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

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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); 1–9. ©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 EMLA-ALK lung cancer (12). In addition, a phase I clinical trial of
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 $1 \times 10^5$ 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.

HGF production
Cells ($1 \times 10^6$) 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 $-80 \degree$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>
<thead>
<tr>
<th>Cell line</th>
<th>EGFR mutation</th>
<th>Met amplification</th>
<th>HGF production (ng/10^5 cells/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-9</td>
<td>E746_A750del</td>
<td>–</td>
<td>&lt;0.03</td>
</tr>
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<td>E746_A750del</td>
<td>–</td>
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<td>HCC827</td>
<td>E746_A750del</td>
<td>–</td>
<td>&lt;0.03</td>
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<td>E746_A750del</td>
<td>+</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>H1975</td>
<td>L858R/T790M</td>
<td>–</td>
<td>&lt;0.03</td>
</tr>
</tbody>
</table>

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.
**Cell proliferation assay**

Cell growth was measured using the MTT dye reduction method (17). Tumor cells (2 × 10^3 cells/100 mL/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/lcr-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) × width (mm).
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$_2$O$_2$ 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 Pharmin-gen). 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...
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
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.
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.
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 EGF 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 Ab1 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 EGF 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.

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Published OnlineFirst July 25, 2012; DOI: 10.1158/1535-7163.MCT-12-0195
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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, 22121010A01) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References
Molecular Cancer Therapeutics

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Mol Cancer Ther  Published OnlineFirst July 25, 2012.

Updated version  Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0195

Supplementary Material  Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/07/25/1535-7163.MCT-12-0195.DC1

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