) Survival time of SCID mice bearing MSTO-211H cells. Kaplan-Meier survival curves are displayed. The mice (N=5/group) were treated with cetuximab (0.05 mg/mouse i.t. on day 7) or in combination with IL-2 (30 IU/ml i.t. on day 7). Intrathoracic administration of cetuximab significantly prolonged the survival time of the mice compared to that of the control group. *P<0.01 compared to the control group; **P<0.01 compared to the control group; ¶, P=0.28 compared to the cetuximab monotherapy group.

expression in MPM cells is enough to mediate ADCC activity and that IL-2 is capable of enhancing this activity.

Effect of cetuximab and IL-2 on SCID mice bearing MSTO-211H cells. To test the antitumor effects of cetuximab *in vivo*, we used an orthotopic implantation mouse model as a clinically relevant animal model. In this model, cells from the mesothelioma cell line MSTO-211H were implanted into the thoracic cavity of SCID mice, which possess robust NK cell activity (36). The implanted mice were treated with cetuximab by direct administration into the thoracic cavity with and without IL-2 on day 7, and then sacrificed on day 21 as described in Materials and methods. To determine the optimal dose of cetuximab for the treatment of SCID mice bearing MSTO-211H, we first determined the survival times of the mice using various amounts of cetuximab (0.5 mg/mouse, 0.05 mg/mouse and 0.005 mg/mouse i.t. on day 7). This preliminary experiment showed that there was no statistically significant difference in survival time between the mice (data not shown). In addition, in a separate study that evaluated the pharmacokinetics of cetuximab in nude mice bearing human colon carcinoma xenografts, the efficacious range for antitumor activity was demonstrated to be 0.04-1 mg/mouse (37). We therefore administered cetuximab at a dose of 0.05 mg/mouse in our subsequent *in vivo* experiments to ensure biological activity yet minimize side effects. As shown in Fig. 4A, cetuximab inhibited intrathoracic mesothelioma growth in the mice, and this inhibition was markedly enhanced by IL-2 co-administration. This inhibitory effect of cetuximab alone

and its enhancement by the addition of IL-2 was confirmed by quantitative measurements of the tumor area using Scion Image Software (Fig. 4B). Furthermore, intrathoracic administration of cetuximab significantly prolonged the survival of the mice, and the combination of cetuximab with IL-2 tended to improve survival (Fig. 4C). These results suggest that cetuximab exerts antitumor effects against MPM cells in the presence of the mouse effector system, and that ADCC activity is highly involved in this effect.

Discussion

In the present study, we evaluated cetuximab as a novel molecular targeting agent for MPM. We found that cetuximab induces potent ADCC activity but not growth inhibition against MPM cell lines. Cetuximab-induced ADCC activity has several characteristics that are relevant to clinical therapeutic applications. First, low concentrations of cetuximab are sufficient to induce maximum ADCC activity. Second, the low EGFR expression levels on MPM cells, which are scored as 1+ by IHC, could be sufficient for maximum ADCC activity mediated by cetuximab. Third, *ex vivo* IL-2 treatment of PBMCs can enhance cetuximab-mediated ADCC activity against MPM cell lines. Finally, intrathoracic administration of cetuximab in an orthotopic implantation mouse model significantly inhibited tumor growth and prolonged the survival time in the presence of the mouse effector system. These data indicate the important role of ADCC activity in the mechanism of action of cetuximab against cancer cells and underscore the promising potential of cetuximab as a new class of therapeutic agent for use against MPM.

In this study, we examined the correlation between the number of EGFRs on the cell surface and cetuximab-induced ADCC activity in MPM cells and found that there is a logarithmic relationship between them. This finding is in agreement with our previous observations and those reported by others in relation to other cancers. The ADCC activities of trastuzumab (38), anti-Ep-CAM antibody (39) and cetuximab (27) have been reported to weakly correlate with the logarithm of the number of target cell surface antigens in breast or lung cancer cells. The correlation observed in this study indicates that low EGFR expression levels could be sufficient for maximum ADCC activity of cetuximab against MPM cells and that an increase in the expression level of EGFR has no obvious effect on ADCC activity.

Our results indicate the possible usefulness of EGFR IHC as a predictive marker of the effectiveness of cetuximab-mediated ADCC activity against MPM cells. We demonstrated that the demarcation point of the EGFR expression level to achieve maximum ADCC activity is between EHMES-1 (6.54×10^3 EGFR molecules/cell) and MSTO-211H (1.42×10^4 EGFR molecules/cell), both of which are scored as 1+ by IHC. Therefore, near-maximum ADCC activity could be expected as long as the cells are stained by IHC, independent of the strength of the staining. This feature could circumvent a common weak point of IHC, as the semi-quantitative (40) nature of IHC makes it prone to inter-observer scoring error. In addition, IHC is superior to other methods for measuring EGFR levels in clinical specimens, such as a ligand binding assay (41) and quantitative flow cytometry (42), because it does not require isolation of cells from fresh tissue or special equipment. Therefore, IHC might be useful if it is scored simply as negative or positive when assessing a tumor sample for predicting the effectiveness of the ADCC activity of cetuximab.

We have demonstrated that cetuximab-mediated ADCC activity against MPM cell lines is enhanced in response to IL-2. This lymphokine is normally produced by T-lymphocytes and augments the function of effector cells, such as B cells, NK cells, T cells and monocytes (43). The combination of IL-2 and a therapeutic monoclonal antibody has been explored extensively in the case of rituximab (44,45) and trastuzumab (46-49) and has been reported to enhance ADCC activity *in vitro* (50) or *in vivo* using mouse xenograft models (51). Based on these fundamental studies, several preclinical trials of these combination therapies have been conducted, including rituximab for B cell non-Hodgkin's lymphoma (52) and trastuzumab for HER2-overexpressing cancer (53,54). Therefore, our observation that IL-2 enhances cetuximab-meditated ADCC activity against MPM cell lines might lend support to the future concurrent use of cetuximab and IL-2 in patients with MPM.

In our study, we showed that intrathoracic administration of cetuximab significantly inhibited tumor growth and prolonged the survival of mice. Several lines of evidences suggest that the anti-MPM effects of cetuximab *in vivo* are dependent on ADCC activity. First, murine spleen cells derived from SCID mice have been reported to induce ADCC activity against melanoma cells treated with cetuximab, though the effect is not as potent as that by parental mouse monoclonal antibodies against EGFR (26). In addition, several reports have described that the mouse effector system can induce the ADCC activity of human IgG1 antibody (55,56). Taken together, it can be concluded that mouse effector cells can bind to the Fc portion of human IgG to some extent, exerting some level of ADCC activity, if not its full activity. Second, our preliminary observation that there was no difference in survival times between mice treated with different amounts of cetuximab is strikingly similar to the dose-effector relationship of cetuximab-induced ADCC activity shown *in vitro*; that is, a cetuximab concentration in excess of 0.25 mg/ml is sufficient for maximum ADCC activity *in vitro*, and higher concentrations have no effect on the activity. Third, we used C.B-17 SCID mice, which lack mature T- and B-lymphocytes but possess robust NK cell activity (36). We have shown in a previous report that only NK cells are major effectors of cetuximab-mediated ADCC activity that is augmented by IL-2 (27). In parallel, IL-2 co-administration with cetuximab significantly inhibited MPM tumor growth in our model. Considering these data, we believe that our successful treatment of MPM in the SCID mouse model reflects cetuximab-induced ADCC activity and that future efforts to enhance this ADCC activity with effective adjuvants, such as cytokines (57), would be of vital importance.

This is the first study to investigate intrathoracic treatment by cetuximab for MPM. Because mesothelioma tends to remain localized in the pleural cavity for a long time, the development of local treatments would be promising. For this purpose, the orthotopic mouse model of MPM would be ideal for the evaluation of cetuximab, because MPM cells mimic the clinical behavior and progression of human MPM in this model (32). Local treatment with antitumor drugs offers a theoretical advantage, because the tumor is exposed directly to higher drug concentrations, while a lower incidence of toxic side effect can be expected. To date, several local treatments have been reported as successful in combination with other therapeutic modalities, such as surgery.

These local treatments include intrathoracic chemotherapy (58), chemohyperthermia (59), and intraoperative photodynamic therapy (IPDT) (60). Our proposed combination therapy using cetuximab with IL-2 is preferable for the local treatment of MPM, because IL-2 causes serious side effects such as vascular leakage syndrome and systemic immuno-suppression if administered systemically (61). Local treatment may not be expected to cure MPM patients, but the improved local control of MPM in the thoracic cavity in combination with systemic treatment could offer potential benefits for MPM patients.

In this study, cetuximab treatment significantly inhibited intrathoracic MPM tumor growth, and the addition of IL-2 enhanced this activity. However, contrary to our expectations, the combined use of cetuximab and IL-2 did not improve survival of the mice compared to cetuximab alone. There are 2 possible explanations for this result. First, the number of mice we used in each group (N=5) was not enough to detect the difference. Second, the effects on tumor growth might not directly affect the survival of the mice due to distant metastasis or the side effects of IL-2, such as cardiac failure (62). Therefore, further study is warranted to determine the cause of death in these mice and to explore more effective and less toxic combination-use of IL-2.

In conclusion, cetuximab induces ADCC activity against EGFR-expressing MPM cell lines. Near-maximum ADCC activity was observed in cells with very weak EGFR expression levels, which was detectable as faint IHC staining. ADCC activity is enhanced at any EGFR expression level in the presence of low doses of IL-2. Intrathoracic administration of cetuximab in SCID mice bearing MSTO-211H cells significantly inhibited tumor growth and prolonged survival of the mice. These observations suggest the possible use of cetuximab as a novel and effective therapeutic agent that could be used in combination therapies for patients with MPM.

Acknowledgements

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Prognostic Value of Aquired Resistance-related Molecules in Japanese Patients with NSCLC Treated with an EGFR-TKI

HIDETAKA URAMOTO[1], TADAAKI YAMADA[2], SEIJI YANO[2], NOBUYUKI KONDO[3], SEIKI HASEGAWA[3] and FUMIHIRO TANAKA[1]

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Abstract. *Background: Most patients with lung cancer experience relapse, although epidermal growth factor receptor (EGFR) of tyrosine kinase inhibitor (TKI) have an astounding effect on tumors with EGFR activating mutations. It is therefore is critical to determine the mechanism for resistance to such agents and the prognostic value of acquired resistance-related molecules to EGFR- TKI. Materials and Methods: Tumor specimens were taken from 19 matched specimens before and after treatment with gefitinib. A retrospective multi-institutional study analyzed the correlation between patient survival and acquired resistance-related molecules in non-small cell lung cancer (NSCLC) samples that possessed sensitive EGFR mutations (7 cases: exon 19 deletion, and 12 cases: exon 21 point mutation). The status of the epidermal growth factor receptor (EGFR) and KRAS genes were investigated by polymerase chain reaction (PCR)-based analyses. Real-time PCR assays were used to evaluate MET gene amplification. The expression of hepatocyte growth factor (HGF) and changes in the epithelial-mesenchymal transition (EMT) status including the expression of E-cadherin and γ-catenin as epithelial markers, and vimentin and fibronectin as mesenchymal markers, were evaluated by immunohistochemistry. Results: Eight of the gefitinib refractory tumors exhibited a secondary threonine-to-methionine mutation at codon 790 in EGFR (T790M). All of the tumors had wild type KARS gene expression. No MET amplification was detected in any of the samples. A strong expression of HGF was detected in eight of specimens at post-treatment. A change in the EMT status between pre-and post-treatment was found in five cases. The 5-year survival rate of patients with and without T790M was 86.7% and 13.3%, respectively (p=0.020). The 5-year overall survival (OS) rate for patients with overexpresion andfor those with weak expression of HGF was 75.0% and 22.2%, respectively (p=0.259). In addition, the 5-year OS rate for patients with unchanged and changed EMT status was 83.3% and 40.0%, respectively (p=0.123). Conclusion: The current results showed that the presence of T790M was associated with favorable survival. On the other hand, the patients with weak HGF expression and EMT change tended to have a poor survival. The current patients' selection might be changed by discrimination of acquired resistance-related molecules in patients with NSCLC treated with an EGFR-TKI.*

Molecular-targeted drug therapy has been promoted because the selection of patients by genetic markers can increase the therapeutic response for patients with non-small cell lung cancer (NSCLC) (1). However, despite an initial response to treatment with EGFR-TKIs in specific patients, the majority of patients eventually experience a progression of their disease (2, 3). Understanding the mechanisms of resistance to treatment can provide a method for overcoming such resistance.

Explanations for the resistance to EGFR-TKI include the T790M mutation in exon 20 of the EGFR, MET amplification, overexpression of HGF, changes in the EMT status, and others (4-8). However, few studies have investigated resistance-related genes in EGFR-TKI-resistant specimens from a translational viewpoint because of the clinical difficulty of re-biopsy. Therefore, a detailed study using matched specimens from both pre- and post-treatment is essential. This is the first comprehensive analysis of prognostic markers for molecules related the acquired resistance in such pre- and post-treatment specimens to elucidate their prognostic value.

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Key Words: Lung adenocarcinoma, prognosis, EGFR, mutation, T790M, resistance, HGF, EMT.

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Table I. *Summary of the patients exhibiting acquired resistance to gefitinib.*

Case	Gender	Age (years)[a]	Smoking status	Stage[b]	Previous chemotherapy	Response to gefitinib	TTP (days)	Survival (days)
1	M	58	Never	IIIB	Yes	PR	191	2488+
2	M	55	Never	IIIB	No	PR	174	2165
3	F	54	Never	IIIB	Yes	SD	368	2961
4	F	70	Never	IA	Yes	PR	60	1629
5	F	65	Current	IIIB	No	PR	110	2073
6	M	53	Current	IIIA	Yes	PR	352	2410
7	F	84	Never	IIB	No	PR	295	619
8	F	57	Never	IIA	No	SD	210	3568+
9	F	76	Never	IV	No	SD	221	597
10	F	85	Never	IIIA	No	CR	210	575
11	F	52	Never	IIIB	No	PR	233	2222+
12	F	87	Never	IIIA	Yes	SD	88	136+
13	F	79	Never	IIIA	No	PR	166	359
14	F	70	Never	IV	Yes	PR	773	1113+
15	M	59	Never	IV	Yes	PR	792	613
16	F	76	Never	IV	No	PR	290	1234+
17	F	62	Never	IIIB	Yes	PR	254	826
18	M	63	Never	IB	Yes	CR	1041	1258
19	F	79	Never	IV	Yes	PR	259	734

[a]At beginning of gefitinib. [b]at first presentation. TTP: Time to progression after gefitinib therapy. +Patients were alive at the time of analysis. CR: complete response, PR: partial response, SD: stable disease.

Materials and Methods

Patients and their characteristics. The characteristics of the 19 patients are listed in Table I. There were five male and 14 female patients. The tumor stage was classified according to the new TNM Classification for Lung Cancer (9). Six patients developed recurrent disease after surgery for primary tumors and three cases underwent incomplete resection. Ten patients were advanced cases. Therefore, the pathological stage was adopted for the surgical cases, and the clinical stage for the 10 non-surgical cases. All cases of diseases were controlled for gefitinib at first (Table I).

The Institutional Review Board approved informed consent for the use of the tumor tissue specimens was obtained either from the patients or from their legal guardians. All patients received 250 mg gefitinib every day. The treatment was continued until the disease progressed. Prior chemotherapy had been administrated to 10 patients. The tumor samples were collected before treatment with gefitinib from surgically resected specimens from primary tumors except for those which were from two metastatic lymph nodes. Refractory tumors were obtained from pulmonary metastases (5 cases), lymph node metastases (4 cases), skin metastasis (2 cases), pleural effusion (4 cases), primary tumors (3 cases), and liver metastasis (1 case). All of the specimens were stained with hematoxylin and eosin for the histopathological diagnosis or cytology, and were confirmed to be adenocarcinoma except for one adenosquamous carcinoma (case 14).

The objective response of the patients was evaluated using the response evaluation in solid tumors (RECIST) criteria, and Routine clinical and laboratory assessments and chest X-rays were performed biweekly and computed tomographic (CT) scans were performed one month after the start of gefitinib and every three months thereafter. Imaging studies (bone scans and brain imaging) were performed every three months after the initiation of gefitinib treatment. The response to the initial gefitinib treatment was a complete response (CR) in 2 cases, a partial response (PR) in 13 cases, and stable disease (SD) in 4 cases. The time to progression (TTP) ranged from 60 to 1041 days. The mean follow-up period from the date of administration of gefitinib to the date of death or last known contact was 1452 days, with a range from 136 to 3568 days.

Analyses of gene expression status of resistance-related molecules in sensitive and resistant tumors. Genomic DNA was extracted from each tumor and the EGFR mutations in exons 19-21 were examined by sequencing using previously described methods (10). The KRAS mutations were investigated by PCR-based analyses (11). MET gene copy numbers were determined by real-time PCR assays (12). The status of HGF was also investigated by using previously described methods (11). The EMT status was also examined using a previously described method. Briefly, immunohistochemical (IHC) staining was used to analyze the protein expression of E-cadherin and γ-catenin as epithelial markers, and vimentin and fibronectin as mesenchymal markers. The up-regulation of mesenchymal markers or down-regulation of epithelial markers in acquired samples was defined as a change in the EMT (13). Fresh malignant cells in the pleural effusion were fixed in an alcohol-based liquid (CytoRich Blue preservatives: BD Diagnostics, Burlington, USA) by thin-layer preparations followed by immunohistochemistry.

Statistical analyses. The Kaplan-Meier method was used to estimate the probability of survival, and survival differences were analyzed by using the log-rank test. Differences were considered to be statistically significant for p-values <0.05. The data were analyzed

Table II. *Summary of the gene expression status of resistance -related molecules in sensitive and resistant tumors.*

Case	Pretreated specimen[a]	Posttreated specimen[b]	EGFR[c]	T790M	KRAS	MET	*HGF*[c]	EMT[f]
1	T	Pulmonary metastasis	19/19	-/+	w/w	-/-	W/S	+
2	T	Pulmonary metastasis	19/19	-/+	w/w	-/-	W/S	+
3	T	LN	21/21	-/+	w/w	-/-	W/S	-
4	T	Liver metastasis	21/21	-/-	w/w	-/-	W/W	+
5	LN	Pleural effusion	19/19	-/+	w/w	-/-	W/ n.e	n.e
6	T	LN	19/19	-/+	w/w	-/-	W/S	-
7	T	Pleural effusion	21/21	-/-	w/w	-/-	W/ n.e	n.e
8	T	LN	21/21	-/+	w/w	-/-	W/S	-
9	T	T	19/19	-/+	w/w	-/-	W/S	-
10	T	Skin metastasis	21/21	-/-	w/w	-/-	W/W	+
11	LN	LN	21/21	-/-	w/w	-/-	S/W	-
12	T	Pleural effusion	21/21	-/-	w/w	-/-	S/W	n.e
13	T	Skin metastasis	21/21	-/-	w/w	-/-	W/S	+
14	T	T	19/19	-/+	n.e	-/-	W/W	-
15	T	Pulmonary metastasis	21/21	-/-	n.e	n.e	n.e/n.e	n.e
16	T	T	21/21	-/-	n.e	n.e	n.e/ S	n.e
17	T	Pulmonary metastasis	19/19	-/-	n.e	n.e	n.e/ W	n.e
18	T	Pulmonary metastasis	21/21	-/-	n.e	n.e	n.e/ W	n.e
19	T	Pleural effusion	21/21	-/-	n.e	n.e	n.e/n.e	n.e

A gene expression status of sensitive and resistant tumors, respectively. 19: exon19 deletion, 21: exon21 L858R, [d]w: wild-type, n.e: not evaluated. [e]W: weak, S: strong, [f]The change in EMT status from the tumor before reatment with gefitinib to the lesion after t g T: Primary lung tumor, LN: lymph node metastasis.

using the Stat View software package (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Gene expression status of resistance-related molecules in sensitive and resistant tumors. All of the tumors exhibited EGFR mutations. Twelve showed a substitution of arginine for leucine at codon 858 (L858R) in exon 21 and seven had a deletion in exon 19 of EGFR in the pre-treated with gefitinib tumors (Table II). Eight of the gefitinib refractory tumors exhibited a secondary T790M mutation, which had not been detected in the tumors before the gefitinib treatment. All of the tumors had wild-type expression of the KRAS gene at codon 12 both before and after the treatment with gefitinib. No MET amplification was detected in any of the samples. Strong expression of HGF was detected in eight of the specimens at post-treatment. A change in the EMT status between pre-and post-treatment were found in five cases.

Influence of gene expression status of resistance -related molecules on overall survival. Neither chemotherapy prior to the administration of EGFR-TKI, nor subsequent chemotherapy after treatments, was associated with any statistically significant difference in survival. The 5 year survival rate of patients with T790M and those without was 86.7% and 13.3%, respectively (p=0.020). The 5-year overall survival rate of patients with over expression and weak those with expression of HGF was 75.0% and 22.2%, respectively (p=0.259). In addition, the 5-year overall survival rate of patients with unchanged and those with changed EMT status was 83.3% and 40.0%, respectively (p=0.123) (Figure 1).

Discussion

There are complicated relationships among acquired resistance-related genes including the EGFR T790 mutation, the overexpression of HGF, and changes in the EMT status (7). However, the prognostic values of these factors remain unclear. This study uncovered three important findings.

Firstly, the presence of T790M in EGFR was associated with favorable survival. A small fraction of tumor cells harboring the T790M mutation might be enriched during the proliferation after drug treatment (14) and the germline EGFR mutation T790M was found in a family with multiple cases of NSCLC (15). Moreover, the T790M mutation in the primary tumor was found significantly more frequently in advanced tumors than in early-stage tumors (14). These phenomena suggest the growth advantage of cells carrying T790M. However, contrary to expectations, T790M may also be a useful marker for predicting a favorable prognosis in Japanese patients treated by an EGFR-TKI, which was consistent with previous data and of another group with a relatively short median follow up (16, 17), and with *in vitro* data (18). This might be due to a difference in the biological significance between the resected primary tumor

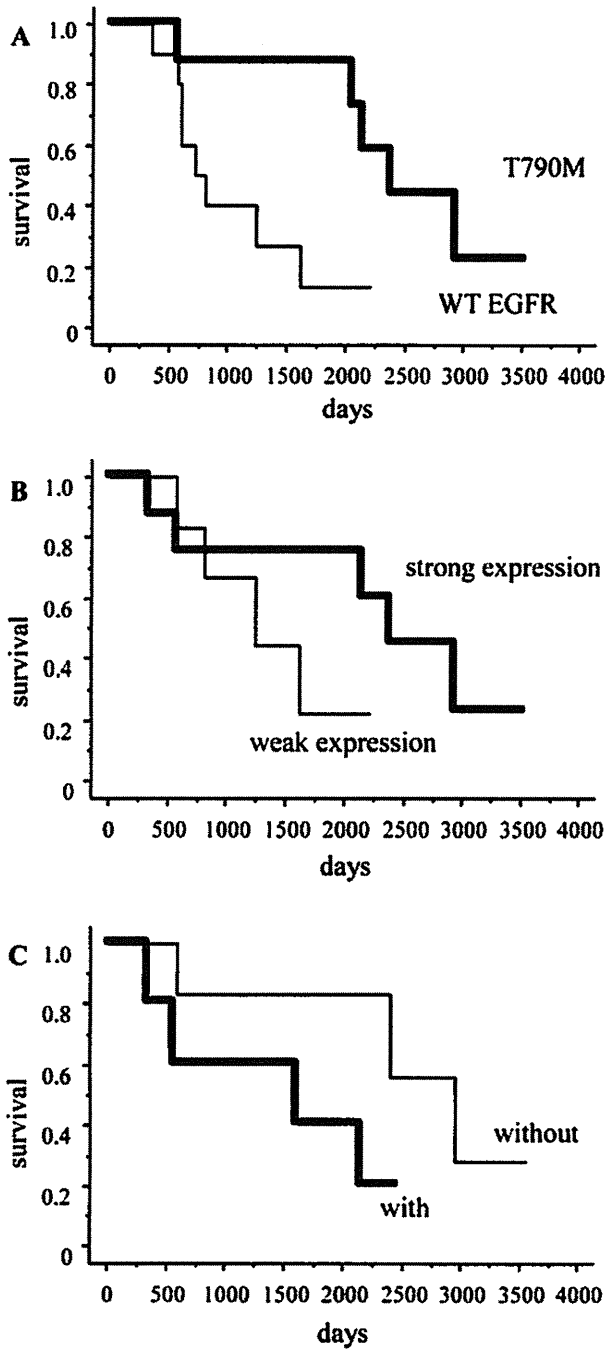

Figure 1. *Kaplan-Meier survival curves stratified according to the T790M of EGFR status (A) HGF expression (B) and EMT status (C). Survival was calculated from the date of gefitinib treatment to either the date of death or the last known contact.*

and the unresectable tumor treated by gefitinib. Most of the current post-treatment specimens were from metastatic or recurrent lesions. In fact, Molinari *et al.* reported that there are differences between primary tumors and metastases with respect to the EGFR pathway deregulation mechanisms implying a different response to EGFR targeted treatment (19). Other reasons might include the aggressive behavior in cases without T790M possessing other gene alterations independent of the EGFR mutation, leading to their poorer prognosis. Therefore, the absence of T790M after progression, likely indicates some other those of resistance mechanism, which might be associated with earlier development of new metastatic disease sites and of a poorer performance status, contributing to the shorter survival of these patients (17). The fact, disease flares sometimes develop following the discontinuation of TKI therapy (20), thus suggesting that a proportion of cells in an apparently resistant tumor cell population remain sensitive to EGFR inhibition (21).

Secondly, in this study patients with HGF overexpression had a tendency towards a more favorable prognosis than those who did not. A high pretreatment serum HGF level was associated with poor clinical outcomes in another study of patients with NSCLC treated with EGFR-TKI (22). This discrepancy could be related in the difference of sampling for specimens, such as pre- or post-treatment or the assays used for detection. In fact, the opposite survival curve was also found by analyzing pretreatment tumor-biopsy specimens for T790M mutation (23). Interestingly, five of seven tumors with T790M had HGF overexpression. On the other hand, only two of eight cases without T790M exhibited HGF overexpression. Therefore, HGF might interred with T790M in the EGFR signaling axis (8, 24). For that matter, pretreatment plasma HGF levels have no correlation with tissue immunoreactivity for HGF (25).

Thirdly, EMT changes were associated with poor survival. These findings seem to be reasonable, because the EMT is an important contributor to the invasion and metastasis of epithelial cell-derived cancer (26). Interestingly, *in vitro* studies demonstrate that benzo(a)pyrene, a chemical fumed cigarette smoke seems to induce EMT in lung cancer cells (27). In fact, nonsmokers with lung cancer have a more favorable prognosis than smokers (28).

In summary, these findings suggest that not only T790M but also HGF and a change in the EMT status might be associated with prognosis in Japanese patients treated with an EGFR-TKI. This analysis has the inherent limitations of a retrospective study and imbalances in the patients' characteristics cannot be excluded given the small number of patients with limited biopsies. Nevertheless, the results may represent an important issue, since understanding the mechanisms of treatment resistanceallows the possibility of establishing of personalization treatment.

Conflicts of interest

Dr. Uramoto and Dr. Tanaka have received research grants to belong to institute from NIPPON ZOKI, Taisho Pharmaceutical Co, Pfizer

Inc, Mitsubishi Tanabe Pharma Corporation, Bristol-Myers Squibb, Sanofi, and Chugai Pharma. Dr. Yano has received a research grant from Chugai Pharma, and lecture fees from Chugai Pharma and Astrazeneca.

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The novel phosphoinositide 3-kinase–mammalian target of rapamycin inhibitor, BEZ235, circumvents erlotinib resistance of *epidermal growth factor receptor* mutant lung cancer cells triggered by hepatocyte growth factor

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Acquired resistance to epidermal growth factor receptor–tyrosine kinase inhibitors (EGFR–TKIs), such as gefitinib and erlotinib, is a critical problem in the management of patients with *EGFR* mutant lung cancer. Several mechanisms have been reported involved in this acquired resistance, including hepatocyte growth factor (HGF) activation of an alternative pathway. PI3K and mTOR are downstream molecules of receptor tyrosine kinases, such as EGFR and Met, and are thought to be ideal targets for controlling various tumor types. We assessed whether BEZ235, a dual inhibitor of PI3K and mTOR, could overcome the EGFR–TKI resistance induced by HGF in an *EGFR* mutant lung cancer model. Exogenous and endogenous HGF triggered resistance to erlotinib in the PC-9 and HCC827, *EGFR* mutant lung cancer cell lines. BEZ235 alone inhibited the viability of PC-9 and HCC827 cells *in vitro*, irrespective of the presence or the absence of HGF. Using a xenograft model of severe combined immunodeficient mice with HGF-gene-transfected PC-9 cells (PC-9/HGF), we found that BEZ235 inhibited tumor growth, whereas erlotinib did not. BEZ235 monotherapy also inhibited the phosphorylation of Akt and p70S6K/S6RP, downstream molecules of PI3K and mTOR, respectively, as well as suppressing tumor-cell proliferation and angiogenesis of PC-9/HGF tumors. These results suggest that BEZ235, even as monotherapy, may be useful in managing HGF-induced EGFR–TKI resistance in *EGFR* mutant lung cancer.

Key words: HGF, EGFR-TKI resistance, PI3K, mTOR, *EGFR* mutation

Abbreviations: EGFR–TKI: epidermal growth factor receptor–tyrosine kinase inhibitor; HGF: hepatocyte growth factor; mTOR: mammalian target of rapamycin; PI3K: phosphoinositide 3-kinase; p70S6K: p70S6 kinase; S6RP: S6 ribosomal protein; VEGF: vascular endothelial growth factor

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

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Mutations activating epidermal growth factor receptor (EGFR), such as deletions of exon 19 and the L858R point mutation in exon 21, have been highly associated with clinical responses to the EGFR tyrosine kinase inhibitors (TKIs), gefitinib and erlotinib.[1–3] Almost all patients, however, develop acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR–TKIs) after varying periods of time.[4] Thus, there is urgent need to develop effective therapies for these patients.

Recent studies have identified the molecular mechanisms of acquired resistance to EGFR–TKIs, including (*i*) gatekeeper mutations in *EGFR*, such as the T790M second mutation[5,6]; (*ii*) activation of bypass signaling caused by *Met* amplification,[7,8] hepatocyte growth factor (HGF) overexpression[9] or Gas6-Axl activation[10]; (*iii*) activation of downstream molecules (PTEN loss or PIK3CA mutation)[11,12]; (*iv*) small-cell lung cancer transformation[13] and (*v*) epithelial-to-mesenchymal transition.[14,15] We found that HGF overexpression triggers resistance by activating the Met/PI3K/Akt pathway,[9] a mechanism that may be involved in both intrinsic and acquired resistance to EGFR–TKIs.[9] For example, HGF overexpression was detected in 29 and 61% of tumor specimens from a Japanese cohort with *EGFR* mutant lung cancers and intrinsic and acquired resistance, respectively, to EGFR–TKIs.[16] Other studies have also reported a high frequency of

What's new?

Hepatocyte growth factor (HGF) activation of the alternative Met/PI3K/Akt signaling pathway is a common mechanism underlying the development of resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in EGFR mutant lung cancer. Here, the novel PI3K and mTOR dual inhibitor, BEZ235, was found to control the growth of tumor cells with HGF-mediated resistance to the TKI erlotinib both in vitro and in vivo. The results demonstrate BEZ235's potential for the management of HGF-induced TKI resistance in EGFR mutant lung cancer.

HGF overexpression in tumors with acquired resistance.[17,18] These findings indicate that management of HGF-triggered resistance is important for more successful treatment of patients with *EGFR* mutant lung cancer.

PI3K plays crucial roles in many cellular processes, including cell proliferation, survival, differentiation, motility and angiogenesis.[19–23] PI3K mediates signals from receptor tyrosine kinases, including EGFR and Met,[24] phosphorylating Akt and activating mTOR and downstream molecules, such as p70S6K and eukaryotic translation initiation factor 4E-binding protein 1, thereby regulating cell growth and gene transcription.[20–23,25,26] As activation of the PI3K pathway has been reported in various types of tumors, including *EGFR* mutant lung cancer,[19,21–24] PI3K and mTOR are also thought to be ideal therapeutic targets. We previously reported that PI3K inhibition with PI-103, a PI3K and mTOR inhibitor that will not enter clinical trials, resensitized *EGFR* mutant lung cancer cells to EGFR–TKI, even in the presence of HGF.[27] However, PI-103 monotherapy did not inhibit growth of EGFR–TKI-resistant tumors triggered by HGF, indicating the need to develop better PI3K inhibitors for clinical use.

BEZ235 is an orally available dual inhibitor of PI3K and mTOR that is being evaluated in phase I/II trials.[22,26,28] BEZ235 reversibly inhibits the catalytic activities of Class I PI3K and mTOR kinases by competitively inhibiting ATP binding.[28] We assessed whether BEZ235 can circumvent the EGFR–TKI resistance triggered by HGF in *EGFR* mutant lung cancer cells and we analyzed the mechanisms underlying its activity.

Material and Methods

Cell culture

The *EGFR* mutant human lung adenocarcinoma cell lines PC-9[29] and HCC827[7] were purchased from Immuno-Biological Laboratories (Takasaki, Gunma, Japan) and the American Type Culture Collection (ATCC; Manassas, VA), respectively. Human HGF-gene transfectant (PC-9/HGF) and vector control (PC-9/Vec) cells were established as described previously.[9] These cell lines were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 units/mL streptomycin, and 2 mmol/L glutamine. All cells were passaged for <3 months before renewal from frozen, early-passage stocks. Cells were regularly screened for mycoplasma using a MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, ME). These cell lines were authenticated at the laboratory of the National Institute of Biomedical Innovation (Osaka, Japan) by short-tandem repeat analysis. Human dermal microvessel endothelial cells (HMVECs) were maintained in HuMedia-MvG with growth supplements (purchased from Kurabo, Osaka, Japan in October 2012) and used for *in vitro* assay at passages 2–5.

Reagents

Erlotinib, BEZ235 and PI-103 were purchased from Selleck Chemicals, Houston, TX.

Cell viability assay

Cell viability was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] dye reduction method.[30] Tumor cells (2×10^3 cells/100 μL/well) in RPMI1640 medium with 10% FBS were plated into 96-well plates and cultured with indicated compounds for 72 hr, followed by the addition of 50 μL of MTT solution (2 mg/mL; Sigma, St. Louis, MO) to each well and further incubation for 2 hr. The medium was removed, and the dark blue crystals in each well were dissolved in 100 μL dimethyl sulfoxide. The absorbance of the wells was measured with a microplate reader at test and reference wavelengths of 550 and 630 nm, respectively. Percent growth was reported relative to untreated controls. Each experiment contained at least triplicate samples and was performed at least three times.

Antibodies and Western blotting

Cells were lysed in cell lysis buffer containing a phosphatase and proteinase inhibitor cocktail (Sigma, St. Louis, MO), and the protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Total protein (40 μg) was resolved by SDS-polyacrylamide gel (Bio-Rad, Hercules, CA) electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). After four washes, the membranes were incubated with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 hr at room temperature, followed by incubation overnight at 4°C with primary antibodies (Abs) to pEGFR (Y1068), pMet (Y1234/Y1235), Met (25H2), p-Akt (Ser473), Akt, p-p70S6K, p70S6K, p-S6RP and S6RP, each in 1:1000 dilution (Cell Signaling Technology, Danvers, MA); and primary Abs to human EGFR (1 μg/mL), p-ERK1/ERK2 and ERK1/ERK2, each diluted 1/1,000 (R&D Systems, Minneapolis, MN). After washing three times, the membranes were incubated for 1 hr

at room temperature with species-specific horseradish peroxidase-conjugated secondary Abs. Immunoreactive bands were visualized using SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Each experiment was performed at least three times independently.

Xenografts in severe combined immunodeficient mice

Suspensions of PC-9/Vec and PC-9/HGF cells (3×10^6) were injected subcutaneously into the backs of 5-week-old female severe combined immunodeficient (SCID) mice (Clea, Tokyo, Japan). After 7 days, when the tumors were >5 mm in diameter, the mice were randomly allocated into groups of 6–10 each and treated with BEZ235 (20 mg/kg/day) and/or erlotinib (25 mg/kg/day) by oral gavage. Tumor volume was calculated daily, using the formula, volume (mm^3) = width^2 × length/2). All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (Approval number: AP-081088).

Histological analyses

Proliferating cells were detected by incubating tissue sections with Ki-67 Ab (Clone MIB-1; DAKO, Glostrup, Denmark). Antigen was retrieved by microwaving tissue sections in 10 mM citrate buffer (pH = 6.0). After incubation with secondary Ab and treatment with the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA), peroxidase activity was visualized using the diaminobenzidine (DAB) reaction. To analyze microvessel density, frozen sections (5 μm thick) of xenograft tumors were fixed with cold acetone and washed with PBS. After blocking endogenous peroxidase activity with 3% aqueous H_2O_2 solution for 10 min, the sections were incubated with 5% normal horse serum, washed and incubated overnight at 4°C with anti-mouse-CD31 (clone MEC13.3, BD Bioscience, San Jose, California) Ab. After washing with PBS, the sections were incubated with peroxidase-conjugated anti-

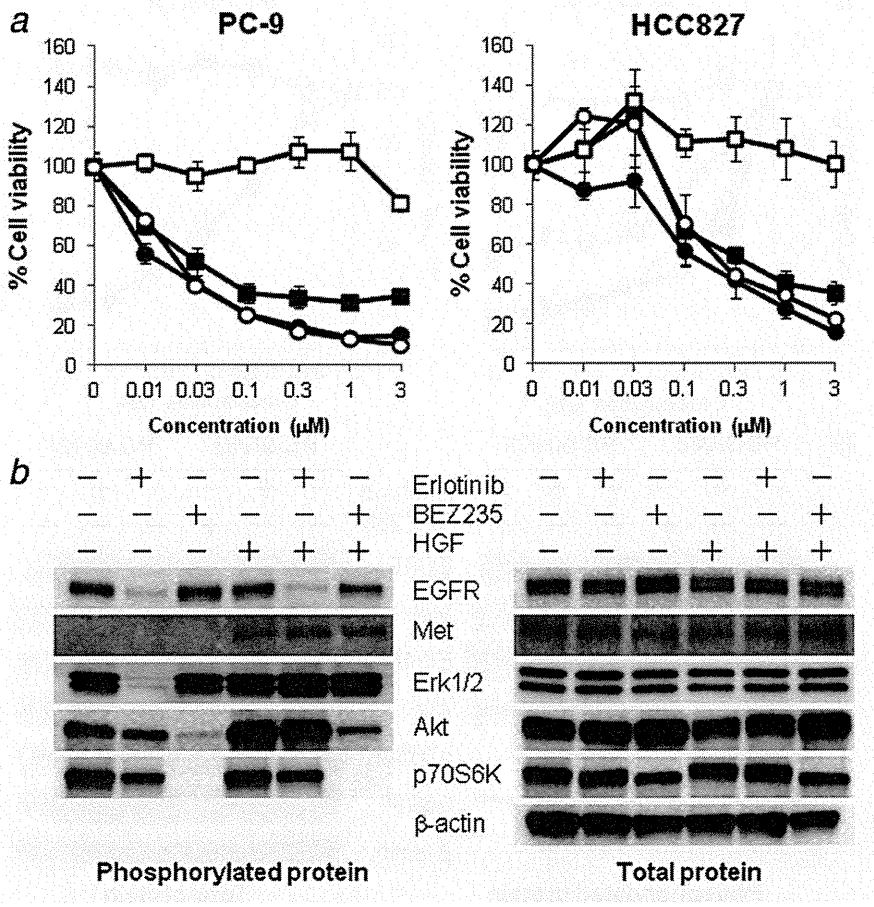

Figure 1. BEZ235 inhibited the viability of *EGFR* mutant lung cancer cells, irrespective of the presence of exogenous HGF. (*a*) PC-9 and HCC827 cells (2×10^3 cells/well) were incubated with various concentrations of drugs and/or HGF (20 ng/mL)(■: erlotinib, □: erlotinib + HGF, ●: BEZ235, ○: BEZ235 + HGF). Bars show standard deviation (SD). (*b*) PC-9 cells were treated with incubated with or without erlotinib (0.3 μM) or BEZ235 (0.3 μM) for 1 hr, followed by the addition of HGF (20 ng/mL) or PBS for 10 min. The cell lysates were harvested and subjected to Western blotting. The data shown are representative of three independent experiments with similar results.