Clinical Cancer Research

Reciprocal and Complementary Role of *MET* Amplification and *EGFR* T790M Mutation in Acquired Resistance to Kinase Inhibitors in Lung Cancer

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Abstract

Purpose: In epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) therapy for lung cancer patients, acquired resistance develops almost inevitably and this limits the improvement in patient outcomes. *EGFR* T790M mutation and *MET* amplification are the two main mechanisms underlying this resistance, but the relationship between these two mechanisms is unclear. In this study, we explored their relationship using *in vitro* models and autopsy specimens.

Experimental Design: Erlotinib-resistant HCC827 (HCC827ER) cells were developed by chronic exposure to erlotinib at increasing concentrations. HCC827EPR cells were also developed by chronic exposure to erlotinib in the presence of PHA-665,752 (a MET TKI). The erlotinib-resistant mechanisms of these cells were analyzed. In addition, 33 autopsy tumor samples from 6 lung adenocarcinoma patients harboring multiple gefitinib-refractory tumors were analyzed.

Results: HCC827ER developed *MET* amplification, and clinically relevant resistance occurred at ≥4-fold *MET* gene copy number gain (CNG). By contrast, HCC827EPR developed T790M without *MET* CNG. Of six patients harboring gefitinib-refractory tumors, three exhibited T790M only, one exhibited *MET* amplification only, and the other two exhibited T790M and/or *MET* amplification depending on the lesion sites. In these gefitinib-refractory tumors, T790M developed in 93% (14 of 15) of tumors without *MET* gene CNGs, in 80% (4 of 5) of tumors with moderate *MET* gene CNGs (<4-fold), and in only 8% (1 of 13) of tumors with *MET* amplification (≥4-fold).

Conclusions: These results indicate a reciprocal and complementary relationship between T790M and *MET* amplification and the necessity of concurrent inhibition of both for further improving patient outcomes. *Clin Cancer Res; 16(22); 5489–98. ©2010 AACR.*

Non–small cell lung cancers harboring activating mutations of the epidermal growth factor receptor (*EGFR*) gene are addicted to the EGFR pathway and are very sensitive to small molecule EGFR tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib (1–7). Despite dramatic initial responses, however, acquired resistance develops almost inevitably after a median of ~10 months (8), and this limits the improvement in patient outcomes. The secondary *EGFR* mutation, substitution of threonine to methionine at codon 790 (the "gatekeeper" residue, T790M), and the amplification of the *MET* gene are the two main molecular mechanisms responsible for acquired resistance to EGFR-TKIs (9–12).

This resistance is postulated to develop from the selection of pre-existing minor resistant clones harboring either the T790M mutation (13) or the *MET* amplification (14), although therapy-naïve tumors rarely harbor these alterations (15–17). In this context, it seems that these cells are destined to develop each resistant mechanism even before EGFR-TKI treatment begins. For example, the HCC827 lung adenocarcinoma cell line reproducibly acquires resistance by *MET* amplification to gefitinib (11) or an irreversible pan-ERBB kinase inhibitor, PF00299804 (14). By contrast, different laboratories have shown that the PC9 cell line always develops resistance because of T790M secondary mutations (18–20). To delay or to avoid the emergence of resistance, it is reasonable to treat patients with agents that are effective against specific resistant mechanisms as part of the initial systemic therapies (14).

In the present study, we chronically exposed HCC827 lung adenocarcinoma cells to increasing concentrations of erlotinib in the absence or the presence of a MET-TKI.

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Translational Relevance

EGFR T790M mutation and *MET* amplification are the two main molecular mechanisms responsible for acquired resistance to gefitinib or erlotinib in patients with non–small cell lung cancer harboring a mutation in the *EGFR* gene. The present study is the first to observe a reciprocal and complementary relationship between these resistant mechanisms. In *in vitro* analyses we show that the HCC827 lung adenocarcinoma cell line could develop either of the resistant mechanisms against erlotinib. In addition, 33 tumors from 6 patients who died after developing acquired resistance to gefitinib were analyzed. In these gefitinib-refractory tumors, the T790M mutation developed in 93% (14 of 15) of tumors without *MET* gene copy number gains (CNG), in 80% (4 of 5) of tumors with moderate *MET* gene CNGs (<4-fold), and in only 8% (1 of 13) of tumors with *MET* amplification (≥4-fold). These results indicate that concurrent inhibition of both mechanisms seems to be essential for improving patient outcomes further.

We also examined multiple sites of recurrent tumors for *EGFR* T790M mutation and *MET* amplification in samples from autopsies of six patients who had showed acquired resistance to gefitinib after an initial good response to identify any *in vivo* relationship between these two mechanisms responsible for resistance.

Materials and Methods

Cell culture and reagents

The *EGFR* mutant human lung adenocarcinoma cell line HCC827 (del E746_A750) was the kind gift of Dr. Adi F. Gazdar (Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas) and was cultured in RPMI1640 medium supplemented with 5% fetal bovine serum (FBS)

and 1× antibiotic-antimycotic solution (Invitrogen) at 37°C in a humidified incubator with 5% CO_2.

Erlotinib was kindly provided by Hoffmann-La Roche, Inc. (Nutley, NJ). The selective MET inhibitor PHA-665,752 and the irreversible EGFR-TKI CL-387,785 were purchased from Tocris Bioscience and Calbiochem, respectively.

Generation of *in vitro* drug-resistant HCC827 cells

Erlotinib-resistant HCC827 (HCC827ER) cells were developed by chronic, repeated exposure to erlotinib at increasing concentration from 5 nmol/L to 2 μmol/L as described previously (11). The erlotinib concentration was increased stepwise when the cells resumed proliferation, similar to the pattern in untreated parental cells. Erlotinib/PHA-665,752–resistant HCC827 (HCC827EPR) cells were also developed by chronic, repeated exposure to erlotinib at increasing concentrations in the presence of 1 μmol/L PHA-665,752. The identity of the HCC827ER cells and HCC827EPR cells was confirmed by analyzing the short tandem repeat (STR) profile using the Cell ID System (Promega).

Cell proliferation assay

Cell proliferation was measured using TetraColor ONE (Seikagaku-kogyo) according to the manufacturer's instructions. Briefly, tumor cells ($3 × 10^3$) were plated into each well of 96-well flat-bottomed plates and grown in RPMI1640 with 5% FBS. After 24 hours, DMSO, erlotinib, PHA-665,752, CL-387,785, or a combination of these drugs was added to achieve the indicated drug concentration, and the cells were incubated for an additional 72 hours. A colorimetric assay was done after addition of 10 μL TetraColor ONE in each well, and the plates were incubated at 37°C for 1 hour. The absorbance was read at 450 nm using a multiplate reader. Percent growth was determined relative to untreated controls.

Phospho-receptor tyrosine kinase array analysis

A Human Phospho-RTK Array Kit (R&D Systems) was used to measure the relative level of tyrosine phosphorylation of receptor tyrosine kinases (RTK). The membranes contained spotted antibodies corresponding to 42 distinct

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Table 1. Clinical characteristics of patients treated with gefitinib

Patient	Age	Sex	PY	Disease status	Lines	Response	TTF (months)	OS (months)
1	57	F	0	Stage IV*	3	PR	13.8	48.5
2	48	F	0	Stage IV	1	PR	11.0	12.2
3	58	M	34	Stage IV	2	PR	14.5	23.7
4	75	M	0	Stage IV	2	PR	43.9	63.0
5	93	F	0	Stage IV	1	PR	14.8	30.9
6	62	M	26	Stage IV	2	PR	9.1	32.9

Abbreviations: PY, pack years (smoking status); TTF, time to treatment failure; OS, overall survival; PR, partial response.
*Postsurgical recurrence.
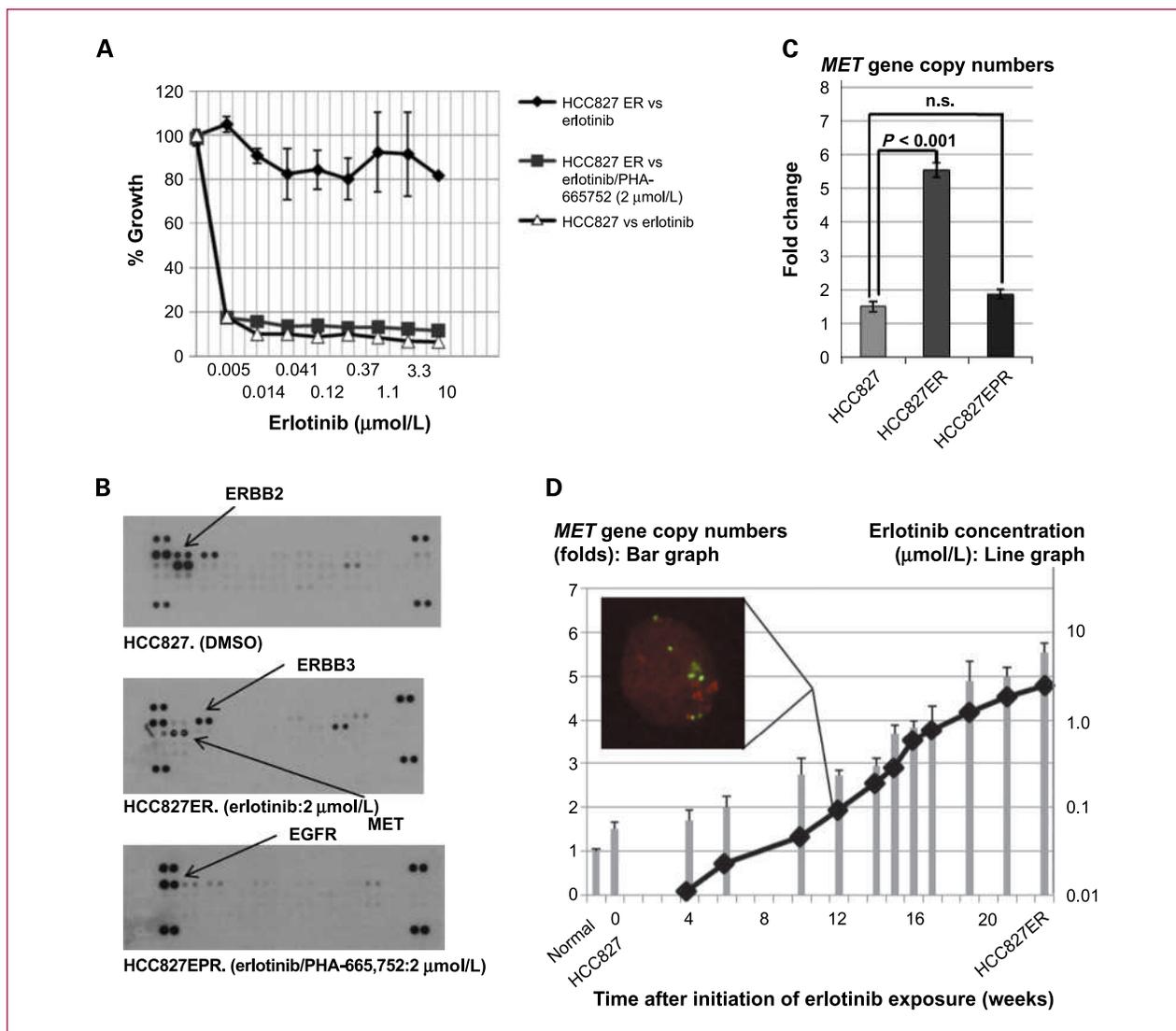
Fig. 1. Amplified *MET* gene caused erlotinib resistance in HCC827ER cells but not in HCC827EPR cells. A, HCC827ER cells were resistant to erlotinib, and PHA-665,752 restored erlotinib sensitivity. HCC827 or HCC827ER cells were incubated for 24 hours and for an additional 72 hours with the indicated concentrations of erlotinib with or without 2 μmol/L PHA-665,752, and cell growth was determined. B, activated RTKs identified by the Human Phospho-RTK Array Kit. Whole-cell extracts from HCC827, HCC827ER, and HCC827EPR exposed for 24 hours to the indicated drug(s) were incubated in the RTK arrays, and the phosphorylation status was determined by subsequent incubation with a horseradish peroxidase–conjugated phosphor-tyrosine detection antibody. Each RTK was spotted in duplicate and the pairs of dots in each corner are the positive controls. C, *MET* gene was amplified in HCC827ER cells but not in HCC827EPR cells. *MET* gene copy numbers were measured by quantitative real-time PCR. Normal genomic DNA was used as a standard sample. D, *MET* gene copy numbers in HCC827ER progenitor cells. Relative *MET* gene copy numbers (columns) were measured by real-time quantitative PCR in HCC827ER and their progenitor cells with incomplete erlotinib resistance. One division on the abscissa indicates 1 week after initiation of erlotinib exposure; left ordinate, the *MET* gene copy number; right ordinate, erlotinib concentration (μmol/L) at the each time. *MET* gene copy number data are presented as the mean ± SD of triplicate experiments. Hybridization of MET/CEP7 probe set with HCC827ER80 cells is also shown.

RTKs and both positive and negative controls. HCC827, HCC827ER, and HCC827EPR cells were cultured in 10-cm plates in RPMI1640 with 5% FBS until subconfluent. The media were changed to 5% FBS containing DMSO, 2 μmol/L erlotinib, and a combination of 2 μmol/L erlotinib/PHA-665,752, respectively, for 24 hours, and the cells were lysed by NP-40 lysis buffer according to the manufacturer's protocol. The arrays were blocked with blocking buffer and incubated with 450 μg of cell lysate overnight

at 4°C. The arrays were washed, incubated with a horseradish peroxidase–conjugated phospho-tyrosine detection antibody, treated with ECL solution, and exposed to film.

Preparation of DNA and RNA

Genomic DNA was extracted using a FastPure DNA Kit (Takara Bio) according to the manufacturer's protocol. Total RNA was prepared using a mirVana miRNA Isolation Kit (Qiagen), according to the manufacturer's protocol.

Random-primed, first-strand cDNA was synthesized from 10 µg of total RNA using Superscript II (Invitrogen) according to the manufacturer's instructions.

Mutation analysis

Mutation analysis of exons 18 to 21 of the *EGFR* gene, exons 1 to 2 of the *KRAS* gene, and exon 20 of the human epidermal growth factor receptor 2 (*HER2*) gene was done by direct sequencing after one-step reverse transcriptase-PCR (RT-PCR) using the Qiagen OneStep Reverse Transcription-PCR Kit (Qiagen) using total RNA as reported previously (17, 21). In the clinical autopsy samples, the *EGFR* mutation was analyzed using the Cycleave PCR technique and fragment analysis as described previously (22). Use of both methods enabled us to detect three types of G719 point mutations: exon 19 deletion mutations, exon 20 insertion mutations, and T790M, L858R, or L861Q point mutations.

Gene copy number analysis

The copy number of the *MET* gene relative to a *LINE-1* repetitive element was measured by quantitative real-time PCR using the SYBR Green Method (Power SYBR Green PCR Master Mix; Qiagen) with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) as described previously (11, 17). PCR was done in triplicate for each primer set. HCC827 incomplete erlotinib-resistant cells were analyzed for genomic status of *MET* by fluorescence *in situ* hybridization (FISH) using a D7S522 probe and chromosome 7 centromere probe (CEP7) purchased from Vysis and following the protocol described previously (11). The copy number of the *EGFR* gene relative to *LINE-1* was analyzed in the same way using primers for *EGFR* exon 21 that was described previously (2). *LINE-1* was used as the internal control because the copy number of *LINE-1* is reported to be similar in normal
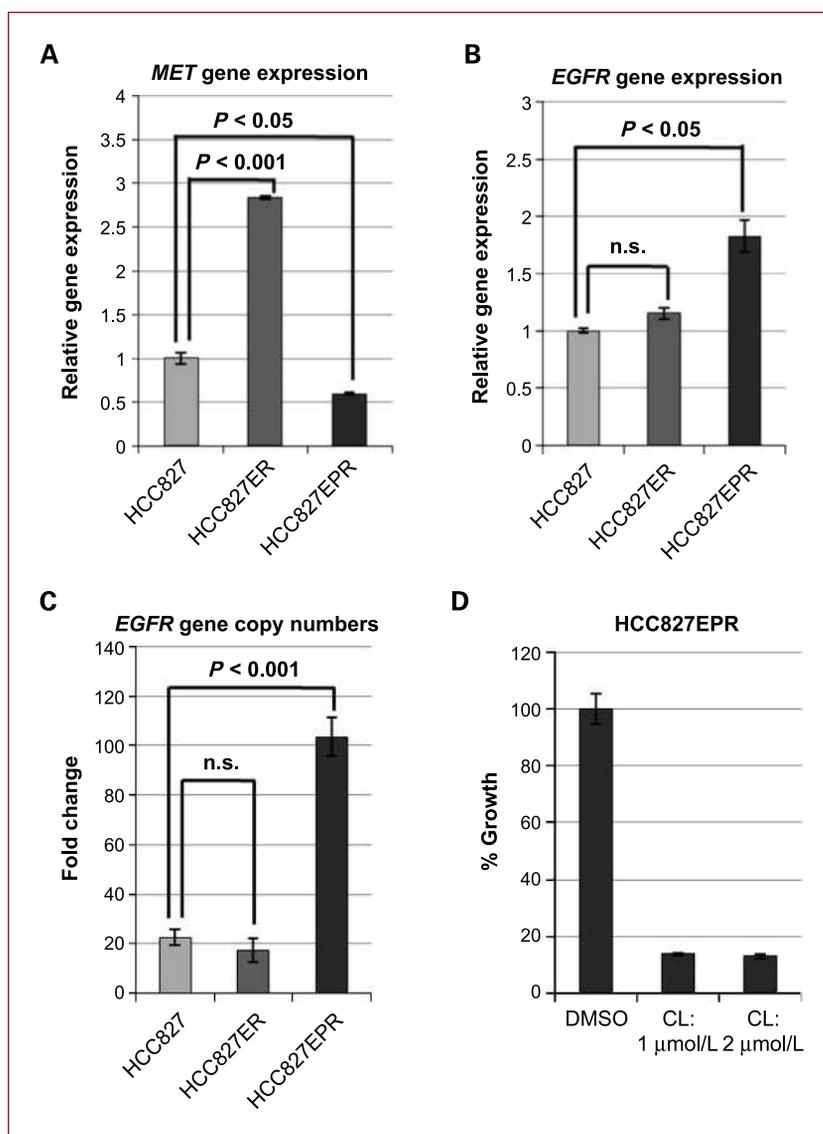
Fig. 2. Increased dependency on EGFR in HCC827EPR cells. A, *MET* gene expression increased in HCC827ER cells but decreased in HCC827EPR cells. B, *EGFR* gene expression increased in HCC827EPR cells. Quantitative real-time RT-PCR was done using validated TaqMan probes. The assays were done in triplicate, and the expression level of 18S rRNA was used as the internal control. n.s., not significant. C, the *EGFR* gene was amplified in HCC827EPR cells but not in HCC827ER cells. *EGFR* gene copy number was determined by quantitative real-time PCR. Normal genomic DNA was used as the standard sample. D, HCC827EPR cells were sensitive to CL-387,785. HCC827EPR cells were incubated for 24 hours and for an additional 72 hours with the indicated concentrations of CL-387,785 or DMSO, and cell growth was measured.

Fig. 3. HCC827EPR cells were resistant to erlotinib and/or PHA-665,752 and harbored the T790M mutation. A, HCC827 cells were sensitive to erlotinib (E) but not to PHA-665,752 (PHA). B, HCC827EPR cells were resistant to erlotinib and to the combination of erlotinib and PHA-665,752. HCC827 and HCC827EPR cells were incubated for 24 hours and for an additional 72 hours with indicated concentrations of drug(s), and cell growth was determined. C, HCC827EPR cells but not HCC827 cells harbored the T790M mutation. Antisense strands of sequencing chromatograms for *EGFR* mRNA are shown. Black arrow, C to T substitution at nucleotide 2,369 (G to A on the antisense strand), which results in the T790M mutation. D, Western blot analysis of EGFR and MET in HCC827 and HCC827EPR cells. Expression of β-actin was used as the control.
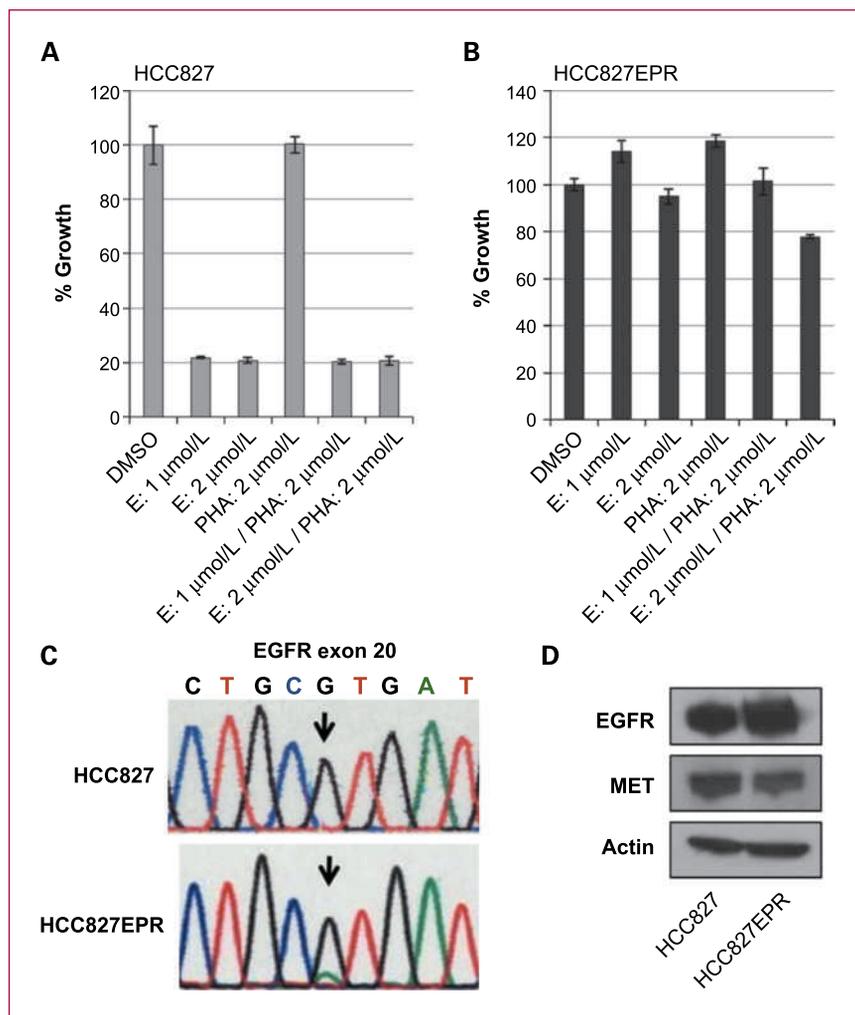
and cancerous cells (23). Normal genomic DNA was used as a standard sample.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was done using first-strand cDNA with TaqMan probes and TaqMan Universal PCR Master Mix (Applied Biosystems). TaqMan probes for *EGFR* and *MET* were purchased from Applied Biosystems, and the amplification was done using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Quantification was done in triplicate, and the expression levels of 18S rRNA were used as the internal control. The expression value for each resistant cell line was calculated relative to that of the HCC827 parent cells.

Antibodies and Western blot analysis

Anti-EGFR and anti-MET antibodies were purchased from Cell Signaling Technology. Anti-β-actin antibody was purchased from Sigma. Preparation of total cell lysates and immunoblotting were carried out as described

previously (24). Briefly, cells were cultured until subconfluent and lysed in SDS sample buffer and homogenized. Total cell lysate (30 ug) was subjected to SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Following blocking with 5% nonfat dry milk, the membranes were incubated with the primary antibody, washed with PBS, reacted with the secondary antibody, treated with ECL solution, and exposed to film.

Clinical autopsy samples

Autopsy samples from six lung adenocarcinoma patients harboring multiple gefitinib-refractory tumors were included. All patients responded to gefitinib monotherapy and experienced disease progression while on continuous treatment with gefitinib. These patients met the recently proposed criteria for acquired resistance to EGFR-TKIs (25). Approval from the institutional review board of Higashihiroshima Medical Center for the use of the tumor tissue specimens was obtained from the legal guardians of the patients. The patients' characteristics

Patient	Primary	IM	LNs	Liver	Ad-G	Oment	Pleura
1	NA	DEL/T	DEL/T	DEL/m	—	DEL/T/M	—
2	DEL/T/m	—	DEL/M	DEL/T/m	DEL/T/m	—	—
3	DEL/M	DEL/M*	—	DEL/M	DEL/M	DEL/M	—
4	NE	—	—	—	—	—	DEL/T
5	L858R/T	—	—	—	—	—	L858R/T
6	DEL	DEL/T*	DEL/T[†]	—	—	—	—

Table 2. *EGFR* mutational status and *MET* gene amplification in each primary or metastatic lesion

(*Continued on the following page*)

are summarized in Table 1. There were three men and three women. Four patients were nonsmokers and two were smokers. One patient had recurrent disease after surgery (patient 1), whereas five patients were nonsurgical cases (patients 2-6). The initial tumor responses to gefitinib were assessed according to the Response Evaluation Criteria in Solid Tumors (26).

Statistical analyses

Statistical analysis was carried out using StatView version 5.01 (SAS Institute). $P < 0.05$ was considered significant. All tests were two-sided.

Results

MET amplification causes resistance to erlotinib in HCC827ER cells

We first generated *in vitro* clones of HCC827 cells that were resistant to erlotinib (designated as HCC827ER) by growing cells in increasing concentrations of erlotinib to a final concentration of 2 μmol/L for up to 6 months, as described previously (11, 14, 27). HCC827ER was >2,000 times as resistant to erlotinib as the parental HCC827. Proliferation declined by <20% in HCC827ER cells incubated at erlotinib concentrations up to 10 μmol/L, whereas only 10% of parental HCC827 cells survived after exposure to 14 nmol/L erlotinib (Fig. 1A). The RTK array of HCC827ER cells showed activation of MET and ERBB3 in the presence of 2 μmol/L erlotinib (Fig. 1B), which was similar to that observed in a previous study (11). The *MET* gene copy number of HCC827ER cells assessed by quantitative real-time PCR was a 5.5-fold gain compared with normal DNA (Fig. 1C). We also used quantitative real-time PCR to confirm that the increased gene dose led to increased *MET* gene expression (Fig. 2A). On the other hand, no secondary mutations, including T790M, in exons 18 to 21 of the *EGFR* gene or a mutation in exons 1 to 2 of the *KRAS* gene were detected in HCC827ER cells. The contribution of *MET* amplification to erlotinib resistance was confirmed by the observation that a MET inhibitor, PHA-665,752, restored erlotinib sensitivity in HCC827ER cells (Fig. 1A).

Clinically relevant erlotinib resistance occurs at a 4-fold *MET* amplification

MET gene copy number was monitored in the developing HCC827ER cells. The *MET* gene copy number increased in proportion to erlotinib resistance (Fig. 1D). To distinguish small gains in *MET* gene copy number across all cells in the pool from an increase in the percentage of highly *MET*-amplified cells in the population, we did FISH of HCC827ER80 cells (HCC827 cells that acquired resistance to 80 nmol/L concentration of erlotinib) and identified that most of the cells harbored moderate *MET* gene copy number gains. When *MET* gene copy number had increased by >4-fold, the cells were able to proliferate in the presence of micromolar concentrations of a TKI, which is achievable clinically (e.g., the maximum drug concentration for a dose of 300 mg gefitinib and of 150 mg erlotinib was 0.85 μmol/L and 4.0 μmol/L, respectively; refs. 28, 29).

Generation of HCC827EPR cells

We then asked what would happen when we treated HCC827 cells with increasing concentrations of erlotinib in the presence of a MET inhibitor. We generated erlotinib-resistant HCC827 cells in the same way up to a final concentration of 2 μmol/L in the presence of 1 μmol/L PHA-665,752 for up to 9 months. We first confirmed the identity of the resultant resistant HCC827 cells to erlotinib plus PHA-665,752 (designated as HCC827EPR) by analyzing 10 loci of STR profiling and comparing them with the 9 loci of STR data of HCC827 provided by the American Type Culture Collection. The evaluation values of each pair of cell lines, HCC827 versus HCC827ER, HCC827 versus HCC827EPR, and HCC827ER versus HCC827EPR, were all 1.0, indicating complete identity of all analyzed STR loci.

HCC827 parental cells were resistant to the treatment with PHA-665,752 alone (Fig. 3A). The HCC827EPR cells were also resistant to 2 μmol/L erlotinib plus 2 μmol/L PHA-665,752 and could be maintained in medium with 2 μmol/L of both drugs. In contrast to the parental HCC827 cells, HCC827EPR cells were resistant to erlotinib alone, PHA-665,752 alone, and the combination of both drugs in the growth-inhibition assay (Fig. 3A and B). The RTK array did not detect activated RTKs except for EGFR

Table 2. *EGFR* mutational status and *MET* gene amplification in each primary or metastatic lesion (Cont'd)

Kidney	Chest	Ret-P	Skin	Thyroid	Bowel	Heart	Bone
	—	DEL/T	DEL/M	—	—	—	—
DEL/T/m	—	—	—	DEL/T	—	—	—
DEL/M	DEL/M	—	—	—	DEL/M	DEL/M	—
—	—	—	—	—	—	—	DEL/T
—	L858R/T	—	—	—	—	—	—
—	—	—	—	—	—	—	—

Abbreviations: IM, intrapulmonary metastasis; LN, mediastinal or hilar lymph nodes; Ad-G, adrenal gland; Oment, omentum; Chest, chest wall; Ret-P, retroperitoneum; Bowel, small intestine; NA, not available; DEL, exon 19 deletion mutation; T, T790M mutation; m, *MET* gene copy number gain (2- to 4-fold compared with normal); M, *MET* gene amplification (≥4-fold); NE, not evaluable because of lack of viable tumor cells.
*Two independent intrapulmonary metastatic lesions were analyzed and both harbored the same genetic alterations.
†Three independent lymph nodes, 10R, 7, and 4L, were analyzed and all harbored the same genetic alterations.

under the inhibition of 2 μmol/L erlotinib and 2 μmol/L PHA-665,752 (Fig. 1B). In addition, the *MET* gene copy number did not increase in HCC827EPR cells (Fig. 1C).

T790M mutation and increased *EGFR* gene copy number developed in HCC827EPR cells

We next sequenced exons 18 to 21 of the *EGFR* gene of HCC827EPR cells and identified the T790M mutation in addition to a homozygous 15 bp deletion in exon 19 (Fig. 3C). The existence of the T790M mutation in HCC827EPR cells but not in HCC827 parental cells was also confirmed by the Cycleave PCR technique (ref. 22; data not shown). The T790M mutation was detected in all three subclones obtained by single cell cloning of HCC827EPR cells. No secondary mutation in exons 1 to 2 of the *KRAS* gene or exon 20 of the *HER2* gene was detected (data not shown). Gene expression analysis revealed significantly increased *EGFR* gene expression (Fig. 2B) and decreased *MET* gene expression (Fig. 2A) in HCC827EPR cells compared with HCC827 cells, and these were consistent with Western blot analysis (Fig. 3D). We next analyzed *EGFR* gene copy number in HCC827 cells and in the resistant cells. HCC827 cells originally harbored 20 times the gene copy number compared with normal DNA (Fig. 2C), confirming the results of a previous study (30). HCC827EPR cells showed a further 5-fold *EGFR* gene amplification (>100-fold gene copy number) compared with the parental HCC827 cells, whereas the gene copy number was similar in HCC827ER cells and HCC827 cells (Fig. 2C). Addition of the irreversible EGFR-TKI CL-387,785 inhibited growth of HCC827EPR cells (Fig. 2D), showing that HCC827EPR cells were still dependent on signaling from the EGFR pathway.

### Analysis of multiple gefitinib-refractory tumors obtained from autopsy

Thirty-four gefitinib-refractory lesions produced after an initial good response to gefitinib were available from the six patients. One sample contained almost no viable tumor cells and the resultant 33 lesions were evaluated by molecular analysis (Table 2). *MET* amplification was defined as a copy number gain (CNG) of the *MET* gene of ≥4-fold, on the basis of the *in vitro* data (described above) and previous studies (11, 14). A CNG of the *MET* gene of <4-fold was defined as a moderate *MET* gene CNG.

Each patient harbored the identical activating mutations of the *EGFR* gene in their tumors (five patients with an exon 19 deletion and one with L858R; Table 2). As the mechanism of acquired resistance, 31 of 33 lesions had T790M and/or *MET* amplification. Nine lesions from patient 3 all had *MET* amplification without T790M. By contrast, all two lesions from patient 4, all three lesions from patient 5, and five of six lesions from patient 6 had T790M without *MET* amplification. Interestingly, the lesions from patients 1 and 2 exhibited T790M and/or *MET* amplification depending on the lesion sites. Ten of the 12 gefitinib-refractory lesions from patients 1 and 2 exhibited one of the two resistance mechanisms. The liver tumor from patient 1 had only a minor degree of *MET* CNG (3.2-fold), whereas the metastatic lesion from the omentum of patient 1 harbored both resistant mechanisms. Moderate *MET* CNGs were found in five lesions obtained only from these two patients (designated "m" in Table 2) but not in other patients, suggesting that the tumors in these two patients had the ability to develop *MET* amplification. We compared the relationship between the presence of T790M and *MET* gene copy number. The T790M mutation developed in 93% (14 of 15) of tumors without *MET* gene CNGs, in 80% (4 of 5) of tumors with moderate *MET* gene CNGs, and in only 8% (1 of 13) of tumors with *MET* amplification (Fig. 4A). This finding suggests that there was a reciprocal and complementary relationship between *MET* amplification and the T790M mutation.

Discussion

We found that HCC827 became resistant to erlotinib because of *MET* amplification, which is similar to the
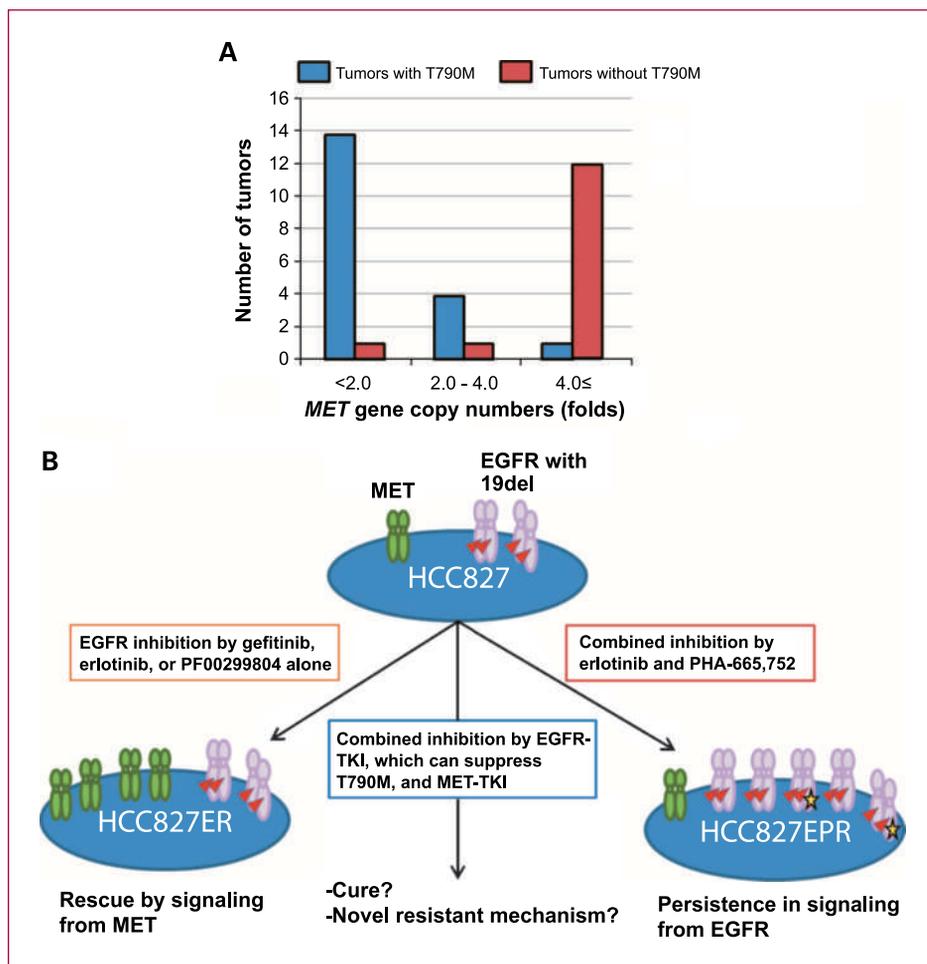
Fig. 4. Reciprocal relationship between *MET* amplification and T790M mutation. A, tumor numbers with or without T790M mutation by *MET* gene copy numbers in gefitinib-refractory tumors obtained from autopsy. B, schema of the difference of escape hatches of HCC827 cells depending on the selection pressure differences by molecular target drug(s). All amplified EGFR alleles harbor exon 19 deletion mutation in HCC827 cells, and a part of them in HCC827EPR cells acquired T790M mutation (yellow stars).

acquired resistance to gefitinib (11) or to an irreversible pan-ERBB kinase inhibitor, PF00299804 (14). We also found that the *MET* CNG increased in proportion to erlotinib resistance and that a ≥4-fold *MET* CNG compared with normal DNA was an apparent threshold for the development of clinically relevant TKI resistance. This observation is consistent with the observation that a moderate *MET* CNG (<4-fold) could coexist with T790M but that *MET* gene amplification (≥4-fold) and T790M were almost mutually exclusive in our autopsy analysis.

On the other hand, PC9 (exon 19 deletion) and H3255 (L858R) are known to develop resistance to EGFR-TKIs through T790M (18–20, 31). These phenomena are explained by the existence of minor clones with such alterations before EGFR-TKI treatment. Supporting this hypothesis further, Turke et al. found 0.06% to 0.14% of *MET*-amplified minor subclones in HCC827 but not in PC9 or H3255 cells (14). In addition, Inukai and Maheswaran showed that patients with *EGFR* mutations had shorter progression-free survival when the tumor had a very small amount of T790M before the EGFR-TKI therapy (13, 32). Thus, it seems that these cell lines are destined to use either mechanism to overcome EGFR-TKIs. It is inter-

esting that HCC827 cells developed the T790M mutation when exposed to increasing concentrations of erlotinib under the inhibition of MET signaling (Fig. 4B), although it took about 1.5 times longer compared with erlotinib alone. The origin of the T790M allele in HCC827 cells is not clear, although this is the case with other *in vitro* gefitinib-resistant models used to develop the T790M mutation (PC9 and H3255 cell lines; refs. 18, 31). More sensitive methods might be able to detect the presence of minor clones with the T790M mutation in these cell lines before the start of EGFR-TKI treatment.

No studies have investigated the mechanisms responsible for the acquired resistance to EGFR-TKI therapy in multiple sites of metastases obtained from autopsy. The autopsy samples allowed us to see the ultimate pictures of resistance and to examine multiple organ sites simultaneously. Thirty-one of 33 lesions harbored the T790M mutation and/or *MET* amplification. We also found an inverse relationship between the presence of T790M and *MET* gene copy number, suggesting a complementary role of the two mechanisms in the acquisition of resistance. This is consistent with a previous report; one of the patients with acquired resistance to EGFR-TKI harbored

two tumors, one with *MET* amplification only and the other with moderate *MET* CNG and T790M (12). The incidence of the T790M mutation and *MET* amplification as mechanisms responsible for the acquired resistance to EGFR-TKIs was reported to be ~50% and ~20%, respectively (33). However, our present results suggest that the incidence of these two mechanisms is higher in the later phase and that overcoming these two mechanisms would be the key to improving patient outcomes further.

The factors that determine which mechanism will be used by tumor cells for overcoming EGFR-TKIs are not clear. One may speculate that the balance between positive and negative regulators of the MET pathway in the microenvironment of the tumor cells determines the mechanisms of resistance. Hepatocyte growth factor, a ligand for MET, has been shown recently to induce transient and reversible resistance to EGFR-TKIs (34, 35) and to facilitate *in vitro MET* amplification in the development of stable acquired resistance to EGFR-TKIs (14). On the other hand, there are several negative regulators of the HGF-MET axis. One example is to increase MET degradation by Cbl-mediated ubiquitination or another mechanism. Overexpression of LRIG1, a transmembrane leucine-rich repeat and immunoglobulin-like domain-containing protein, destabilizes MET and impairs the ability to respond to hepatocyte growth factor (36). Another possibility is a negative regulator of MET-induced cell behavior, such as Abl tyrosine kinase, which functions as a negative regulator of MET-induced cell motility via phosphorylation of the adapter protein CrkII (37).

In conclusion, we observed a reciprocal, complementary relationship between *MET* amplification and the *EGFR* T790M mutation in both an *in vitro* erlotinib-resistant model (illustrated in Fig. 4B) and in our analysis of gefitinib-refractory tumors obtained from autopsy samples. Molecular target therapy prolongs the overall survival in lung cancer patients with an *EGFR* mutation (38), and the development of the concurrent inhibition therapy might be essential for the further improvement.

Disclosure of Potential Conflicts of Interest

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Reciprocal and Complementary Role of *MET* Amplification and *EGFR* T790M Mutation in Acquired Resistance to Kinase Inhibitors in Lung Cancer

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