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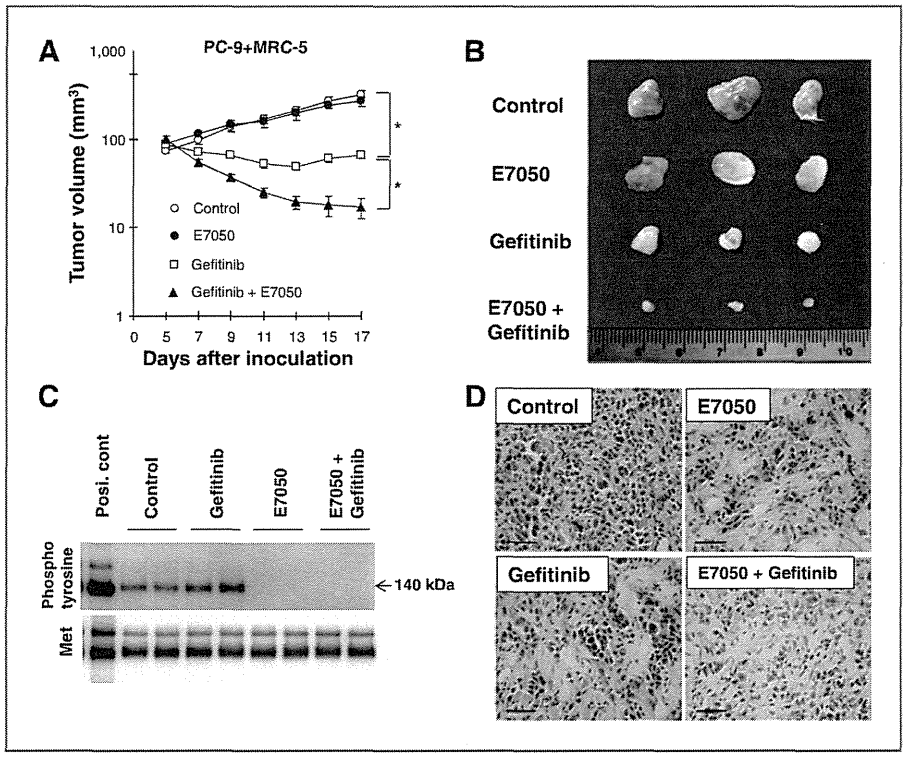

Figure 5. E7050 circumvents HGF-induced resistance when combined with gefitinib *in vivo*. A, PC-9 cells (5×10^6) with or without MRC-5 cells (5×10^6) were inoculated subcutaneously into SCID mice ($N = 6$) on day 0. The mice began treatment with oral gefitinib (25 mg/kg/d) and/or E7050 (50 mg/kg/d), on day 4. The tumor area was measured every 3 or 4 days and the tumor volume was calculated as described in Materials and Methods. Bars show SE of the means ± SD. $^*P < 0.01$. B, macroscopic appearance of treated tumors harvested on day 17. C, expression of phosphorylated Met in harvested tumors. Met protein was immunoprecipitated by anti-Met antibody. Then, phosphorylated Met and Met protein were detected by anti-phosphotyrosine antibody and anti-Met antibody, respectively. D, expression of phosphorylated Akt in the harvested tumors. Frozen sections were immunohistochemically stained with anti-phospho Akt antibody. Original magnification, ×200.

HCC827 cells. Unexpectedly, when we cultured HCC827 cells with gefitinib and HGF for 30 days, we found that the percentage of cells with *Met* amplification was not increased. The reason we failed to detect expansion of clones with *Met* amplification, however, remains unclear. Transfection of the *HGF* gene into HCC827 cells produced HCC827/HGF cells, which constitutively produce HGF. These cells, however, were selected in the presence of geneticin but not gefitinib, with several clones showing amplification of *Met* (data not shown). Therefore, this phenomenon may be unique to a population of *EGFR* mutant lung cancer cells observed only under selection pressure with gefitinib plus an as yet unknown concentration of HGF.

Met was shown to be constitutively phosphorylated in human lung cancer cell lines, with the degree of phosphorylation not always correlated with susceptibility to EGFR-TKIs (33). Indeed, previous studies reported that the level of Met phosphorylation was higher in HCC827 cells than in other *EGFR* mutant cell lines (9, 10, 13, 29). Similar to these results, we also observed that the level of Met phosphorylation was higher in HCC827 cells than in PC-9 and Ma-1 cells (Supplementary Fig. S4). Although the bands for pMet in our study seem to be weaker than those in a previous study (34), ours and previous studies constantly showed that Met phosphorylation in HCC827 cells was higher than that in other *EGFR* mutant cells. Although the difference in the intensity of pMet bands between our study and the previous is unclear, it might be due to minor differences in experimental conditions, including the exposure time at Western blot and the cell culture conditions. With regard to HGF-triggered EGFR-TKI resistance, previous studies also support our findings that although HCC827 cells were highly sensitive to EGFR-TKIs, further Met activation or phosphorylation resulted in inducing resistance to EGFR-TKIs (10, 29, 35). We confirmed that knockdown of Met by siRNA canceled HGF-induced resistance in HCC827 cells (9). Moreover, it was reported that *Met* amplification resulted in increased level of Met phosphorylation and caused resistance to EGFR-TKIs in HCC827 cells (8). This accumulating evidence indicates that constitutive Met phosphorylation is insufficient and further activation by HGF or *Met* amplification may be necessary to induce EGFR-TKI resistance in HCC827 cells. Therefore, there may be a threshold level for Met phosphorylation to sufficiently cause EGFR-TKI resistance.

E7050 inhibits both Met and VEGFR2 kinases (16). *In vitro*, PC-9 and HCC827 cells express little VEGFR2 (data not shown). E7050 did not significantly inhibit the growth of these cell lines, and the anti-VEGF antibody bevacizumab did not augment the susceptibility of these cell lines to gefitinib (data not shown). These results suggest that the *in vitro* antitumor effects of E7050, when combined with gefitinib and HGF, may be largely due to Met inhibition. *In vivo*, we found that very high concentrations of HGF, obtained by *HGF* gene transfection into cancer cells, increased intratumor vessel density (submitted for publication elsewhere). However, HGF concentrations were

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lower in our xenograft model of mixed PC-9 and MRC-5 cells (fibroblasts) than in xenograft tumors produced by HGF gene–transfected lung cancer cells. We observed no difference in intratumor vessel density between tumors induced by PC-9 cells alone and tumors induced by PC-9 and MRC-5 cells (Supplementary Fig. S5). In addition, E7050 did not affect significantly the vessel density in tumors induced by PC-9 and MRC-5 cells. Collectively, these observations suggest that the antitumor effects of E7050 in this resistance model may not be predominantly due to angiogenesis inhibition.

The secondary T790M mutation in *EGFR* is the most prominent mechanism of acquired resistance to EGFR-TKIs in *EGFR* mutant lung cancer, with this mutation detected in about 50% of these patients (4). The T790M mutation increases the affinity of EGFR for ATP, decreasing the binding of EGFR to EGFR-TKIs and inducing resistance to the latter agents (36). *EGFR* mutant lung cancer cells with the T790M secondary mutation, however, remain susceptible to EGFR-mediating signaling and are thought to be manageable by inhibition of EGFR-mediated signaling (37). Preclinical studies have shown that next-generation EGFR-TKIs, irreversible TKIs, and mutant EGFR-selective TKIs have activity against gefitinib-resistant tumors with EGFR T790M secondary mutation (21–23). However, several irreversible EGFR-TKIs, including BIBW2992 (38) and HKI-272 (39), failed to meet primary endpoints in clinical trials of patients with EGFR-TKI–refractory lung cancer. High concentrations of HGF have been frequently detected in tumors with *EGFR*-T790M secondary mutations showing acquired resistance (10, 40, 41). In addition, we found previously (11) and confirmed here that HGF induces resistance to irreversible EGFR-TKIs in *EGFR* mutant lung cancer cells. Taken together, these observations suggest that HGF expressed in tumors with acquired resistance and *EGFR* T790M secondary mutations induce resistance to irreversible EGFR-TKI. As E7050 reversed the resistance to irreversible and mutant-selective EGFR-TKIs, it may augment the therapeutic efficacy of next-generation EGFR-TKIs in *EGFR* mutant lung cancer patients with acquired resistance to the *EGFR* T790M secondary mutation. These ideas further illustrate the necessity of methods to select patients who develop EGFR-TKI resistance due to HGF.

In conclusion, we have presented preclinical evidence showing that a new Met kinase inhibitor, E7050, may overcome HGF-induced resistance in *EGFR* mutant lung cancer. Further evaluation of E7050 in clinical trials is warranted to improve the outcomes of patients with *EGFR* mutant lung cancer.

Disclosure of Potential Conflicts of Interest

T. Uenaka and T. Nakagawa are employees of Eisai Co. S. Yano has received a commercial research grant from Eisai Co. and honoraria from speaker's bureau from Chugai Pharma.

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References

1. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
2. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
3. Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. Lancet Oncol 2010;11:121–8.
4. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 2010;362:2380–8.
5. Jackman D, Pao W, Riely GJ, Engelman JA, Kris MG, Janne PA, et al. Clinical definition of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. J Clin Oncol 2010;28:357–60.
6. Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
7. Pao W, Miller VA, Politi KA, Janne PA, Kocher O, Meyerson M, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005;2:e73.
8. Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039–43.
9. Yano S, Wang W, Li Q, Matsumoto K, Sakurama H, Nakamura T, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma cells with EGF receptor mutations. Cancer Res 2008;68:9479–87.
10. Turke AB, Zejnullahu K, Wu YL, Song Y, Dias-Santagata D, Lifshits E, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell 2010;17:77–88.
11. Yamada T, Matsumoto K, Wang W, Li Q, Nishioka Y, Sekido Y, et al. Hepatocyte growth factor reduces susceptibility to an irreversible epidermal growth factor receptor inhibitor in EGFR-T790M mutant lung cancer. Clin Cancer Res 2010;16:174–83.
12. Matsumoto K, Nakamura T. Hepatocyte growth factor and the Met system as a mediator of tumor-stromal interactions. Int J Cancer 2006;119:477–83.
13. Wang W, Li Q, Yamada T, Matsumoto K, Matsumoto I, Oda M, et al. Crosstalk to stromal fibroblasts induces resistance of lung cancer to EGFR tyrosine kinase inhibitors. Clin Cancer Res 2009;15:6630–8.
14. Donev IS, Wang W, Yamada T, Li Q, Takeuchi S, Matsumoto K, et al. Transient PI3K inhibition induces apoptosis and overcomes HGF-

Published OnlineFirst February 8, 2012; DOI: 10.1158/1078-0432.CCR-11-1171

mediated resistance to EGFR-TKIs in EGFR mutant lung cancer. Clin Cancer Res 2011;17:2260–9.
15. Kim ES, Salgia R. MET pathway as a therapeutic target. J Thorac Oncol 2009;4:444–7.
16. Nakagawa T, Tohyama O, Yamaguchi A, Matsushima T, Takahashi K, Funasaka S, et al. E7050: a dual c-Met and VEGFR-2 tyrosine kinase inhibitor promotes tumor regression and prolongs survival in mouse xenograft models. Cancer Sci 2010;101:210–5.
17. Montesano R, Matsumoto K, Nakamura T, Orci L. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell 1991;67:901–8.
18. Green LM, Reade JL, Ware CF. Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. J Immunol Methods 1984;70:257–68.
19. Nishioka Y, Yano S, Fujiki F, Mukaida N, Matsushima K, Tsuruo T, et al. Combined therapy of multidrug-resistant human lung cancer with anti-P-glycoprotein antibody and monocyte chemoattractant protein-1 gene transduction: the possibility of immunological overcoming of multidrug resistance. Int J Cancer 1997;71:170–7.
20. Cappuzzo F, Marchetti A, Skokan M, Rossi E, Gajapathy S, Felicioni L, et al. Increased MET gene copy number negatively affects survival of surgically resected non-small-cell lung cancer patients. J Clin Oncol 2009;27:1667–74.
21. Navab R, Liu J, Seiden-Long I, Shih W, Li M, Bandarchi B, et al. Co-overexpression of Met and hepatocyte growth factor promotes systemic metastasis in NCI-H460 non-small cell lung carcinoma cells. Neoplasia 2009;11:1292–300.
22. Li D, Ambrogio L, Shimamura T, Kubo S, Takahashi M, Chilieac LR, et al. BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. Oncogene 2008;27: 4702–11.
23. Kobayashi S, Ji H, Yuza Y, Meyerson M, Wong KK, Tenen DG, et al. An alternative inhibitor overcomes resistance caused by a mutation of the epidermal growth factor receptor.Cancer Res 2005;65: 7096–101.
24. Kwak EL, Sordella R, Bell DW, Godin-Heymann N, Okimoto RA, Brannigan BW, et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. Proc Natl Acad Sci U S A 2005;102:7665–70.
25. Zhou W, Ercan D, Chen L, Yun CH, Capellletti M, Cortot AB, et al. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. Nature 2009;462:1070–4.
26. Stabile LP, Rothstein ME, Keohavong P, Lenzner D, Land SR, Gaither-Davis AL, et al. Targeting of both EGFR and c-Met pathways results in additive inhibition of lung tumorigenesis in transgenic mice. Cancers 2010;2 2153–70.
27. Siegfried JM, Weissfeld LA, Singh-Kaw P, Weyant RJ, Testa JR, Landreneau RJ. Association of immunoreactive hepatocyte growth factor with poor survival in resectable non-small cell lung cancer. Cancer Res 1997;57:433–9.
28. Ma PC, Tretiakova MS, MacKinnon AC, Ramnath N, Johnson C, Dietrich S, et al. Expression and mutational analysis of MET in human solid cancers. Genes Chromosomes Cancer 2008;47: 1025–37.
29. Benedettini E, Sholl LM, Peyton M, Reilly J, Ware C, Davis L, et al. Met activation in non-small cell lung cancer is associated with de novo resistance to EGFR inhibitors and the development of brain metastasis. Am J Pathol 2010;177:415–23.
30. Kasahara K, Arao T, Sakai K, Matusmoto K, Sakai A, Kimura H, et al. Impact of serum hepatocyte growth factor on treatment response to epidermal growth factor receptor tyrosine kinase inhibitors in patients with non-small cell lung adenocarcinoma. Clin Cancer Res 2010;16: 4616–24.
31. Tanaka H, Kimura T, Kudoh S, Mitsuoka S, Watanabe T, Suzumura T, et al. Reaction of plasma hepatocyte growth factor levels in non-small cell lung cancer patients treated with EGFR-TKIs. Int J Cancer 2011;129:1410–6.
32. Shojaei F, Lee JH, Simmons BH, Wong A, Esparza PA, Plumlee PA, et al. HGF/c-Met acts as an alternative angiogenic pathway in sunitinib-resistant tumors. Cancer Res 2010;70:10090–100.
33. Kubo T, Yamamoto H, Lockwood WW, Valencia I, Soh J, Peyton M, et al. MET gene amplification or EGFR mutation activate MET in lung cancers untreated with EGFR tyrosine kinase inhibitors. Int J Cancer 2009;124:1778–84.
34. Guo A, Villén J, Kornhauser J, Lee KA, Stokes MP, Rikova K, et al. Signaling networks assembled by oncogenic EGFR and c-Met. Proc Natl Acad Sci U S A 2008;105:692–7.
35. Okamoto W, Okamoto I, Tanaka K, Hatashita E, Yamada Y, Kuwata K, et al. TAK-701, a humanized monoclonal antibody to hepatocyte growth factor, reverses gefitinib resistance induced by tumor-derived HGF in non-small cell lung cancer with an EGFR mutation. Mol Cancer Ther 2010;9:2785–92.
36. Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. Proc Natl Acad Sci U S A 2008; 105:2070–5.
37. Nguyen KS, Kobayashi S, Costa DB. Acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancers dependent on the epidermal growth factor receptor pathway. Clin Lung Cancer 2009;10:281–9.
38. Miller VA, Hirsh V, Cadranel J, Chen YM, Park K, Kim SW, et al. Phase IIB/III double-blind randomized trial of AFATINIB (BIBW 2992), an irreversible inhibitor of EGFR/HER1 and HER2) + best supportive care (BSC) versus placebo plus BSC in patients with NSCLC failing 1-2 lines of chemotherapy and ERLOTINIB or GEFITINIB (LUX-lung 1). Ann Oncol 2010;21 Suppl 8:viii1–viii2.
39. Sequist LV, Besse B, Lynch TJ, Miller VA, Wong KK, Gitlits B, et al. Neratinib, an irreversible pan-ErbB receptor tyrosine kinase inhibitor: results of a phase II trial in patients with advanced non-small-cell lung cancer. J Clin Oncol 2010;28:3076–83.
40. Onitsuka T, Uramoto H, Nose N, Takenoyama M, Hanagiri T, Sugio K, et al. Acquired resistance to gefitinib: the contribution of mechanisms other than the T790M, MET, and HGF status. Lung Cancer 2010;68: 198–203.
41. Yano S, Yamada T, Takeuchi S, Tachibana K, Minami Y, Yatabe Y, et al. Hepatocyte growth factor expression in EGFR mutant lung cancer with intrinsic and acquired resistance to tyrosine kinase inhibitors in a Japanese cohort.J Thorac Oncol 2011;6:2011–7.

Hsp90 Inhibition Overcomes HGF-Triggering Resistance to EGFR-TKIs in EGFR-Mutant Lung Cancer by Decreasing Client Protein Expression and Angiogenesis

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Introduction: The three major clinically relevant mechanisms of acquired resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in EGFR mutant lung cancer are a second mutation in the *EGFR* gene (T790M), *Met* amplification, and increased expression of hepatocyte growth factor (HGF). Heat shock protein90 (Hsp90) is a 90 kDa molecular chaperone for proteins that include EGFR, Met, and echinoderm microtubule-associated proetin-like-4-the anaplastic lymphoma kinase. Here, we determined whether inhibition of Hsp90 could overcome HGF-triggered EGFR-TKI resistance in *EGFR* mutant lung cancer cells.

Methods: The effects of the Hsp90 inhibitor 17-demethoxygeldanamycin (17-DMAG) on the growth of lung cancer cells resistant to the EGFR-TKI were examined in the presence and absence of HGF, and in cells transfected with the *HGF* gene in vitro and in vivo.

Results: EGFR-TKI erlotinib did not inhibit the growth of *HGF*-gene transfected Ma-1 (Ma-1/HGF) cells and H1975 cells, containing the *EGFR* L858R and T790M mutations, respectively. Erlotinib also did not inhibit the growth of PC-9 and Ma-1 cells, with deletions in *EGFR* exon19, in the presence of HGF. However, 17-DMAG induced apoptosis and markedly inhibited the growth of these cell lines, even in the presence of HGF. This inhibition by 17-DMAG was associated with decreased expression of EGFR and Met in tumor cells. An *in vivo* model of HGF-triggered erlotinib-resistance, which used Ma-1/HGF cells, showed that 17-DMAG markedly suppressed tumor growth by decreasing angiogenesis and increasing apoptosis.

Conclusions: Hsp90 inhibitors may overcome HGF-triggered resistance to EGFR-TKIs and may result in more successful treatment of patients with *EGFR*-mutant lung cancers.

Key Words: HGF, EGFR-TKI resistance, Hsp90, Lung cancer, EGFR mutation.

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Lung cancers with mutations that activate epidermal growth factor receptor (EGFR) such as exon 19 deletions and exon 21 L858R point mutations are sensitive to the EGFR-tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib.[1] Although clinical trials have shown that these EGFR-TKIs enhance progression-free survival, compared with conventional chemotherapy, in patients with *EGFR* mutant lung cancers,[2–5] the vast majority of responders relapse after developing resistance to EGFR-TKIs.[1,6] Among the clinically relevant mechanisms by which these tumors develop resistance to EGFR-TKIs are the T790M secondary mutation in *EGFR*,[6,7] *Met* gene amplification,[8] and hepatocyte growth factor (HGF) overexpression,[9] with HGF overexpression being the most frequently detected of the three in our cohort study of Japanese patients with acquired resistance to EGFR-TKIs.[10] Moreover, HGF overexpression is frequently accompanied by the T790M second mutation[10,11] and/or *MET* gene amplification[10] in these tumors, indicating that HGF is an important therapeutic target for overcoming tumor resistance to EGFR-TKIs.

Several strategies have been proposed to overcome this acquired resistance. For example, new generation EGFR-TKIs, such as irreversible EGFR-TKIs and TKIs selective for mutant *EGFR*, have been developed to treat T790M associated resistance.[12–14] Combined treatment with an EGFR-TKI and a Met-TKI may overcome resistance associated with *Met* amplification.[15] Another approach involves the disruption of heat shock protein 90 (Hsp90) function, because many mutant oncoproteins require Hsp90 for maturation and conformational stability and are degraded after Hsp90 inhibition.[16] Hsp90 inhibitors, including geldanamycin and its derivative 17-demethoxygeldanamycin (17-DMAG), have been reported effective in overcoming EGFR-TKI resistance caused by T790M second mutation and *Met* amplification.[17,18] However, the effect of Hsp90 inhibition on HGF-induced resistance to EGFR-TKIs is still not known. We therefore evaluated the effect of Hsp90

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inhibition on HGF-induced resistance to EGFR-TKIs in *EGFR*-mutant lung cancer cell lines in vitro and in vivo.

MATERIALS AND METHODS

Cell Cultures and Reagents

The *EGFR*-mutant human lung adenocarcinoma cell lines PC-9 (del E746_A750) and HCC827, with deletions in *EGFR* exon 19, were purchased from Immuno-Biological Laboratories Co. (Takasaki, Gunma, Japan) and the American Type Culture Collection (Manassas, VA), respectively. Ma-1 cells with an EGFR exon 19 deletion, HCC827ER cells with deletions in *EGFR* exon 19 and *Met* amplification,[19] and H1975 cells with the L858R/T790M double mutation,[20] were kindly provided by Dr. E. Shimizu (Tottori University, Tottori, Japan), Dr. T. Mitsudomi (Aichi Cancer Center Hospital, Nagoya, Japan), and Dr. Y. Sekido (Aichi Cancer Center Research Institute, Nagoya, Japan), respectively. Human HGF-gene transfectant (Ma-1/HGF) and vector control (Ma-1/Vec) cells were established as described.[9] All of these cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. All cells were passaged for less than 3 months before renewal from frozen, early-passage stocks. Cells were regularly screened for mycoplasma, using MycoAlert Mycoplasma Detection Kits (Lonza, Rockland, ME). Erlotinib, CL-387,785, and 17-DMAG were obtained from Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan), Calbiochem (San Diego, CA), and Selleck Chemicals (Houston, TX), respectively. Human recombinant HGF was prepared as described.[21]

HGF Production in Cell Culture Supernatants

Cells (2×10^5) were cultured in 2 ml of RPMI 1640 or Dulbecco's modified eagle medium with 10% FBS for 24 hours, washed with phosphate-buffered saline (PBS), and incubated for 48 hours in RPMI 1640 with 10% FBS. The culture media were harvested and centrifuged, and the supernatants were stored at −70°C until analysis. HGF was measured by enzyme-linked immunosorbent assay (Immunis HGF EIA; B-Bridge International, Mountain View, CA; limit of detection, 0.1 ng/ml) according to the manufacturer's instructions. All samples were assayed in triplicate. Color intensity was measured at 450 nm with a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves.

Cell Growth Assay

Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method.[22] Tumor cells, plated at 2×10^3/100 μl RPMI 1640 plus 10% FBS per well in 96-well plates, were incubated for 24 hours; erlotinib, CL-387,785, 17-DMAG, and/or HGF were added to each well, and incubation was continued for an additional 72 hours. Cell growth was measured with MTT solution (2 mg/ml; Sigma, St. Louis, MO), as described.[9] Each experiment was performed at least three times, each with triplicate samples.

Antibodies and Western Blotting

Protein aliquots of 25 μg each were resolved by sodium dodecyl sulfate polyacrylamide gel (Bio-Rad, Hercules, CA) electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad). After washing four times, the membranes were incubated with Blocking One (Nacalai Tesque, Inc., Kyoto, Japan) for 1 hour at room temperature and overnight at 4°C with primary antibodies to -actin (13E5), MET (25H2), phospho-Met (anti-p-Met, Y1234/Y1235; 3D7), p-EGFR (Y1068), Akt or p-Akt (S473) (Cell Signaling Technology, Beverly, MA), human EGFR (1 μg/ml), human/mouse/rat ERK1/ERK2 (0.2 μg/ml), and p-ERK1/ERK2 (T202/Y204; 0.1 μg/ml) (R&D Systems, Minneapolis, MN). After three washes, the membranes were incubated for 1 hour at room temperature with species-specific horseradish-peroxidase–conjugated secondary antibodies. Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL), an enhanced chemiluminescent substrate. Each experiment was performed at least three times independently.

Cell Apoptosis Assay

Cell apoptosis induced by erlotinib and 17-DMAG was measured with an Annexin V-fluorescein isothiocyanate Apoptosis Detection Kit I (BD Biosciences, San Jose, CA) in accordance with the manufacturer's directions.[9] Cells were analyzed on a FACSCalibur flow cytometer with CellQuest Software (Becton Dickinson, Franklin Lakes, NJ).

Xenograft Studies in Severe Combined Immunodeficiency Mice

Suspensions of Ma-1/Vec and Ma-1/HGF cells (5×10^6) were injected subcutaneously into the backs of 5-week-old female severe combined immunodeficiency mice (Clea, Tokyo, Japan). After 7 days, the mice were randomized to (1) no treatment (control group), (2) oral erlotinib (20 mg/kg/day), (3) intraperitoneal 17-DMAG (10 mg/kg/day in water), or (4) oral erlotinib plus intraperitoneal 17-DMAG. Tumor size was measured twice per week, and tumor volume was calculated, in mm^3, as $width^2 \times length/2$. All animal experiments complied with the guidelines for the Institute for Experimental Animals, Advanced Science Research Center, Kanazawa University, Kanazawa, Japan (approval no. AP-081088).

Histological Analyses

Apoptotic cells were detected by terminal deoxynucleotidyl transferase (TUNEL)–mediated nick end labeling staining, using the DeadEnd Fluorometric TUNEL system (Promega, Madison, WI). Briefly, formalin-fixed, paraffin-embedded sections (4 μm thick) were deparaffinized and tissues were permeabilized with protease K solution. The samples were equilibrated, and DNA strand breaks were labeled with fluorescein-12-2-deoxy-uridine-5-triphosphate by adding a nucleotide mixture and TUNEL. The reaction was stopped by the addition of saline sodium citrate, and the localized green fluorescence of apoptotic cells was detected by fluorescence microscopy (×400). Proliferating cells were detected by incubating tissue sections with Ki-67 antibody (Clone MIB-1; DAKO Corp., Glostrup, Denmark). Antigen was retrieved by microwaving tissue sections in 10 mM citrate buffer (pH 6.0). After incubation with secondary antibody and treatment with the Vectastain ABC Kit (Vector Laboratories,

Burlingame, CA), peroxidase activity was visualized using the diamino benzidine (DAB) reaction. To analyze microvessel density, frozen sections (5-μm thick) of xenograft tumors were fixed with cold acetone and washed with PBS. After blocking endogenous peroxidase activity with 3% aqueous H_2O_2 solution for 10 minutes, the sections were incubated with 5% normal horse serum, washed, and incubated overnight at 4°C with antimouse-CD31 (clone MEC13.3, BD Bioscience) antibody. After washing with PBS, the sections were incubated with peroxidase conjugated antirat IgG (Cell Signaling Technology) for 40 minutes. Peroxidase activity was visualized with DAB reactions. The sections were counterstained with hematoxylin.

EGFR was assessed immunohistochemically, using the EGFR pharmDx kit (DAKO), as recommended by the manufacturer. To detect Met protein, formalin-fixed paraffin-embedded tissue sections were incubated with a 1:100 dilution of polyclonal rabbit anti-MET antibody overnight at 4°C after microwave antigen retrieval in 0.01 M citrate buffer (pH 6.0). After incubation with secondary antibody and treatment with the Vectastain ABC Kit), peroxidase activity was visualized using DAB reactions.

Quantification of Immunohistochemistry and Immunofluorescence

The five areas containing the highest numbers of stained cells within a section were selected for histologic quantitation by light or fluorescent microscopy at 400-fold magnification. All results were independently evaluated by two investigators (H.K. and T.N.).

Statistical Analysis

Between-group comparisons were assessed by two-tailed Student's *t* tests. All analyses were performed using GraphPad Software (GraphPad Software, Inc., La Jolla, CA). A p value < 0.001 was considered statistically significant.[23]

RESULTS

17-DMAG Inhibits Growth of *EGFR*-Mutant Lung Cancer Cells in the Presence of HGF

PC-9, HCC827, and Ma-1 are human lung adenocarcinoma cell lines with deletions of exon 19 of *EGFR*, resulting in constitutive activation of this gene. These three cell lines were sensitive to erlotinib, whereas HGF induced erlotinib resistance (Fig. 1*A, B, D*). The Hsp90 inhibitor 17-DMAG alone markedly inhibited the growth of these three cells lines, with an IC_{50} (half maximal inhibitory concentration) lower than that of erlotinib in PC-9 (0.01 μmol/l versus 0.03 μmol/l) and Ma-1 (0.01 μmol/l versus 0.03 μmol/l) cells and a similar IC_{50} in HCC827 (0.01 μmol/l) cells. Under these experimental conditions, HGF did

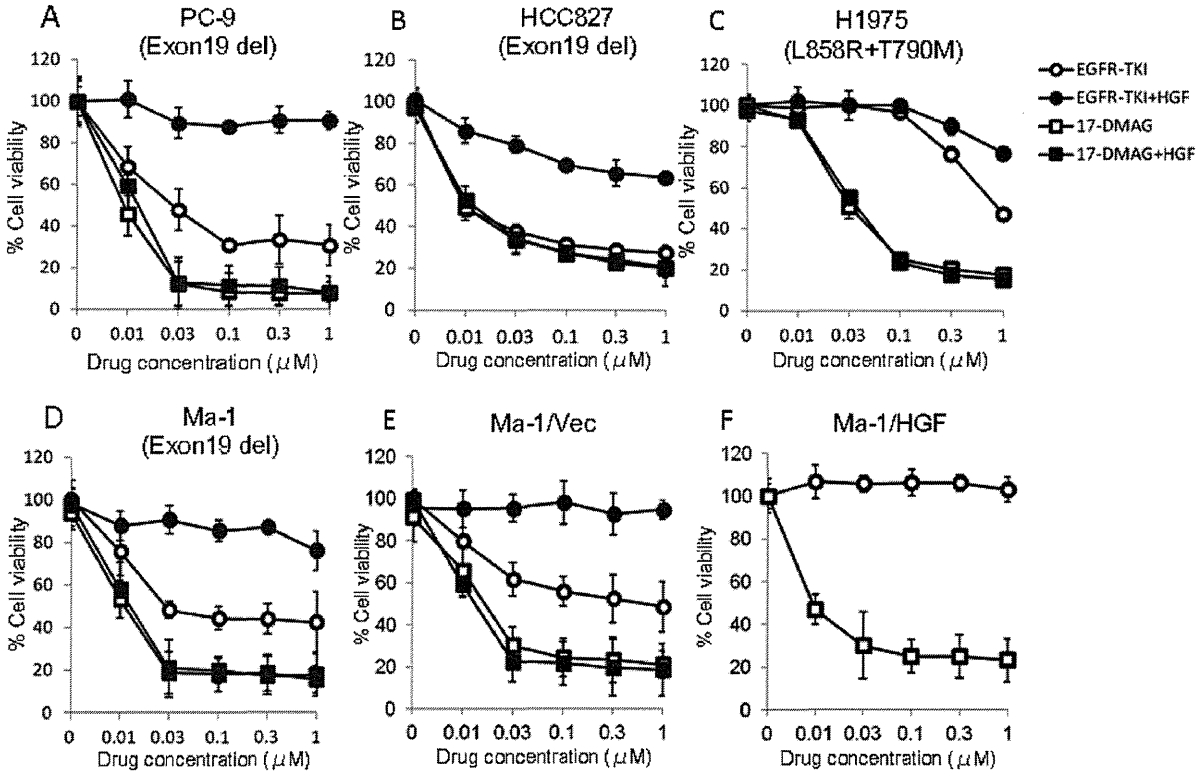

FIGURE 1. 17-DMAG suppresses the in vitro growth of EGFR mutant lung cancer cells in the presence of HGF. Tumor cells were continuously treated with increasing concentrations of EGFR-TKI, erlotinib (PC-9, HCC827, Ma-1, Ma-1/Vec, and Ma-1/HGF), CL-387,785 (H1975), or 17-DMAG, with or without HGF (20 ng/ml), and cell growth was determined after 72 hours by MTT assay. Data shown are representative of five independent experiments. Error bars indicate SD of triplicate cultures. EGFR, epidermal growth factor receptor; HGF, hepatocyte growth factor; TKI, tyrosine kinase inhibitor.

not decrease the sensitivity of the three cell lines to 17-DMAG. In parallel experiments, we used H1975, a human lung adenocarcinoma cell line with both an exon 20 T790M gatekeeper mutation and an exon 21 L858R mutation in *EGFR*. Although these cells were resistant to erlotinib (data not shown), they were sensitive to an irreversible EGFR-TKI CL-387,785,[24] as described (Fig. 1*C*). We found that 17-DMAG alone inhibited the growth of H1975 cells, with an IC_{50} much lower than that of CL-387,785 (0.03 μmol/l versus 0.5 μmol/l); and also inhibited the growth of HCC827ER cells, with a deletion of exon 19 of *EGFR* and *Met* gene amplification, with an IC_{50} of 0.03 μmol/l (Supplemental Figure 1, Supplemental Digital Content, http://links.lww.com/JTO/A267). HGF did not decrease the sensitivity of H1975 and HCC827ER cells to 17-DMAG. Incubation of PC-9, H1975, and HCC827ER cells, harboring mutant EGFR, with the combination of 17-DMAG plus EGFR-TKI showed that 17-DMAG did not sensitize these cells to EGFR-TKI treatment in vitro (Supplemental Figures 1 and 2, Supplemental Digital Content, http://links.lww.com/JTO/A267). Taken to-gether, these results suggest that 17-DMAG may overcome HGF-induced resistance to reversible and irreversible EGFR TKIs in lung cancer cells containing an *EGFR* activating and gatekeeper mutation, respectively.

Our previous study demonstrated that, in patients with non–small-cell lung cancer, HGF is detected primarily in cancer cells with acquired resistance to EGFR-TKIs, suggesting that the production of HGF by these cells occurs via an autocrine mechanism. To further explore the effect of 17-DMAG on autocrine HGF, we generated stable HGF-gene transfectants in Ma-1 cells (Ma-1/HGF); as a control, we generated Ma-1/Vec cells transfected with vector alone. Ma-1/HGF cells secreted high concentrations of HGF (27.8 ± 0.9 ng/ml), whereas the concentrations of HGF secreted by Ma-1 and Ma-1/Vec cells were under the limit of detection (Supplemental Figure 3, Supplemental Digital Content, http://links.lww.com/JTO/A267). In addition, Ma-1/HGF cells became resistant to erlotinib. We found that 17-DMAG inhibited the growth of both Ma-1/Vec and Ma-1/HGF cells, with IC_{50}s of 0.01 μmol/l for both (Fig. 1*E* and 1*F*), whereas the combination of 17-DMAG plus erlotinib did not inhibit the growth of Ma-1/HGF cells, indicating that 17-DMAG did not sensitize these cells to erlotinib in vitro (Supplemental Figure 2, Supplemental Digital Content, http://links.lww.com/JTO/A267). These findings indicated that 17-DMAG circumvented resistance to HGF via both autocrine and paracrine mechanisms.

17-DMAG Reduces Met Protein Expression and Inhibits Downstream Pathways Even in the Presence of HGF

To explore the molecular mechanism by which 17-DMAG inhibited cell growth, even in the presence of HGF, we examined the protein expression and phosphorylation status of Met, EGFR, and their downstream molecules (PI3K/Akt and ERK1/2) in PC-9 and Ma-1 cells by Western blotting (Fig. 2). PC-9 and Ma-1 cells expressed

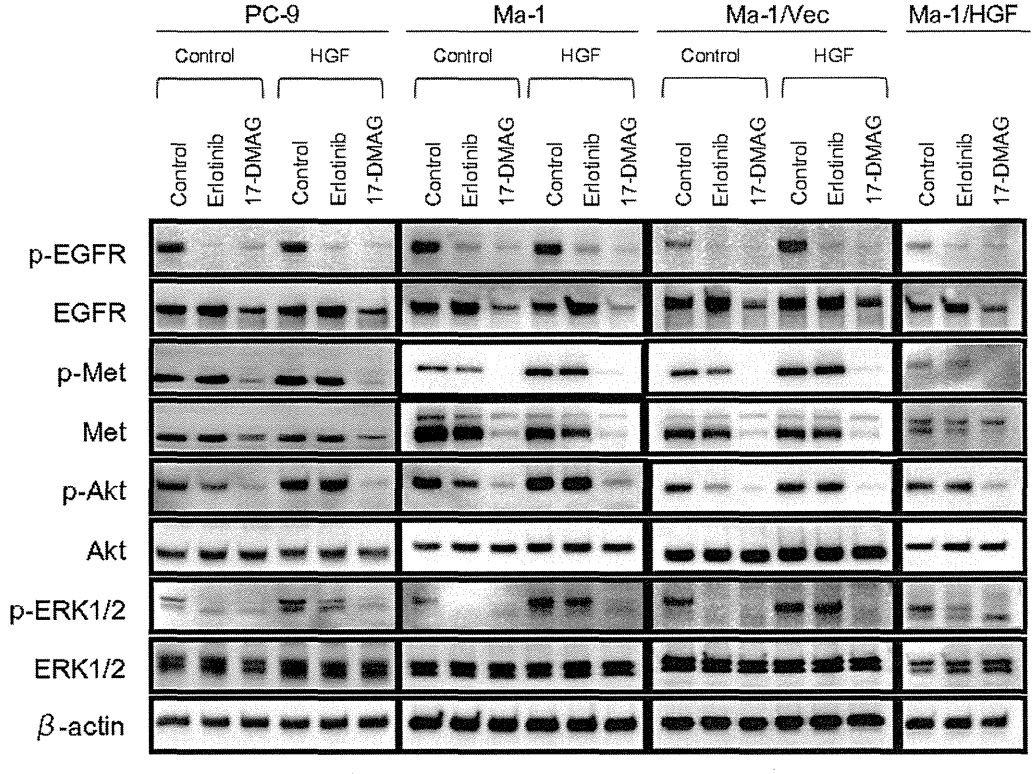

FIGURE 2. 17-DMAG suppresses EGF and Met protein expression and phosphorylation even in the presence of HGF. PC-9, Ma-1, Ma-1/Vec, and Ma-1/HGF tumor cells were treated with or without erlotinib (0.3 μmol/l) or 17-DMAG (0.3 μmol/l) for 24 hours, and then stimulated with or without HGF (20 ng/ml) for 10 minutes. The resultant cells were lysed, and the indicated proteins were detected by immunoblotting. EGF, epidermal growth factor; HGF, hepatocyte growth factor.

EGFR and Met proteins, both of which were phosphorylated, as were the downstream molecules Akt and ERK1/2. Although HGF alone did not affect the phosphorylation of EGFR, it stimulated the phosphorylation of Met, thereby activating Akt and ERK1/2. In the absence of HGF, erlotinib inhibited the phosphorylation of EGFR, but not of Met, thereby inhibiting the phosphorylation of Akt and ERK1/2. In the absence of HGF, erlotinib inhibited the phosphorylation of EGFR, but not of Met, thereby inhibiting the phosphorylation of Akt and ERK1/2. In the presence of HGF, erlotinib failed to inhibit the phosphorylation of Met, Akt, and ERK1/2, although it inhibited EGFR phosphorylation.[9] 17-DMAG decreased the expression of EGFR and Met proteins and inhibited their phosphorylation and the phosphorylation of Akt and ERK1/2, irrespective of the presence of HGF. Similar results were observed in Ma-1/HGF cells. These results suggested that 17-DMAG overcomes resistance to HGF, predominantly by inhibiting the expression of EGFR and Met proteins, and then inhibits the expression of the downstream proteins Akt and ERK1/2.

17-DMAG Induces Apoptosis of *EGFR*-Mutant Lung Cancer Cells Even in the Presence of HGF

Flow-cytometry analyses with Annexin V further confirmed that erlotinib induced apoptosis of Ma-1/Vec cells in the absence, but not the presence, of HGF (Supplemental Figure 4, Supplemental Digital Content, http://links.lww.com/JTO/A267). In contrast, 17-DMAG induced apoptosis in both the presence and absence of HGF. Moreover, 17-DMAG, but not erlotinib, induced apoptosis of Ma-1/HGF cells. These findings indicated that 17-DMAG induces apoptosis and reduces cell growth even in the presence of HGF.

17-DMAG Overcomes HGF-Induced Erlotinib Resistance In Vivo

We next evaluated whether 17-DMAG could overcome HGF-induced resistance to erlotinib in vivo. Oral administration of erlotinib and/or 17-DMAG markedly inhibited the growth of Ma-1/Vec-tumors (Fig. 3*A* and 3*C*). In contrast,

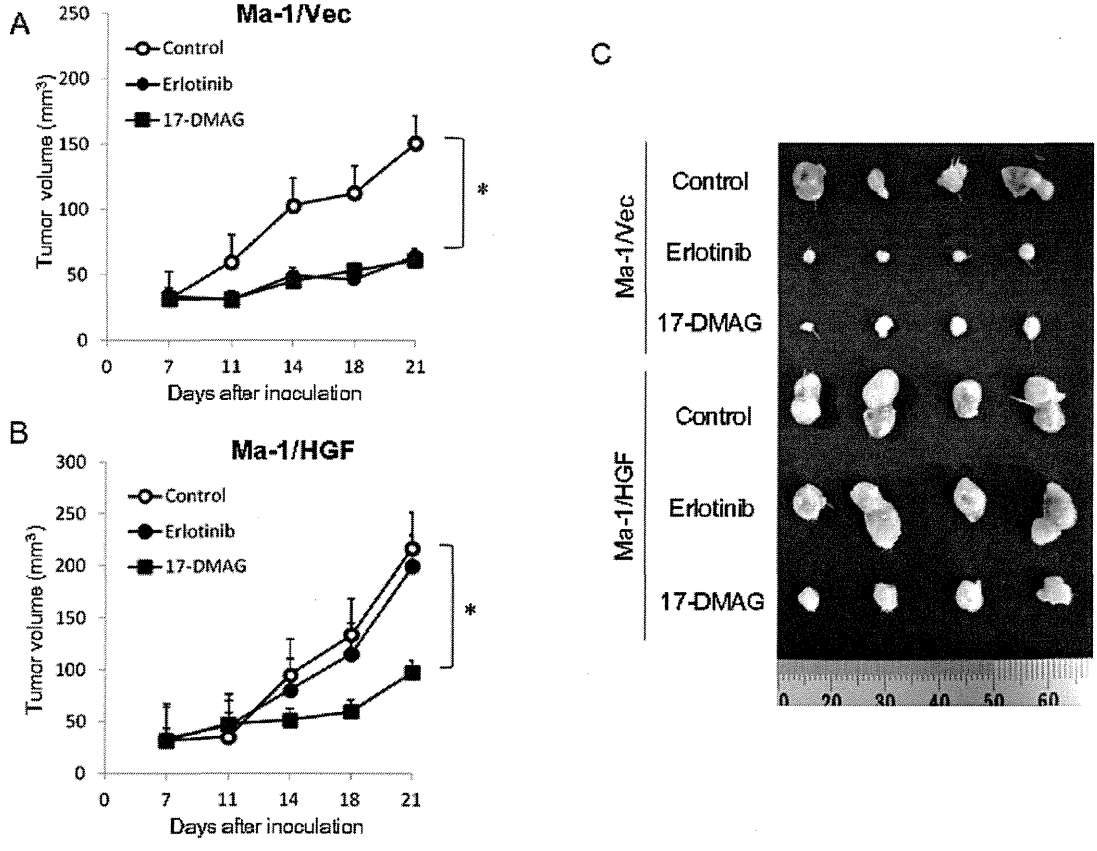

FIGURE 3. 17-DMAG overcomes HGF-induced erlotinib resistance in vivo. Ma-1/Vec (*A*) or Ma-1/HGF (*B*) cells (5×10^6 each) were inoculated subcutaneously into SCID mice on day 0. Mice received oral erlotinib (20 mg/kg/day) or intraperitoneal 17-DMAG (10 mg/kg/day), starting on day 7. Tumor size was measured twice a week and tumor volumes were calculated as described in Materials and Methods. Error bars indicate SEs of six tumors. C, Macroscopic appearances of representative tumors harvested on day 21. *$p < 0.01$ compared with the control group (Student's *t* test). 17-DMAG, 17-Dimethylaminoethylamino-17-demethoxygeldanamycin; HGF, hepatocyte growth factor; SCID, severe combined immunodeficiency.

erlotinib failed to inhibit the growth of Ma-1/HGF-tumors, indicating that HGF induced resistance to erlotinib in vivo. Under these experimental conditions, 17-DMAG markedly suppressed the growth of Ma-1/HGF tumors, whereas the combination of 17-DMAG plus erlotinib did not sensitize to erlotinib (Supplemental Figure 5, Supplemental Digital Content, http://links.lww.com/JTO/A267). These results indicated that 17-DMAG may overcome HGF-induced resistance to EGFR-TKIs in vivo (Fig. 3*B* and 3*C*).

17-DMAG Inhibits EGFR and Met Expression and Angiogenesis In Vivo

We finally assessed angiogenesis and expression of EGFR and Met proteins in Ma-1/HGF-tumors treated with or without erlotinib or 17-DMAG. Control tumor cells expressed both EGFR and Met. Although treatment with erlotinib did not affect their expression of EGFR or Met, treatment with 17-DMAG inhibited their expression of EGFR and Met, indicating the mode of action of 17-DMAG. Moreover, treatment with 17-DMAG, but not erlotinib, increased the number of apoptotic cells. However, the numbers of proliferating control, erlotinib-treated, and 17-DMAG-treated cells were similar (Fig. 4, Fig. 5*A* and 5*B*, Supplemental Figure 6, Supplemental Digital Content, http://links.lww.com/JTO/A267). Moreover, 17-DMAG treatment inhibited the angiogenesis of Ma-1/HGF tumors (Fig. 5*C*, Supplemental Figure 7, Supplemental Digital Content, http://links.lww.com/JTO/A267). These results suggested that 17-DMAG treatment inhibited tumor growth by inducing tumor-cell apoptosis, presumably by affecting the expression of EGFR and Met and by decreasing angiogenesis.

DISCUSSION

We have shown here that inhibition of Hsp90 by 17-DMAG could reduce the levels of EGFR and Met proteins and angiogenesis even in the presence of HGF, thus inhibiting the growth of *EGFR*-mutant lung cancer cells in vitro and in vivo. These findings suggest that HGF-induced EGFR-TKI-resistance in *EGFR*-mutant lung cancer could be overcome by Hsp90 inhibition.

Hsp90 is involved in stabilizing various oncogene products, including mutant EGFR and echinoderm microtubule-associated proetin-like-4-the anaplastic lymphoma kinase (EML4-ALK) fusion protein.[25,26] Hsp90 stabilizes various types of mutant EGFR, including proteins with exon 19 deletions and the T790M and L858R mutations,[17] and amplified Met.[18] We found that Hsp90 is also involved in the expression of Met stimulated by HGF, suggesting that inhibition of Hsp90 may overcome all three major mechanisms of resistance to EGFR-TKIs in *EGFR*-mutant lung cancers, including the T790M gatekeeper mutation, *Met* amplification, and increased expression of HGF. Furthermore, Hsp90 inhibitors are likely effective in EML4-ALK lung cancers,[27–29] overcoming acquired resistance to ALK inhibitors caused by *ALK* gene amplification and the L1196M gatekeeper mutation in *ALK*.[29] Thus, Hsp90 may be an ideal target in patients with lung cancers containing oncogene alterations, such as *EGFR*

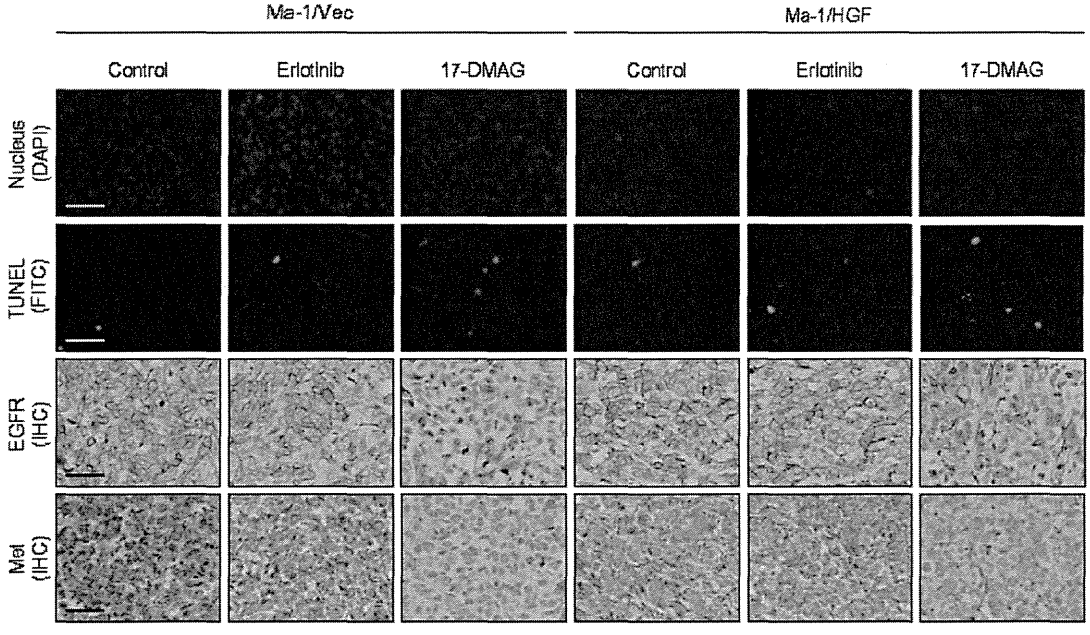

FIGURE 4. 17-DMAG inhibits EGFR and Met expression in vivo. Ma-1/Vec or Ma-1/HGF cells (5×10^6 each) were inoculated subcutaneously into SCID mice on day 0. Mice received oral erlotinib (20 mg/kg/day) or intraperitoneal 17-DMAG (10 mg/kg/day), starting on day 7. Two hours after treatment on day 11, the mice were sacrificed, and their tumors were harvested and examined histologically. Sections were stained with DAPI (nuclear stain), TUNEL (FITC), EGFR (IHC), and Met (IHC), respectively. Bars indicate 50 μm. 17-DMAG, 17-demethoxygeldanamycin; EGF, epidermal growth factor; HGF, hepatocyte growth factor; SCID, severe combined immunodeficiency; DAPI, 4′,6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase; FITC, fluorescein isothiocyanate; EGFR, epidermal growth factor receptor; IHC, immunohistochemistry.

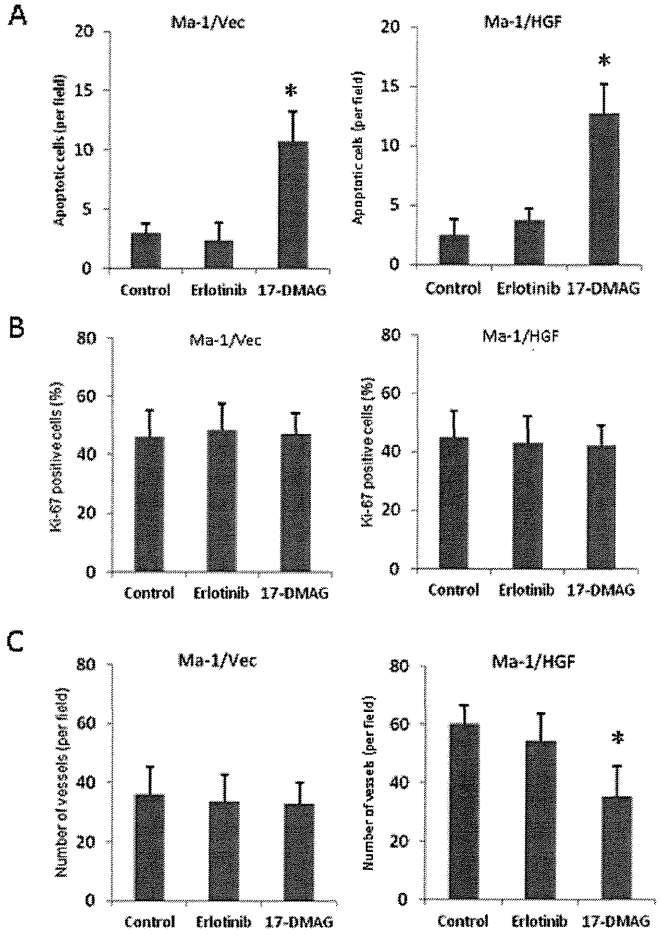

FIGURE 5. Quantification of apoptotic and proliferative cells, and of angiogenesis in vivo. *A,* Apoptotic cells, as determined by the TUNEL assay as described in Materials and Methods. *B,* Tumor-cell proliferation, as determined by the Ki-67-positive proliferation index (percentage of Ki-67-positive cells). C, Microvessel density, as determined by staining with antimouse-CD31 (clone MEC13.3, BD Bioscience) antibody as described in Materials and Methods. *Columns,* mean of five areas; *bars,* SD. *$p < 0.05$ compared with the control group (Student's *t* test). TUNEL, terminal deoxynucleotidyl transferase.

mutations and *EML4-ALK* gene translocations, before and after acquiring resistance to kinase inhibitors such as gefitinib, erlotinib, and crizotinib. Conversely, the efficacy of 17-DMAG was restricted not only to the erlotinib resistance, but also to EGFR wild-type cell lines independent of HGF treatment (data not shown), indicating that Hsp90 inhibitor is a general modifier of antitumor activity in combination with other anticancer drugs, and the agent could be used for overcoming the resistance. These findings also indicated that the combination of trastuzumab and Hsp90 inhibitor has antitumor activity in patients with trastuzumab-refractory HER-2 overexpressing breast cancer.[30]

Clarification of molecular mechanisms is essential for developing treatment strategies. To overcome resistance caused by HGF-triggered activation of the Met/PI3K/Akt pathway, it is necessary to simultaneously block two signaling pathways, those associated with mutant EGFR and HGF-Met. The HGF-Met pathway may be inhibited by several mechanisms, including HGF neutralization, Met kinase inhibition, and inhibition of downstream molecules.[31] The EGFR and HGF-Met pathways can be inhibited by two compounds. For example, an anti-HGF neutralizing antibody and an HGF antagonist (NK4), when combined with EGFR-TKIs, dramatically reversed HGF-induced resistance in vitro and in vivo.[32] Moreover, transient but intensive inhibition of PI3K/Akt by PI3K inhibitors and gefitinib successfully overcame HGF-induced EGFR-TKI resistance in vitro and in vivo.[33] The feasibility of these strategies, however, should be evaluated carefully in clinical trials. Hsp90 inhibition may have an advantage, however, because inhibition by a single agent may be sufficient. Recently reported results of early-phase clinical trials have shown the feasibility of several Hsp90 inhibitors.[28,34,35]

HGF is a mediator that regulates multiple biological functions, including cell motility and invasion.[36] Moreover, HGF plays an important role in angiogenesis by cooperating with vascular endothelial growth factor .[37] We previously reported that HGF stimulated vascular endothelial growth factor production by EGFR-mutant lung cancer cells, thereby facilitating angiogenesis and tumor growth in xenograft models. Tumors produced by HGF-gene transfected (Ma-1/HGF) cells were more vascularized and grew faster than those produced by control (Ma-1/Vec) cells. Moreover, 17-DMAG treatment inhibited the angiogenesis of Ma-1/HGF, but not Ma-1/Vec tumors, suggesting that 17-DMAG may overcome HGF-induced angiogenesis. Thus, HGF inhibition would suppress angiogenesis, thereby inhibiting tumor progression. Therefore, in HGF-triggered, EGFR-TKI-resistant lung cancers, Hsp90 inhibitors may control tumor growth not only by decreasing client proteins, EGFR and Met, but also by inhibiting HGF-induced angiogenesis.

Our findings, however, indicate that Hsp90 inhibitors have limitations. Although our in vivo experiments showed that 17-DMAG reduced tumor growth significantly, it failed to shrink tumors produced by Ma-1/Vec and Ma-1/HGF cells. These results are in agreement with previous findings, showing that treatment with the Hsp90 inhibitor, IPI-504, markedly suppressed the growth of H3122 tumor cells containing the *EML4-ALK* fusion gene, but that these tumors regrew during treatment.[26] Further experiments are warranted to clarify this mechanism; such experiments are now ongoing in our laboratory.

In conclusion, we demonstrated that Hsp90 inhibition may overcome HGF-triggered resistance to EGFR-TKIs in EGFR-mutant lung cancer. Several early-phase clinical trials have shown the efficacy and feasibility of Hsp90 inhibitors in lung cancer patients. Further clinical development of Hsp90 inhibitors is warranted for EGFR-mutant lung cancer patients who become refractory to EGFR-TKIs.

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REFERENCES

1. Pao W Miller VA. Epidermal growth factor receptor mutations, small-molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. *J Clin Oncol* 2005;23:2556–2568.
2. Maemondo M, Inoue A, Kobayashi K, et al.; North-East Japan Study Group. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 2010;362:2380–2388.
3. Mitsudomi T, Morita S, Yatabe Y, et al.; West Japan Oncology Group. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* 2010;11:121–128.
4. Zhou C, Wu YL, Chen G, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. *Lancet Oncol* 2011;12:735–742.
5. Rosell R, Gervais R, Vergnenegre A, et al. Erlotinib versus chemotherapy (CT) in advanced non-small cell lung cancer (NSCLC) patients (p) with epidermal growth factor receptor (EGFR) mutations: Interim results of the European Erlotinib Versus Chemotherapy (EURTAC) phase III randomized trial. *J Clin Oncol* 2011;29:abstract 7503.
6. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005;2:e73.
7. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005;352:786–792.
8. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316:1039–1043.
9. Yano S, Wang W, Li Q, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 2008;68:9479–9487.
10. Yano S, Yamada T, Takeuchi S, et al. Hepatocyte growth factor expression in EGFR mutant lung cancer with intrinsic and acquired resistance to tyrosine kinase inhibitors in a Japanese cohort. *J Thorac Oncol* 2011;6:2011–2017.
11. Onitsuka T, Uramoto H, Nose N, et al. Acquired resistance to gefitinib: the contribution of mechanisms other than the T790M, MET, and HGF status. *Lung Cancer* 2010;68:198–203.
12. Engelman JA, Zejnullahu K, Gale CM, et al. PF00299804, an irreversible pan-ERBB inhibitor, is effective in lung cancer models with EGFR and ERBB2 mutations that are resistant to gefitinib. *Cancer Res* 2007;67:11924–11932.
13. Janne PA, Schellens JH, Engelman JA, et al. Preliminary activity and safety results from a phase I clinical trial of PF-00299804, an irreversible pan-HER inhibitor, in patients (pts) with NSCLC. *J Clin Oncol* 2008;26:abstract 8027.
14. Wong KK, Fracasso PM, Bukowski RM, et al. A phase I study with neratinib (HKI-272), an irreversible pan ErbB receptor tyrosine kinase inhibitor, in patients with solid tumors. *Clin Cancer Res* 2009;15:2552–2558.
15. Tang Z, Du R, Jiang S, et al. Dual MET-EGFR combinatorial inhibition against T790M-EGFR-mediated erlotinib-resistant lung cancer. *Br J Cancer* 2008;99:911–922.
16. Trepel J, Mollapour M, Giaccone G, Neckers L. Targeting the dynamic HSP90 complex in cancer. *Nat Rev Cancer* 2010;10:537–549.
17. Shimamura T, Li D, Ji H, et al. Hsp90 inhibition suppresses mutant EGFR-T790M signaling and overcomes kinase inhibitor resistance. *Cancer Res* 2008;68:5827–5838.
18. Wang S, Pashtan I, Tsutsumi S, Xu W, Neckers L. Cancer cells harboring MET gene amplification activate alternative signaling pathways to escape MET inhibition but remain sensitive to Hsp90 inhibitors. *Cell Cycle* 2009;8:2050–2056.
19. Suda K, Murakami I, Katayama T, et al. Reciprocal and complementary role of MET amplification and EGFR T790M mutation in acquired resistance to kinase inhibitors in lung cancer. *Clin Cancer Res* 2010;16:5489–5498.
20. Yun CH, Mengwasser KE, Toms AV, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc Natl Acad Sci USA* 2008;105:2070–2075.
21. Nakamura T, Nishizawa T, Hagiya M, et al. Molecular cloning and expression of human hepatocyte growth factor. *Nature* 1989;342:440–443.
22. Green LM, Reade JL, Ware CF. Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J Immunol Methods* 1984;70:257–268.
23. Chou TC Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984;22:27–55.
24. Yamada T, Matsumoto K, Wang W, et al. Hepatocyte growth factor reduces susceptibility to an irreversible epidermal growth factor receptor inhibitor in EGFR-T790M mutant lung cancer. *Clin Cancer Res* 2010;16:174–183.
25. Sawai A, Chandarlapaty S, Greulich H, et al. Inhibition of Hsp90 down-regulates mutant epidermal growth factor receptor (EGFR) expression and sensitizes EGFR mutant tumors to paclitaxel. *Cancer Res* 2008;68:589–596.
26. Normant E, Paez G, West KA, et al. The Hsp90 inhibitor IPI-504 rapidly lowers EML4-ALK levels and induces tumor regression in ALK-driven NSCLC models. *Oncogene* 2011;30:2581–2586.
27. Chen Z, Sasaki T, Tan X, et al. Inhibition of ALK, PI3K/MEK, and HSP90 in murine lung adenocarcinoma induced by EML4-ALK fusion oncogene. *Cancer Res* 2010;70:9827–9836.
28. Sequist LV, Gettinger S, Senzer NN, et al. Activity of IPI-504, a novel heat-shock protein 90 inhibitor, in patients with molecularly defined non-small-cell lung cancer. *J Clin Oncol* 2010;28:4953–4960.
29. Katayama R, Khan TM, Benes C, et al. Therapeutic strategies to overcome crizotinib resistance in non-small cell lung cancers harboring the fusion oncogene EML4-ALK. *Proc Natl Acad Sci USA* 2011;108:7535–7540.
30. Modi S, Stopeck AT, Gordon MS, et al. Combination of trastuzumab and tanespimycin (17-AAG, KOS-953) is safe and active in trastuzumab-refractory HER-2 overexpressing breast cancer: a phase I dose-escalation study. *J Clin Oncol* 2007;25:5410–5417.
31. Yano S, Wang W, Li Q, et al. HGF-MET in resistance to EGFR tyrosine kinase inhibitors in lung cancer. *Curr Signal Transduct Ther* 2011;6:228–233.
32. Wang W, Li Q, Yamada T, et al. Crosstalk to stromal fibroblasts induces resistance of lung cancer to epidermal growth factor receptor tyrosine kinase inhibitors. *Clin Cancer Res* 2009;15:6630–6638.
33. Donev IS, Wang W, Yamada T, et al. Transient PI3K inhibition induces apoptosis and overcomes HGF-mediated resistance to EGFR-TKIs in EGFR mutant lung cancer. *Clin Cancer Res* 2011;17:2260–2269.
34. Solit DB, Osman I, Polsky D, et al. Phase II trial of 17-allylamino-17-demethoxygeldanamycin in patients with metastatic melanoma. *Clin Cancer Res* 2008;14:8302–8307.
35. Pacey S, Wilson RH, Walton M, et al. A phase I study of the heat shock protein 90 inhibitor alvespimycin (17-DMAG) given intravenously to patients with advanced solid tumors. *Clin Cancer Res* 2011;17:1561–1570.
36. Nakamura T, Sakai K, Nakamura T, Matsumoto K. Hepatocyte growth factor twenty years on: Much more than a growth factor. *J Gastroenterol Hepatol* 2011;26 Suppl 1:188–202.
37. Bohonowych JE, Gopal U, Isaacs JS. Hsp90 as a gatekeeper of tumor angiogenesis: clinical promise and potential pitfalls. *J Oncol* 2010;2010:412985.

Hepatocyte Growth Factor Induces Resistance to Anti-Epidermal Growth Factor Receptor Antibody in Lung Cancer

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Introduction: Epidermal growth factor receptor (EGFR) is an attractive drug target in lung cancer, with several anti-EGFR antibodies and small-molecule inhibitors showing efficacy in lung cancer patients. Patients, however, may develop resistance to EGFR inhibitors. We demonstrated previously that hepatocyte growth factor (HGF) induced resistance to EGFR tyrosine kinase inhibitors in lung cancers harboring *EGFR* mutations. We therefore determined whether HGF could induce resistance to the anti-EGFR antibody (EGFR Ab) cetuximab in lung cancer cells, regardless of EGFR gene status.

Methods: Cetuximab sensitivity and signal transduction in lung cancer cells were examined in the presence or absence of HGF, HGF-producing fibroblasts, and cells tranfected with the HGF gene in vitro and in vivo.

Results: HGF induced resistance to cetuximab in H292 (EGFR wild) and Ma-1(EGFR mutant) cells. Western blotting showed that HGF-induced resistance was mediated by the Met/Gab1/Akt signaling pathway. Resistance of H292 and Ma-1 cells to cetuximab was also induced by coculture with lung fibroblasts producing high levels of HGF and by cells stably transfected with the HGF gene. This resistance was abrogated by treatment with anti-HGF neutralizing antibody.

Conclusions: HGF-mediated resistance is a novel mechanism of resistance to EGFR Ab in lung cancers, with fibroblast-derived HGF inducing cetuximab resistance in H292 tumors in vivo. The involvement of HGF-Met-mediated signaling should be assessed in acquired resistance to EGFR Ab in lung cancer, regardless of EGFR gene status.

Key Words: Hepatocyte growth factor, Lung cancer, Anti-EGFR antibody, Drug resistance.

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Lung cancer is one of the most prevalent malignancies and the leading cause of malignancy-related deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for ~80% of cases of lung cancers. The median survival of patients with metastatic NSCLC is 8 to 10 months, even if treated with the most active combination of conventional chemotherapeutic agents.[1,2]

Epidermal growth factor receptor (EGFR) is a cell surface receptor tyrosine kinase that transduces growth signals through dimerization with the HER of family receptors. As EGFR is highly expressed in a variety of human tumors,[3,4] it is regarded as an attractive target for the development of therapeutic agents. EGFR overexpression has been observed in 40 to 80% of NSCLCs and is associated with tumor development.[3,5,6] Recent clinical trials have demonstrated that NSCLC patients with *EGFR* mutants had good outcomes with the EGFR tyrosine kinase inhibitors (EGFR-TKIs) gefitinib and erlotinib.[7–9] Overall, however, EGFR-TKIs, with or without standard chemotherapy, have not yielded good outcomes in patients with NSCLC.[10,11]

Cetuximab, a chimeric human-mouse anti-EGFR IgG1 monoclonal antibody (EGFR Ab), has shown clinical success in patients with colorectal and head and neck cancers.[12–15] Because EGFR mutations in NSCLCs were associated with sensitivity to gefitinib but not to cetuximab,[16] cetuximab was hypothesized to be effective in NSCLC patients overall, regardless of EGFR mutations. Clinical trials showed that the addition of cetuximab to platinum-based chemotherapy resulted in significantly longer survival time in patients with EGFR-positive advanced NSCLC than chemotherapy alone and that first-cycle skin rash may be a surrogate clinical marker for the efficacy of cetuximab.[17,18] Nevertheless, other phase III clinical trials demonstrated that the addition of cetuximab to chemotherapy did not significantly improve clinical outcomes in patients with advanced NSCLC and that efficacy parameters was not correlated with K-Ras mutation status or with any EGFR-related biomarker, mutation, protein expression, or gene copy number.[19,20] Thus, treatment with cetuximab may have only limited effects in patients with EGFR-positive NSCLC, and the identification of resistance biomarkers to cetuximab may improve the efficacy of cetuximab in these patients.

We previously showed that hepatocyte growth factor (HGF) induces resistance to reversible or irreversible EGFR-TKIs by activating Met, restoring the phosphorylation of downstream MAPK-extracellular signal-regulated kinase1/2 (ERK1/2) and PI3K-Akt pathways in lung cancers with mutant EGFR.[21,22] We sought to determine whether HGF could induce resistance to EGFR Ab in lung cancer cells, regardless of the presence or absence of EGFR mutations.

MATERIALS AND METHODS

Cell Lines and Reagents

The H292 human lung adenocarcinoma cell line, which expresses wild type EGFR, was purchased from the American Type Culture Collection (Manassas, VA). The Ma-1 human lung adenocarcinoma cell line with an EGFR-activating mutation (deletion in exon 19) was kindly provided by Dr. Eiji Shimizu (Tottori University, Yonago, Japan). The MRC-5 lung embryonic fibroblast cell line was obtained from RIKEN Cell Bank. H292 and Ma-1 cells were cultured in RPMI 1640 medium and MRC-5 (P 25–30) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), each supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (50 μg/mL), in a humidified CO_2 incubator at 37°C.

Cetuximab was obtained from Merck Serono (Darmstadt, Germany) and erlotinib hydrochloride from Chugai Pharmaceutical Co., Ltd (Tokyo, Japan). BIBW2992, BEZ235, and AZD6244 were purchased from Seleck Chemicals (Houston, TX). SU11274 was purchased from Calbiochem (San Diego, CA). Recombinant EGF, Amphiregulin, PDGF-AA, and IGF-1 were obtained from R&D Systems (Minneapolis, MN). Recombinant HGF was prepared as described.[23–25] Its purity, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein staining, was 96.4% and >98%, respectively. Goat anti-human HGF neutralizing antibody and control goat IgG were purchased from R&D Systems.

Cell Proliferation Assay

Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) dye reduction method.[26] Tumor cells at 80% confluence were harvested, seeded at 2×10^3 cells per well in 96-well plates and incubated in RPMI 1640 for 24 hours. Several concentrations of cetuximab, erlotinib, BIBW2992, SU11274, BEZ235, AZD6244, goat anti-human HGF neutralizing antibody, control goat IgG, and/or HGF, EGF, Amphiregulin, PDGF-AA, and IGF-1 were added to each well, and incubation was continued for a further 72 hours. A 50 μL aliquot of MTT solution (2 mg/mL; Sigma, St. Louis, MO) was added to each well, followed by incubation for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 100 μL of DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric, Ibaraki, Japan) at test and reference wavelengths of 550 and 630 nm, respectively. The percentage growth is shown relative to untreated controls. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.

Antibodies and Western Blotting

Tumor cells were incubated in 10 mL of RPMI 1640 with 10% FBS in the presence or absence of cetuximab (1 μg/mL) for 1 hour, and then in the presence or absence of cetuximab (1 μg/mL) and/or HGF (20 ng/mL) for 15 minutes. The cells were washed twice with phosphate-buffered saline (PBS), harvested in cell lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride), and flash-frozen on dry ice. After allowing the cells to thaw, the lysates were collected with a rubber scraper, sonicated, and centrifuged at 14000×*g* for 20 minutes at 4°C. The total protein concentration of each lysate was measured using a Pierce BCA Protein Assay Kit (Pierce, Rockford, IL). For Western blotting assays, cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, Hercules, CA) and the proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with Blocking One solution (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature and incubated at 4°C overnight with antibodies to Met (25H2), phospho-Met (Y1234/Y1235) (3D7), Gab1, phospho-Gab1 (Tyr627), PTEN (138G6), ErbB3, phospho-ErbB3, Akt, phospho-Akt (Ser473), or β-actin (13E5; each at 1:1000 dilution; Cell Signaling Technology, Danvers, MA), or with antibodies to human EGFR (1 μg/mL), human/mouse/rat ERK1/ERK2 (0.2 μg/mL), or phospho-ERK1/ERK2 (T202/Y204) (0.1 μg/mL) (R&D Systems). After washing three times, the membranes were incubated for 1 hour at room temperature with secondary Ab (horseradish peroxidase-conjugated species-specific Ab). Immunoreactive bands were visualized with SuperSignal West Dura Extended Duration Substrate Enhanced Chemiluminescent Substrate (Pierce). Each experiment was performed at least three times independently.

RNA Interference

Duplexed Stealth RNAi (Invitrogen), targeted against nucleotides 1014-1038 of the *EGFR* gene (5′-CGGAATAGGTATTGGTGAATTTAAA-3′), and Stealth RNAi Negative Control Low GC Duplex 3 (Invitrogen) were used for RNA interference (RNAi) assay. Briefly, aliquots of 1×10^5 cells in 2 mL of antibiotic-free medium were plated on six-well plates and incubated at 37°C for 24 hours. The cells were then transfected with siRNA (250 pmol) or scramble RNA using Lipofectamine 2000 (5 μL) in accordance with the manufacturer's instructions (Invitrogen). After 24 hours, the cells were washed twice with PBS and incubated with or without recombinant human HGF (20 ng/mL) for an additional 15 minutes in antibiotic-containing medium. These cells were then used for Western blotting assay as described above. *EGFR* knockdown was confirmed by Western blotting. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.

HGF Production in Cell Culture Supernatant

Cells (2×10^5) were cultured in 2 mL of RPMI 1640 or DMEM with 10% FBS for 24 hours, washed with PBS and incubated for 48 hours with or without cetuximab (1 μg/mL) in

RPMI 1640 or DMEM with 10% FBS. The culture media were harvested and centrifuged, and the supernatants were stored at −70°C until analysis. HGF was measured by enzyme-linked immunosorbent assay (Immunis HGF EIA; B-Bridge International, Mountain View, CA; limit of detection, 0.1 ng/mL), according to the manufacturer's recommendations. All samples were assayed in triplicate. Color intensity was measured at 450 nm with a spectrophotometric plate reader. Growth factor concentrations were determined by comparison with standard curves.

Coculture of Lung Cancer Cells with Fibroblasts

Cells were cocultured in Transwell chambers separated by filters of 8 μm pore size. Tumor cells (8×10^3 cells/700 μL) with or without cetuximab (1 μg/mL) were placed in the lower chambers, and the human fibroblast MRC-5 (1×10^4 cells/300 μL) cell lines, with or without 2 hours of pretreatment with control IgG (2 μg/mL) or anti-HGF neutralizing antibody (2 μg/mL), were placed in the upper chambers. After coculture for 72 hours, the upper chambers were removed, 200 μL of MTT solution (2 mg/mL; Sigma) was

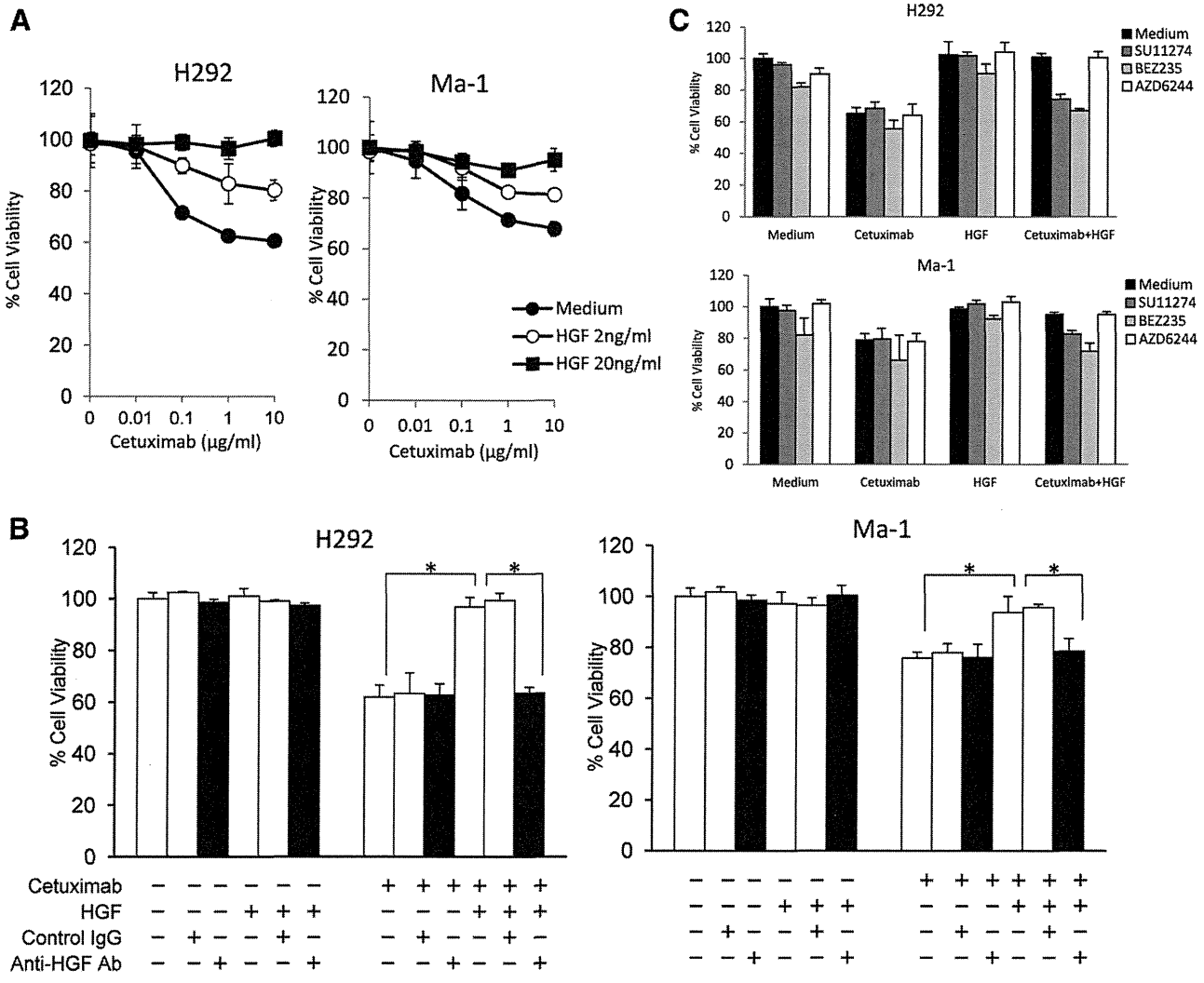

FIGURE 1. HGF induces resistance to anti-EGFR antibody in lung adenocarcinoma cells regardless of *EGFR* gene status. *A*, H292 cells, containing wild-type *EGFR*, and Ma-1 cells, containing *EGFR* with an exon19 deletion, were incubated with the anti-EGFR antibody cetuximab, at various concentrations and/or with 0, 2, and 20 ng/mL HGF for 72 hours, with cell growth determined by the MTT assay. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently. *B*, HGF (20 ng/mL) was pretreated with control IgG (2 μg/mL) or anti-HGF antibody (2 μg/mL) at 37°C for 1 hour. The resultant solutions were added to cultures of H292 and Ma-1 cells in the presence or absence of cetuximab (1 μg/mL), and cell growth was determined as in *A*. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently. *$p < 0.05$ (Mann-Whitney *U* test). *C*, HGF (20 ng/mL) was pretreated with SU11274 (1 μM), BEZ235 (0.001 μM), or AZD6244 (1 μM) at 37°C for 1 hour. The resultant solutions were added to cultures of H292 and Ma-1 cells in the presence or absence of cetuximab (1 μg/mL), and cell growth was determined as in *A*. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.

added to each well, and the cells were incubated for 2 hours at 37°C. The media were removed and the dark blue crystals in each well were dissolved in 400 μL of DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric) at test and reference wavelengths of 550 and 630 nm, respectively. The percentage growth is shown relative to untreated controls. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.

HGF Gene Transfection

One day before transfection, aliquots of 1×10^5 Ma-1 cells in 1 mL of antibiotic-free medium were plated on six-well plates. The full-length HGF cDNA cloned into a BCMGSneo expression vector (25-A) was transfected using Lipofectamine 2000 in accordance with the manufacturer's instructions. After 24 hours, the cells were washed with PBS and incubated for an additional 72 hours in antibiotic-containing medium. The cells were selected in G418 sulfate (Calbiochem, La Jolla, CA). After the limiting dilution, HGF-producing cells, Ma-1/HGF, were established. HGF production by Ma-1/HGF was confirmed by enzyme-linked immunosorbent assay.

Xenograft Studies in SCID Mice

Suspensions of H292 cells (5×10^6) with or without MRC-5 cells (5×10^6) were injected subcutaneously into the backs of 5-week-old female SCID mice (Japan Clea). After 4 days (tumors diameter >4 mm), mice were randomly allocated into groups of six animals to receive intraperitoneal cetuximab (4 mg/kg, twice/wk) or vehicle. Tumor size was measured with digital calipers, and tumor volume was calculated as $0.5 \times \text{length} \times (\text{width})^2$. All animal experiments complied with the Guidelines for the Institute for Experimental Animals, Kanazawa University Advanced Science Research Center (approval no. AP-081088).

Statistical Analysis

The statistical significance of differences was analyzed by one-way analysis of variance. All statistical analyses were performed using GraphPad Prism Ver. 4.01 (GraphPad Software, Inc., San Diego, CA). $p < 0.05$ was considered statistically significant.

RESULTS

HGF Induces Resistance to Anti-EGFR Antibody in Lung Cancer Cells Regardless of EGFR Gene Status

We first tested the effects of the anti-EGFR Ab cetuximab against H292 cells, an NSCLC cell line with wild type in EGFR, and Ma-1 cells, an NSCLC cell line harboring the exon 19 deletion in the EGFR gene, resulting in its constitutive activa-

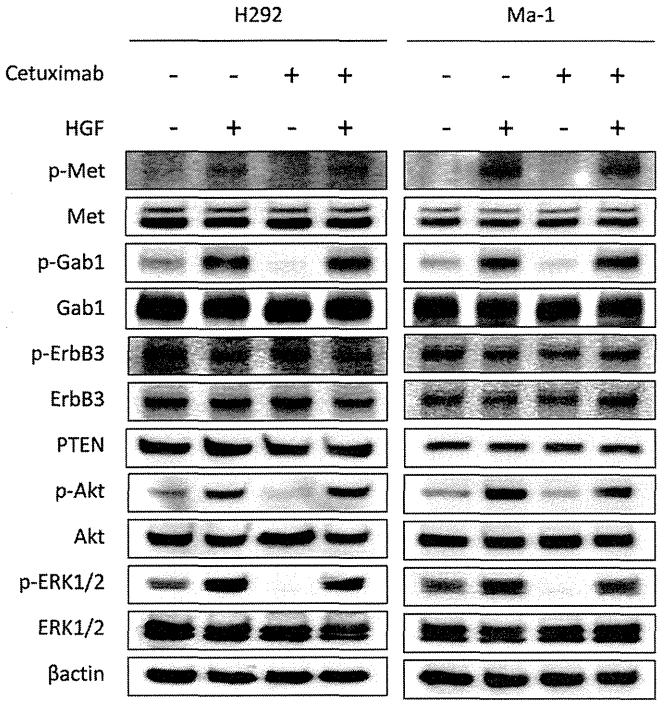

FIGURE 3. HGF induces resistance of lung adenocarcinoma cells to cetuximab by restoring phosphorylation of Akt and ERK1/2. Tumor cells were treated with or without cetuximab (1 μg/mL) for 1 hour and incubated in the presence or absence of cetuximab (1 μg/mL) and/or HGF (20 ng/mL) for 15 minutes. The cells were lysed, and the indicated proteins were detected by immunoblotting. The results shown are representative of three independent experiments.

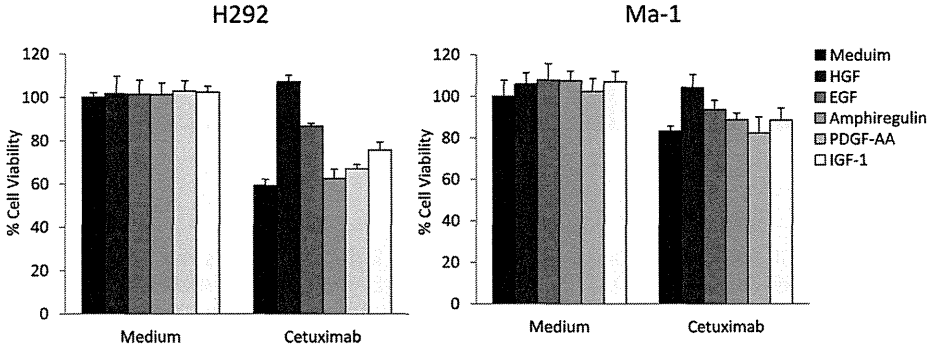

FIGURE 2. HGF was most potent in induction of cetuximab resistance of lung adenocarcinoma cells. H292 and Ma-1 cells were incubated with or without cetuximab (1 μg/mL) and/or 20 ng/mL of HGF, EGF, Amphiregulin, PDGF-AA, or IGF-I. Cell growth was determined after 72 hours of treatment. The percentage of growth is shown relative to untreated controls. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently.

tion. We found that both cell lines were sensitive to cetuximab (Figure 1*A*). Although HGF (20 ng/mL) alone had no effect on the growth of these lung cancer cells, HGF (at 2 and 20 ng/mL) markedly and dose-dependently induced the resistance of both H292 and Ma-1 cells to cetuximab (Figure 1*A*). Moreover, HGF (20 ng/mL) also significantly enhanced the resistance of H292 cells to the reversible EGFR-TKI erlotinib and the irreversible EGF-TKI BIBW2992 (Supplemental Figure 1*A*, *B*, http://links.lww.com/JTO/A195). These effects of HGF were abrogated by an anti-HGF neutralizing antibody (2 μg/mL) but not by control IgG (2 μg/mL) (Figure 1*B*). In addition, these effects of HGF were abrogated by Met-TKI SU11274 (1 μM) and phosphatidylinositol 3-kinase (PI3K) inhibitor BEZ235 (0.001 μM) but not by MEK inhibitor AZD6244 (1 μM) (Figure 1*C*). We also examined the effects of other receptor's ligands, including EGF, Amphiregulin, PDGF-AA, and IGF-I. Although EGF and IGF-1 tend to induce resistance of H292 and Ma-1 cells to cetuximab, HGF showed the strongest effect in induction of resistance to cetuximab in H292 and Ma-1 cells (Figure 2).

HGF-Induced Cetuximab Resistance Is Mediated by the Restoration of Gab1, Akt, and ERK1/2 Phosphorylation

To investigate the molecular mechanism by which HGF induces resistance to cetuximab in lung cancer cells, we assessed the level of expression and phosphorylation of Met protein and downstream molecules by Western blotting. We found that both H292 and Ma-1 cells expressed Met, PTEN, Gab1, and ErbB3 proteins, with Met, Gab1, and ErbB3 being phosphorylated to at least some extent. We also found that Akt and ERK1/2, molecules downstream to these receptors, were phosphorylated. Cetuximab inhibited the phosphorylation of Akt and ERK1/2 but not of ErbB3, Met, and Gab1. HGF alone did not affect the phosphorylation of ErbB3, but it stimulated the phosphorylation of Met and Gab1. Even in the presence of cetuximab, HGF restored the phosphorylation of Met, Gab1, Akt, and ERK1/2 (Figure 3), indicating that HGF-induced resistance to cetuximab is mediated by Met/Gab1/Akt signaling.

Specific Down-Regulation of EGFR Also Induces Phosphorylation of Gab1, Akt, and ERK1/2 Induced by HGF

Using *EGFR*-specific siRNA to knockdown the expression of *EGFR* in H292 cells, we found that the phosphorylation of Akt and ERK1/2 was also inhibited. However, down-regulation of EGFR expression by *EGFR*-specific siRNA had no effect on the HGF-induced phosphorylation of Met, Gab1, Akt, and ERK1/2 (Supplemental Figure 1*C*), indicating that HGF also induces resistance to EGFR knockdown by restoring the Akt and ERK1/2 signaling pathway.

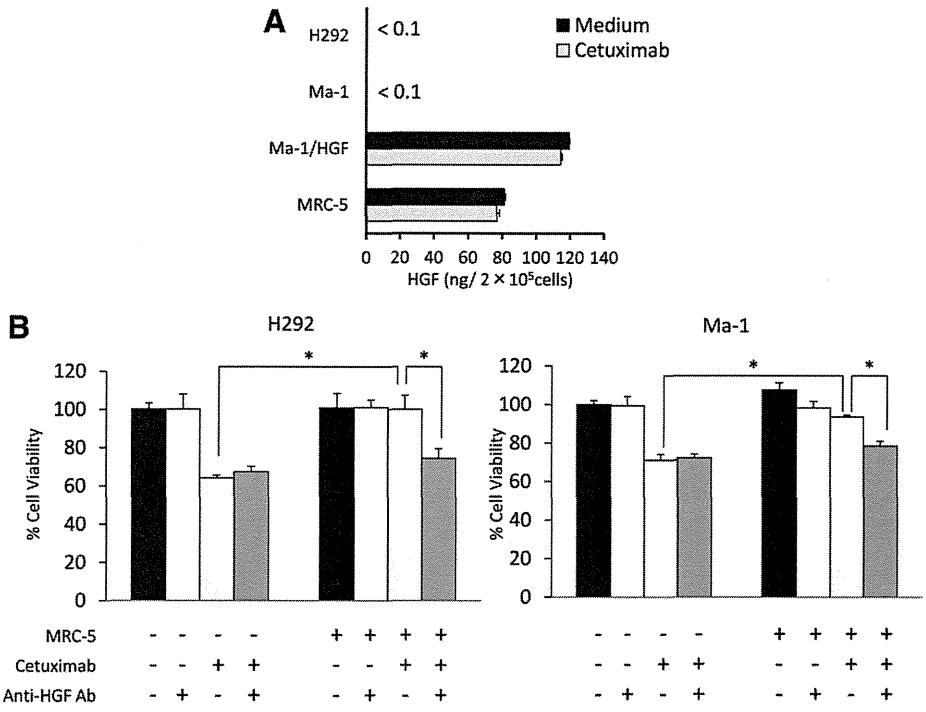

FIGURE 4. Fibroblast-derived HGF induces resistance to cetuximab in lung cancer cells regardless of *EGFR* gene status. *A*, HGF production by lung cancer cell lines (H292, Ma-1) and human embryonic lung fibroblasts (MRC-5). Cells were incubated for 48 hours with or without cetuximab (1 μg/mL), the culture supernatants were harvested, and their concentrations of HGF were determined by enzyme-linked immunosorbent assay (ELISA). All samples were assayed in triplicate. *B*, Coculture with fibroblasts induced cetuximab resistance in lung cancer cells. Lung cancer H292 and Ma-1 cell lines were cocultured with MRC-5 cells and anti-HGF neutralizing antibody (2 μg/mL) in the presence or absence of cetuximab (1 μg/mL) for 72 hours, and lung cancer cell growth was determined after 72 hours by the MTT assay. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently. *$p < 0.05$ (Mann-Whitney *U* test).

Fibroblast-Derived HGF Induces Cetuximab Resistance in Lung Cancer Cells

Because host microenvironments can have a profound effect on the chemosensitivity of cancers and because stromal fibroblasts are the major source of HGF,[27] we assayed HGF production by human fibroblast cell lines. We found that the human embryonic lung-derived fibroblast cell line MRC-5 secreted high levels of HGF into the supernatant. In contrast, H292 and Ma-1 cells did not secrete detectable levels of HGF into the culture supernatant (Figure 4*A*). To further investigate whether the resistance of H292 and Ma-1 cells to cetuximab may be affected by crosstalk with stromal fibroblasts, we cocultured these lung cancer cells with MRC-5 cells using Transwell systems. We found that coculture of H292 and Ma-1 cells with MRC-5 cells did not significantly affect the proliferation of the former. In the presence of MRC-5 cells, however, H292 and Ma-1 cells became resistant to cetuximab, a resistance abrogated by treatment with anti-HGF neutralizing antibody (2 μg/mL) (Figure 4*B*). These results indicate that fibroblast-derived HGF could induce cetuximab resistance in lung cancer cells regardless of *EGFR* gene status.

Cell-Derived HGF Induces Cetuximab Resistance in Lung Cancer Cells

To determine whether cell-derived HGF induces cetuximab resistance in lung cancer cells, we established Ma-1/HGF cells, which stably express human HGF (Figure 4*A*). Unlike Ma-1 cells, Ma-1/HGF cells were resistant to cetuximab (Figure 5*A*), but this resistance was abrogated by treatment with anti-HGF neutralizing antibody (2 μg/mL) (Figure 5*B*). When we assayed the level of expression and phosphorylation status of Met protein and its downstream molecules by Western blotting, we found that Met, but not Gab1, was phosphorylated in Ma-1/HGF cells. We could not specify the reason for weak phosphorylation in Gab1 in Ma-1/HGF cells, compared with Ma-1 cells stimulated with HGF. Akt and ERK1/2, the molecules downstream of these receptors, were also phosphorylated. Addition of cetuximab to Ma-1/HGF cells had no effect on the phosphorylation of Akt and ERK1/2 (Figure 5*C*), indicating that cell-derived HGF could induce cetuximab resistance of lung cancer cells by means of the Akt and ERK1/2 signaling pathways.

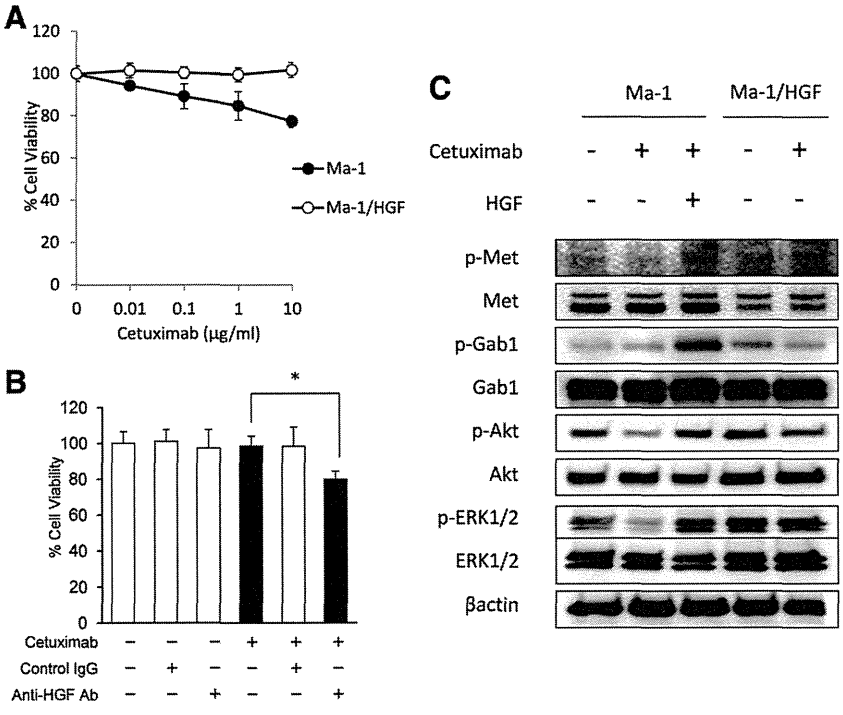

FIGURE 5. Cell-derived HGF induces resistance to cetuximab in lung cancer cells through phosphorylation of Akt and ERK1/2. *A*, Ma-1 and Ma-1/HGF cells were incubated for 72 hours with increasing concentrations of cetuximab, and cell growth was determined by the MTT assay. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently. *B*, Ma-1/HGF cells were incubated for 72 hours with or without cetuximab (1 μg/mL) and/or control IgG (2 μg/mL) in the presence or absence of anti-HGF neutralizing antibody (2 μg/mL), and cell growth was determined by the MTT assay. Each experiment included triplicate determinations, and each experiment was repeated at least three times independently. $*p < 0.05$ (Mann-Whitney *U* test). *C*, Ma-1 and Ma-1/HGF cells were incubated with or without cetuximab (1 μg/mL) for 1 hour and in the presence or absence of cetuximab (1 μg/mL) and/or HGF (20 ng/mL) for 15 minutes. The cells were lysed, and the indicated proteins were detected by immunoblotting. Results shown are representative of three independent experiments.

HGF Derived from Fibroblasts Induces In Vivo Cetuximab Resistance of Lung Cancer Cells with Wild-Type EGFR

To investigate whether the cetuximab sensitivity of lung cancer cells with wild-type EGFR could be affected by fibroblasts in vivo, we inoculated H292 cells, with or without MRC-5 cells, into SCID mice subcutaneously as described.[28] The tumors in mice injected with H292 and MRC-5 cells grew slightly faster than those in mice injected with H292 cells alone. Cetuximab treatment, beginning on day 4, caused marked regression of tumors in mice injected with H292 cells alone. The same treatment prevented the enlargement of tumors in mice injected with H292 and MRC-5 cells, but it did not cause tumor regression, indicating that these tumors were resistant to cetuximab treatment in vivo (Figure 6). These results indicate that HGF, produced presumably by fibroblasts (MRC-5), induced in vivo cetuximab resistance in lung cancer cells with wild-type EGFR.

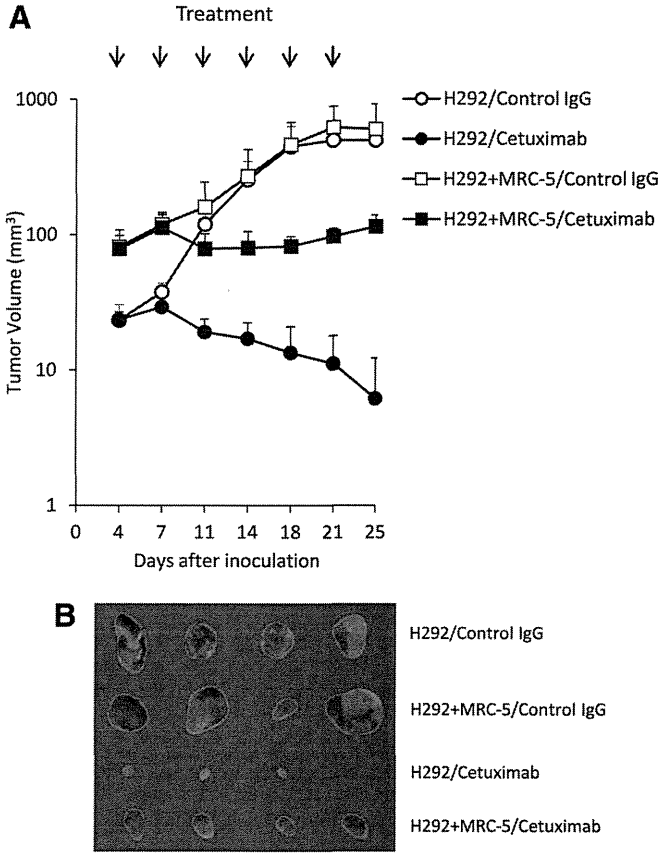

FIGURE 6. HGF derived from fibroblasts induces in vivo resistance to cetuximab in lung cancer cells with *EGFR* wild type. *A*, Suspensions of H292 cells (5×10^6) with or without MRC-5 cells (5×10^6) were inoculated subcutaneously into SCID mice on day 0. Mice received intraperitoneal cetuximab (4 mg/kg, twice/wk) or vehicle, starting on day 4. Tumor sizes were measured every 3 or 4 days and tumor volumes were calculated as described in Materials and Methods. Data shown are the representative of two independent experiments. Error bars indicate standard errors of six mice. *B*, Macroscopic appearances of representative tumors harvested on day 25.

DISCUSSION

EGFR is an attractive target for treatment of various cancers including lung cancer. Several agents targeting EGFR have been developed, including anti-EGFR antibodies and small-molecule EGFR-TKIs. Most patients who show a dramatic response to initial treatment, however, acquire resistance to these agents after varying periods of time. To overcome this resistance to EGFR inhibitors, it is necessary to clarify its molecular mechanisms.

Among the mechanisms associated with resistance to reversible EGFR-TKIs in NSCLCs with EGFR-activating mutations are the T790M secondary mutation in *EGFR*[29,30] and *Met* amplification.[31] NSCLC resistance to the anti-EGFR antibody cetuximab has been associated with increased PTEN instability, *Met* amplification, and strong activation of Met.[32,33]

We focused on the role of HGF in NSCLC resistance to cetuximab, in cell lines with or without EGFR mutations. HGF was originally identified as a mitogenic protein for hepatocytes and has been shown to have pleiotropic biological activities.[23] We and other researchers previously demonstrated that HGF induces resistance to reversible and irreversible EGFR-TKIs in lung cancer cells harboring EGFR-activating mutations by activating its receptor Met and the downstream Gab1/Akt pathway.[21,22,34] In addition, HGF induced resistance to cetuximab in colorectal cancer cells through Met activation.[35] Thus, HGF may be a candidate molecule for resistance to EGFR inhibitors in cancers, including colorectal and lung cancers.

We found that HGF induced resistance to cetuximab in lung cancer cells, both in vitro and in vivo. We also found that HGF acted by restoring the Met/Gab1/Akt pathway in these cells and that inhibitors of *HGF*, *Met*, and *PI3K* could overcome this resistance. Several HGF-Met targeting agents are being tested clinically. These compounds may be promising in overcoming resistance to cetuximab.

EGFR mutations have been shown to correlate with the efficacy of EGFR-TKIs in several clinical trials of patients with NSCLC. Anti-EGFR agents, including erlotinib[36] and cetuximab,[17] have shown particular efficacy in patients with wild-type EGFR. To our knowledge, this report is the first to show that HGF induced resistance to EGFR blocking, both with EGFR inhibitors and gene knockdown, in NSCLCs with EGFR wild type. These findings suggest that HGF induces resistance to EGFR inhibitors in NSCLC containing both wild-type and mutant *EGFR*. However, further studies are required to determine which type of resistance, primary and/or acquired, is involved in the HGF-triggered resistance to cetuximab in NSCLC.

Several factors have been reported to be biomarkers for the effectiveness of cetuximab in patients with colorectal cancer, including K-Ras mutation, levels expression of EGFR ligands, and absence of PTEN.[37–39] In contrast, biomarkers predictive of the effects of cetuximab in patients with NSCLC have not yet been established. Moreover, the cetuximab is less effective in NSCLC than in colorectal cancer. Thus,

dependence on EGFR signaling for growth and survival and subsequent EGFR inhibition may differ in NSCLC and colorectal cancer, indicating a need for novel, tumor-specific approaches for the use of cetuximab in NSCLC.

Serum and plasma concentrations of HGF may predict the effects of EGFR-TKIs in NSCLC, regardless of EGFR gene status.[40–42] We showed that resistance to cetuximab was also induced by crosstalk with HGF-producing fibroblast cell lines (paracrine model) and by stable transfection of an HGF gene (autocrine model). Therefore, HGF in the cancer microenvironment may be predictive of sensitivity to cetuximab in NSCLC. Further studies are needed to identify markers predictive of cetuximab benefit in NSCLC containing wild-type EGFR.

In summary, we have described a novel mechanism of resistance to cetuximab in lung cancer cells, irrespective of EGFR gene status. HGF induced resistance to cetuximab by activating the Gab1/Akt pathway through phosphorylation of Met. This resistance was also induced by transfecting cells with the HGF gene, resulting in an autocrine effect, and by coculture with HGF-producing fibroblasts, suggesting that microenvironments may be associated with resistance to cetuximab. Thus, regardless of EGFR gene status, the HGF-Met-triggering pathway may be of importance in lung cancer resistance to cetuximab.

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REFERENCES

1. Schiller JH, Harrington D, Belani CP, et al; Eastern Cooperative Oncology Group. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* 2002;346:92–98.
2. Ohe Y, Ohashi Y, Kubota K, et al. Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan. *Ann Oncol* 2007; 18:317–323.
3. Salomon DS, Brandt R, Ciardiello F, et al. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995;19:183–232.
4. Rusch V, Baselga J, Cordon-Cardo C, et al. Differential expression of the epidermal growth factor receptor and its ligands in primary non-small cell lung cancers and adjacent benign lung. *Cancer Res* 1993;53: 2379–2385.
5. Grandis JR, Sok JC. Signaling through the epidermal growth factor receptor during the development of malignancy. *Pharmacol Ther* 2004; 102:37–46.
6. Merrick DT, Kittelson J, Winterhalder R, et al. Analysis of c-ErbB1/epidermal growth factor receptor and c-ErbB2/HER-2 expression in bronchial dysplasia: evaluation of potential targets for chemoprevention of lung cancer. *Clin Cancer Res* 2006;12:2281–2288.
7. Maemondo M, Inoue A, Kobayashi K, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 2010;362:2380–2388.
8. Mitsudomi T, Morita S, Yatabe Y, et al; West Japan Oncology Group. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* 2010;11:121–128.
9. Zhou C, Wu YL, Chen G, et al. Efficacy results from the randomised phase III OPTIMAL (CTONG 0802) study comparing first-line erlotinib versus carboplatin (CBDCA) plus gemcitabine (GEM), in Chinese advanced non-small-cell lung cancer (NSCLC) patients (pts) with EGFR activating mutations. *Ann Oncol* 2010;21(Suppl 8):viii6 (Abstract #LBA13).
10. Giaccone G, Herbst RS, Manegold C, et al. Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a phase III trial–INTACT 1. *J Clin Oncol* 2004;22:777–784.
11. Herbst RS, Giaccone G, Schiller JH, et al. Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a phase III trial–INTACT 2. *J Clin Oncol* 2004;22:785–794.
12. Jonker DJ, O'Callaghan CJ, Karapetis CS, et al. Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 2007;357:2040–2048.
13. Vermorken JB, Trigo J, Hitt R, et al. Open-label, uncontrolled, multicenter phase II study to evaluate the efficacy and toxicity of cetuximab as a single agent in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck who failed to respond to platinum-based therapy. *J Clin Oncol* 2007;25:2171–2177.
14. Kawaguchi Y, Kono K, Mimura K, et al. Cetuximab induce antibody-dependent cellular cytotoxicity against EGFR-expressing esophageal squamous cell carcinoma. *Int J Cancer* 2007;120:781–787.
15. Mendelsohn J, Baselga J. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. *J Clin Oncol* 2003;21:2787–2799.
16. Mukohara T, Engelman JA, Hanna NH, et al. Differential effects of gefitinib and cetuximab on non-small-cell lung cancers bearing epidermal growth factor receptor mutations. *J Natl Cancer Inst* 2005;97:1185–1194.
17. Pirker R, Pereira JR, Szczesna A, et al. Cetuximab plus chemotherapy in patient with advanced non–small-cell lung cancer (FLEX): an open-label randomised phase III trial. *Lancet* 2009;373:1525–1531.
18. Gatzemeier U, von Pawel J, Vynnychenko I, et al. First-cycle rash and survival in patients with advanced non-small-cell lung cancer receiving cetuximab in combination with first-line chemotherapy: a subgroup analysis of data from the FLEX phase 3 study. *Lancet Oncol* 2011;12: 30–37.
19. Khambata-Ford S, Harbison CT, Hart LL, et al. Analysis of potential predictive markers of cetuximab benefit in BMS099, a phase III study of cetuximab and first-line taxane/carboplatin in advanced non-small-cell lung cancer. *J Clin Oncol* 2010;28:918–927.
20. Lynch TJ, Patel T, Dreisbach L, et al. Cetuximab and first-line taxane/carboplatin chemotherapy in advanced non-small-cell lung cancer: results of the randomized multicenter phase III trial BMS099. *J Clin Oncol* 2010;28:911–917.
21. Yano S, Wang W, Li Q, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. *Cancer Res* 2008;68:9479–9487.
22. Yamada T, Matsumoto K, Wang W, et al. Hepatocyte growth factor reduces susceptibility to an irreversible epidermal growth factor receptor inhibitor in EGFR-T790M mutant lung cancer. *Clin Cancer Res* 2010; 16:174–183.
23. Nakamura T, Nishizawa T, Hagiya M, et al. Molecular cloning and expression of human hepatocyte growth factor. *Nature* 1989;42:440–443.
24. Seki T, Ihara I, Sugimura A, et al. Isolation and expression of cDNA for different forms of hepatocyte growth factor from human leukocytes. *Biochem Biophys Res Commun* 1990;72:321–327.
25. Tomioka D, Maehara N, Kuba K, et al. Inhibition of growth, invasion, and metastasis of human pancreatic carcinoma cells by NK4 in an orthotopic mouse model. *Cancer Res* 2001;61:7518–7524.
26. Green LM, Reade JL, Ware CF. Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. *J Immunol Methods* 1984;70:257–268.
27. Matsumoto K, Nakamura T. Hepatocyte growth factor and the Met system as a mediator of tumor-stromal interactions. *Int J Cancer* 2006; 119:477–483.
28. Wang W, Li Q, Yamada T, et al. Crosstalk to stromal fibroblasts induces resistance of lung cancer to epidermal growth factor receptor tyrosine kinase inhibitors. *Clin Cancer Res* 2009;15:6630–6638.

29. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* 2005;2:e73.
30. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. *N Engl J Med* 2005;352:786–792.
31. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* 2007;316:1039–1043.
32. Kim SM, Kim JS, Kim JH, et al. Acquired resistance to cetuximab is mediated by increased PTEN instability and leads cross-resistance to gefitinib in HCC827 NSCLC cells. *Cancer Lett* 2010;296:150–159.
33. Krumbach R, Schüler J, Hofmann M, et al. Primary resistance to cetuximab in a panel of patient-derived tumour xenograft models: activation of MET as one mechanism for drug resistance. *Eur J Cancer* 2011;47:1231–1243.
34. Turke AB, Zejnullahu K, Wu YL, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. *Cancer Cell* 2010;17:77–88.
35. Liska D, Chen CT, Bachleitner-Hofmann T, et al. HGF rescues colorectal cancer cells from EGFR inhibition via MET activation. *Clin Cancer Res* 2011;17:472–482.
36. Tsao MS, Sakurada A, Cutz JC, et al. Erlotinib in lung cancer—molecular and clinical predictors of outcome. *N Engl J Med* 2005;353:133–144.
37. Khambata-Ford S, Garrett CR, Meropol NJ, et al. Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. *J Clin Oncol* 2007;25:3230–3237.
38. Loupakis F, Pollina L, Stasi I, et al. PTEN expression and KRAS mutations on primary tumors and metastases in the prediction of benefit from cetuximab plus irinotecan for patients with metastatic colorectal cancer. *J Clin Oncol* 2009;27:2622–2629.
39. Frattini M, Saletti P, Romagnani E, et al. PTEN loss of expression predicts cetuximab efficacy in metastatic colorectal cancer patients. *Br J Cancer* 2007;97:1139–1145.
40. Kasahara K, Arao T, Sakai K, et al. Impact of serum hepatocyte growth factor on treatment response to epidermal growth factor receptor tyrosine kinase inhibitors in patients with non-small cell lung adenocarcinoma. *Clin Cancer Res* 2010;16:4616–4624.
41. Tanaka H, Kimura T, Kudoh S, et al. Reaction of plasma hepatocyte growth factor levels in non-small cell lung cancer patients treated with EGFR-TKIs. *Int J Cancer* 2011;129:1410–1416.
42. Han JY, Kim JY, Lee SH, et al. Association between plasma hepatocyte growth factor and gefitinib resistance in patients with advanced non-small cell lung cancer. *Lung Cancer*. 2011;74:293–299.