

Figure 6. Ligand-triggered resistance to ALK inhibitors is abrogated by inhibitors of both HGF-Met and EGFR. A–C, the EGFR inhibitors erlotinib and cetuximab abrogated EGFR ligand-induced crizotinib resistance in EML4-ALK lung cancer cells. H2228 and H3122 cells were treated for 72 hours with or without crizotinib (100 nmol/L) and/or EGF (100 ng/mL), TGF- α (100 ng/mL), or HB-EGF (10 ng/mL) in the presence or absence of erlotinib (1 μ mol/L) or cetuximab (2 μ g/mL). Cell growth was determined by MTT assays. *, $P < 0.01$ (one-way ANOVA). Each experiment included triplicate determinations, and each experiment was repeated at least 3 times independently. D, Met-TKI E7050 or Anti-HGF antibody abrogated HGF-induced TAE684 resistance in EML4-ALK lung cancer cells. H2228 and H3122 cells were treated for 72 hours with or without TAE684 (100 nmol/L) and/or HGF (50 ng/mL) in the presence or absence of E7050 (1 μ mol/L) or anti-HGF-neutralizing antibody (2 μ g/mL). Cell growth was determined by MTT assays. *, $P < 0.01$ (one-way ANOVA). Each experiment included triplicate determinations, and each experiment was repeated at least 3 times independently.

Met (5). Selective ALK inhibitors are expected to be effective against *EML4-ALK* lung cancer cells, even after acquiring *ALK* amplification and *ALK* second mutations and becoming refractory to crizotinib (7, 35). Our findings, however, suggest that HGF-triggered resistance may be directed against selective ALK inhibitors, not crizotinib. Future clinical trials with selective ALK inhibitors may reveal the class of ALK inhibitors that is more beneficial for *EMK4-ALK* lung cancer patients.

EML4-ALK- and *EGFR*-mutant lung cancers show dramatic responses to ALK inhibitors and *EGFR*-TKIs, respectively (5, 36, 37). Complete responses, however, are rarely achieved, despite these cells express the target (*EML4-ALK* or mutant *EGFR*) of the drug. Low expression of BIM, a proapoptotic molecule, may explain, at least in part, the variations in sensitivity of *EGFR*-mutant lung cancer to *EGFR*-TKIs (38). This heterogeneous sensitivity may also be explained by HGF, as HGF is expressed more or less equally in *EGFR*-mutant lung tumors sensitive to *EGFR*-

TKIs (32). Therefore, *EGFR* ligands in *EML4-ALK* lung tumors may be involved in their heterogeneous response to crizotinib. It is also curious whether ligand-triggered resistance is an independent mechanism or one that provided partial resistance when combined with another mechanism. Because crizotinib is expected to be approved in Japan to treat *EML4-ALK* lung cancer in 2012, we are planning a study to assess this possibility in clinical specimens.

In conclusion, we found that receptor ligands, such as *EGFR* ligands and HGF, could cause resistance to the ALK inhibitors crizotinib and/or TAE684 by activating bypass survival signals. These ligands and growth factors may be produced by host stromal cells, which constitute the cancer microenvironment. Paracrine HGF from stromal fibroblasts may also trigger resistance to *EGFR*-TKIs in *EGFR*-mutant lung cancer cells by activating bypass signals (22). Collectively, these observations suggest that paracrine receptor activation by the microenvironment

may be an important mechanism inducing resistance to molecular targeted drugs in oncogene-activated lung cancer cells. These findings suggest that targeting of receptor ligands may result in more successful therapy in lung cancer.

Disclosure of Potential Conflicts of Interest

S. Yano received honoraria from Chugai Pharma and AstraZeneca and research fundings from Chugai Pharma and Eisai co., Ltd. H. Mano received honoraria from Pfizer Inc., and T. Nakagawa and T. Uenaka are employees of Eisai co., Ltd. The other authors disclosed no potential conflicts of interest.

Authors' Contributions

Conception and design: T. Yamada, S. Takeuchi, S. Yano

Development of methodology: T. Yamada

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Yamada, H. Mano

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Kita, T. Nakagawa, T. Uenaka

Writing, review, and/or revision of the manuscript: T. Yamada, S. Yano

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Nakade, S. Nanjo, T. Nakamura, K. Matsumoto, M. Soda, H. Mano, S. Yano
Study supervision: S. Yano

Acknowledgments

The authors thank Dr. Jeffrey A. Engelman (Massachusetts General Hospital Cancer Center) for providing the H3122 cells.

Grant Support

This study was supported by grants-in-aid for cancer research (T. Yamada, 23790902 and S. Yano, 21390256) and scientific research on innovative areas "Integrative Research on Cancer Microenvironment Network" (S. Yano, 22112010A01) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 21, 2011; revised March 28, 2012; accepted April 17, 2012; published OnlineFirst May 2, 2012.

References

- Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature* 2007;448:561-6.
- Horn L, Pao W. EML4-ALK: honing in on a new target in non-small-cell lung cancer. *J Clin Oncol* 2009;27:4232-5.
- Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, Holmes AJ, et al. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. *Clin Cancer Res* 2008;14:4275-83.
- Choi YL, Takeuchi K, Soda M, Inamura K, Togashi Y, Hatano S, et al. Identification of novel isoforms of the EML4-ALK transforming gene in non-small cell lung cancer. *Cancer Res* 2008;68:4971-6.
- Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010;363:1693-703.
- Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T, et al. ALK Lung Cancer Study Group. EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med* 2010;363:1734-9.
- Katayama R, Khan TM, Benes C, Lifshits E, Ebi H, Rivera VM, et al. Therapeutic strategies to overcome crizotinib resistance in non-small cell lung cancers harboring the fusion oncogene EML4-ALK. *Proc Natl Acad Sci U S A* 2011;108:7535-40.
- Sasaki T, Okuda K, Zheng W, Butrynski J, Capelletti M, Wang L, et al. The neuroblastoma-associated F1174L ALK mutation causes resistance to an ALK kinase inhibitor in ALK-translocated cancers. *Cancer Res* 2010;70:10038-43.
- Doebbele RC, Pilling AB, Aisner DL, Kutateladze TG, Le AT, Weickhardt AJ, et al. Mechanisms of resistance to crizotinib in patients with ALK gene rearranged non-small cell lung cancer. *Clin Cancer Res* 2012;18:1472-82.
- Katayama R, Shaw AT, Khan TM, Mino-Kenudson M, Solomon BJ, Halmos B, et al. Mechanisms of acquired crizotinib resistance in ALK-rearranged lung Cancers. *Sci Transl Med* 2012;4:120ra17.
- Sasaki T, Koivunen J, Ogino A, Yanagita M, Nikiforow S, Zheng W, et al. A novel ALK secondary mutation and EGFR signaling cause resistance to ALK kinase inhibitors. *Cancer Res* 2011;71:6051-60.
- Heuckmann JM, Hölzel M, Sos ML, Heynck S, Balke-Want H, Koker M, et al. ALK mutations conferring differential resistance to structurally diverse ALK inhibitors. *Clin Cancer Res* 2011;17:7394-401.
- McAllister SS, Weinberg RA. Tumor-host interactions: a far-reaching relationship. *J Clin Oncol* 2010;28:4022-8.
- Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. *Nat Rev Cancer* 2009;9:239-52.
- Seruga B, Zhang H, Bernstein LJ, Tannock IF. Cytokines and their relationship to the symptoms and outcome of cancer. *Nat Rev Cancer* 2008;8:887-99.
- Janku F, Stewart DJ, Kurzrock R. Targeted therapy in non-small-cell lung cancer—is it becoming a reality? *Nat Rev Clin Oncol* 2010;7:401-14.
- Yasumoto K, Yamada T, Kawashima A, Wang W, Li Q, Donev IS, et al. The EGFR ligands amphiregulin and heparin-binding egf-like growth factor promote peritoneal carcinomatosis in CXCR4-expressing gastric cancer. *Clin Cancer Res* 2011;17:3619-30.
- Matsumoto K, Nakamura T. Hepatocyte growth factor and the Met system as a mediator of tumor-stromal interactions. *Int J Cancer* 2006;119:477-83.
- Masuya D, Huang C, Liu D, Nakashima T, Kameyama K, Haba R, et al. The tumour-stromal interaction between intratumoral c-Met and stromal hepatocyte growth factor associated with tumour growth and prognosis in non-small-cell lung cancer patients. *Br J Cancer* 2004;90:1555-62.
- Meert AP, Martin B, Delmotte P, Berghmans T, Lafitte JJ, Mascaux C, et al. The role of EGF-R expression on patient survival in lung cancer: a systematic review with meta-analysis. *Eur Respir J* 2002;20:975-81.
- Yamada T, Matsumoto K, Wang W, Li Q, Nishioka Y, Sekido Y, et al. Hepatocyte growth factor reduces susceptibility to an irreversible epidermal growth factor receptor inhibitor in EGFR-T790M mutant lung cancer. *Clin Cancer Res* 2010;16:174-83.
- Wang W, Li Q, Yamada T, Matsumoto K, Matsumoto I, Oda M, et al. Crosstalk to stromal fibroblasts induces resistance of lung cancer to epidermal growth factor receptor tyrosine kinase inhibitors. *Clin Cancer Res* 2009;15:6630-8.
- Soda M, Takada S, Takeuchi K, Choi YL, Enomoto M, Ueno T, et al. A mouse model for EML4-ALK-positive lung cancer. *Proc Natl Acad Sci U S A* 2008;105:19893-7.
- Nakamura Y, Azuma M, Okano Y, Sano T, Takahashi T, Ohmoto Y, et al. Upregulatory effects of interleukin-4 and interleukin-13 but not interleukin-10 on granulocyte/macrophage colony-stimulating factor production by human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1996;15:680-7.
- Koren HS, Anderson SJ, Larrick JW. *In vitro* activation of a human macrophage-like cell line. *Nature* 1979;279:328-31.
- Kuniyasu H, Yano S, Sasaki T, Sasahira T, Sone S, Ohmori H. Colon cancer cell-derived high mobility group 1/amphotericin induces growth inhibition and apoptosis in macrophages. *Am J Pathol* 2005;166:751-60.
- Nakagawa T, Tohyama O, Yamaguchi A, Matsushima T, Takahashi K, Funasaka S, et al. E7050: a dual c-Met and VEGFR-2 tyrosine kinase

- inhibitor promotes tumor regression and prolongs survival in mouse xenograft models. *Cancer Sci* 2010;101:210–5.
28. Montesano R, Matsumoto K, Nakamura T, Orci L. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* 1991;67:901–8.
 29. Matsumoto K, Nakamura T, Sakai K, Nakamura T. Hepatocyte growth factor and Met in tumor biology and therapeutic approach with NK4. *Proteomics* 2008;8:3360–70.
 30. Yano S, Wang W, Li Q, Matsumoto K, Sakurama H, Nakamura T, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 2008;68:9479–87.
 31. Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer Cell* 2010;17:77–88.
 32. Yano S, Yamada T, Takeuchi S, Tachibana K, Minami Y, Yatabe Y, et al. Hepatocyte growth factor expression in *EGFR* mutant lung cancer with intrinsic and acquired resistance to tyrosine kinase inhibitors in a Japanese cohort. *J Thorac Oncol* 2011;6:2011–7.
 33. Stabile LP, Rothstein ME, Keohavong P, Lenzner D, Land SR, Gaither-Davis AL, et al. Targeting of both the c-Met and EGFR pathways results in additive inhibition of lung tumorigenesis in transgenic mice. *Cancers (Basel)* 2010;2:2153–70.
 34. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316:1039–43.
 35. Sakamoto H, Tsukaguchi T, Hiroshima S, Kodama T, Kobayashi T, Fukami TA, et al. CH5424802, a selective ALK inhibitor capable of blocking the resistant gatekeeper mutant. *Cancer Cell* 2011;19:679–90.
 36. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Sobue H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 2010;362:2380–8.
 37. Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. West Japan Oncology Group. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* 2010;11:121–8.
 38. Faber AC, Corcoran RB, Ebi H, Sequist LV, Waitman BA, Chung E, et al. BIM expression in treatment-naïve cancers predicts responsiveness to kinase inhibitors. *Cancer Discovery* 2011;1:352–65.

Combined Therapy with Mutant-Selective EGFR Inhibitor and Met Kinase Inhibitor for Overcoming Erlotinib Resistance in EGFR-Mutant Lung Cancer

Takayuki Nakagawa^{1,5}, Shinji Takeuchi¹, Tadaaki Yamada¹, Shigeki Nanjo¹, Daisuke Ishikawa¹, Takako Sano¹, Kenji Kita¹, Takahiro Nakamura², Kunio Matsumoto², Kenichi Suda³, Tetsuya Mitsudomi³, Yoshitaka Sekido⁴, Toshimitsu Uenaka⁵, and Seiji Yano¹

Abstract

Although the EGF receptor tyrosine kinase inhibitors (EGFR-TKI) erlotinib and gefitinib have shown dramatic effects against *EGFR* mutant lung cancer, patients become resistant by various mechanisms, including gatekeeper *EGFR*-T790M mutation, *Met* amplification, and HGF overexpression, thereafter relapsing. Thus, it is urgent to develop novel agents to overcome EGFR-TKI resistance. We have tested the effects of the mutant-selective EGFR-TKI WZ4002 and the mutant-selective Met-TKI E7050 on 3 *EGFR* mutant lung cancer cell lines resistant to erlotinib by different mechanisms: PC-9/HGF cells with an exon 19 deletion, H1975 with an L858R mutation, and HCC827ER with an exon 19 deletion, with acquired resistance to erlotinib because of HGF gene transfection, gatekeeper T790M mutation, and *Met* amplification, respectively. WZ4002 inhibited the growth of H1975 cells with a gatekeeper T790M mutation, but did not inhibit the growth of HCC827ER and PC-9/HGF cells. HGF triggered the resistance of H1975 cells to WZ4002, whereas E7050 sensitized HCC827ER, PC-9/HGF, and HGF-treated H1975 cells to WZ4002, inhibiting EGFR and Met phosphorylation and their downstream molecules. Combined treatment potently inhibited the growth of tumors induced in severe-combined immunodeficient mice by H1975, HCC827ER, and PC-9/HGF cells, without any marked adverse events. These therapeutic effects were associated with the inhibition of EGFR and Met phosphorylation *in vivo*. The combination of a mutant-selective EGFR-TKI and a Met-TKI was effective in suppressing the growth of erlotinib-resistant tumors caused by gatekeeper T790M mutation, *Met* amplification, and HGF overexpression. Further evaluations in clinical trials are warranted. *Mol Cancer Ther*; 11(10); 2149–57. ©2012 AACR.

Introduction

The EGF receptor tyrosine kinase inhibitors (EGFR-TKI) gefitinib and erlotinib have shown marked therapeutic effects against non-small cell lung cancer (NSCLC) with *EGFR* activating mutations, such as exon 19 deletions and L858R point mutations (1). Almost all tumors, however, acquire resistance to EGFR-TKIs after varying periods of time. Among the molecular mechanisms of this acquired resistance to EGFR-TKIs are gatekeeper mutations in *EGFR* (i.e., a T790M second mutation) and bypass

signaling caused by *Met* amplification or hepatocyte growth factor (HGF) overexpression (2–5). In addition, *PIK3CA* mutations and transformation to SCLC have been found to contribute to EGFR-TKI resistance in a subpopulation of tumors (6). All of these alterations have been detected in clinical specimens from patients with *EGFR* mutant lung cancer who became resistant to EGFR-TKIs (6, 7), indicating the urgent need to develop effective therapies for these patients.

Several strategies have been proposed to overcome T790M-mediated resistance, including treatment with BIBW2992, an irreversible EGFR-TKI, and an anti-EGFR antibody (8), and treatment with Hsp90 inhibitors (9) and mutant-selective EGFR-TKIs (10). Mutant-selective EGFR-TKIs have shown activity not only against tumors harboring exon19 deletions and the L858R mutation, but against tumors with the T790M resistance mutation. In preclinical models, the combination of a Met-TKI and an EGFR-TKI was shown effective in overcoming resistance caused by *Met* amplification (11). Among the Met-TKIs being evaluated in clinical trials is crizotinib, which also inhibits ALK and has been approved by the U.S. Food and Drug Administration to treat patients with *EML4-ALK* lung cancer (12). In addition, a phase I clinical trial of

Authors' Affiliations: Divisions of ¹Medical Oncology and ²Tumor Dynamics and Regulation, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa; ³Departments of Thoracic Surgery, Aichi Cancer Center Hospital; ⁴Division of Molecular Oncology, Aichi Cancer Center Research Institute, Nagoya, Aichi; and ⁵Tsukuba Research Laboratories, Eisai Co., Ltd., Ibaraki, Japan

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Corresponding Author: Seiji Yano, Division of Medical Oncology, Cancer Research Institute, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-0934, Japan. Phone: 81-76-265-2780; Fax: 81-76-234-4524; E-mail: syano@staff.kanazawa-u.ac.jp

doi: 10.1158/1535-7163.MCT-12-0195

©2012 American Association for Cancer Research.

E7050, a dual inhibitor of Met and VEGF receptor 2 (VEGFR)-2, was recently completed (13).

Although the combination of a mutant-selective EGFR-TKI and a Met-TKI can theoretically overcome the resistance caused by all 3 mechanisms, this effect has not yet been assessed. We therefore analyzed whether the combination of the mutant-selective EGFR-TKI WZ4002 and the Met-TKI E7050 could inhibit the growth of cells with the 3 types of EGFR-TKI resistance mechanisms.

Materials and Methods

Cell lines and reagents

PC-9 cells, an *EGFR* mutant human lung adenocarcinoma cell line, were obtained from Immunobiological Laboratories Co., Ltd. The HCC827ER (14) cell line was kindly provided by Dr. Tetsuya Mitsudomi (Aichi Cancer Center Hospital); and the H1975 cell line was kindly provided by Dr. John D. Minna (University of Texas Southwestern Medical Center, Dallas, TX). HCC827 cells were obtained from the American Type Culture Collection. All cell lines were maintained in RPMI1640 medium supplemented with 10% FBS, 100 units/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L L-glutamine. The characteristics of these cell lines are summarized in Table 1. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks. Cells were regularly screened for *Mycoplasma* using the MycoAlert Mycoplasma Detection Kit (Lonza Rockland Inc). The cell lines were authenticated at the laboratory of the National Institute of Biomedical Innovation by short-tandem repeat analysis. WZ4002 was purchased from Selleck Chemicals; erlotinib hydrochloride was obtained from Biovision Inc; and E7050 was obtained from Eisai Co., Ltd. Recombinant HGF was prepared as described (15). The chemical structures of WZ4002 and E7050 are shown in Fig. 1B.

HGF gene transfection

One day before transfection, aliquots of 1×10^5 PC-9 cells in 1 mL of antibiotic-free medium were added to each well of a 6-well plate. The cells were subsequently transfected with full-length *HGF* cDNA cloned into the BCMGSneo expression vector (16) using Lipofectamine

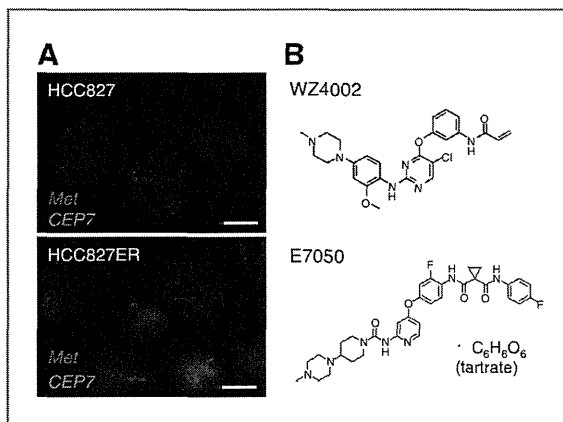


Figure 1. Characteristics of the cell lines used in this study. A, representative interphase FISH analysis of HCC827 and erlotinib-resistant HCC827ER cancer cell lines. The red signal indicates the *Met* gene, and the green signal indicates the *CEP7* (control) gene. *Met* is amplified in HCC827ER but not in HCC827 cells. Bar, 10 µm. B, chemical structures of WZ4002 and E7050.

2000, in accordance with the manufacturer's instructions. After incubation for 24 hours, the cells were washed with PBS and incubated for an additional 72 hours in antibiotic-containing medium, followed by selection in G418 sulfate (Calbiochem). After limiting dilution, an HGF-producing cell line, PC-9/HGF, was established.

HGF production

Cells (1×10^5) were cultured in RPMI-1640 medium containing 10% FBS for 24 hours, washed with PBS, and incubated for 24 hours in 1 mL of RPMI1640 medium containing 10% FBS. The culture medium was harvested and centrifuged, and the supernatant was stored at -80°C until analysis. HGF concentrations were measured by IMMUNIS HGF EIA (Institute of Immunology, Tokyo, Japan) as recommended by the manufacturer. All samples were assayed in duplicate. Color intensity was measured at 450 nm using a spectrophotometric plate reader. HGF concentrations were determined by comparison with a standard curve. The limit of detection was 30 pg/mL.

Table 1. Genetic status of EGFR and Met and HGF expression in the culture medium of each cell line

Cell line	EGFR mutation	Met amplification	HGF production (ng/ 10^5 cells/24 h)
PC-9	E746_A750del	–	<0.03
PC-9/Vec	E746 A750del	–	<0.03
PC-9/HGF#4	E746 A750del	–	1.57 ± 0.07
PC-9/HGF#5	E746 A750del	–	103.64 ± 4.73
HCC827	E746 A750del	–	<0.03
HCC827ER	E746 A750del	+	<0.03
H1975	L858R/T790M	–	<0.03

Cell proliferation assay

Cell growth was measured using the MTT dye reduction method (17). Tumor cells (2×10^3 cells/100 μL /well) were plated into each well of 96-well plates in RPMI-1640 medium containing 10% FBS. After incubation for 24 hours, various reagents were added to each well, and the plates were incubated for an additional 72 hours. To each well was added 50 μL of MTT (2 mg/mL; Sigma), and incubation was continued for a further 2 hours. The media containing MTT solution were removed, and the dark blue crystals in each well were dissolved in 100 μL dimethyl sulfoxide. The absorbance of each well at 570 nm was measured with a microplate reader, with the percentage growth shown relative to untreated controls.

Western blot analysis

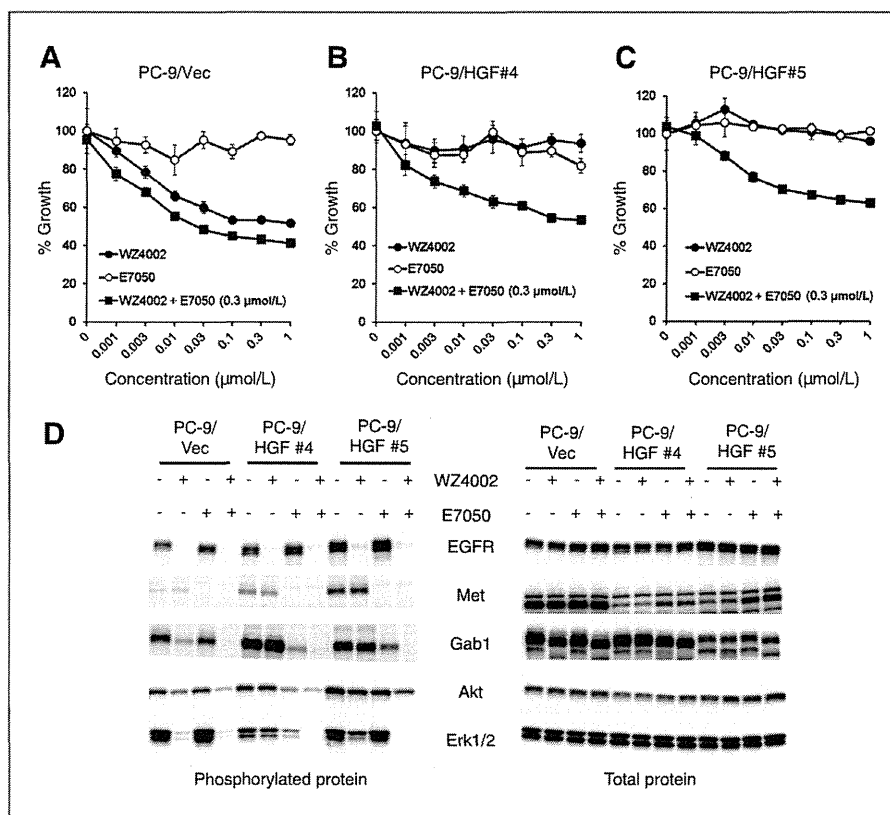
Culture cells and subcutaneous tumors were lysed in cell lysis buffer containing phosphatase inhibitor cocktail and proteinase inhibitor cocktail (Sigma), and the protein concentrations were determined using the BCA Protein Assay Kit (Pierce Biotechnology). Total protein (20–40 μg) was subjected to SDS-PAGE (Bio-Rad) under reducing conditions and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with TBS containing 0.05% Tween-20 and either 5% skim milk or 5% bovine serum albumin and incubated with

antibodies to phospho-Met (Y1234/Y1235; D26), phospho-EGFR (Y1068), ErbB3 (1B2), phospho-ErbB3 (Tyr1289; 21D3), Gab1, phospho-Gab1 (Y627; C32H2), Akt, and phospho-Akt (Ser473; Cell Signaling Technology); Met (C-28; Santa Cruz Biotechnology Inc); and phospho-Erk1/2 (T202/Y204), Erk1/2, and EGFR (R&D Systems). After washing 3 times, the membranes were incubated for 1 hour at room temperature with species-specific horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce Biotechnology). Each experiment was carried out at least 3 times independently.

Subcutaneous xenograft models

Severe-combined immunodeficient (SCID) mice (C.B-17/1cr-scid/scid Jcl, male, 5–6 weeks old) were obtained from CLEA Japan Inc. Cultured tumor cells (3×10^6 cells/0.1 mL/head) were implanted subcutaneously into the flanks of mice. When tumor volumes reached 100 to 200 mm^3 , the mice were randomized and treated once daily with 25 mg/kg of WZ4002 and/or E7050, doses that inhibit the phosphorylation of EGFR and Met, respectively, in mouse models (9, 12). Each tumor was measured in 2 dimensions, and the volume was calculated using the formula: tumor volume (mm^3) = $1/2 \times \text{length (mm)} \times$

Figure 2. HGF triggers resistance to mutant-selective EGFR in NSCLC cell lines harboring *EGFR*-activating mutations. PC-9/Vec (A), PC-9/HGF#4 (B), and PC-9/HGF#5 (C) cells (2×10^3 cells per well) were incubated with various concentrations of WZ4002, with or without E7050, for 72 hours. Cell growth was determined by the MTT assay. D, PC-9/Vec, PC-9/HGF#4, and PC-9/HGF#5 cells were incubated with WZ4002 (0.3 $\mu\text{mol/L}$) and/or E7050 (0.3 $\mu\text{mol/L}$) for 1 hour. The cell lysates were harvested and the phosphorylation of indicated proteins was determined by Western blot analysis.



width (mm)². All animal experiments were complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval No. AP-081088).

Immunohistochemical staining

Sections of 5 μm thickness were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. After antigen retrieval, endogenous peroxidase activity was blocked by incubation in 3% aqueous H₂O₂ for 10 min. Following treatment with 5% normal horse serum, the sections were incubated with primary antibodies to phospho-Met (Y1234/Y1235), phospho-EGFR (Y1068; Cell Signaling Technology), Ki-67 (MIB-1; Dako Cytomation), and mouse CD31 (MEC13.3; BD Pharmingen). After probing with species-specific biotinylated secondary antibodies, the sections were incubated for 30 minutes with avidin-biotinylated peroxidase complex (ABC) using a Vectastain ABC kit (Vector Laboratories). The DAB (3,3'-diaminobenzidine tetrahydrochloride) Liquid System (Dako Cytomation) was used to detect immunostaining.

Statistical analysis

Between group differences were analyzed by one-way ANOVA. All statistical analyses were carried out using GraphPad Prism Ver. 4.01 (GraphPad Software, Inc), with $P < 0.05$ considered statistically significant.

Results

HGF triggers resistance to mutant-selective EGFR-TKI WZ4002

PC-9 cells, a human lung cancer cell line with an *EGFR* exon 19 deletion and highly sensitive to gefitinib and erlotinib (18), were transfected with a human *HGF* gene, yielding the human HGF overexpressing clones (Table 1), PC-9/HGF#4 and PC-9/HGF#5. Although PC-9/Vec cells, a vector control, were sensitive to EGFR-TKIs, such as erlotinib and WZ4002 (Fig. 2A, Supplementary Fig. S1A), PC-9/HGF cells were resistant to both (Fig. 2B and C, Supplementary Fig. S1B and S1C).

E7050 is an orally bioavailable small molecule that inhibits Met and VEGFR-2 activity (13). Although E7050 alone did not affect the viability of PC-9/Vec and PC-9/HGF cells (Fig. 2A–C), E7050 sensitized PC-9/HGF cells to WZ4002 (Fig. 2B and C). In assessing the effects of HGF overexpression on tumor cell migration, we found that HGF overexpression enhanced the migration activity of PC-9 cells, whereas WZ4002 had no effect against the motility of PC-9/Vec and PC-9/HGF#5 cells. In contrast E7050 inhibited the migration of PC-9/HGF#5 cells induced by HGF overexpression (Supplementary Fig. S2).

In exploring signal transduction status by Western blot analysis, we found that WZ4002 inhibited EGFR phosphorylation in PC-9/Vec cells, suppressing the phosphorylation of Erk and Akt (Fig. 2D). Although Met protein expression was downregulated in HGF overexpressing

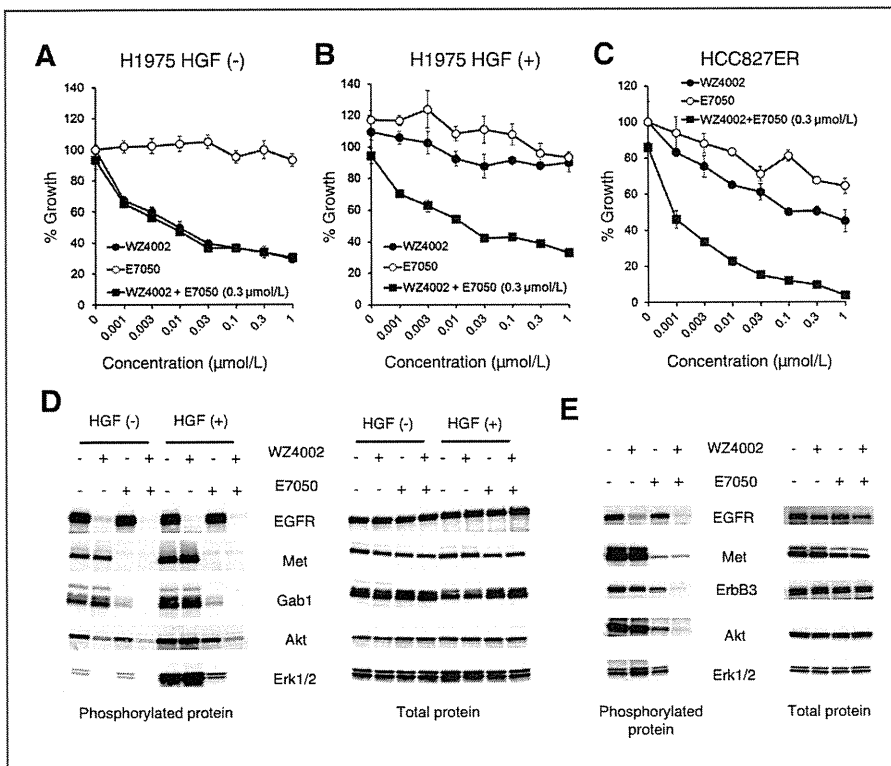


Figure 3. The combination of a mutant-selective EGFR-TKI and a Met inhibitor overcomes several erlotinib-resistant mechanisms. H1975 cells (2×10^3 cells per well) were incubated with various concentrations of erlotinib, with or without E7050, in the absence (A) or presence (B) of HGF (40 ng/mL). C, HCC827ER cells (2×10^3 cells per well) were incubated with various concentrations of WZ4002 with or without E7050 for 72 hours. Cell growth was determined by the MTT assay. D, H1975 cells were incubated with WZ4002 (0.3 $\mu\text{mol/L}$) and/or E7050 (0.3 $\mu\text{mol/L}$) for 1 hour. After stimulation with HGF (40 ng/mL) for 10 minutes, the cell lysates were harvested and the phosphorylation of indicated proteins was determined by Western blot analysis. E, HCC827ER cells were incubated with WZ4002 (0.3 $\mu\text{mol/L}$) and/or E7050 (0.3 $\mu\text{mol/L}$) for 1 hour. The cell lysates were harvested and the phosphorylation of the indicated proteins was determined by Western blot analysis.

PC-9/HGF cells, Met was more highly phosphorylated in these cells than in PC-9/Vec cells, presumably because of the overexpression of HGF. In contrast to the results observed in PC-9/Vec cells, WZ4002 inhibited EGFR, but not Erk and Akt phosphorylation in PC-9/HGF cells, whereas the combination of WZ4002 and E7050 inhibited the phosphorylation of Erk and Akt in these cells. Moreover, these alterations in phosphorylation persisted for at least 72 hours (Supplementary Fig. S3). These effects of WZ4002 and/or E7050 were apparently cytostatic, because treatment with these agents did not reduce cell numbers or activate apoptotic signals (Supplementary Fig. S4). Consistent with our previous reports (4, 19), these results suggest that HGF activates the Met–Akt pathway and triggers resistance to the mutant-selective EGFR inhibitor WZ4002 in EGFR-mutant lung tumors. Furthermore, its combination with the Met-TKI E7050 resensitizes these cells to WZ4002.

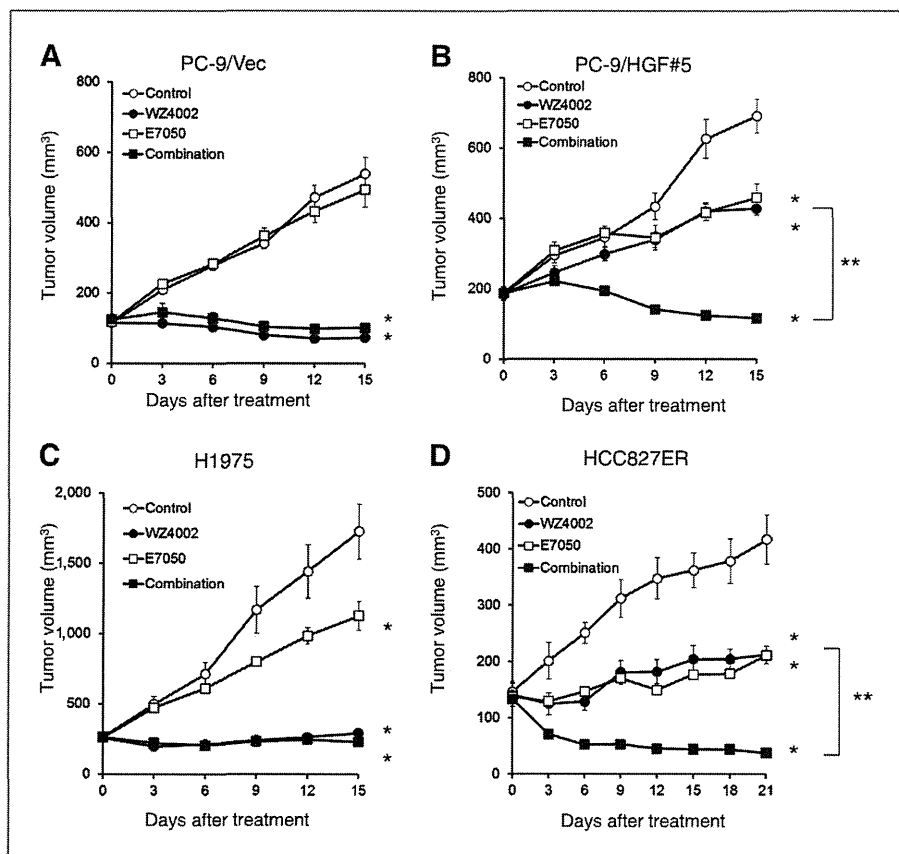
Combined use of WZ4002 and E7050 sensitizes EGFR mutant lung cancer cells with an EGFR-T790M secondary mutation or Met amplification

H1975 is a human lung cancer cell line with mutations in EGFR exons 21 (L858R) and 20 (T790M) and is refractory to gefitinib and erlotinib (20). We found that H1975 cells were also sensitive to the mutant-selective EGFR-TKI

WZ4002 (Fig. 3A, Supplementary Table S1), whereas exogenously added HGF (40 ng/mL) triggered resistance to WZ4002 (Fig. 3B, Supplementary Table S1). This HGF-triggered resistance was totally abrogated by E7050 (Fig. 3B, Supplementary Table S1). Western blot analysis showed that WZ4002 inhibited EGFR phosphorylation, thereby suppressing the phosphorylation of Akt and Erk. HGF did not restore EGFR phosphorylation, but did restore and enhance Akt and Erk phosphorylation (Fig. 3D). E7050 inhibited HGF-induced Akt and Erk phosphorylation. The combination of E7050 and WZ4002 inhibited the phosphorylation of EGFR and Met even in the presence of HGF, thereby diminishing the phosphorylation of Akt and Erk (Fig. 3D). These results indicate that HGF can induce resistance of EGFR mutant lung cancer cells with the T790M gatekeeper mutation to mutant-selective EGFR-TKIs, as well as to reversible EGFR-TKIs, by activating the Met–Akt pathway, and that E7050 can resensitize these cells to WZ4002 by inhibiting Met activation induced by HGF.

Met amplification has also been associated with acquired resistance to EGFR-TKIs in EGFR mutant lung cancer (3). HCC827ER cells, which were established after continuous exposure to erlotinib, showed amplification of Met and were resistant to erlotinib (Fig. 1A, Supplementary Fig. S1E, Supplementary Table S1). Both WZ4002 and

Figure 4. Antitumor activity of WZ4002 and/or E7050 in mouse xenograft models of human tumors. SCID mice-bearing PC-9/Vec (A), PC-9/HGF#5 (B), H1975 (C), or HCC827ER (D) tumors were administered 25 mg/kg WZ4002 and/or E7050 once daily for 14 to 21 days. Tumor volume was measured using calipers on the indicated days. Mean \pm SE tumor volumes are shown for groups of 4 to 5 mice. *, $P < 0.05$ versus control; **, $P < 0.05$ versus WZ4002 by one-way ANOVA.



E7050 inhibited the viability of HCC827ER cells, but their effects were marginal (Fig. 3C). In the presence of E7050 (0.3 $\mu\text{mol/L}$), WZ4002 inhibited the viability of HCC827ER cells in a dose-dependent manner. Western blot analysis revealed that, although WZ4002 did not alter the phosphorylation status of ErbB3, Erk, and Akt, E7050 slightly inhibited the phosphorylation of ErbB3 and Akt compared with controls, and the combination of WZ4002 and E7050 markedly inhibited the phosphorylation of ErbB3, Akt, and Erk (Fig. 3E). These results indicate that this combination successfully inhibited the phosphorylation of EGFR and Met, thereby suppressing downstream ErbB3/Akt signals and resensitizing HCC827ER cells. These *in vitro* data suggest that combined treatment with a mutant-selective EGFR-TKI and a Met-TKI may overcome erlotinib resistance caused by an EGFR-T790M secondary mutation, Met amplification and HGF overexpression.

Combined treatment with WZ4002 and E7050 overcomes various resistance mechanisms *in vivo*

We assessed the efficacy of the combination of WZ4002 and E7050 in 3 resistance models. In the HGF-induced resistance model, we compared the sensitivity of tumors established by PC-9/Vec and PC-9/HGF#5 cells. Treatment with WZ4002 completely inhibited the growth of PC-9/Vec tumors, whereas treatment with E7050 did not (Fig. 4A). Treatment with either agent alone slightly suppressed the growth of PC-9/

HGF#5 tumors (Fig. 4B). Importantly, the combination of WZ4002 and E7050 reduced the size of PC-9/HGF#5 tumors (Fig. 4B). These results suggest that HGF can induce resistance to WZ4002 *in vivo* and that this resistance can be overcome by E7050.

In the second model, the growth of tumors induced by H1975 cells, which carry a T790M second mutation, was inhibited by WZ4002 (Fig. 4C), but not erlotinib (Supplementary Fig. S2F). E7050 also slightly inhibited the growth of H1975 tumors, whereas the combination of E7050 and WZ4002 did not further inhibit tumor growth in this model. These results suggest that WZ4002 monotherapy may be sufficient to overcome erlotinib resistance induced by the T790M second mutation, as reported previously (10).

In the third model, in which resistance was induced by Met amplification, monotherapy with either WZ4002 or E7050 partially inhibited the growth of HCC827ER tumors (Fig. 4D), consistent with our *in vitro* findings (Fig. 3C), whereas combined treatment markedly reduced tumor size (Fig. 4D). During treatment with WZ4002 or E7050, either alone or in combination, there were no macroscopic changes or evidence of severe loss in body weight (Supplementary Fig. S5).

Collectively, these *in vivo* data suggest that the combination of a mutant-selective EGFR-TKI and a Met-TKI may overcome erlotinib resistance caused by a T790M second mutation, Met amplification or HGF overexpression.

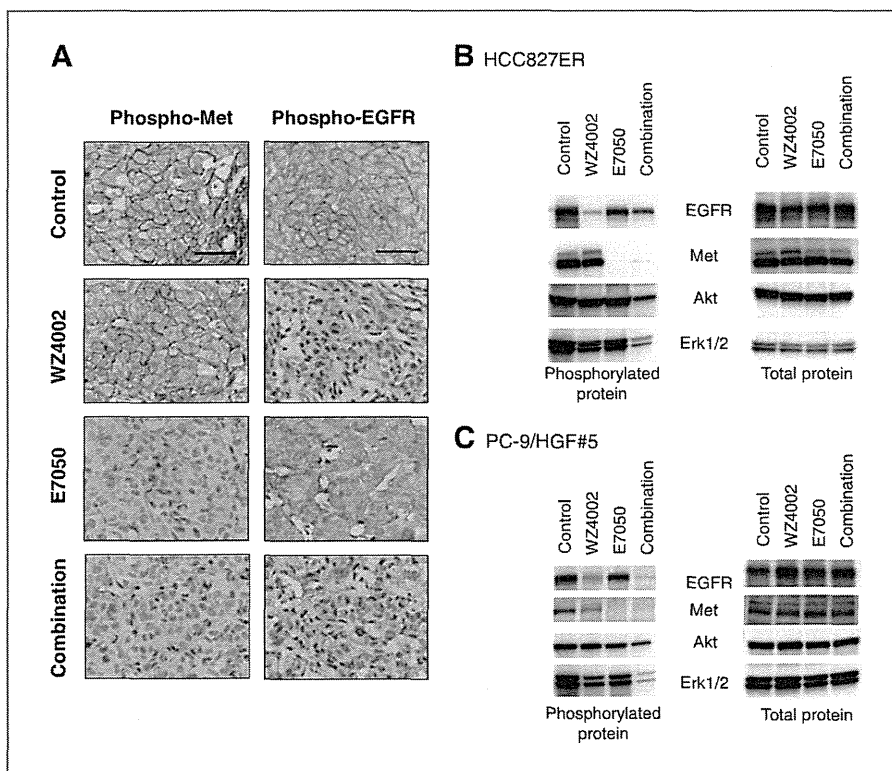


Figure 5. Effect of WZ4002 with or without E7050 on erlotinib-resistant tumors. A, representative images of HCC827ER tumors immunohistochemically stained with antibodies to phosphorylated Met and phosphorylated EGFR. Bar, 50 μm . B and C, HCC827ER (B) and PC-9/HGF#5 (C) tumors were resected from the mice 4 hours after administration of WZ4002 and/or E7050 (25 mg/kg each), and the relative levels of proteins in the tumor lysates were determined by Western blot analysis.

Therapeutic effects of WZ4002 and E7050 are associated with the inhibition of EGFR and Met phosphorylation *in vivo*

To evaluate the effects of combined treatment with WZ4002 and E7050 on resistance caused by *Met* amplification and HGF overexpression, we analyzed the phosphorylation status of target molecules in the tumors. Immunohistochemical analysis showed that Met and EGFR were phosphorylated in the plasma membranes of HCC827ER cells (Fig. 5A). Treatment with WZ4002 or E7050 inhibited the phosphorylation of EGFR or Met, respectively, whereas combined treatment inhibited the phosphorylation both (Fig. 5A), a result confirmed by Western blot analysis (Fig. 5B). Western blot analysis of downstream signals showed that combined treatment with WZ4002 and E7050 showed greater inhibition of Erk and Akt phosphorylation than observed in control cells or cells treated with each agent alone, even *in vivo* (Fig. 5B). We observed similar findings in PC-9/HGF#5 tumors (Fig. 5C). These results indicate that the antitumor effects of WZ4002 and E7050 are associated with the inhibition of both EGFR and Met phosphorylation, even *in vivo*.

We further evaluated tumor cell proliferation by Ki-67 staining. We observed active cell proliferation in HCC827ER tumors, with a 52% proliferation index (Fig. 6A and B). Monotherapy with WZ4002 or E7050 slightly decreased the percentage of Ki-67-positive proliferating tumor cells, with proliferation indices of 45% and 38%, respectively (Fig. 6A and B). Combined treatment with WZ4002 and E7050 markedly decreased the percentage of

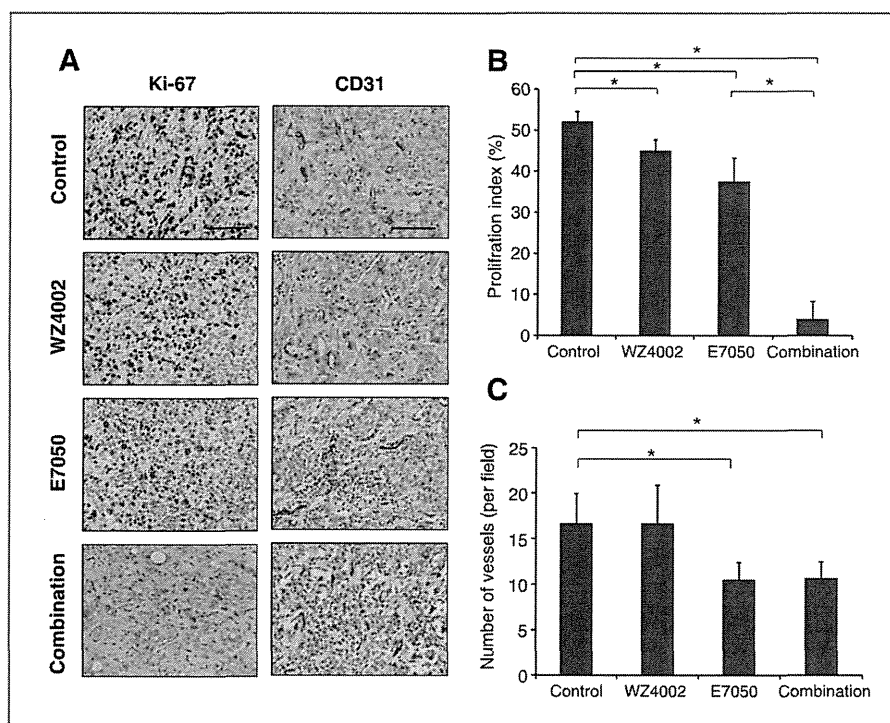
Ki-67-positive proliferating tumor cells to 4%, consistent with the marked inhibition of Erk and Akt phosphorylation. We also examined whether treatment with WZ4002 and/or E7050 induced apoptosis *in vivo* in PC-9/HGF#5 and HCC827ER tumors by terminal deoxynucleotidyl transferase dUTP nick end labeling. Combined treatment did not increase the number of apoptotic cells compared with cells of monotreated or control mice (Supplementary Fig. S6), suggesting that combination treatment has a cytostatic effect.

Because E7050 can inhibit VEGFR-2, we also evaluated the effects of E7050 on angiogenesis. CD31 staining showed that E7050, with or without WZ4002, significantly decreased the vascular density of HCC827ER tumors. The ability of E7050 to inhibit VEGFR-2 also suggests a link between its antitumor and antiangiogenesis effects. These findings also suggest that the combination of the mutant-selective EGFR-TKI inhibitor WZ4002 and the Met-TKI inhibitor E7050 can overcome erlotinib resistance resulting from mutant EGFR, Met inhibition, and VEGFR-2 inhibition.

Discussion

Recent prospective studies have demonstrated that the EGFR-TKIs gefitinib and erlotinib are associated with a high response rate and prolong progression-free survival in patients with EGFR mutant lung cancer (1, 21). Responders to these agents, however, later relapse after acquiring EGFR-TKI resistance, making it urgent to develop novel therapeutic agents that can overcome acquired resistance

Figure 6. Effect of WZ4002 with or without E7050 on tumor cell proliferation and microvessel density. A, representative images of HCC827ER tumors immunohistochemically stained with antibodies to human Ki-67 and mouse CD31. Bar, 200 μ m. B, mean \pm SD proliferation index of HCC827ER tumor sections, as determined by immunohistochemical staining with anti-Ki-67 antibody. *, $P < 0.05$ by one-way ANOVA. C, mean \pm SD blood vessel density in HCC827ER tumor sections by immunohistochemical staining with anti-CD31 antibody. *, $P < 0.05$ by one-way ANOVA.



to EGFR-TKIs. To our knowledge, this study is the first to show that the combination of a mutant-selective EGFR-TKI and a Met-TKI is effective against tumor cell lines refractory to erlotinib by 3 different mechanisms: an *EGFR* gatekeeper T790M mutation, *Met* amplification, and HGF overexpression.

We previously reported that HGF induces resistance in *EGFR* mutant lung cancer cells to treatment not only with the reversible EGFR-TKIs gefitinib and erlotinib, but to treatment with the irreversible EGFR-TKI CL-387,785 (4, 22). We have shown here that both endogenously and exogenously produced HGF could induce resistance to mutant-selective EGFR-TKI, whereas the addition of the Met-TKI E7050 successfully abrogated HGF-triggered resistance *in vitro* and *in vivo*. These observations indicate that HGF is a crucial factor in cellular resistance to various classes of EGFR-TKIs, suggesting the need to combine EGFR-TKIs with HGF-Met inhibitors to overcome the HGF-induced resistance to the former.

Gatekeeper mutations, including the T315I mutation in *Abl* associated with resistance to imatinib (23), the L1196M mutation in *ALK* associated with resistance to crizotinib (24), and the T790M mutation in *EGFR* associated with resistance to gefitinib and erlotinib (5), are common mechanisms by which tumor cells acquire resistance to molecularly targeted drugs. Although irreversible EGFR-TKIs, including BIBW2992, have been developed to overcome T790M-mediated resistance to gefitinib and erlotinib (25), recent clinical trials have failed to show that monotherapy with irreversible EGFR-TKIs has benefits in patients with NSCLC refractory to gefitinib or erlotinib (26). This may be due, at least in part, to the low selectivity of this class of compounds to wild-type and mutant EGFR. WZ4002 is a mutant-selective EGFR-TKI with high activity against EGFR with both sensitive (e.g., exon 19 deletion and L858R) and resistant (T790M) mutations. Nevertheless, because HGF overexpression was frequently observed in tumors with the gatekeeper T790M mutation (7, 27), monotherapy with a mutant-selective EGFR-TKI may not be sufficient to inhibit the growth of tumors with acquired resistance to gefitinib and erlotinib. Our findings suggest that the combination of a Met-TKI and a mutant-selective EGFR-TKI may be effective in controlling these resistant tumors.

Met amplification is also associated with acquired resistance to gefitinib and erlotinib in mutant *EGFR* lung cancer (3). Overexpressed Met protein resulting from gene amplification utilizes ErbB3 as an adaptor protein and mediates survival signals to the PI3K-Akt pathway (3). Theoretically, erlotinib resistance because of *Met* amplification would not be abrogated by inhibiting its ligand (HGF), for example, with anti-HGF or single-chain Met antibody (MetMAB; ref. 28). When combined with WZ4002, however, the orally active Met-TKI E7050 efficiently abrogated erlotinib resistance because of *Met* amplification. E7050 is now being evaluated in clinical trials.

Recent studies have revealed molecular mechanisms of resistance to molecularly targeted agents (29), and many candidate drugs that could overcome this resistance have been developed (30). A biopsy following the development of resistance may result in the accurate molecular diagnosis of resistance mechanisms and the use of appropriate agents (6). Difficulties in molecular diagnosis may arise, however, when multiple lesions progress after becoming resistant. In some patients, the recurrent tumors may be caused by the same resistance mechanism, whereas, in other patients, these tumors may be caused by several mechanisms (7, 14); thus, rebiopsy of a single lesion may be insufficient for an accurate molecular diagnosis. Because acquired resistance to EGFR-TKIs in most patients with *EGFR* mutant lung cancer is likely caused by a gatekeeper T790M mutation, *Met* amplification, or HGF overexpression, the combination of a mutant-selective EGFR-TKI and a Met-TKI, which can overcome all 3 resistance mechanisms, may be a practical approach in the treatment of these patients. This strategy, however, may not be effective against tumors with other EGFR-TKI resistance mechanisms, such as *PIK3CA* mutation and transformation to SCLC. Alternative strategies are therefore needed to overcome these EGFR-TKI resistance mechanisms.

Both EGFR and HGF-Met play crucial roles in maintaining normal homeostasis. Treatment with EGFR inhibitors, such as EGFR-TKIs and anti-EGFR antibodies, has been associated with several types of adverse events, including skin rash, diarrhea, and acute lung disease (31, 32). HGF-Met has been reported to play a protective role against lung fibrosis (33). Therefore, continuous inhibition of both EGFR and HGF-Met may cause serious adverse events. Because mutant-selective EGFR inhibitors are less effective at inhibiting wild-type than mutant EGFR and are therefore likely to be less cytotoxic than gefitinib or erlotinib (10), the combination of a mutant-selective EGFR-TKI and a Met-TKI may optimize both antitumor efficacy and safety. The efficacy and safety of combination treatment should be evaluated in clinical trials.

Disclosure of Potential Conflicts of Interest

S. Yano received honoraria from Chugai Pharmaceutical Co., Ltd. and AstraZeneca. S. Yano received research funding from Chugai Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd. and Eisai Co., Ltd. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: T. Nakagawa, S. Takeuchi, S. Nanjo, S. Yano
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Nakagawa, D. Ishikawa, K. Kita, K. Suda, T. Mitsudomi

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Nakagawa, T. Sano

Writing, review, and/or revision of the manuscript: T. Nakagawa, Y. Sekido, T. Uenaka, S. Yano

Development of methodology: T. Yamada

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Nakamura, K. Matsumoto, K. Suda, Y. Sekido, T. Uenaka, S. Yano

Study supervision: T. Yamada, S. Yano

Grant Support

This study was supported by Grants-in-Aid for Cancer Research (S. Yano, 21390256) and Scientific Research on Innovative Areas "Integrative Research on Cancer Microenvironment Network" (S. Yano, 22112010A01) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 22, 2012; revised June 18, 2012; accepted July 15, 2012; published OnlineFirst July 25, 2012.

References

- Pao W, Chmielecki J. Rational, biologically based treatment of EGFR-mutant non-small-cell lung cancer. *Nat Rev Cancer* 2010;10:760-74.
- Kobayashi S, Boggon TJ, Dayaram T, Janne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005;352:786-92.
- Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316:1039-43.
- Yano S, Wang W, Li Q, Matsumoto K, Sakurama H, Nakamura T, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 2008;68:9479-87.
- Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005;2:e73.
- Sequist LV, Waltman BA, Dias-Santagata D, Digumarthy S, Turke AB, Fidias P, et al. Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* 2011;3:75ra26.
- Yano S, Yamada T, Takeuchi S, Tachibana K, Minami Y, Yatabe Y, et al. Hepatocyte growth factor expression in EGFR mutant lung cancer with intrinsic and acquired resistance to tyrosine kinase inhibitors in a Japanese cohort. *J Thorac Oncol* 2011;6:2011-7.
- Regales L, Gong Y, Shen R, de Stanchina E, Vivanco I, Goel A, et al. Dual targeting of EGFR can overcome a major drug resistance mutation in mouse models of EGFR mutant lung cancer. *J Clin Invest* 2009;119:3000-10.
- Shimamura T, Li D, Ji H, Haringsma HJ, Liniker E, Borgman CL, et al. Hsp90 inhibition suppresses mutant EGFR-T790M signaling and overcomes kinase inhibitor resistance. *Cancer Res* 2008;68:5827-38.
- Zhou W, Ercan D, Chen L, Yun CH, Li D, Capelletti M, et al. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. *Nature* 2009;462:1070-4.
- Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer Cell* 2010;17:77-88.
- Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 2010;363:1693-703.
- Nakagawa T, Tohyama O, Yamaguchi A, Matsushima T, Takahashi K, Funasaka S, et al. E7050: a dual c-Met and VEGFR-2 tyrosine kinase inhibitor promotes tumor regression and prolongs survival in mouse xenograft models. *Cancer Sci* 2010;101:210-5.
- Suda K, Murakami I, Katayama T, Tomizawa K, Osada H, Sekido Y, et al. Reciprocal and complementary role of MET amplification and EGFR T790M mutation in acquired resistance to kinase inhibitors in lung cancer. *Clin Cancer Res* 2010;16:5489-98.
- Matsumoto K, Nakamura T, Sakai K, Nakamura T. Hepatocyte growth factor and Met in tumor biology and therapeutic approach with NK4. *Proteomics* 2008;8:3360-70.
- Nishioka Y, Yano S, Fujiki F, Mukaida N, Matsushima K, Tsuruo T, et al. Combined therapy of multidrug-resistant human lung cancer with anti-P-glycoprotein antibody and monocyte chemoattractant protein-1 gene transduction: the possibility of immunological overcoming of multidrug resistance. *Int J Cancer* 1997;71:170-7.
- Green LM, Reade JL, Ware CF. Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J Immunol Methods* 1984;70:257-68.
- Okabe T, Okamoto I, Tamura K, Terashima M, Yoshida T, Satoh T, et al. Differential constitutive activation of the epidermal growth factor receptor in non-small cell lung cancer cells bearing EGFR gene mutation and amplification. *Cancer Res* 2007;67:2046-53.
- Wang W, Li Q, Takeuchi S, Yamada T, Koizumi H, Nakamura T, et al. Met kinase inhibitor E7050 reverses three different mechanisms of hepatocyte growth factor-induced tyrosine kinase inhibitor resistance in EGFR mutant lung cancer. *Clin Cancer Res* 2012;18:1663-71.
- Kwak EL, Sordella R, Bell DW, Godin-Heymann N, Okimoto RA, Brannigan BW, et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. *Proc Natl Acad Sci U S A* 2005;102:7665-70.
- Mitsudomi T, Yatabe Y. Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensitivity in lung cancer. *Cancer Sci* 2007;98:1817-24.
- Yamada T, Matsumoto K, Wang W, Li Q, Nishioka Y, Sekido Y, et al. Hepatocyte growth factor reduces susceptibility to an irreversible epidermal growth factor receptor inhibitor in EGFR-T790M mutant lung cancer. *Clin Cancer Res* 2010;16:174-83.
- Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, et al. High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. *Blood* 2002;99:3472-5.
- Choi YL, Soda M, Yamashita Y, Ueno T, Takashima J, Nakajima T, et al. ALK Lung Cancer Study Group. EML4-ALK mutations in lung cancer that confer resistance to ALK inhibitors. *N Engl J Med* 2010;363:1734-9.
- Li D, Ambrogio L, Shimamura T, Kubo S, Takahashi M, Chirieac LR, et al. BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. *Oncogene* 2008;27:4702-11.
- Wong KK, Fracasso PM, Bukowski RM, Lynch TJ, Munster PN, Shapiro GI, et al. A phase I study with neratinib (HKI-272), an irreversible pan ErbB receptor tyrosine kinase inhibitor, in patients with solid tumors. *Clin Cancer Res* 2009;15:2552-8.
- Uramoto H, Shimokawa H, Hanagiri T, Kuwano M, Ono M. Expression of selected gene for acquired drug resistance to EGFR-TKI in lung adenocarcinoma. *Lung Cancer* 2011;73:361-5.
- Okamoto W, Okamoto I, Tanaka K, Hatashita E, Yamada Y, Kuwata K, et al. TAK-701, a humanized monoclonal antibody to hepatocyte growth factor, reverses gefitinib resistance induced by tumor-derived HGF in non-small cell lung cancer with an EGFR mutation. *Mol Cancer Ther* 2010;9:2785-92.
- Barouch-Bentov R, Sauer K. Mechanisms of drug resistance in kinases. *Expert Opin Investig Drugs* 2011;20:153-208.
- Giaccone G, Wang Y. Strategies for overcoming resistance to EGFR family tyrosine kinase inhibitors. *Cancer Treat Rev* 2011;37:456-64.
- Neal JW, Heist RS, Fidias P, Temel JS, Huberman M, Marcoux JP, et al. Cetuximab monotherapy in patients with advanced non-small cell lung cancer after prior epidermal growth factor receptor tyrosine kinase inhibitor therapy. *J Thorac Oncol* 2010;5:1855-8.
- Ku GY, Haaland BA, de Lima Lopes G Jr. Gefitinib vs. chemotherapy as first-line therapy in advanced non-small cell lung cancer: meta-analysis of phase III trials. *Lung Cancer* 2011;74:469-73.
- Panganiban RA, Day RM. Hepatocyte growth factor in lung repair and pulmonary fibrosis. *Acta Pharmacol Sin* 2011;32:12-20.

ORIGINAL ARTICLE

Endoscopic Findings of Upper Gastrointestinal Lesions in Patients with Pancreatic Cancer

Koushiro Ohtsubo¹, Hiroyuki Watanabe^{1,2}, Hisatsugu Mouri¹,
Kaname Yamashita¹, Kazuo Yasumoto¹, Seiji Yano¹

¹Division of Medical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Japan.

²Internal Medicine, Fukuiken Saiseikai Hospital, Fukui, Japan

ABSTRACT

Context Pancreatic cancer is often complicated with upper gastrointestinal lesions. However, there have been few endoscopic studies in pancreatic cancer patients. We retrospectively investigated the upper gastrointestinal lesions in patients with pancreatic cancer who underwent upper gastrointestinal endoscopy. **Methods** Upper gastrointestinal endoscopy was performed in 75 patients with pancreatic cancer between 2003 and 2010. We examined upper gastrointestinal lesions, such as gastroduodenal invasion, ulcers, esophagogastric varices, radiation-induced gastroduodenal mucosal lesions, and portal hypertensive gastropathy. **Results** Among the 53 patients with pancreatic cancer who underwent upper gastrointestinal endoscopy at diagnosis, 23 gastrointestinal lesions were observed in 20 patients (38%) as follows: gastroduodenal invasion (n=11), esophagogastric varices (n=7), gastroduodenal ulcers (n=3), portal hypertensive gastropathy (n=1) and duodenal metastasis (n=1). Among the 75 patients with pancreatic cancer, 56 gastrointestinal lesions were identified in 46 patients (61%) during the clinical course as follows: gastroduodenal invasion (n=20), esophagogastric varices (n=14), radiation-induced gastroduodenal mucosal lesions (n=9), gastroduodenal ulcers (except radiation-induced ulcers) (n=8), portal hypertensive gastropathy (n=3), duodenal metastasis (n=1), and gastrointestinal bleeding from unknown primary site (n=1). Twenty-nine (52%) of the 56 gastrointestinal lesions showed symptoms related to the lesions. Fifteen (27%) lesions were accompanied by upper gastrointestinal bleeding. Fourteen (25%) lesions developed according to the progression of pancreatic cancer. **Conclusion** We should pay attention to upper gastrointestinal lesions in patients with pancreatic cancer.

INTRODUCTION

Pancreatic cancer is often complicated with upper gastrointestinal lesions. Lethal outcomes are sometimes encountered in daily practice. Upper gastrointestinal lesions related to pancreatic cancer have been reported in the English language literature [1, 2, 3, 4, 5, 6, 7, 8, 9]. However, there have been few reports of endoscopic studies in pancreatic cancer patients [2, 5, 9]. In addition, although the endoscopic findings at diagnosis have been reported previously [9], endoscopic upper gastrointestinal lesions that developed during the clinical course of pancreatic cancer have not been reported previously.

We investigated the upper gastrointestinal lesions in patients with pancreatic cancer who underwent upper gastrointestinal endoscopy.

Received February 26th, 2012 - Accepted May 14th, 2012

Key words Endoscopy; Esophageal and Gastric Varices; Neoplasm Invasiveness; Pancreatic Neoplasms; Ulcer

Abbreviations NSAID: nonsteroidal anti-inflammatory drug

Correspondence Koushiro Ohtsubo

Division of Medical Oncology; Cancer Research Institute; Kanazawa University; 13-1 Takaramachi; Kanazawa 920-0934; Japan

Phone: +81-76.265.2794; Fax: +81-76.234.4524

E-mail: ohtsubo@staff.kanazawa-u.ac.jp

METHODS

Patients

We encountered 112 patients with pancreatic cancer between 2003 and 2010. We retrospectively reviewed the upper gastrointestinal lesions in 75 patients who underwent upper gastrointestinal endoscopy. Patients consisted of 50 men and 25 women ranging in age from 29 to 89 (median 64) years. The tumor locations of the pancreatic cancer were the pancreatic head in 39 cases and the body and/or tail in 37 cases because one patient had two tumors, one in the pancreatic head and one in the body/tail. Clinical staging at diagnosis was Stage IB in 1 case (1.3%), Stage IIA in 2 cases (2.7%), Stage IIB in 3 cases (4.0%), Stage III in 24 cases (32.0%), and Stage IV in 45 cases (60.0%) according to the TNM classification, 6th edition [10]. Five patients (6.7%) with pancreatic cancer underwent pancreaticoduodenectomy or distal pancreatectomy. Sixty-nine patients (92.0%) were treated with chemotherapy by gemcitabine and/or S-1. Eighteen patients (24.0%) received radiotherapy combined with chemotherapy (gemcitabine and/or S-1) before or after chemotherapy.

Table 1. Incidence of upper gastrointestinal lesions at diagnosis ^a.

	Total (n=53 ^a)	Head (n=27)	Body/tail (n=27)	P value
Gastroduodenal invasion	11 (20.8%)	8 (26.9%)	3 (11.1%)	0.175
Esophagogastric varices	7 (13.2%)	1 (3.7%)	6 (22.2%)	0.100
Ulcers	3 (5.7%)	1 (3.7%)	2 (7.4%)	1.000
Portal hypertensive gastropathy	1 (1.9%)	0	1 (3.7%)	1.000
Other (duodenal metastasis)	1 (1.9%)	0	1 (3.7%)	1.000
Total	23 (43.4%)	10 (37.0%)	13 (48.1%)	0.583

^a 53 patients underwent upper gastrointestinal endoscopy at diagnosis: one patient had two tumors in the pancreatic head and body/tail

Endoscopic Findings

We examined the incidence of upper gastrointestinal lesions, such as gastroduodenal invasion, ulcers, esophagogastric varices, radiation-induced gastro-duodenal mucosal lesions, and portal hypertensive gastropathy. Gastroduodenal invasion was judged as erosion, ulcers, or stricture due to direct invasion of pancreatic cancer. In this study, extrinsic compression was excluded because the findings were indefinite between endoscopists and seldom susceptible to bleeding. Gastroduodenal ulcers were defined as ulcerative lesions except for direct invasion of pancreatic cancer. Esophagogastric varices and portal hypertensive gastropathy were diagnosed according to the General Rules for Recording Endoscopic Findings of Esophagogastric Varices (2nd edition) [11]. Radiation-induced gastroduodenal ulcers and gastritis were defined as ulcerative and reddish or erosive lesions post-radiotherapy, respectively. In this study, these conditions were termed radiation-induced gastroduodenal mucosal lesions.

The symptoms associated with gastrointestinal bleeding were defined as hematemesis, melena, and/or progression of anemia due to upper gastrointestinal lesions. The symptoms related to upper gastrointestinal lesions were defined as abdominal pain, nausea vomiting, and/or appetite loss in addition to the symptoms of gastrointestinal bleeding.

Upper gastrointestinal lesions were independently reviewed and verified by two experienced investigators. If the opinions were different between the two investigators, a consensus was reached by further discussion.

ETHICS

The written informed consent was obtained from each patient and the study protocol conforms to the ethical guidelines of the “World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects” adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964 and amended by the 59th WMA General Assembly, Seoul, South Korea, October 2008, as reflected in a priori approval by our institutional review committee.

STATISTICS

Frequencies were used as descriptive statistics and the Pearson chi-square and the Fisher’s exact tests were applied. Statistical analyses were made by means of the Stat Mate (version IV, ATMS, Tokyo, Japan) statistical software. Two-tailed P values less than 0.05 were considered significant.

RESULTS

Total Findings

Of the 75 patients with pancreatic cancer, 53 (70.7%) underwent upper gastrointestinal endoscopy at diagnosis. In these 53 patients (54 tumors), 23 upper gastrointestinal lesions were observed in 20 patients (37.7%) (Table 1). Gastroduodenal invasion was observed in 11 (20.8%), esophagogastric varices in 7 (13.2%), and gastroduodenal ulcers in 3 (5.7%). Portal hypertensive gastropathy and duodenal metastasis were identified in one case each (1.9%).

On the other hand, during the clinical course of pancreatic cancer in the 75 patients (76 tumors) with

Table 2. Incidence of upper gastrointestinal lesions during the clinical course ^a.

	Total (n=75 ^a)	Head (n=39)	Body/tail (n=37)	P value
Gastroduodenal invasion	20 (26.7%)	16 (41.0%)	4 (10.8%)	0.004
Esophagogastric varices	14 (18.7%)	3 (7.7%)	11 (29.7%)	0.018
Radiation-induced gastroduodenal mucosal lesions	9 (12.0%)	6 (15.4%)	3 (8.1%)	0.481
Ulcers (except for radiation-induced)	8 (10.7%)	6 (15.4%)	2 (5.4%)	0.263
Portal hypertensive gastropathy	3 (4.0%)	1 (2.6%)	2 (5.4%)	0.610
Other ^b	2 (2.7%)	1 (2.6%)	1 (2.7%)	1.000
Total	56 (74.7%)	33 (84.6%)	23 (62.2%)	0.037

^a 75 patients with pancreatic cancer were evaluated: one patient had two tumors in the pancreatic head and body/tail

^b Duodenal metastasis and gastrointestinal bleeding of unknown primary site in one case, respectively

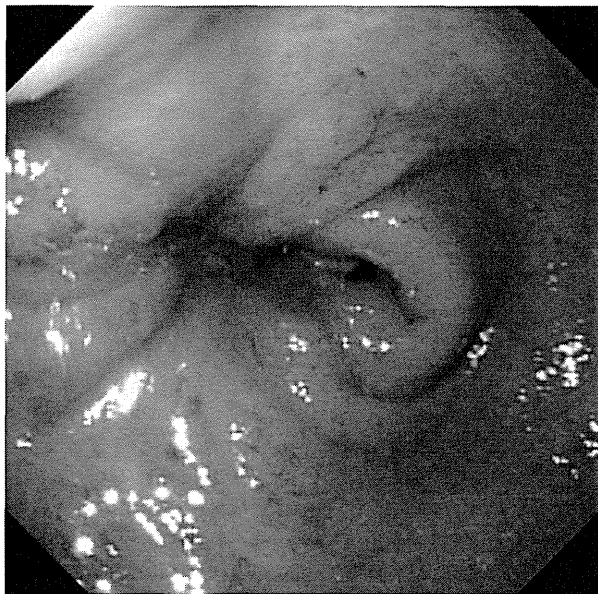


Figure 1. Case of pancreatic head cancer with duodenal invasion.



Figure 2. Case of pancreatic head cancer with esophageal varices.

pancreatic cancer, 56 upper gastrointestinal lesions were observed in 46 patients (61.3%) (Table 2). Gastroduodenal invasion was observed in 20 patients (26.7%), esophagogastric varices in 14 (18.7%), radiation-induced gastroduodenal mucosal lesions in 9 (12.0%), gastroduodenal ulcers (except radiation-induced ulcers) in 8 (10.7%), and portal hypertensive gastropathy in 3 (4.0%). Duodenal metastasis and gastrointestinal bleeding of unknown primary site were identified in one patient each (1.3%).

Gastroduodenal Invasion

Gastroduodenal invasion was observed in the cases with lesions located in the pancreatic head significantly more frequently than in those located in the body and/or tail: 16 of 39 cases (41.0%) vs. 4 of 37 (10.8%) during the clinical course ($P=0.004$, Fisher's exact test), although significant difference was not recognized at diagnosis (Tables 1 and 2; Figure 1). The involved organs were as follows: the stomach in 2 cases (10.0%), in which all tumors were located in the pancreatic body and/or tail; duodenal bulb in 2 cases (10.0%), in which all tumors were located in the pancreatic head; descending part of the duodenum in 13 cases (65.0%), in which all tumors were located in the pancreatic head; and horizontal part of the duodenum in 3 cases (15.0%), in which 1 and 2 tumors were located in the pancreatic head and body, respectively. Malignant cells were confirmed in 9 (52.9%) of 17 patients in whom endoscopic biopsy was performed. Although gastrointestinal bleeding has not been identified in patients with gastric invasion, that developed in 4 out of 18 patients with duodenal invasion (22.2%). One of these 4 patients died of repeated gastrointestinal bleeding.

Esophagogastric Varices

Unlike gastroduodenal invasion, esophagogastric varices were observed in the cases with lesions located in the pancreatic body and/or tail significantly more frequently than in those with lesions in the head (3 of 39 cases (7.7%) vs. 11 of 37 cases (29.7%) during the clinical course ($P=0.018$, Fisher's exact test)), although significant difference was not recognized at diagnosis (Tables 1 and 2; Figure 2). The involved organs were as follows: the esophagus in 6 cases (42.9%), in which 2 tumors were located in the pancreatic head and 4 in the body and/or tail; stomach in 5 cases (35.7%), in which all tumors were located in the pancreatic body or tail; and both the esophagus and stomach in 3 cases



Figure 3. Case of pancreatic head cancer with radiation-induced gastric ulcer.

(21.4%), in which 1 and 2 tumors were located in the pancreatic head and body, respectively. Gastric varices were found in 8 cases and were located in cardia in 1 case (12.5%), in fornix in 5 cases (62.5%), and both cardia and fornix in 2 cases (25.0%). Portal venous systems were invaded in all 14 patients with esophagogastric varices. The splenic vein was involved in all 11 patients with pancreatic body and/or tail cancer, but not in any of those with pancreatic head cancer. F1 varices were observed in 7 of the 14 patients (50.0%), and F2 varices in another 7 patients (50.0%), while no F3 varices were identified. The red color sign was detected in 5 of the 14 patients (35.7%). Only 2 patients with F2 varices and red color sign presented with bleeding from esophagogastric varices. Although 1 of these 2 patients received endoscopic injection sclerotherapy, he died of repeated gastrointestinal

bleeding. The other patient did not receive endoscopic treatment because variceal bleeding was controlled conservatively. There were no patients in whom endoscopic treatment for esophagogastric varices without bleeding was performed.

Radiation-Induced Gastroduodenal Mucosal Lesions

Thirteen (72.2%) of the 18 patients treated with radiotherapy underwent upper gastrointestinal endoscopy post-radiotherapy. Radiation-induced gastroduodenal ulcers developed in 5 (38.5%) of these patients (Figure 3). The involved organs were as follows: the stomach in 3 cases, in which 1 and 2 tumors were located in the pancreatic body and pancreatic head, respectively; duodenum in 1, in which the tumor was located in the pancreatic head; and both stomach and duodenum in 1, in which the tumor was located in the pancreatic head. In addition, radiation-induced gastritis developed in 4 patients (30.8%) in whom 2 tumors were located in the pancreatic head and 2 in the body. A total of 9 (50.0%) of the 18 patients suffered radiation-induced gastroduodenal mucosal lesions. All 9 patients with them received proton pump inhibitor (PPI; n=5) or histamine H₂-receptor antagonist (H₂ blocker; n=4) after radiotherapy. Upper gastrointestinal bleeding was observed in 3 (33.3%) out of these 9 patients (1 with gastric ulcer and 2 with gastritis). Upper gastrointestinal bleeding developed 3 weeks after radiotherapy in 1 patient with radiation-induced gastric ulcer, and 5 or 7 months after radiotherapy in 2 patients with radiation-induced gastritis. Argon plasma coagulation therapy was effective for hemostasis in 2 patients with radiation-induced gastritis (Figure 4ab). The other patient with radiation-induced ulcer did not receive endoscopic treatment because bleeding was controlled conservatively.

Gastroduodenal Ulcers

Of the 8 patients with gastroduodenal ulcers (except radiation-induced ulcers), the involved organs were as follows: the stomach in 4 cases (50.0%), in which 2 tumors were located in the pancreatic head and 2 in pancreatic body; and duodenum in 4 (50.0%), in which all tumors were located in the pancreatic head. Ulcers were induced by biliary drainage device in 3 cases (37.5%) (self-expandable metallic stent in 2, and endoscopic nasobiliary drainage in 1) and nonsteroidal anti-inflammatory drugs (NSAIDs) in 2 cases (25.0%); although 1 of the 2 patients with duodenal ulcer induced by NSAIDs was not treated with PPI or H₂ blocker, the other patient received H₂ blocker before the onset of ulcer. *Helicobacter pylori* infection was confirmed in 2 of the remaining 3 patients. Gastrointestinal bleeding was observed in 4 patients (50.0%) (1 by self-expandable metallic stent, 2 by NSAIDs, and 1 with *Helicobacter pylori* infection). Endoscopic clipping was effective for hemostasis in 1 patient with gastroduodenal ulcers (except radiation-

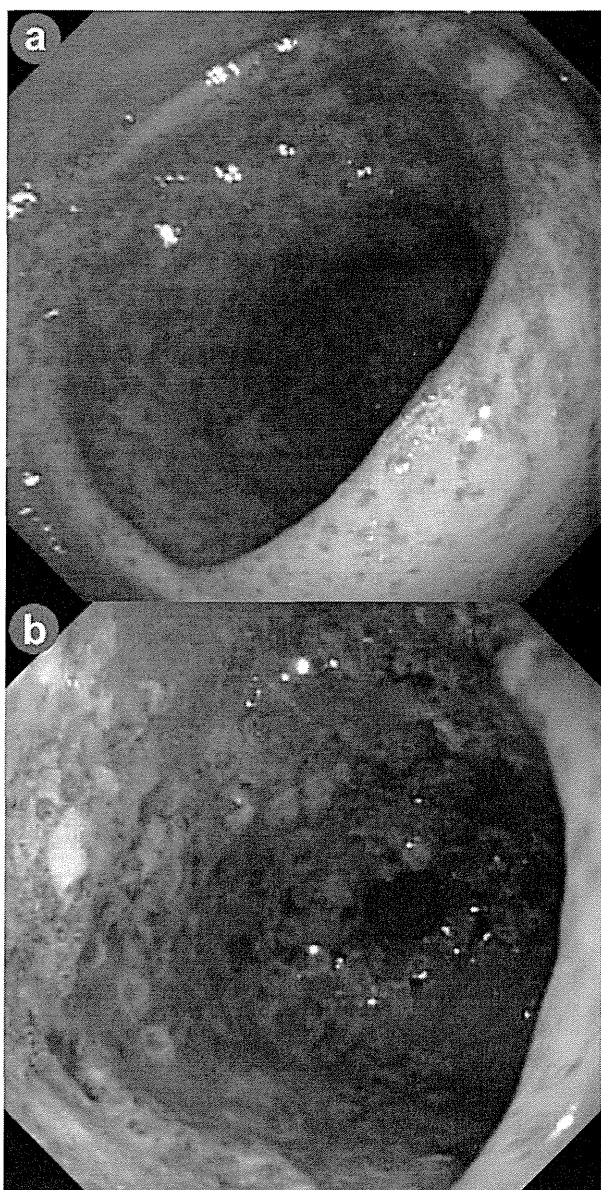


Figure 4 a. Case of pancreatic head cancer with radiation-induced gastritis. b. Argon plasma coagulation therapy was effective in this case.

induced ulcers). The other 3 patients did not receive endoscopic treatment because bleeding was controlled conservatively. In addition, 5 out of the 75 patients with pancreatic cancer had radiation-induced ulcers (6.7%); therefore, the total number of patients with gastroduodenal ulcers accounted to 13 cases (17.3%).

Portal Hypertensive Gastropathy

Portal hypertensive gastropathy was observed in 3 patients (4.0%) in whom tumor locations were the pancreatic head (n=1) and body (n=2). All three patients with portal hypertensive gastropathy also suffered from esophagogastric varices. All esophagogastric varices complicated with portal hypertensive gastropathy presented with F2 varices and red color sign. Upper gastrointestinal bleeding developed in 1 patient with portal hypertensive gastropathy. Although gastrointestinal bleeding was uncontrollable by medication (beta-blocker), percutaneous portal vein stent (10x60 mm; Luminexx®, BARD, Murray Hill, NY, USA) was effective for hemostasis of gastrointestinal bleeding.

Other Findings

One patient with pancreatic tail cancer was accompanied with duodenal metastasis (1.3%). In this case, a flat elevated lesion with ulceration was observed in the duodenal bulb, and the presence of malignant cells was confirmed.

Gastrointestinal bleeding from unknown primary site was identified in 1 patient with pancreatic head cancer (1.3%). He died of sudden gastrointestinal bleeding.

Symptoms Related to Upper Gastrointestinal Lesions

The incidences of symptoms related to upper gastrointestinal lesions were examined. Twenty-nine of 56 lesions (51.8%) presented with symptoms related to the lesions. The symptoms were observed in 12 (60.0%) of 20 patients with gastroduodenal invasion, 2 (14.3%) of 14 with esophagogastric varices, 8 (88.9%) of 9 with radiation-induced gastroduodenal mucosal lesions, 5 (62.5%) of 8 with gastroduodenal ulcers (except radiation-induced ulcers), 1 (33.3%) of 3 with portal hypertensive gastropathy, and 1 out of 1 with gastrointestinal bleeding from unknown primary site. The remaining patient with duodenal metastasis had no

symptoms. The incidences of symptoms related to gastroduodenal invasion (60.0%) and radiation-induced gastroduodenal mucosal lesions (88.9%) were significantly higher than in esophagogastric varices (14.3%) (P=0.013 and P<0.001, respectively; Fisher’s exact test). Fifteen of 56 lesions (26.8%) were accompanied by upper gastrointestinal bleeding. Most of these patients received endoscopic therapy and/or blood transfusion. Gastrointestinal bleeding was observed in 4 (20.0%) of 20 patients with gastroduodenal invasion, 2 (14.3%) of 14 with esophagogastric varices, 3 (33.3%) of 9 with radiation-induced gastroduodenal mucosal lesions, 4 (50.0%) of 8 with gastroduodenal ulcers (except radiation-induced ulcers), 1 (33.3%) of 3 with portal hypertensive gastropathy. In addition, one patient had gastrointestinal bleeding from unknown primary site, while the remaining patient with duodenal metastasis had no gastrointestinal bleeding. There were no significant differences in the incidence of gastrointestinal bleeding between different upper gastrointestinal lesions (P=1.000, P=0.343, P=0.137, and P=0.465, respectively) in comparison with esophagogastric varices. Three of 15 patients in whom bleeding was confirmed endoscopically (1 patient with duodenal invasion, 1 with esophagogastric varices, and 1 of unknown primary site) died of upper gastrointestinal bleeding. In addition, 2 patients in whom upper gastrointestinal endoscopy was not performed at hematemesis (1 patient with duodenal invasion, 1 with unknown primary site) died of upper gastrointestinal bleeding. No significant endoscopic abnormalities were found at diagnosis in 2 patients with gastrointestinal bleeding from unknown primary site. A total of 5 (6.7%) of the 75 patients died of upper gastrointestinal bleeding.

New Upper Gastrointestinal Lesions

As shown in Table 3, new upper gastrointestinal lesions on second or later examinations were observed in 14 (25.0%) of the 56 upper gastrointestinal lesions during the clinical course of pancreatic cancer: 4 cases with gastroduodenal invasion, 1 with esophagogastric varices, 6 with radiation-induced gastroduodenal mucosal lesions, 1 with duodenal ulcer (except radiation-induced ulcers), 1 with portal hypertensive gastropathy, and 1 with gastrointestinal bleeding from an unknown primary site.

DISCUSSION

Upper gastrointestinal lesions related to pancreatic cancer have been reported in the English language literature [1, 2, 3, 4, 5, 6, 7, 8, 9]. However, there have been few endoscopic studies in pancreatic cancer patients [2, 5, 9]. In addition, although the endoscopic findings at diagnosis have been reported previously [9], endoscopic upper gastrointestinal lesions that developed during the clinical course of pancreatic cancer have not been reported previously. In the present study, we investigated the upper gastrointestinal lesions in patients with pancreatic

Table 3. Incidence of new upper gastrointestinal lesions on 2nd or later examination.

	Total (n=56)
Gastroduodenal invasion	4 (7.1%)
Esophagogastric varices	1 (1.8%)
Radiation-induced gastroduodenal mucosal lesions	6 (10.7%)
Ulcers (except for radiation-induced)	1 (1.8%)
Portal hypertensive gastropathy	1 (1.8%)
Gastrointestinal bleeding of unknown primary site)	1 (1.8%)
Total	14 (25.0%)

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 H₂ 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 H₂ 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 H₂ 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

References

- Cubilla A, Fitzgerald PJ. Pancreas cancer. I. Duct adenocarcinoma. A clinical-pathologic study of 380 patients. *Pathol Annu* 1978; 13 Pt 1: 241-89. [PMID 214741]
- Sharon P, Stalnikovicz R, Rachmilewitz D. Endoscopic diagnosis of duodenal neoplasms causing upper gastrointestinal bleeding. *J Clin Gastroenterol* 1982; 4: 35-8. [PMID 6978900]
- Lee P, Sutherland D, Feller ER. Massive gastrointestinal bleeding as the initial manifestation of pancreatic carcinoma. *Int J Pancreatol* 1994; 15: 223-7. [PMID 7930783]
- Mao C, Domenico DR, Kim K, Hanson DJ, Howard JM. Observations on the developmental patterns and the consequences of pancreatic exocrine adenocarcinoma. Findings of 154 autopsies. *Arch Surg* 1995; 130: 125-34. [PMID 7848081]
- Tio TL, Kimmings N, Rauws E, Jansen P, Tytgat G. Endosonography of gastroesophageal varices: evaluation and follow-up of 76 cases. *Gastrointest Endosc* 1995; 42: 145-50. [PMID 7590050]
- Tomita H, Osada S, Matsuo M, Shimokawa K. Pancreatic cancer presenting with hematemesis from directly invading the duodenum: report of an unusual manifestation and review. *Am Surg* 2006; 72: 363-6. [PMID 16676866]
- Mullan FJ, McKelvey ST. Pancreatic carcinoma presenting as bleeding from segmental gastric varices: pitfalls in diagnosis. *Postgrad Med J* 1990; 66: 401-3. [PMID 2371194]
- Smith TA, Brand EJ. Pancreatic cancer presenting as bleeding gastric varices. *J Clin Gastroenterol* 2001; 32: 444-7. [PMID 11319321]
- Shirato I, Nakamura S, Mitsunaga A, Shiratori K. Clinical features of esophago-gastric varices caused by pancreatic cancer (in Japanese with English abstract). *Nippon Shokakibyo Gakkai Zasshi (JJSJG)* 2008; 105: 1186-92. [PMID 18678994]
- Sobin LH, Christian W. UICC. TNM classification of malignant tumours. 6th ed. New York, Wiley-Liss, 2002.
- Tajiri T, Yoshida H, Obara K, *et al*. General rules for recording endoscopic findings of esophagogastric varices (2nd edition). *Dig Endosc* 2010; 22: 1-9. [PMID 20078657]
- Turrill FL, Mikkelsen WP. "Sinistral" (left-sided) extrahepatic portal hypertension. *Arch Surg* 1969; 99: 365-8. [PMID 5306330]
- Okusaka T, Ito Y, Ueno H, *et al*. Phase II study of radiotherapy combined with gemcitabine for locally advanced pancreatic cancer. *Br J Cancer* 2004; 91: 673-7. [PMID 15226765]
- Wilkowski R, Thoma M, Bruns C, Wagner A, Heinemann V. Chemoradiotherapy with gemcitabine and continuous 5-FU in patients with primary inoperable pancreatic cancer. *JOP* 2006; 7: 349-60. [PMID 16832132]
- Burriss HA 3rd, Moore MJ, Andersen J, *et al*. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; 15: 2403-13. [PMID 9196156]
- Ueno H, Okusaka T, Ikeda M, Takezako Y, Morizane C. An early phase II study of S-1 in patients with metastatic pancreatic cancer. *Oncology* 2005; 68: 171-8. [PMID 16006754]

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

JUN KURAI¹, HIROKI CHIKUMI¹, KIYOSHI HASHIMOTO¹, MIYAKO TAKATA¹, TAKANORI SAKO¹, KOSUKE YAMAGUCHI¹, NAOKI KINOSHITA¹, MASANARI WATANABE¹, HIROKAZU TOUGE¹, HARUHIKO MAKINO¹, TADASHI IGISHI¹, HIRONOBU HAMADA², SEIJI YANO³ and EIJI SHIMIZU¹

¹Division of Medical Oncology and Molecular Respiriology, Department of Multidisciplinary Internal Medicine, Faculty of Medicine, Tottori University, Yonago-shi, Tottori-ken 683-8504; ²Graduate School of Health Sciences, Hiroshima University, Minami-ku, Hiroshima 734-8553; ³Division of Medical Oncology, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa 920-0934, Japan

Received June 15, 2012; Accepted July 24, 2012

DOI: 10.3892/ijo.2012.1607

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 ⁵¹Cr 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.

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

Correspondence to: Dr Hiroki Chikumi, Division of Medical Oncology and Molecular Respiriology, Department of Multidisciplinary Internal Medicine, Faculty of Medicine, Tottori University, 36-1 Nishi-cho, Yonago-shi, Tottori-ken 683-8504, Japan
E-mail: chikumi@grape.med.tottori-u.ac.jp

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