

The Role of MET Activation in Determining the Sensitivity to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors

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Abstract

The development of resistance to epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) seems almost inevitable, even in patients with lung cancer that initially respond well to EGFR-TKIs. MET amplification was recently found to be a mechanism of escape from the anticancer effect of EGFR inhibitors. In the present study, we investigated the means whereby MET affects sensitivity to EGFR-TKIs in PC-9 cells. Gefitinib- or erlotinib-resistant sublines were established by exposing the parental PC-9 cell line to chronic, repeated treatments with these drugs. These resistant sublines showed more than 100-fold more resistance to gefitinib and erlotinib and acquired cross-resistance to other EGFR-TKIs. The T790M EGFR mutation was found by pyrosequencing, and this seemed to be the cause of drug resistance. Resistant cells also showed MET activation, although gene amplification was not detected. Furthermore, the induction of MET activity was not found to be associated with sensitivity to EGFR-TKIs. Interestingly, increased passage number without exposure to gefitinib or erlotinib caused MET activation, but this did not affect sensitivity to EGFR-TKIs. In addition, hepatocyte growth factor was found to block the ability of EGFR-TKIs to inhibit MET activation. However, sustained MET activation by hepatocyte growth factor did not modulate the cellular effects of gefitinib or erlotinib. Rather, activated MET enhanced migration and invasion abilities. Summarizing, MET activation may be acquired during cancer cell proliferation and enhances migratory and invasive abilities without affecting cellular sensitivity to EGFR-TKIs. Accordingly, the present study suggests that MET activation caused by factors other than MET gene amplification is

not a suitable surrogate marker of resistance to EGFR-TKIs. (Mol Cancer Res 2009;7(10):1736–43)

Introduction

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) are used to treat advanced non-small cell lung cancer. In theory, these tumors often show dramatic responses when cancer cells are dependent on EGFR signaling for survival. EGFR-TK mutations and increases in *EGFR* gene copy number, as determined by fluorescent *in situ* hybridization, might be predictive of therapeutic response to EGFR-TKIs (1, 2). However, resistance to these drugs invariably develops, and this limits median response duration to 6 to 8 months, although therapeutic response can sometimes persist for more than 2 years (3). Studies undertaken to elucidate the mechanism underlying acquired resistance found that the secondary T790M mutation is an important contributory factor in up to 50% of cases (4, 5). MET amplification was recently found to be involved in escape from the anticancer effects of EGFR inhibitors (6). This amplification caused HCC827 cells to become resistant to gefitinib through the ErbB3-dependent activation of the phosphoinositide 3-kinase-Akt pathway. Furthermore, increased Akt expression and sustained activation, even after treatment with EGFR-TKIs, were common findings in lung cancer cells with acquired resistance. Moreover, when this redundant MET signaling and the stimulation of Akt expression via the EGFR pathway were simultaneously inhibited, apoptosis increased dramatically among resistant cells.

MET and its ligand, hepatocyte growth factor/scatter factor (HGF/SF), are involved in normal development and organogenesis and in the regulation of cell proliferation, migration, and angiogenesis (7–9). Dysregulation of MET signaling through gene amplification, mutation, or autocrine or paracrine activation is now known to be an important feature in various human cancers, especially in terms of the evolution of metastasis (10). Furthermore, the recent development of many MET inhibitors has stimulated broader research on their therapeutic indications in cancer. In particular, Engelman et al. (6) showed that a MET inhibitor could restore cellular sensitivity to EGFR-TKIs. Furthermore, activated MET was found to be a marker of primary resistance to gefitinib in lung cancer patients, which suggests that MET inhibitors might be useful in these patients (11). These findings mean that cancer cells expressing activated MET prior to developing resistance can promptly use the

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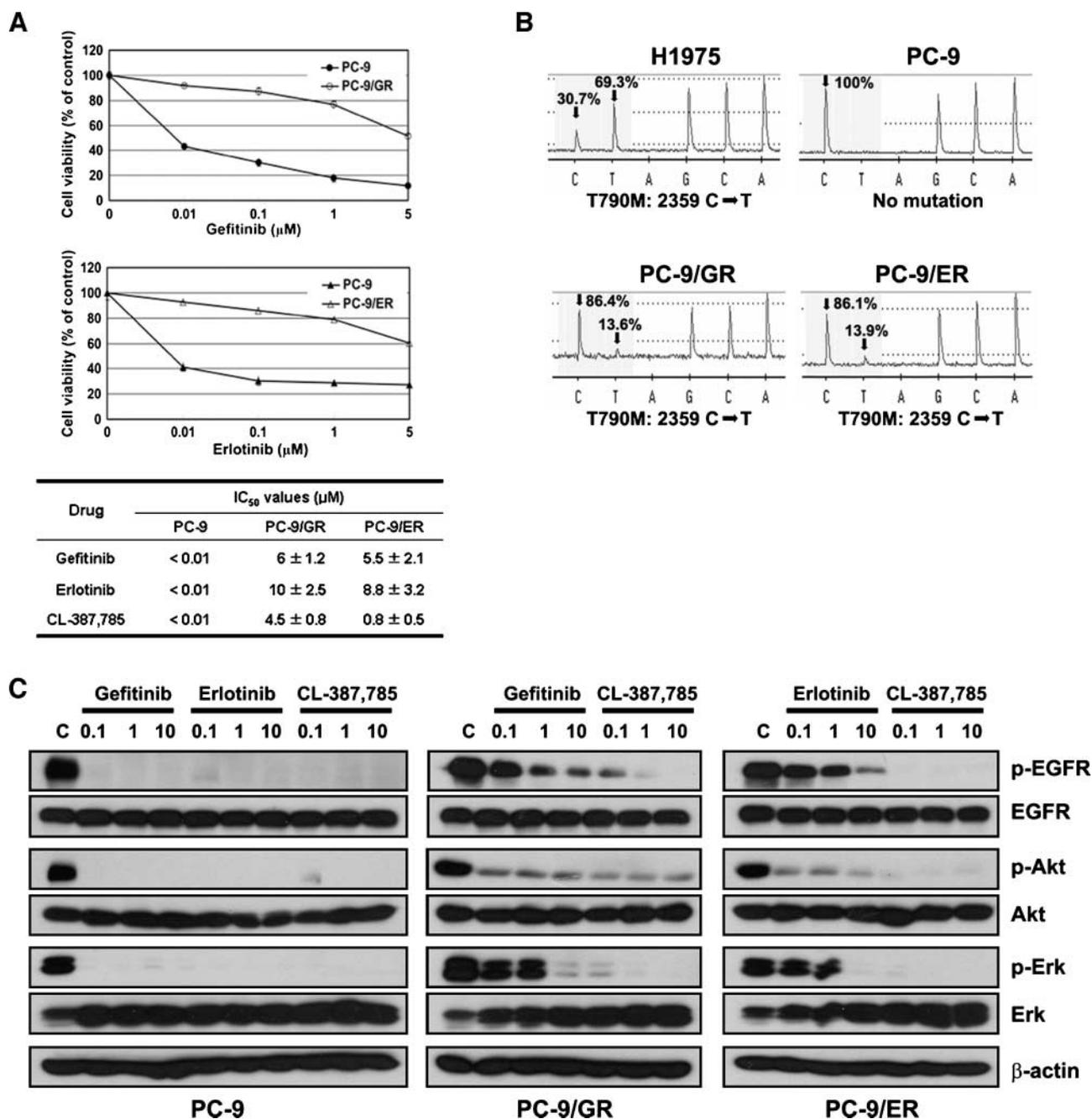


FIGURE 1. Cytotoxic effects of EGFR-TKIs in gefitinib- and erlotinib-resistant cells. **A.** Cells were exposed to gefitinib or erlotinib at different doses for 72 h. The growth inhibition curves of PC-9, PC-9/GR (gefitinib resistant), and PC-9/ER (erlotinib resistant) cells are shown. Cross-resistance to other EGFR-TKIs was determined using MTT assays. Experiments were done in triplicate; bars, SD. **B.** Pyrosequencing of EGFR-TK exon 20 revealed a C-to-T base pair change (arrows) corresponding to T790M. **C.** PC-9, PC-9/GR, and PC-9/ER cells were treated with the indicated concentrations of gefitinib, erlotinib, or CL-387,785 (an EGFR inhibitor) for 12 h. Lysates were Western blotted using the indicated antibodies.

MET pathway if EGFR signaling, which they are dependent on for survival, is blocked.

In this study, we examined the acquisition of resistance to EGFR-TKIs and changes in MET activation during proliferation in PC-9 cells (a non-small cell lung cancer cell line) with a deletion mutation at exon 19 of the *EGFR* gene. We also investigated cross talk between EGFR and MET signaling and the effects of MET activation on sensitivity to EGFR-TKIs.

Results

Gefitinib- and Erlotinib-Resistant Cells Showed Cross-Resistance to Other EGFR-TKIs

Gefitinib- and erlotinib-resistant cells were established by stepwise selection using increasing concentrations of gefitinib or erlotinib (up to a final concentration of 1 μmol/L) over a period of 6 months, as described in Materials and Methods. The gefitinib- and erlotinib-resistant sublines are referred to

as PC-9/GR and PC-9/ER, respectively. As shown in Fig. 1A, these cells were >100 times more resistant to gefitinib and erlotinib than parental cells (gefitinib IC_{50} , 6 $\mu\text{mol/L}$ in PC-9/GR cells and <0.01 $\mu\text{mol/L}$ in PC-9 cells; erlotinib IC_{50} , 10 $\mu\text{mol/L}$ in PC-9/ER cells and <0.01 $\mu\text{mol/L}$ in PC-9 cells).

To investigate the mechanism responsible for this resistance to EGFR-TKIs, we analyzed the sequence of the *EGFR* gene at exons 18 to 21. Although additional mutations were not detected by direct sequencing (data not shown), pyrosequencing confirmed the presence of the T790M mutation in gefitinib- and erlotinib-resistant cells (Fig. 1B), although both resistant cell types contained only a small amount of mutated EGFR (~13-14%) as compared with H1975 cells (~69%). Furthermore, because the emergence of the T790M mutation can increase the affinity of oncogenic EGFR for ATP (12), we examined changes in EGFR signal transduction in the presence of gefitinib and erlotinib. Unlike that observed in parental PC-9 cells, the activities of EGFR, Akt, and extracellular signal-regulated kinase (Erk) in gefitinib- and erlotinib-resistant cells were maintained in the presence of gefitinib or erlotinib. However, CL-387,785 (an irreversible EGFR inhibitor) was considerably more effective than gefitinib or erlotinib at suppressing EGFR activity and its downstream signaling (as determined by Akt and Erk activity assays), although they did not completely inhibit Akt activity in gefitinib-resistant cells (Fig. 1C).

MET Activity Was Elevated in Gefitinib- and Erlotinib-Resistant Cells and in PC-9 Cells at High Passage Numbers but This Did Not Affect Sensitivity to EGFR-TKIs

We used Western blotting to investigate EGFR family members and molecules known to be involved in sensitivity to EGFR-TKIs. As shown Fig. 2A, these moieties were similarly expressed in parental and resistant cells, but MET protein activity and level were significantly higher in gefitinib- and erlotinib-resistant cells than in PC-9 cells. To determine whether MET activity upregulation plays a role in the acquisition of resistance to gefitinib and erlotinib, we examined changes in sensitivity to gefitinib and erlotinib after inhibiting MET with PHA-665752 (a MET inhibitor). However, although combined treatments with PHA-665752 and gefitinib or erlotinib showed some additive effect, these treatments only marginally restored sensitivity to EGFR-TKIs (Fig. 2B).

To develop resistant cells, high passage numbers are inevitable, and unfortunately, cells can acquire different morphologies and functionalities during passage (13, 14). Accordingly, it was possible that the observed alterations in the expression and activation of MET were attributable to passage numbers and not to drug exposure. Thus, we examined MET activity levels in untreated PC-9 cells that had been passaged ≤ 12 , 2 to 36, or ≥ 72 times (referred to as PC-9/LP, PC-9/MP, and PC-9/HP, respectively). These three sublines had similar proliferation rates (Fig. 3C, bottom). As shown in Fig. 3A, increasing PC-9 cell

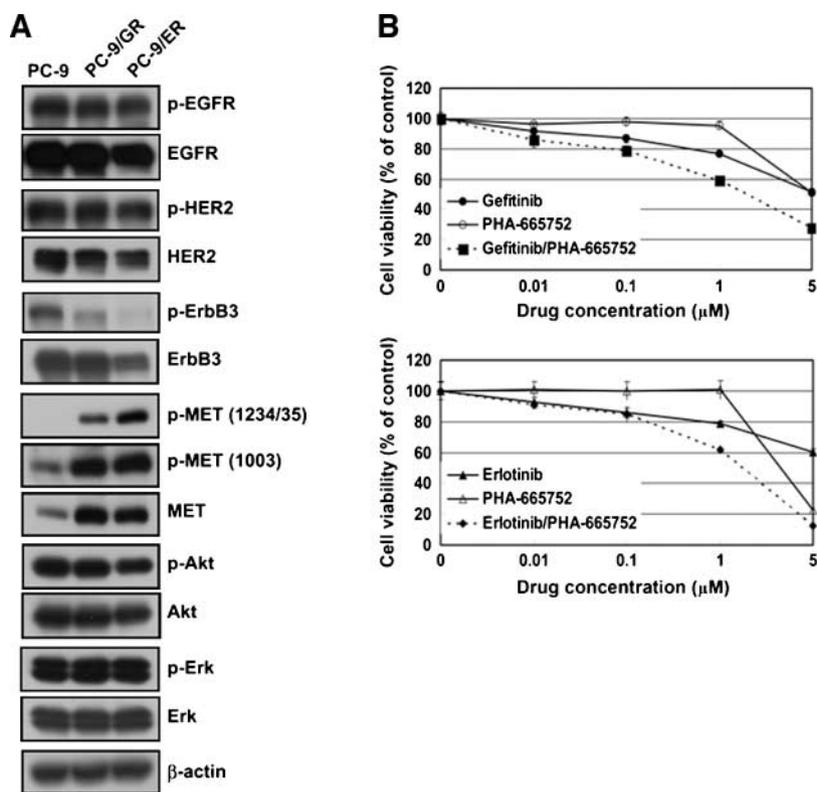


FIGURE 2. Enhanced MET activity in PC-9/GR and PC-9/ER cells was not related to sensitivity to EGFR-TKIs. **A.** The basal expressions of EGFR family and MET and their downstream molecules in PC-9, PC-9/GR, and PC-9/ER cells were determined by Western blotting. **B.** Top graph, PC-9/GR cells were treated with the indicated doses of gefitinib, PHA-665752 (a MET inhibitor), or gefitinib and erlotinib in combination for 72 h. Bottom graph, PC-9/ER cells were treated with the indicated doses of erlotinib, PHA-665752, or gefitinib and erlotinib in combination for 72 h. Cell viabilities were determined using MTT assays.

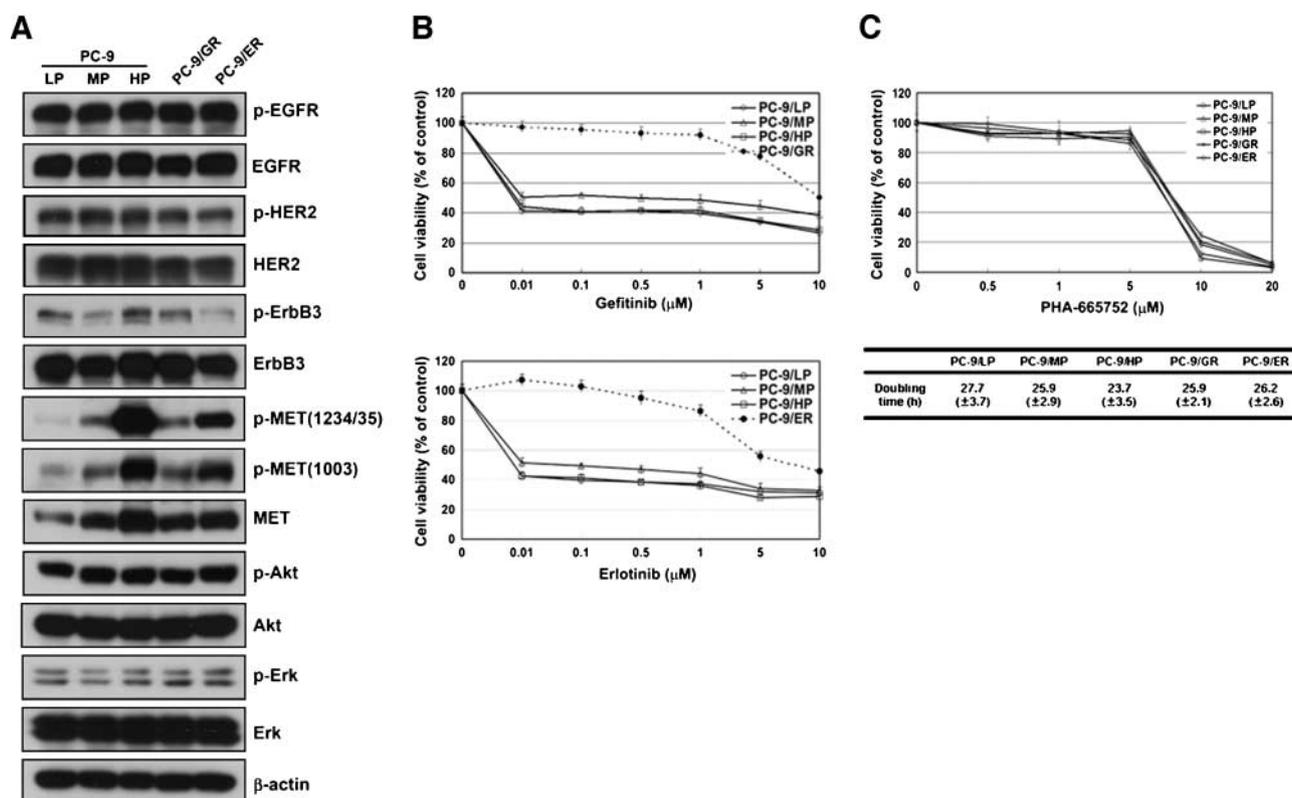


FIGURE 3. MET activity increased with passage number in PC-9 cells, but MET activation did not affect sensitivity to EGFR-TKIs. **A.** The basal levels of EGFR family and MET and their downstream molecules in the indicated cell lines were detected by Western blotting. **B** and **C.** The cells were treated with the indicated doses of gefitinib (*top graph*), erlotinib (*bottom graph*), or PHA-665752 (**C**) for 72 h. Cell viabilities and doubling times were determined using MTT assays. LP, ≤ 12 passages; MP, 20 to 36 passages; HP, ≥ 72 passages.

passage number enhanced both the expression and the activity of MET to a level greater than that in the resistant sublines (PC-9/GR and PC-9/ER). However, these increases did not affect the sensitivity to gefitinib and erlotinib (Fig. 3B). Furthermore, the expression and activity of MET did not alter sensitivity to PHA-665752 (Fig. 3C). These results indicate that MET activation can be spontaneously acquired during proliferation, and that this activation is not involved in therapeutic resistance to gefitinib or erlotinib.

Inhibition of EGFR Decreased MET Activity but Did Not Affect Sensitivity to Gefitinib and Erlotinib

EGFR and MET are frequently coexpressed in cancers, including those of the lungs, head and neck, breast, and colon. Some studies have shown that the transactivation occurs between EGFR and MET (15, 16), but this remains to be proved. To determine whether transactivation between EGFR and MET occurs via a ligand-dependent mechanism in lung cancer cell lines, PC-9/LP and PC-9/HP with different levels of MET activation were treated with EGF and HGF. Both ligands enhanced their receptor activities, but no transactivation was detected (Fig. 4A). The same results were obtained in other non-small cell lung carcinoma cells (Supplementary Fig. S1). Interestingly, despite this lack of transactivation, the inhibition of EGFR with gefitinib or erlotinib decreased MET activity, and these effects were reversed by HGF treatment. However,

the suppression of MET with PHA-665752 did not affect EGFR activity (Fig. 4B). In addition, we evaluated the role of MET in sensitivity to gefitinib and erlotinib using this compensatory effect of HGF to maintain MET activity in the presence of EGFR-TKIs. As might be expected, the sustained MET activity by HGF did not affect sensitivity to gefitinib or erlotinib (Fig. 4C).

Induction of MET Activity in PC-9 Cells Increased Migration and Invasiveness

One of the major cellular effects of MET is that it enhances migration and invasiveness, which is critically required for metastasis. Hence, we investigated whether elevated MET activity during proliferation affects these abilities using Boyden chamber assays. The MET-activated cells were found to migrate more than parental cells (i.e., by 2.1-fold in PC-9/MP cells, 3.6-fold in PC-9/HP cells, 6.2-fold in PC-9/GR cells, and 5.9-fold in PC-9/ER cells; Fig. 5A) and to be more invasive (4.2-fold in PC-9/MP cells, 7.8-fold in PC-9/HP cells, 20-fold in PC-9/GR cells, and 41-fold in PC-9/ER cells; Fig. 5B). Furthermore, these fold increases were markedly reduced by treating PC-9/MP and PC-9/HP cells with PHA-665752, suggesting that MET activation played a major role in their activation. Moreover, although the same effect was also observed in PC-9/GR and PC-9/ER cells, it was much reduced. In addition, PC-9/GR and PC-9/ER cells exhibited morphologic changes,

such as greater proportions of spindle-shaped cells with or without pseudopodia (Supplementary Fig. S2), which suggests that phenotypic changes other than MET activation contributed to their malignant characteristics.

Discussion

In this study, the T790M mutation was found by pyrosequencing analysis, which showed that the mutation was present in a small number of gefitinib- and erlotinib-resistant PC-9

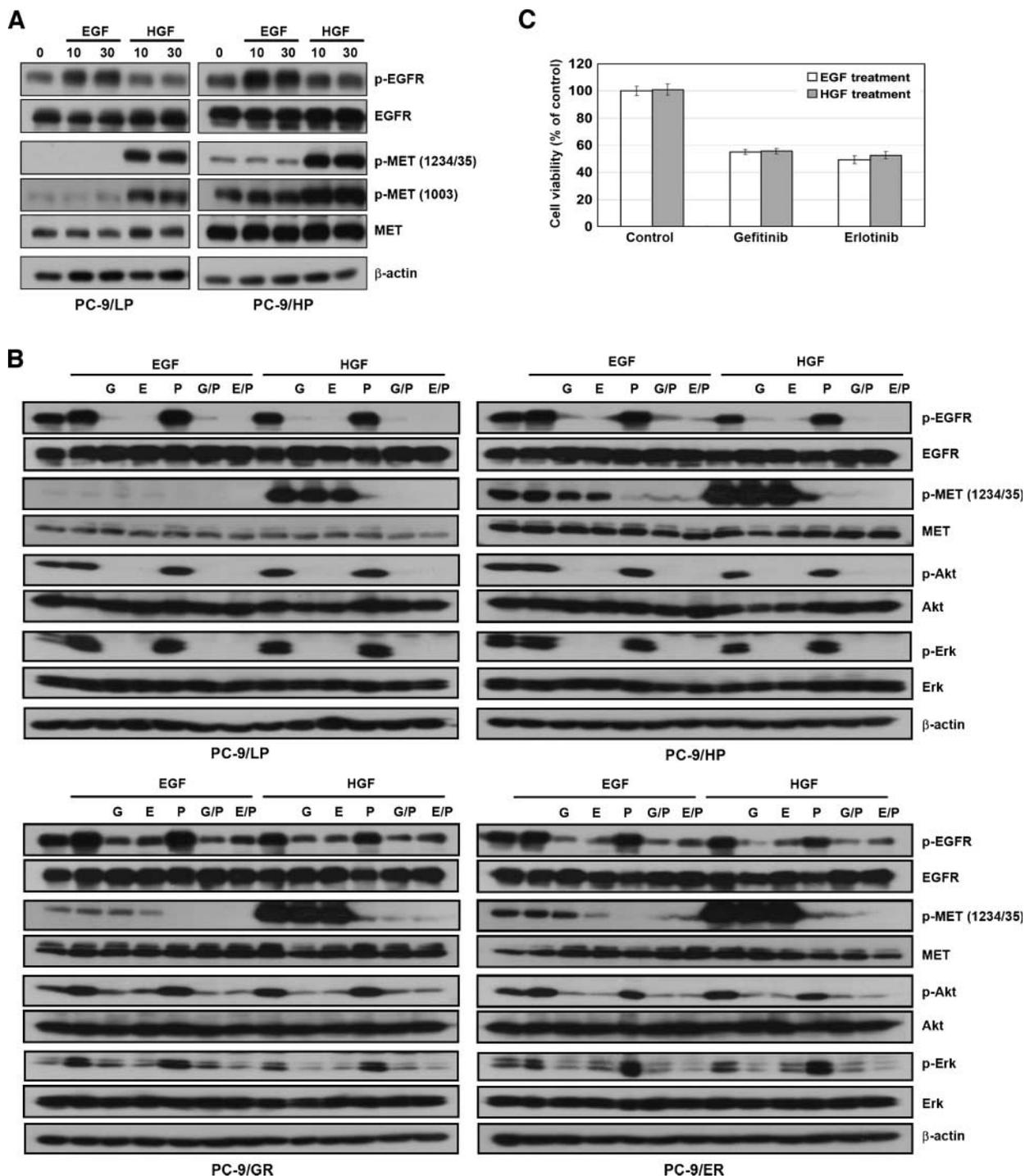


FIGURE 4. EGFR inhibition also decreased MET activity, but this effect was partially reversed by HGF. Cells were incubated for 24 h under serum-starved conditions. **A.** These serum-starved cells were treated with 100 ng/mL EGF or 50 ng/mL HGF for the indicated times and then Western blotted. **B.** Serum-starved cells were treated with gefitinib, erlotinib, or PHA-665752 at 1 μ mol/L for 3 h and then treated with 100 ng/mL EGF or 50 ng/mL HGF for 30 min. The EGFR and MET activities were detected by Western blotting. **C.** Serum-starved PC-9/LP cells were treated with gefitinib or erlotinib at 1 μ mol/L plus EGF or HGF for 24 h. Differences between sensitivities to the drugs were determined using MTT assays. G, gefitinib; E, erlotinib; P, PHA-665752.

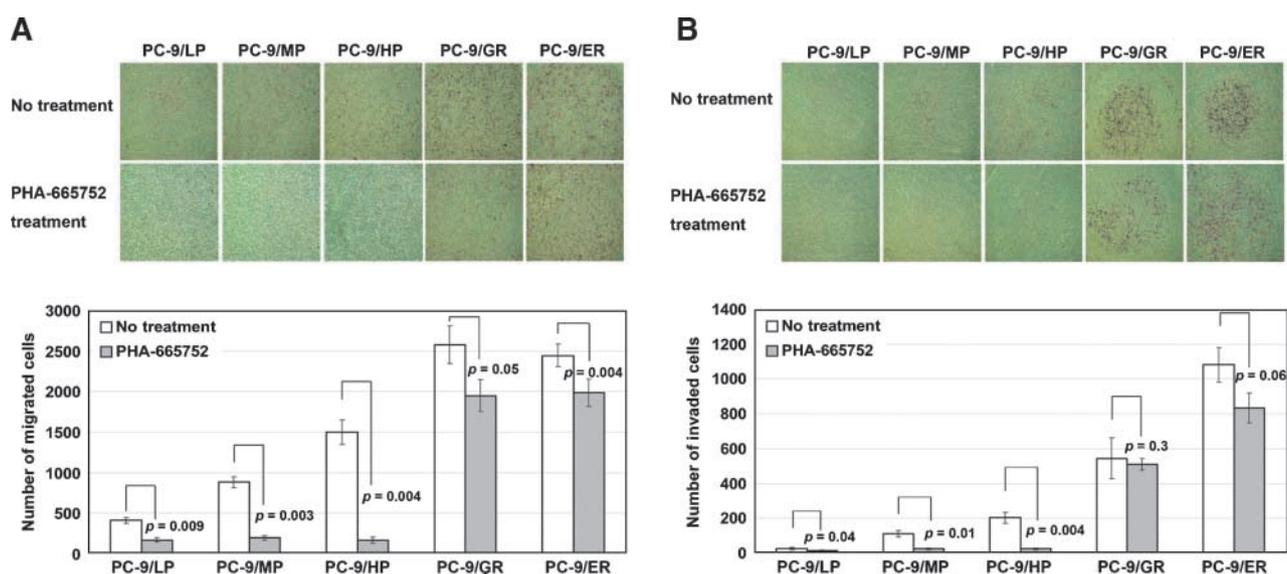


FIGURE 5. Elevated MET activity enhanced migratory and invasive ability. **A** and **B.** Cells were seeded onto either collagen- or Matrigel-coated polycarbonate filters to determine their migratory and invasive potentials, respectively. They were then treated with or without 1 $\mu\text{mol/L}$ PHA-665752 (MET inhibitor) for 48 h in modified Boyden chambers. Cells that migrated through or invaded filters were stained and counted under a light microscope. Experiments were repeated in triplicate; bars, SDs.

cells, which is consistent with previous studies that used this cell line (17). Although EGFR-TKI-resistant cells contained only a low level of the T790M mutation, other studies have shown that this can be sufficient to confer EGFR-TK resistance. It has been suggested that the low fraction of T790M-containing alleles in these resistant cells acts in a dominant manner and enables the continued maintenance of the activities of EGFR, ErbB3/phosphoinositide 3-kinase/Akt, and Erk1/2 (18, 19). These findings are consistent with our observations that gefitinib and erlotinib do not completely inhibit EGFR and its downstream signaling activities in resistant cells. Some authors have suggested that irreversible EGFR inhibitors that bind covalently to EGFR may inhibit EGFR containing the T790M mutation (20, 21). According to our findings, the irreversible EGFR inhibitor CL-387,785 was more potent than gefitinib or erlotinib at inhibiting EGFR and its downstream activities in resistant cells. In particular, the effects of CL-387,785 on erlotinib-resistant cells were similar to its effects on parental cells. In line with these results, CL-387,785 also inhibited the growth of resistant cells more effectively than did gefitinib or erlotinib. Taken together, it seems that the acquisition of the EGFR T790M mutation by resistant cells may lead to resistance to EGFR-TKIs.

MET activation in human tumors can be induced by different mechanisms, such as overexpression (22), structural alterations (7), and HGF-dependent or HGF-independent activation (10). Recently Kubo et al. (23) showed that *MET* gene amplification or EGFR mutation can activate MET in lung cancer. Furthermore, *MET* gene amplification was found in patients with acquired resistance to EGFR-TKIs, and this always led to the activation of MET protein *in vitro*, indicating that MET activation may be a useful surrogate marker of sensitivity to EGFR-TKIs. However, it has not been defined whether MET activation caused by other mechanisms can affect sensitivity to EGFR-TKIs.

In the present study, we found that MET activity and protein levels were elevated in PC-9/GR and PC-9/ER cells. However, this phenomenon was also observed in untreated cells that had undergone a high number of passages. Furthermore, MET expression and activity were found to be elevated in proportion to the number of passages, which raised the possibility that MET activation in resistant cells is not related to drug resistance. We examined this possibility in two ways. First, MET activity was inhibited in resistant cells with PHA-665752, but this failed to restore sensitivity to gefitinib or erlotinib. Second, MET activation by chronic culture in drug-sensitive cells also failed to affect sensitivity to EGFR-TKIs. Nevertheless, our results are at odds with previous reports because MET activation in our resistant cells was not accompanied by *MET* gene amplification (see Supplementary Fig. S3). Kubo et al. also concluded that the effect of gefitinib does not depend on MET status in non-small cell lung carcinoma cell lines, indicating that MET activation is not the only factor that confers resistance. Accordingly, we suggest that increases in MET activation are not associated with cellular sensitivity to EGFR-TKIs if the activation is not driven by *MET* gene amplification.

Cross talk between MET and EGFR may substantially alter growth control during tumorigenesis. In fact, cross talk between these two receptors has been reported to contribute to an oncogenic mechanism (24), cancer growth and invasion (25, 26), and epithelial wound healing (15). In addition, the activations of these two receptors can affect sensitivity to EGFR-TKIs because they initiate similar signal transduction pathways, including the Erk and phosphoinositide 3-kinase/Akt pathways (27, 28). However, we found that there was no cross talk between the two receptors via the ligand-dependent mechanism in all the tested lung cancer cells. Nevertheless, inhibition of EGFR could also have decreased the MET activity, but not vice versa. These phenomena may be explained by a

previous report, in which it was shown that mutated and amplified EGFR can activate MET (23). Although EGFR-TKIs can suppress MET activity, sensitivity to EGFR-TKIs did not change under experimental conditions when MET activity was sustained by administering HGF.

The finding that MET activity was increased in cells with high passage numbers was not altogether surprising because MET is known to play an important role in the acquisition of an aggressive cancer phenotype; especially increased cellular motility (10). The present study shows that cells exhibiting MET activation also had enhanced migratory and invasive abilities, which could possibly be controlled by PHA-665752. However, in PC-9/GR and PC-9/ER cells, the effect of PHA-665752 was incomplete, which suggested that other changes had enhanced malignant characteristics during the acquisition of drug resistance. Furthermore, morphologic changes were also observed in resistant cells, which were reminiscent of epithelial-to-mesenchymal transition. However, because E-cadherin loss and delocalization were not observed, epithelial-to-mesenchymal transition is unlikely to have been responsible for gefitinib and erlotinib resistance, although we previously suggested that epithelial-to-mesenchymal transition might be a mechanism of gefitinib resistance (29). Further investigations on how MET activity increases during cell proliferation and on what morphologic changes are present in resistant cells are needed.

Many MET inhibitors, including monoclonal antibodies and small-molecule TKIs, are currently undergoing clinical trials or preclinical investigations (30), and it is expected that a number of these will be clinically available in the near future. The present study indicates that MET inhibitors could be used to regulate cellular mobility but not overcome resistance to EGFR-TKIs in cells without *MET* gene amplification. Thus, our findings indicate that the status of *MET* gene amplification should be considered in parallel with MET activity when selecting patients for MET inhibitor treatment.

Summarizing, our findings suggest that MET activation may be acquired during cancer cell proliferation, and that this activation enhances migratory and invasive abilities without affecting sensitivity to EGFR-TKIs. Accordingly, our findings suggest that MET activation is not caused by *MET* gene amplification, and that it cannot be used as a surrogate marker of resistance to EGFR-TKIs.

Materials and Methods

Cell Culture and Reagents

The PC-9 cells were a gift from F. Koizumi and K. Nishio (National Cancer Center Hospital, Tokyo, Japan). Cells were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) at 37°C in a 5% CO₂ atmosphere. MTT solution and HGF were purchased from Sigma. EGF and CL-387,785 (EGFR inhibitor) were purchased from Calbiochem. Gefitinib was kindly provided by AstraZeneca. Erlotinib and PHA-665752 (the MET inhibitor) were gifts from Roche and Pfizer, respectively.

Establishment of the Gefitinib- and Erlotinib-Resistant Cell Lines

Gefitinib- and erlotinib-resistant cells were developed by chronic, repeated exposure. Briefly, PC-9 cells were exposed

to 10 nmol/L of gefitinib or erlotinib for 48 h in medium containing 10% fetal bovine serum. They were then washed and cultured in drug-free medium until surviving cells were 80% confluent. These cells were then reexposed to increasing concentrations of gefitinib or erlotinib. Cells that were able to grow in 1 µmol/L gefitinib or erlotinib were obtained 6 mo after initial exposure. The established resistant cell lines were maintained in medium containing 1 µmol/L of gefitinib or erlotinib. For all *in vitro* studies, resistant cells were cultured in drug-free medium for at least 1 wk to eliminate gefitinib and erlotinib. Gefitinib- and erlotinib-resistant cells are referred to as PC-9/GR and PC-9/ER cells, respectively.

MTT Assays

Briefly, cells in the logarithmic growth phase were harvested and seeded onto 96-well plates overnight. Drugs were then added and cells were incubated for the indicated times. Cell viabilities were determined using MTT assays as described by Carmichael et al. (31).

Pyrosequencing Assay for T790M Mutation

Pyrosequencing was done as follows. The PCR amplification primers used were exon 20 EGFR-forward, 5'-ctggcctcctcacac-3', and EGFR-reverse biotinylated primer, 5'-tgtgtcccggacatagcca-3'. Each PCR mix contained forward and reverse primers (each 0.4 pmol), 0.2 mmol each of deoxynucleotide triphosphates, 1.5 mmol/L MgCl₂, 1× PCR buffer, 1.5 units of Immolase DNA polymerase (Bioline), and 5 µL of genomic DNA in a total volume of 50 µL. PCR conditions consisted of initial denaturing at 94°C for 1 min, 50 amplification cycles (95°C for 20 s, 58°C for 20 s, and 72°C for 40 s), and a final extension at 72°C for 1 min. The PCR products so obtained were electrophoresed in 2% agarose gel to confirm successful amplification of PCR products, 20 µL of which were sequenced using a Pyrosequencing PSQ96 HS System (Biotage) according to the manufacturer's instructions. The pyrosequencing primer sequence was 5'-ccgtgcagctcatca-3'.

Immunoblot Analysis

Proteins from cell lysates were prepared as previously described (32), separated by SDS-PAGE, and then electrotransferred onto Immobilon membranes (Millipore). These membranes were subsequently probed with specific antibodies for p-EGFR (Tyr1173), EGFR (1005), HER2 (C-18), ErbB3 (C-17), MET (C-12), p-Akt (Ser473), Akt (C-20), p-Erk (E-4), Erk (K-23), and β-actin (C4), which were obtained from Santa Cruz Biotechnology. p-HER2 (Tyr1221/22), p-ErbB3 (Tyr1222), and p-MET (Tyr1234/1235) were purchased from Cell Signaling Technology. p-MET (Tyr1003) was obtained from Invitrogen. Horseradish peroxidase-conjugated antibodies were used as secondary antibodies. Membranes were developed using ECL kits (Amersham Biosciences).

Migration and Invasion Assays

Cell migration and invasion assays were done using a modified Boyden chamber assay (33). Polyvinylpyrrolidone-free polycarbonate filters (6.5-mm diameter, 8 µm pore size polycarbonate membrane) were coated with collagen (0.01%, w/v) for migration assays or with Matrigel (15 µg/filter) for invasion assays. Cells (1 × 10⁵ per chamber) in 200 µL of medium were

placed in the upper chamber, and the lower chamber was filled with 1 mL of serum-free medium supplemented with or without 1 $\mu\text{mol/L}$ of PHA-665752. After incubation for 48 h at 37°C, cells that migrated to or invaded the lower surfaces of filters were stained using Diff-Quick kits (Fisher Scientific) and then counted under a microscope. Results were expressed as means \pm SDs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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