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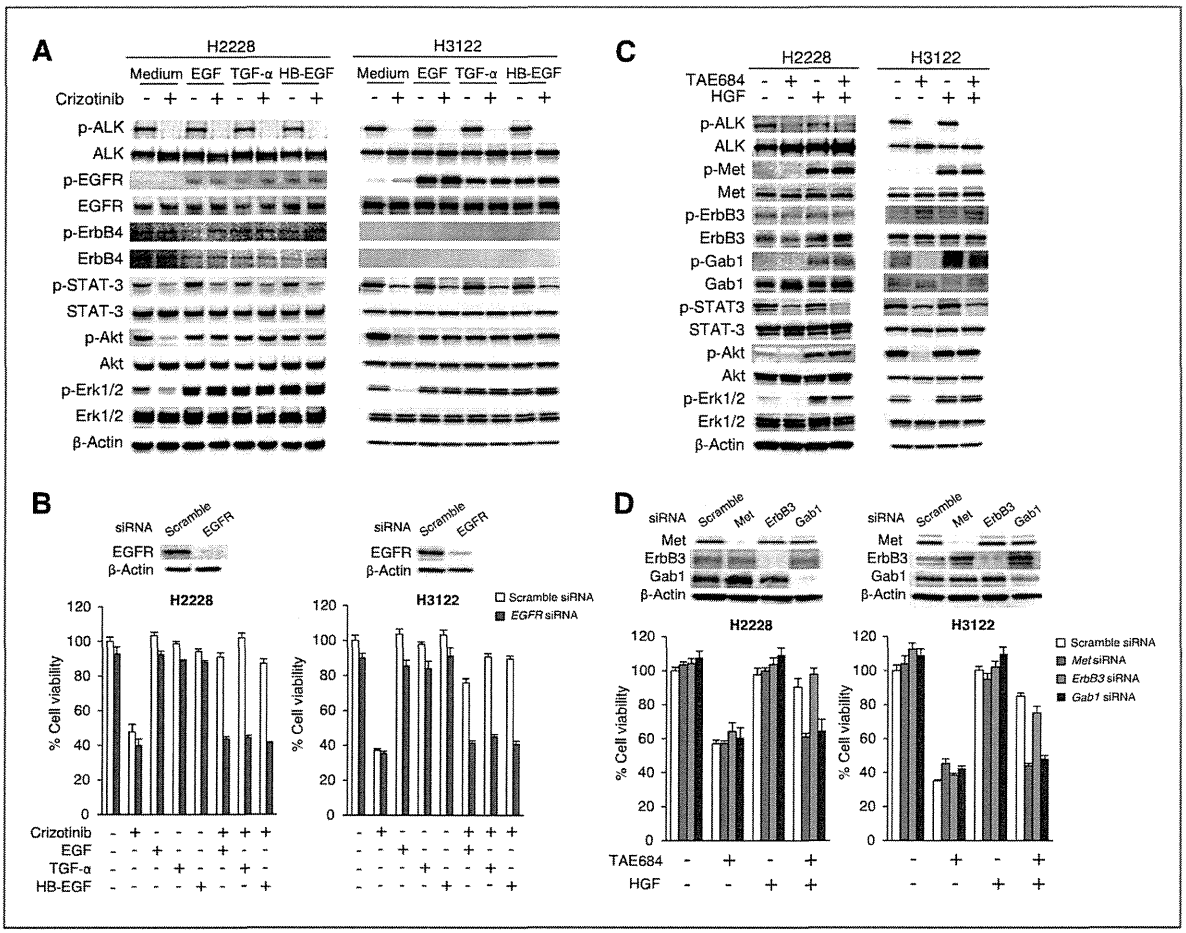

Figure 3. HGF and EGFR ligands trigger ALK inhibitor resistance via Met/Gab1 and EGFR, respectively. A, crizotinib inhibited the phosphorylation of ALK and STAT-3 but not that of EGFR, Akt, and Erk1/2 in the presence of EGF, TGF-α, or HB-EGF. Tumor cells were treated with or without crizotinib (100 nmol/L) for 1 hour and/or EGF (100 ng/mL), TGF-α (100 ng/mL), or HB-EGF (10 ng/mL) for 15 minutes. The cells were lysed and the indicated proteins were detected by immunoblotting. The results shown are representative of 3 independent experiments. B, control or *EGFR*-specific siRNAs were introduced into H2228 and H3122 cells. After 24 hours, the cells were incubated with or without crizotinib (100 nmol/L), and/or EGF (100 ng/mL), TGF-α (100 ng/mL), or HB-EGF (10 ng/mL) for 72 hours and lung cancer cell growth was determined by MTT assays. *EGFR* knockdown was confirmed by immunoblotting. The percentage of growth is shown relative to untreated controls. Each sample was assayed in triplicate, with each experiment repeated at least 3 times independently. C, TAE684 inhibited the phosphorylation of ALK and STAT-3, but not that of Met, Gab1, Akt, and Erk1/2 in the presence of HGF. Tumor cells were treated with or without TAE684 (100 nmol/L) for 1 hour and/or HGF (50 ng/mL) for 15 minutes. The cells were lysed and the indicated proteins were detected by immunoblotting. The results shown are representative of 3 independent experiments. D, control or *Met*, *ErbB3*, or *Gab1*-specific siRNAs were introduced into H2228 and H3122 cells. After 24 hours, the cells were incubated with or without TAE684 (100 nmol/L) and/or HGF (50 ng/mL) for 72 hours and lung cancer cell growth was determined by MTT assays. *Met*, *Gab1*, and *ErbB3* knockdowns were confirmed by immunoblotting. The percentage of growth is shown relative to untreated controls. Each sample was assayed in triplicate, with each experiment repeated at least 3 times independently.

MANA2) and lung epithelial cells (BEAS-2B) produced low or no detectable levels of EGFR ligands or HGF. Interestingly, coculture of H2228 or H3122 cells with fibroblasts (MRC-5) significantly reduced their sensitivity to TAE684, an effect abrogated by anti-HGF antibody (Fig. 4B). Coculture with endothelial cells (HMVEC) also reduced sensitivity to crizotinib, an effect inhibited by anti-EGFR antibody (Fig. 4C).

These results suggested that host stromal cells, such as endothelial cells and fibroblasts, may regulate sensitivity to ALK inhibitors by secreting EGFR ligands and HGF, respectively.

HGF derived from fibroblasts induces TAE684 resistance of EML4-ALK lung cancer cells *in vivo*

To investigate whether sensitivity to TAE684 could be affected by fibroblasts *in vivo*, we subcutaneously inoculated H2228 cells, with or without MRC-5 cells, into SCID mice. The tumors of mice injected with H2228 and MRC-5 cells grew slightly faster than those of mice injected with

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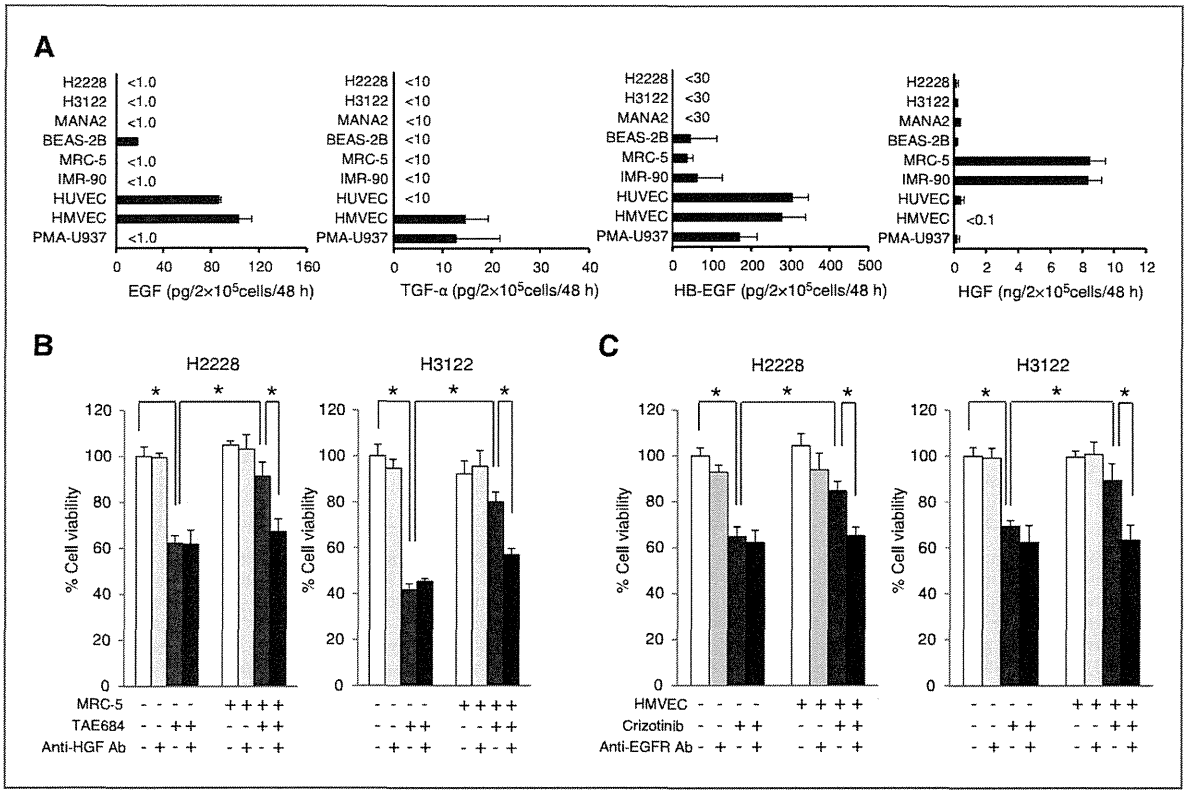

Figure 4. Cross-talk of endothelial cells and fibroblasts reduces sensitivity of EML4-ALK lung cancer cells to ALK inhibitors. A, receptor ligand production was assayed in lung cancer (H2228, H3122, and MANA2), human bronchial epithelial cell (BEAS-2B), fibroblasts (MRC-5 and IMR-90), endothelial cells (HUVEC and HMVEC), and the macrophage differentiated cell line (PMA-U937). The cells were incubated in medium for 48 hours, culture supernatants were harvested, and EGF, TGF-α, HB-EGF, and HGF concentrations were determined by ELISA. All samples were assayed in triplicate. B, H2228 and H3122 cells were cocultured with or without fibroblasts, MRC-5 cells, and/or anti-HGF–neutralizing antibody (2 μg/mL), in the presence or absence of TAE684 (100 nmol/L) for 72 hours, with cell growth determined by MTT assays. *, $P < 0.05$ (one-way ANOVA). Each experiment included triplicate determinations, and each experiment was repeated at least 3 times independently. C, endothelial cell–derived EGFR ligands induced crizotinib resistance in lung cancer cells with EML4-ALK fusion protein, an induction abrogated by blockade of EGFR. H2228 and H3122 cells were cocultured with or without endothelial cells, HMVECs, and/or anti-EGFR–neutralizing antibody (2 μg/mL) in the presence or absence of crizotinib (100 nmol/L) for 72 hours, with cell growth determined as in B. *, $P < 0.05$ (one-way ANOVA). Each experiment included triplicate determinations, with each experiment repeated at least 3 times independently.

H2228 cells alone, but the difference was not statistically significant by day 8 (Fig. 5A). TAE684 treatment, beginning on day 4, caused marked regression of tumors in mice injected with H2228 cells alone, but not of tumors in mice injected with H2228 and MRC-5 cells, indicating that fibroblasts induced resistance to TAE684 *in vivo* (Fig. 5A). We confirmed that HGF was produced by MRC-5 cells *in vivo*. Although the tumors of mice injected with H2228 cells alone did not produce detectable levels of HGF, the tumors of mice injected with H2228 and MRC-5 cells produced high levels of HGF, started on day 4, but decreasing slightly on day 8 (Fig. 5B).

We further analyzed whether coinjection of MRC-5 cells restored the Akt pathway inhibited by TAE684 in the tumors. Western blotting showed that TAE684 treatment inhibited Akt phosphorylation, which was restored by coinjection of MRC-5 cells (Fig. 5C). These results suggested that fibroblasts produced HGF in the tumors and restored Akt phosphorylation as a survival signal, as well as inducing resistance to TAE684 in EML4-ALK lung cancer cells *in vivo*.

Ligand-triggered resistance to ALK inhibitors is abrogated by inhibitors of both HGF-Met and EGFR

To establish novel strategies to treat EGFR ligand- or HGF-triggered resistance to ALK inhibitors, we examined the effect of combinations of ALK inhibitors with EGFR inhibitors (anti-EGFR Abs and reversible EGFR-TKIs) and HGF-Met inhibitors (anti-HGF Abs and Met-TKIs). Combined treatment with erlotinib, a reversible EGFR-TKI and cetuximab, an anti-EGFR Ab, successfully resensitized H2228 and H3122 cells to crizotinib even in the presence of the EGFR ligands, EGF (Fig. 6A), TGF-α (Fig. 6B), and HB-EGF (Fig. 6C). Moreover, the combination of HGF with E7050 (Met-TKI) or anti-HGF Ab resensitized cells to TAE684 (Fig. 6D).

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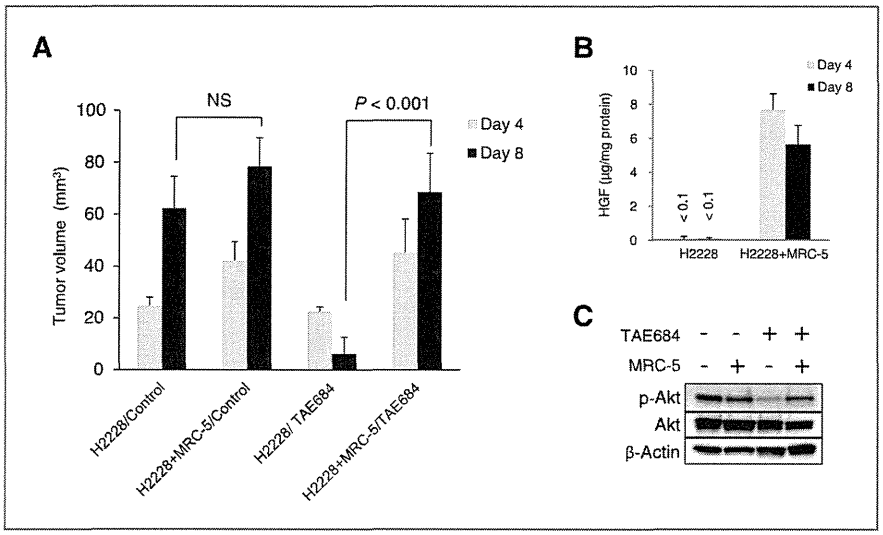

Figure 5. HGF derived from fibroblasts induces TAE684 resistance of EML4-ALK lung cancer cells *in vivo*. A, fibroblast-derived HGF induced TAE684 resistance in H2228 tumors in SCID mice. H2228 cells (5×10^6), with or without MRC-5 cells (5×10^6), were inoculated subcutaneously into SCID mice on day 0. Starting on day 4, mice received oral TAE684 (1.25 mg/kg/d) or vehicle alone, with tumor size measured on days 4 and 8. Tumor volumes were calculated as described in Materials and Methods. Data shown are the representative of 2 independent experiments. Error bars indicate SEs of 6 mice. $P < 0.05$ was considered significant by one-way ANOVA. NS, not significant. B, HGF production by tumor tissues. Tumors were harvested on days 4 and 8 and lysed, and HGFs in the lysates were assayed by ELISA. All samples were assayed in triplicate. C, fibroblast-derived HGF induced TAE684 resistance via the Akt signal pathway *in vivo*. Tumors were harvested 2 hours after treatment on day 7 and lysed, and the lysates were analyzed by immunoblotting with the indicated antibodies, as described in Materials and Methods. The results shown are representative of 2 independent experiments.

Discussion

We have shown here that endothelial cells and fibroblasts, both components of the tumor microenvironment, secreted EGFR ligands and HGF, respectively, causing resistance to the ALK inhibitors crizotinib and/or TAE684 by activating bypass survival signals.

Of the EGFR ligands, EGF and TGF-α bind predominantly to EGFR, whereas HB-EGF binds to EGFR and ErbB4 (17). H2228 cells expressed both EGFR and ErbB4. Our results suggested that the bypass survival signal induced by EGFR ligands is mediated mainly by EGFR, as EGFR ligands markedly activated the phosphorylation of EGFR, not ErbB4. Moreover, knockdown of EGFR abrogated resistance caused by all EGFR ligands tested. EGFR ligand–triggered resistance was canceled by erlotinib or cetuximab, an anti-EGFR Ab, drugs approved for the treatment of patients with NSCLC and colorectal cancer. In addition, AP26113, an inhibitor of both ALK and EGFR, has been reported active against *EML4-ALK* lung cancer cells with amplified *ALK* and secondary mutations (7). Therefore, clinical trials are warranted to evaluate the efficacy and feasibility of combinations of an ALK inhibitor and these EGFR inhibitors to overcome ALK inhibitor resistance.

HGF, the sole ligand of Met (29), is important in EGFR-TKI resistance in *EGFR*-mutant lung cancer. HGF derived from cancer cells or stromal fibroblasts activated Met phosphorylation and stimulated the downstream Akt and Erk1/2 pathways (21, 22, 30) using Gab1, an adaptor protein for Met (31), triggering resistance to both reversible and irreversible EGFR-TKIs. In our Japanese cohort study of patients with *EGFR*-mutant lung cancer, high HGF expression was detected in 61% of tumors with acquired resistance and in 29% of tumors with intrinsic resistance to EGFR-TKIs, suggesting the rationale of targeting HGF to overcome EGFR-TKI resistance (32). We also found that HGF triggered TAE684 resistance by activating Met and stimulating downstream Akt and Erk1/2 pathways using the adaptor protein Gab1. Because many anti-HGF Abs and Met-TKIs are being evaluated in clinical trials, HGF-triggered resistance to selective ALK inhibitors may be controlled by their combinations in the near future.

EGFR and Met have been shown to interact with each other and to mediate redundant signaling in lung cancer cells (33). In *EGFR*-mutant lung cancer cells, *Met* amplification causes EGFR-TKI resistance by triggering bypass survival signals using ErbB3, an adaptor protein (34). Met activation by HGF also triggers resistance to EGFR-TKIs that use Gab1 as an adaptor. In *EML4-ALK* lung cancer cells, both novel ALK second mutations and autocrine EGFR activation causes resistance to ALK inhibitors (11). We found that paracrine HGF and EGFR ligands could trigger ALK inhibitor resistance. Taken together, these findings suggest that signaling by EGFR and Met is crucial for the survival of lung cancer cells with *EGFR* mutations and *EML4-ALK* translocations under inhibition of these driver oncogenes.

We found that resistance to TAE684 was induced by both EGFR ligands and HGF, whereas crizotinib resistance was induced by EGFR ligands alone, a finding that may be due to the dual activities of crizotinib on ALK and

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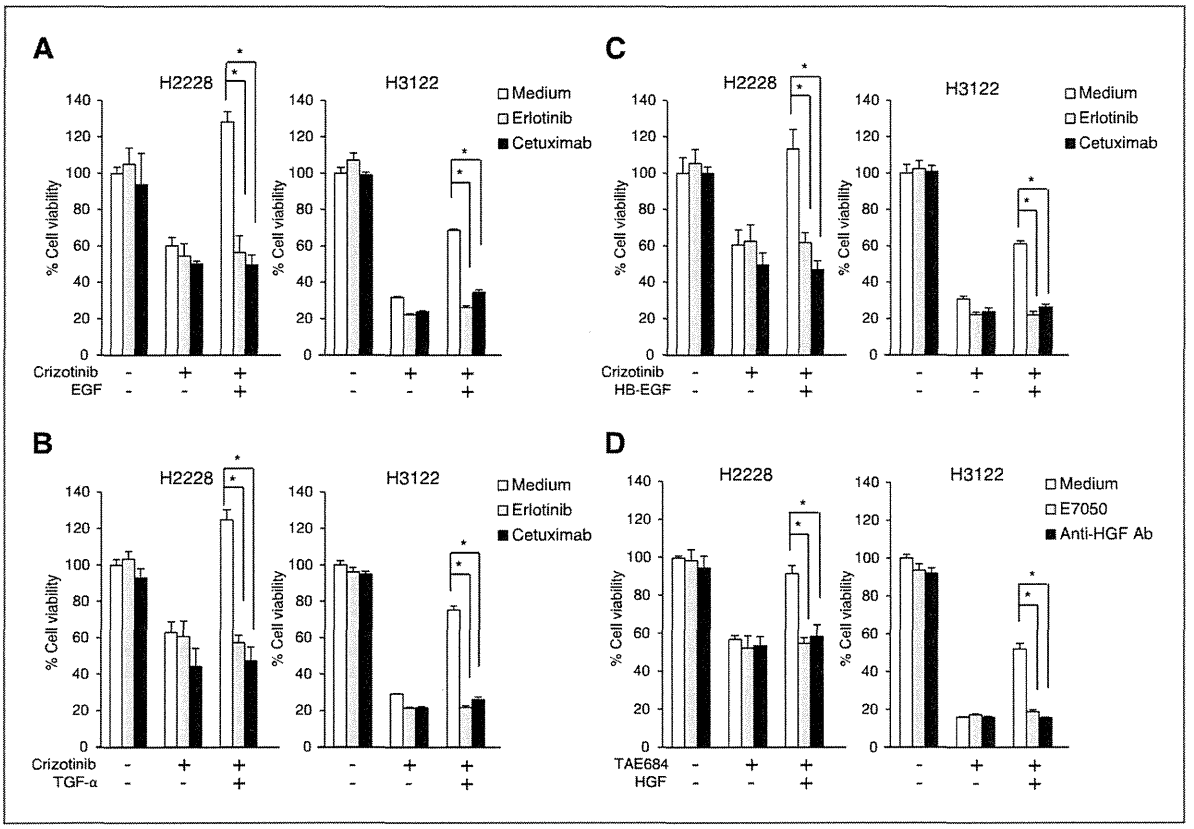

Figure 6. Ligand-triggered resistance to ALK inhibitors is abrogated by inhibitors of both HGF-Met and EGFR. A–C, the EGFR inhibitors erlotinib and cetuximab abrogated EGFR ligand-induced crizotinib resistance in EML4-ALK lung cancer cells. H2228 and H3122 cells were treated for 72 hours with or without 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 μmol/L) or cetuximab (2 μg/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 μmol/L) or anti-HGF–neutralizing antibody (2 μg/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

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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

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Molecular Cancer Therapeutics

Therapeutic Discovery

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 Takeuchi[1], Tadaaki Yamada[1], Shigeki Nanjo[1], Daisuke Ishikawa[1], Takako Sano[1], Kenji Kita[1], Takahiro Nakamura[2], Kunio Matsumoto[2], Kenichi Suda[3], Tetsuya Mitsudomi[3], Yoshitaka Sekido[4], Toshimitsu Uenaka[5], and Seiji Yano[1]

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 [1]Medical Oncology and [2]Tumor Dynamics and Regulation, Cancer Research Institute, Kanazawa University, Kanazawa, Ishikawa; [3]Departments of Thoracic Surgery, Aichi Cancer Center Hospital; [4]Division of Molecular Oncology, Aichi Cancer Center Research Institute, Nagoya, Aichi; and [5]Tsukuba 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

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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 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.

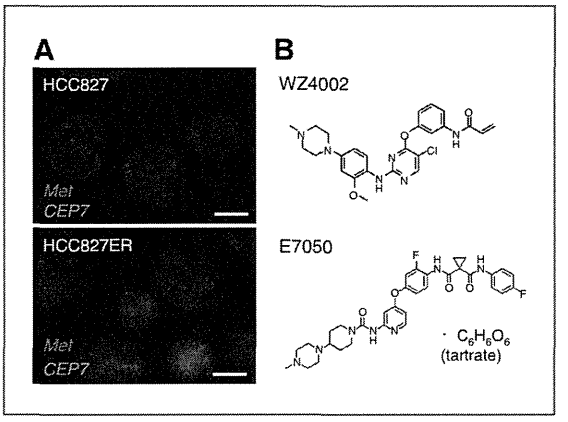

Figure 1. Characteristics of the cell lines used in this study. A, representative interphase FISH analysis of HCC827 and erlotinib-resistant HCC827ER cancer cell lines. The red signal indicates the *Met* gene, and the green signal indicates the *CEP7* (control) gene. *Met* is amplified in HCC827ER but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

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

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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/Icr-*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 × length (mm) ×

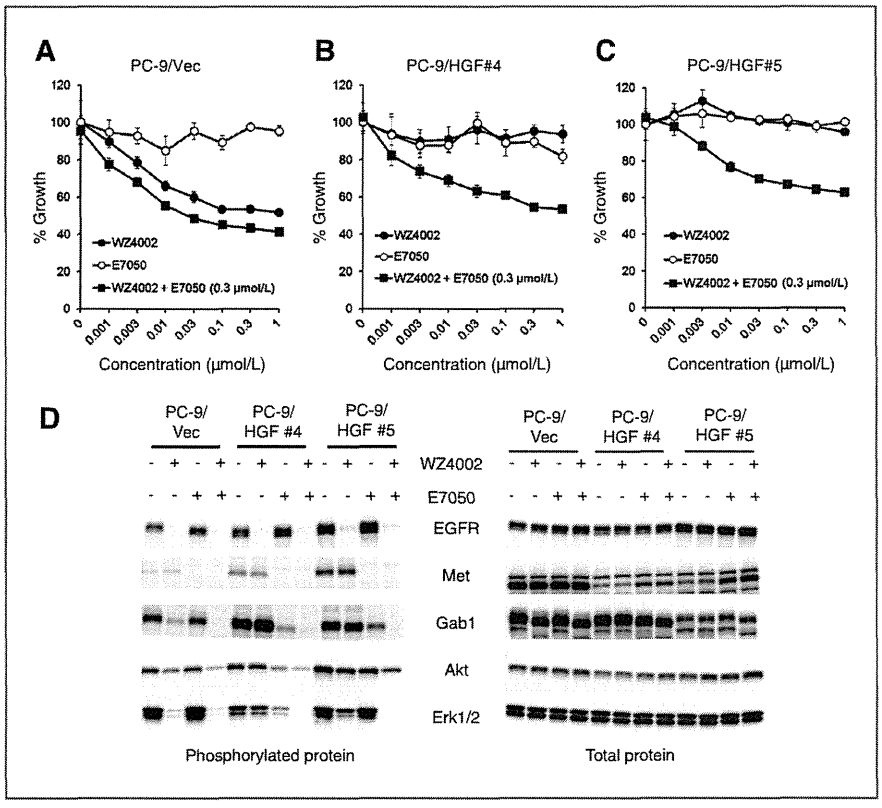

Figure 2. HGF triggers resistance to mutant-selective EGFR in NSCLC cell lines harboring *EGFR*-activating mutations. PC-9/Vec (A), PC-9/HGF#4 (B), and PC-9/HGF#5 (C) cells (2×10^3 cells per well) were incubated with various concentrations of WZ4002, with or without E7050, for 72 hours. Cell growth was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

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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 H_2O_2 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

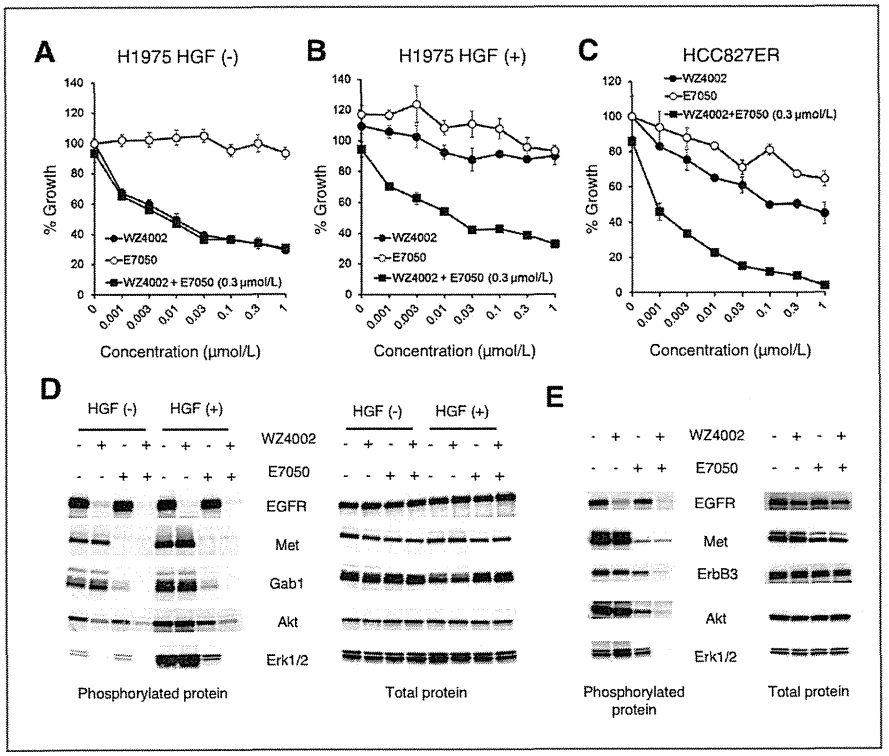

Figure 3. The combination of a mutant-selective EGFR-TKI and a Met inhibitor overcomes several erlotinib-resistant mechanisms. H1975 cells (2×10^3 cells per well) were incubated with various concentrations of erlotinib, with or without E7050, in the absence (A) or presence (B) of HGF (40 ng/mL). C, HCC827ER 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 μmol/L) and/or E7050 (0.3 μmol/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 μmol/L) and/or E7050 (0.3 μmol/L) for 1 hour. The cell lysates were harvested and the phosphorylation of the indicated proteins was determined by Western blot analysis.

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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 and irreversible 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

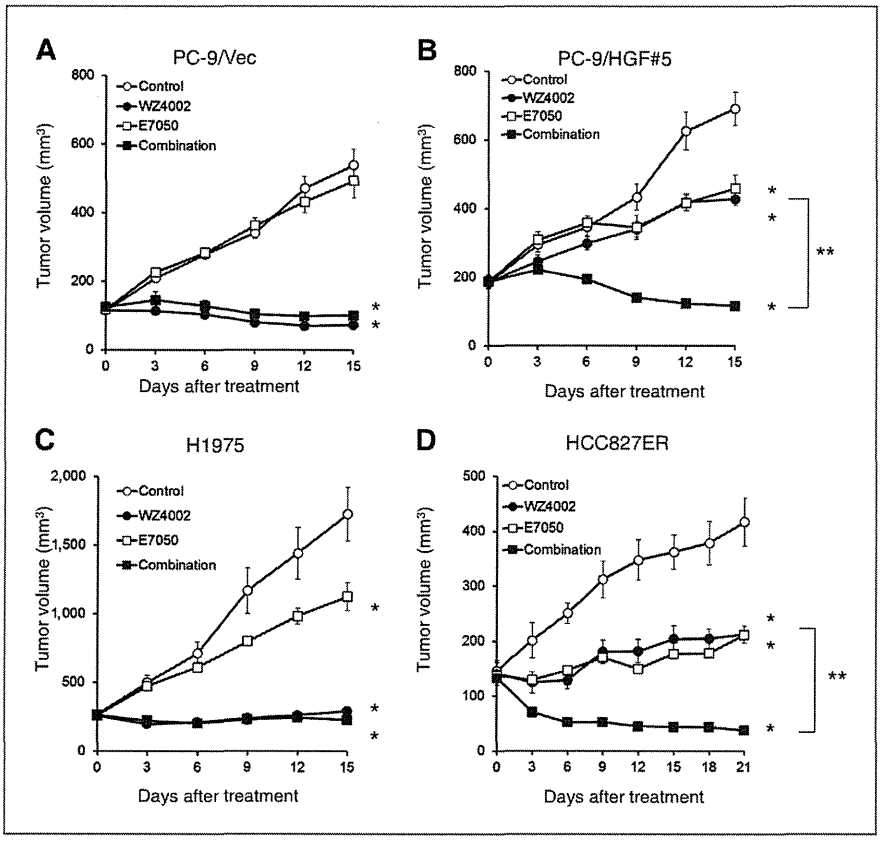

Figure 4. Antitumor activity of WZ4002 and/or E7050 in mouse xenograft models of human tumors. SCID mice-bearing PC-9/Vec (A), PC-9/HGF#5 (B), H1975 (C), or HCC827ER (D) tumors were administered 25 mg/kg WZ4002 and/or E7050 once daily for 14 to 21 days. Tumor volume was measured using calipers on the indicated days. Mean ± 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.

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E7050 inhibited the viability of HCC827ER cells, but their effects were marginal (Fig. 3C). In the presence of E7050 (0.3 μmol/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.

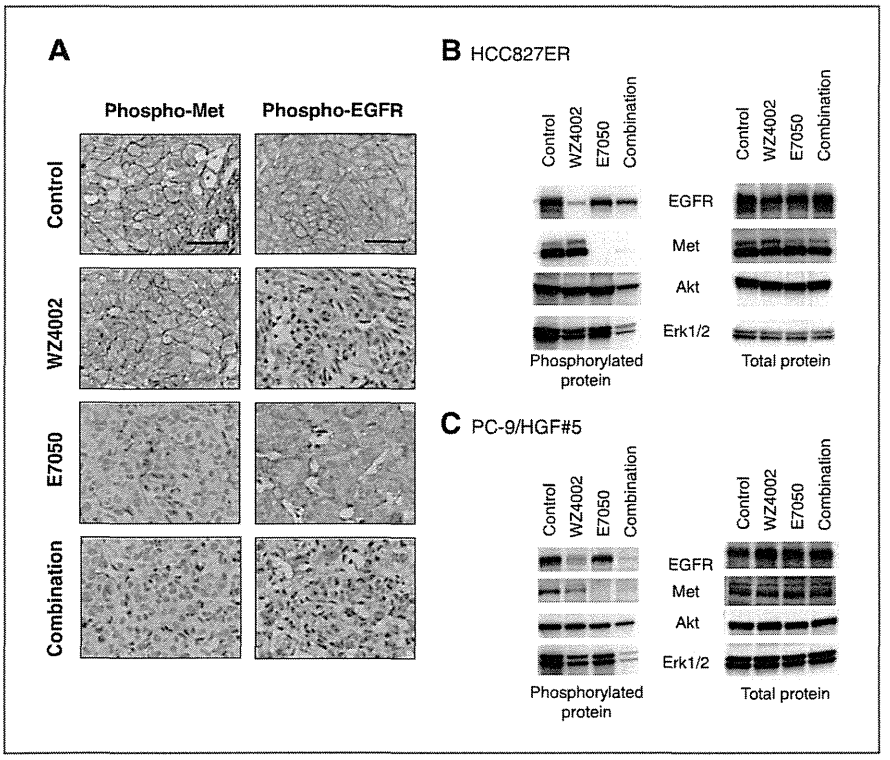

Figure 5. Effect of WZ4002 with or without E7050 on erlotinib-resistant tumors. A, representative images of HCC827ER tumors immunohistochemically stained with antibodies to phosphorylated Met and phosphorylated EGFR. Bar, 50 μm. B and C, HCC827ER (B) and PC-9/HGF#5 (C) tumors were resected from the mice 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.

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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

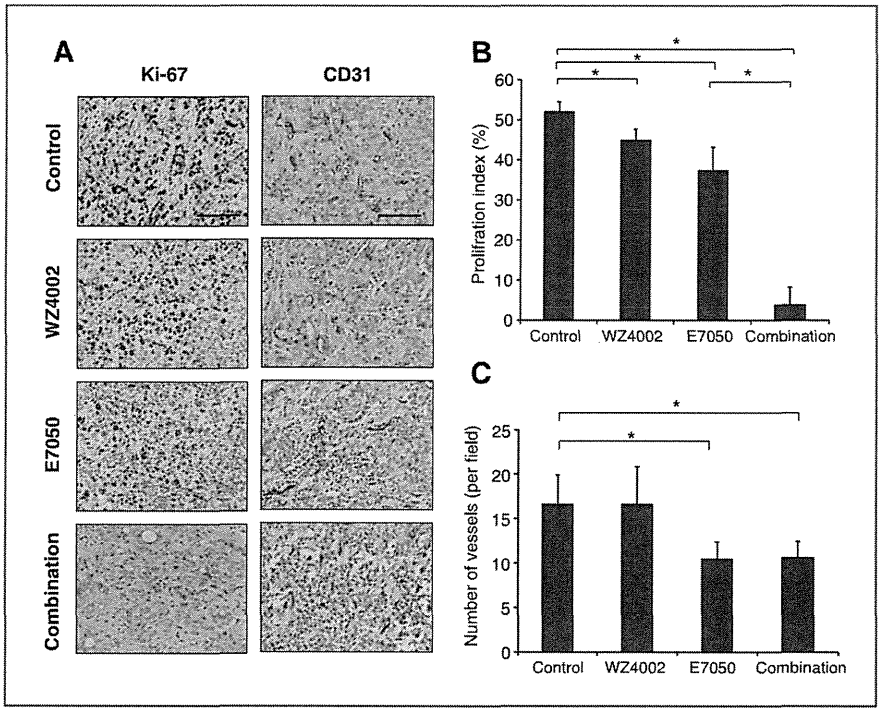

Figure 6. Effect of WZ4002 with or without E7050 on tumor cell proliferation and microvessel density. A, representative images of HCC827ER tumors immunohistochemically stained with antibodies to human Ki-67 and mouse CD31. Bar, 200 μm. B, mean ± SD proliferation index of HCC827ER tumor sections, as determined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

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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

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ORIGINAL ARTICLE

Endoscopic Findings of Upper Gastrointestinal Lesions in Patients with Pancreatic Cancer

Koushiro Ohtsubo[1], Hiroyuki Watanabe[1,2], Hisatsugu Mouri[1], Kaname Yamashita[1], Kazuo Yasumoto[1], Seiji Yano[1]

[1]Division of Medical Oncology, Cancer Research Institute, Kanazawa University. Kanazawa, Japan.
[2]Internal 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.

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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 gastroduodenal 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

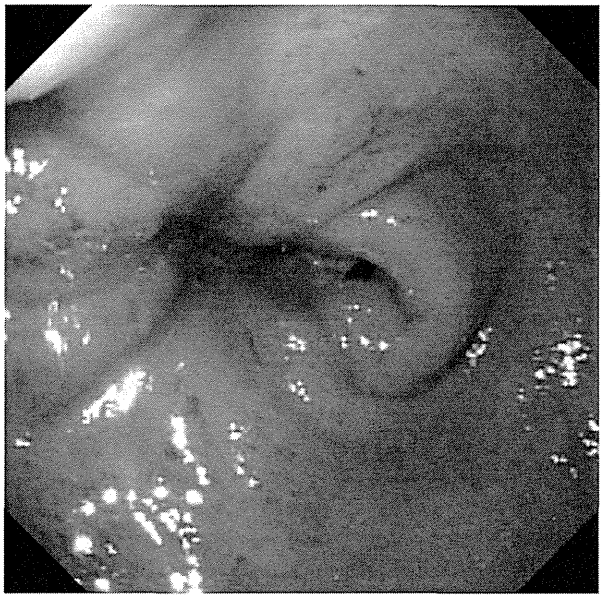

Figure 1. Case of pancreatic head cancer with duodenal invasion.

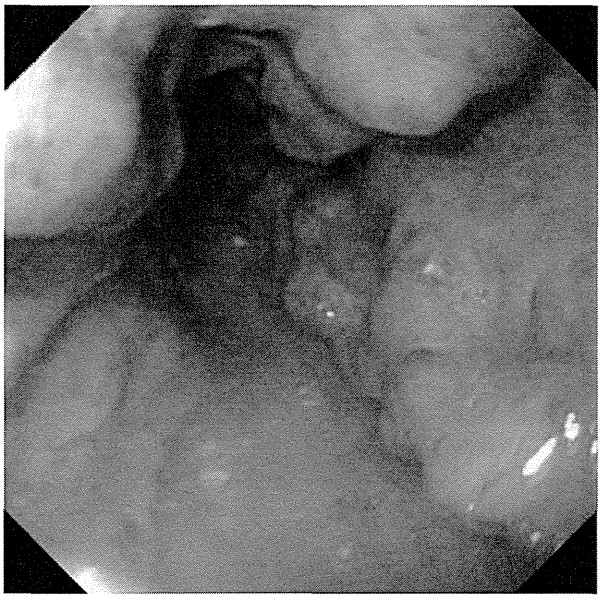

Figure 2. Case of pancreatic head cancer with esophageal varices.

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

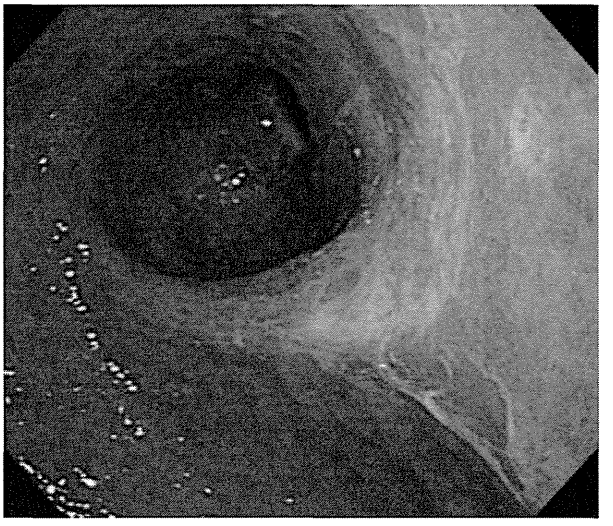

Figure 3. Case of pancreatic head cancer with radiation-induced gastric ulcer.

(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.

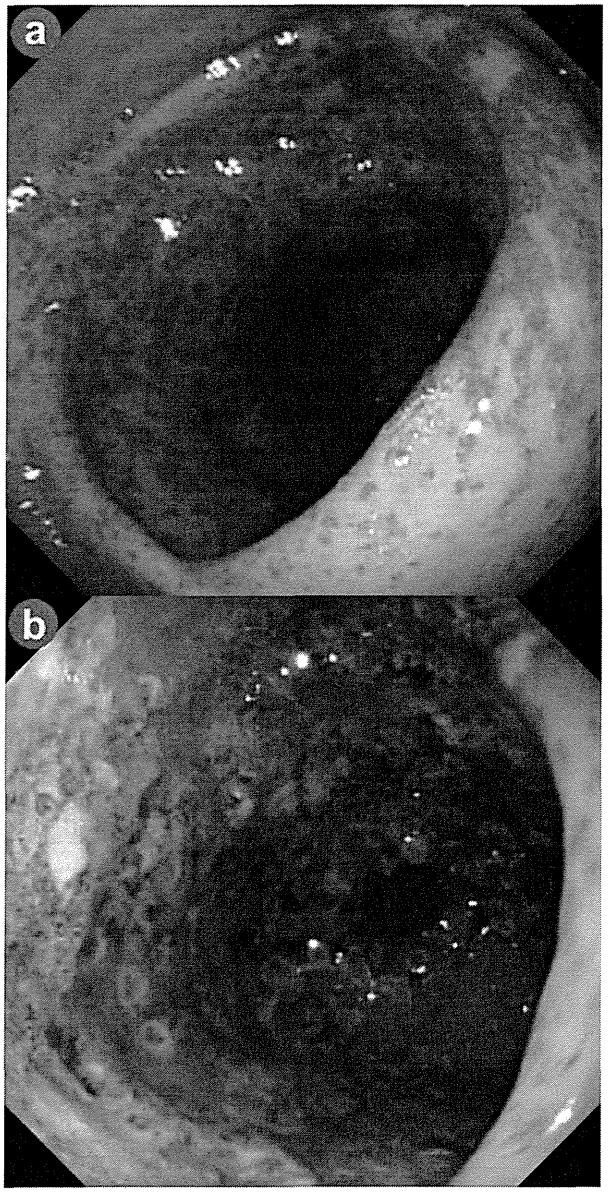

Figure 4 a. Case of pancreatic head cancer with radiation-induced gastritis. **b.** Argon plasma coagulation therapy was effective in this case.

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 H_2-receptor antagonist (H_2 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 H_2 blocker, the other patient received H_2 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-

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, P0.137, 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.

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%)**

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