TABLE 1. Clinicopathologic Data for 60 Patients with Lung Cancer

	ERBB4 Expression (%)		
Characteristic	**High** (n = 16)	**Low** (n = 44)	p
Age (yr)			
>67	7 (43.8)	23 (52.3)	0.559
≤67	9 (56.2)	21 (47.7)	
Sex			
Male	8 (50.0)	31 (70.5)	0.142
Female	8 (50.0)	13 (29.5)	
Pathologic stage			
I, II	13 (81.3)	25 (56.8)	0.130
III, IV	3 (18.7)	19 (43.2)	
Smoking			
Never	6 (37.5)	12 (27.3)	0.445
Current or former	10 (62.5)	32 (72.7)	
Tumor size (cm)			
>3	6 (43.8)	28 (63.6)	0.071
≤3	10 (56.2)	16 (36.4)	
Histology			
Adenocarcinoma	11 (68.9)	32 (72.7)	0.763
Nonadenocarcinoma	5 (31.1)	12 (27.3)	

exclusive, in lung cancers.[6,7] The aims of this study were to identify mutations of the *ERBB4* extracellular and kinase domains in lung cancers and to confirm their mutual exclusivity with mutations in the above genes. Recently, activating point mutations at the *EGFR* extracellular domains were found in 12.9% of glioblastoma.[8] Mutations of the *ERBB4* extracellular domain also have been reported in NSCLC.[4] We considered that there was need for analysis of mutations of the *ERBB4* extracellular domain in East Asian patients with lung cancer. In this mutation analysis, only a Q793Q polymorphism was detected in the *ERBB4* kinase domain. The *ERBB4* polymorphism was analogous to the Q787Q polymorphism of *EGFR*. In East Asia, two mutation analyses of *ERBB4* have been reported.[2,3] Considering those together with this study, 5 of 394 patients with lung cancer (1.3%) harbored *ERBB4* mutations, all of which were present in the *ERBB4* kinase domain.

Gene amplification and expression of the *ERBB* family have been reported in lung cancer. *EGFR* gene amplifications are frequently observed in squamous cell carcinoma with poor prognosis.[9,10] Synchronous protein overexpression of *EGFR* and *ERBB2* significantly predicted increased recurrence risk and decreased survival.[11] In *ERBB4* expression assays of lung cancers, the expression levels are remarkably lower than those of other members of the *ERBB* family, and there is no relationship between *ERBB4* expression and metastasis.[12] Conversely, Starr et al.[13] reported that the proliferation of the *ERBB4*-transfected human adenocarcinoma cell line H1299 was 2-fold higher than that of the parental cells, and in mice injected with the *ERBB4*-transfected cells, the tumors were larger,[13] suggesting that *ERBB4* is associated with metastasis and inferior survival.[13]

As indicated earlier, opinions vary concerning the relationship of *ERBB4* expression with metastasis and prognosis in lung cancers. In this study, the patients with regional lymph node or distant metastasis at diagnosis made up 40% (6 of 16 patients) of the *ERBB4* high-expression group and 44% (20 of 44 patients) of the *ERBB4* low-expression group (p = 0.582). The DFS and OS were not significantly different between the high- and low-expression groups. There was no significant relationship between *ERBB4* expression and metastasis or prognosis, and we did not detect a significant relationship between *ERBB4* expression and any clinicopathologic factors.

In conclusion, mutation of the *ERBB4* kinase domain and CR1 domain were not detected. High *ERBB4* expression was infrequent in Japanese patients with lung cancer, and the clinical significance of *ERBB4* was negligible.

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Biological and clinical significance of *KRAS* mutations in lung cancer: an oncogenic driver that contrasts with *EGFR* mutation

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Abstract *KRAS* and epidermal growth factor receptor (*EGFR*) are the two most frequently mutated proto-oncogenes in adenocarcinoma of the lung. The occurrence of these two oncogenic mutations is mutually exclusive, and they exhibit many contrasting characteristics such as clinical background, pathological features of patients harboring each mutation, and prognostic or predictive implications. Lung cancers harboring the *EGFR* mutations are remarkably sensitive to EGFR tyrosine kinase inhibitors such as gefitinib or erlotinib. This discovery has dramatically changed the clinical treatment of lung cancer in that it almost doubled the duration of survival for lung cancer patients with an *EGFR* mutation. In this review, we describe the features of *KRAS* mutations in lung cancer and contrast these with the features of *EGFR* mutations. Recent strategies to combat lung cancer harboring *KRAS* mutations are also reviewed.

Keywords Lung cancer · Oncogene addiction · Targeted therapy · Personalized medicine

1 Discovery of RAS in human cancers

In the search for the molecular basis of human cancer, an activity that transforms the mouse NIH 3T3 cell line *in vitro* was detected in DNA from human cancer cell lines [1–3]. Later, it turned out that this activity is present in the human homologues of oncogenes of retroviruses found earlier [4–6]. These genes were named *HRAS* or *KRAS* according to the names of these viruses, Harvey- or Kirsten-rat sarcoma viruses. What was most intriguing about these early studies was that the difference between the *RAS* gene present in normal tissue and that in cancer tissue was usually a single missense point mutation at codon 12 [7–9] and less frequently at codons 13 or 61 [10]. The third member of the RAS family gene, *NRAS*, was identified 1 year later in a human neuroblastoma cell line, although its viral homologue was not identified [11, 12]. A certain type of cancer tends to involve a specific type of *RAS* gene; e.g., most *RAS* mutations in lung, colorectal or pancreatic cancer occur in the *KRAS* gene, whereas most *RAS* mutations in bladder cancer occur in the *HRAS* gene [10].

2 Biology of RAS

The *RAS* gene encodes for a small protein with a molecular weight of 21,000 Da with guanosine triphosphatase (GTPase) activity. Because of this activity, RAS protein toggles the guanosine diphosphate (GDP)-bound inactive form to and from the GTP-bound active form. Guanine nucleotide-exchange factors, including SOS1, are recruited to the plasma membrane, where RAS is located, after growth factor binding to cell-surface receptors and stimulate guanine nucleotide dissociation from RAS, which results in increased levels of RAS-GTP. By contrast, RAS is negatively regulated by the catalytic reaction of RAS GTPase-activating proteins (RAS-GAPs), which enhances RAS GTPase activity. The tumor suppressor gene of neurofibromatosis I, *NF1*, also encodes for protein with

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RAS-GAP activity [13]. Oncogenic point mutations impair the intrinsic GTPase activity of RAS and confer resistance to GAPs, thereby causing RAS to accumulate in its active GTP-bound state, which sustains the activation of RAS signaling [14]. GTP-bound active RAS binds to more than 20 effector proteins and stimulates downstream signaling cascades (Fig. 1a, b).

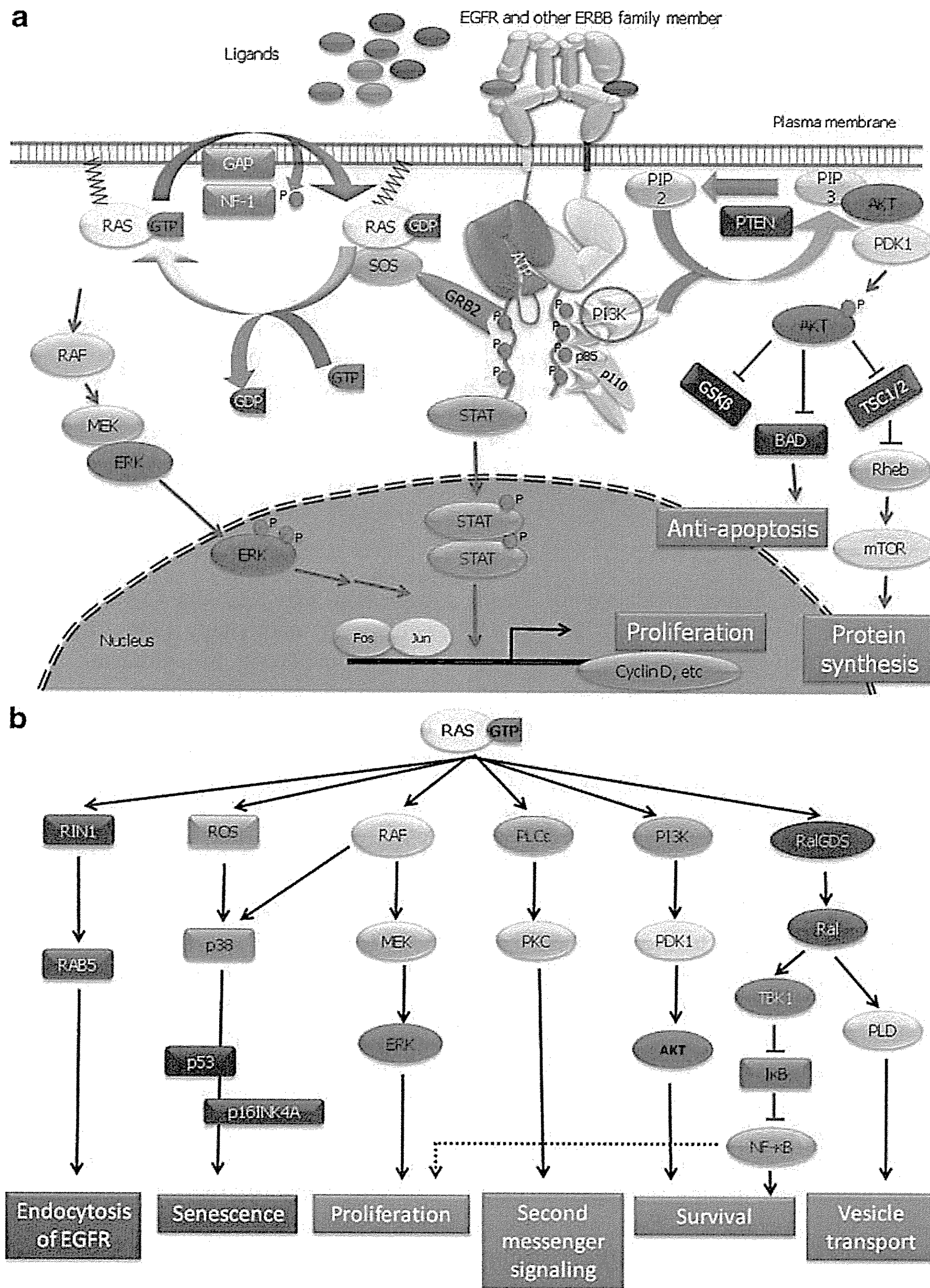
To acquire their biological and transforming activities, RAS proteins must be bound to the inner surface of the plasma membrane by appropriate posttranslational modification [15–17]. Briefly, farnesyltransferase (FTase) catalyzes the reaction that adds a farnesyl isoprenoid lipid to the C-terminal CAAX (where C is cysteine, A is an aliphatic amino acid, and X is any amino acid) motif of RAS, which induces the association between RAS and the intracellular membrane via the farnesyl group. After several other processing steps, one or two palmitic acids are finally added to HRAS, NRAS, or KRAS-4A by palmitoyltransferase just upstream of the CAAX motif [18]. By contrast, the C-terminal electropositive Lys residues in KRAS-4B are sufficient to anchor it in the membrane without being modified by palmitic acid. KRAS-4A and -4B are two splice variants of KRAS protein that are produced by alternative splicing at the C-terminal region of KRAS. The transforming effect of mutant KRAS during lung carcinogenesis *in vivo* is thought to be mediated primarily through Kras-4A because transgenic mice that express only Kras-4B are highly resistant to urethane- or *N*-methyl-*N*-nitrosourea-induced lung tumor formation [19, 20].

Although mutated *RAS* transforms immortalized cell lines such as NIH3T3, it cannot transform primary rat embryo fibroblasts. However, in cooperation with so-called "immortalizing genes", such as *MYC*, *simian virus 40 large T*, or *adenovirus E1A* oncogene, mutated *RAS* can transform cells in primary culture [21–23]. This observation is relevant to the finding that oncogenic RAS has been shown to cause senescence or cell-cycle arrest in primary cells through activation of the TP53 and/or p16INK4A-RB tumor-suppressor pathways [24]. It is thought that p16 is the key factor in determining whether cells become senescent or are transformed in response to RAS activation [25]. The RAS-induced RAF-mitogen-activated protein kinase (MAPK) pathway might also feed into the TP53 pathway by activating the p38 MAPK pathway. RAS activation may also cause p38 activation by increasing the reactive oxygen species level [26] (Fig. 1b). However, *in vivo* expression of oncogenic Kras at levels comparable to those of its endogenous counterparts causes cellular transformation [27], developing multiple lung adenomas (pre-malignant tumors) and a few lung adenocarcinomas. One solution of this discrepancy is that moderate activation of Ras, such as that mimicked in the endogenous Kras mouse models, does not cause an acute p16 response, contrasting with high level Ras ectopic expression in cultured cells, allowing Ras-induced transformation [25]. On the other hand, Collado et al. reported the existence of senescence cells in premalignant tumors but not in malignant ones of Kras transgenic mouse models, suggesting that substantial number of cells in pre-malignant tumors undergo oncogene-induced senescence, but that cells in malignant tumors are unable to do this owing to the loss of oncogene-induced senescence effetors such as p16INK4A or TP53 [28].

Fig. 1 EGFR- and KRAS-signaling pathways. **a** Binding of specific ▶ ligands to the extracellular domain of the EGFR leads to formation of a dimer with another EGFR or another ERBB family member. Dimerization consequently stimulates intrinsic tyrosine kinase activity of the receptors and triggers autophosphorylation of specific tyrosine residues within the cytoplasmic regulatory domain. These phosphorylated tyrosines serve as specific binding sites for several adaptor proteins such as GRB2 and p85 (catalytic subunit of PI3K). Several signal transducers then bind to these adaptors to initiate multiple signaling pathways. The GRB2/SOS complex, brought into the vicinity of the plasma membrane, catalyzes guanine nucleotide exchange of membrane-binding RAS, and GTP-bound RAS activates its downstream signaling cascades presented by the RAS–RAF–MAPK/ERK kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway. Activated ERK is recruited into the nucleus and, finally, a Fos–Jun dimer that makes up the AP1 transcription factor is activated to simulate gene transcription-related cell proliferation such as expression of Cyclin D. The other important downstream pathways are the PI3K–AKT, STAT3, and STAT5 pathways, which mainly confer antiapoptotic effects. **b** RAS-GTP binds to more than 20 effector proteins and stimulates downstream signaling cascades. In addition to the RAF–MEK–ERK pathway, the PI3K–AKT pathway is activated by RAS through the direct interaction of the PI3K catalytic subunit with RAS. Then, 3-phosphoinositide-dependent protein kinase-1 (PDK1) and AKT are activated to transmit antiapoptotic signals. Other effectors include Ral guanine nucleotide-dissociation stimulator (RALGDS) and phospholipase Cε (for review see [75, 96–98]). By contrast, the RAS downstream pathways also include effectors with reported tumor-suppressor activities. We have mentioned the senescence and cell-cycle arrest effects of RAS in the text. Another RAS effector protein RIN1 is reported to trigger endocytosis of growth factor receptors, such as the EGFR, thereby inhibiting RAS signaling (**b**). This figure is based on modifications of figures from Schubbert et al. [99]., Downward et al. [100], and Karnoub et al. [25]

3 *RAS* gene activation in lung cancer

Frequent somatic mutation of the *KRAS* gene in lung cancer was first identified in 1984 [29]. Mutation of the *RAS* gene usually occurs in adenocarcinoma [30], rarely in squamous cell carcinoma, and never in small cell lung cancer [31]. *KRAS* mutation in lung cancer usually occurs at codon 12, occasionally at codon 13 and rarely at codon 61 [30]. Rare instances of *HRAS* or *NRAS* mutations have been reported in lung cancers [31]. *KRAS* mutations occur predominantly in Caucasian patients rather than in East Asians; the incidence of *KRAS* mutation is ~30% in Caucasian patients



and ~10% in East Asian patients with adenocarcinoma [32, 33] (Fig. 2).

Although adenocarcinoma of the lung is thought to be associated less with smoking than is squamous or small cell carcinoma of the lung, a possible association between *KRAS* mutations and smoking exposure was reported in 1991 [34]. We categorized Japanese patients according to the amount of smoke exposure (0, <20, 20–50, and >50 pack-years (PY)) and found that the incidence of *KRAS* mutations increased as smoke exposure increased (6%, 5%, 18%, and 18%, respectively) [35]. This trend was similar to that for *TP53* mutations (29%, 40%, 65%, and 66%, respectively) [35]. Recent large-scale analysis of somatic mutations in lung adenocarcinoma also confirmed the association between *KRAS* mutations and smoking status [36]. By contrast, three studies restricted to Caucasian populations failed to show a significant association between smoking status and *KRAS* mutation,

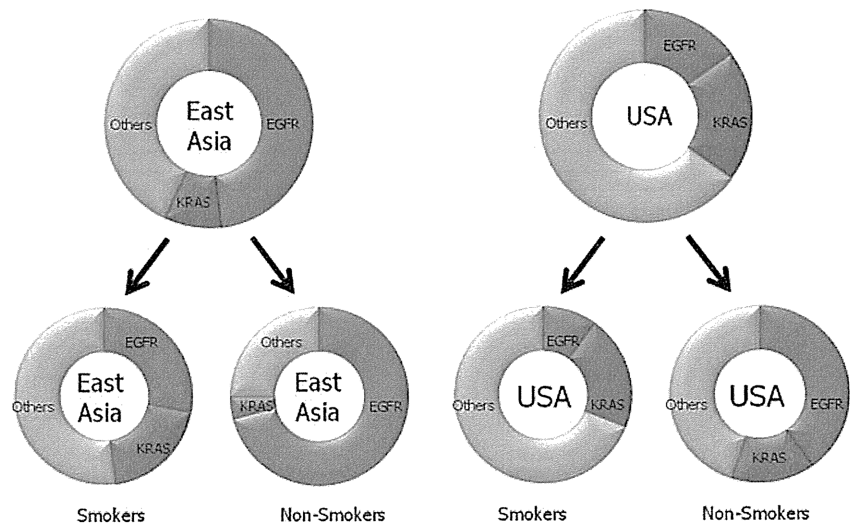


Fig. 2 Incidence of *EGFR* or *KRAS* mutations according to ethnicity and smoking status. Data on ethnicity are from Shigematsu et al. [33]. Data on smoking in Asia are from Kosaka et al. [35] and Tam et al. [101], and those on smoking in the USA are based on Rudin et al. [102]

although a trend toward a higher incidence of *KRAS* mutations was observed in smokers [37–39]. The *KRAS* mutation at codon 12 in lung cancer is characterized by the frequent G-to-T transversions, in contrast to the frequent G-to-A transitions found in colorectal cancer [40]. These transversion mutations of the *KRAS* gene are associated strongly with smoking status even when restricted to Caucasian populations, whereas the transition mutations occur in both former or current and never smokers [39].

Mutant allele specific imbalance (MASI) is a genetic aberration observed often in tumors harboring mutations in proto-oncogenes. The *KRAS* chromosomal locus at 12p12.1 or the *epidermal growth factor receptor* (*EGFR*) locus at 7p11.2 have both been identified as frequent regions of gene amplification in lung cancer by two independent genome-wide studies [41, 42]. Soh et al. reported recently that MASI is frequently (58%) observed in lung cancer with *KRAS* mutation [43]. Of interest, MASI in *KRAS* occurs mainly because of uniparental disomy resulting from the complete loss of the wild-type allele without copy number gain (CNG), whereas MASI in *EGFR* occurs mainly because of CNG [43]. These differences in MASI mechanisms may be relevant to the observation that the wild-type RAS acts like a tumor suppressor in some models [44–46]. *KRAS* mutations or MASI are significantly associated with increased GTP-bound active RAS protein [43].

In terms of histological types, *KRAS* mutations are associated more with mucinous bronchioloalveolar cell carcinoma (BAC) or lung cancer with goblet cell morphology than with non-mucinous BAC [47–50]. For example, Marchetti et al. found *KRAS* mutations in ten of ten mucinous BACs, but in only 34 of 98 non-mucinous adenocarcinomas [49]. Yatabe et al. demonstrated further that a subset of lung adenocarcinomas frequently shows expression of CK20 and CDX2, *KRAS* mutations, and/or goblet cell morphology [50]. These phenotypes are observed commonly in colorectal, pancreatobiliary, and ovarian mucinous carcinomas, suggesting that adenocarcinomas with these features represent one prototype that is independent of the organ of origin [50].

4 Discovery of the *EGFR* mutation in lung cancer

In 2004, activating mutations of the *EGFR* gene were found in a subset of non-small cell lung cancers (NSCLCs), and tumors harboring *EGFR* mutations were shown to be highly sensitive to EGFR tyrosine kinase inhibitors (TKIs) [51–53]. *EGFR* mutations are present mainly in the first four exons of the tyrosine kinase domain of the *EGFR* gene. About 90% of *EGFR* mutations are either small deletions encompassing five amino acids from codons 746 through 750 (ELREA) or missense mutations resulting in leucine-to-arginine substitution at codon 858 (L858R) [54]. There are >20 variant types of deletion: larger deletion, deletion plus point mutation, deletion plus insertion, etc. About 3% of *EGFR* mutations occur at codon 719 and cause substitution of glycine to cysteine, alanine, or serine (G719X) [54]. In addition, about 3% are in-frame insertion mutations in exon 20. It is unusual for more than one type of the common forms of mutation to be present in an individual carcinoma, although there are many examples of rare point mutations, some of which occur with L858R mutation.

EGFR mutations are found predominantly in female, non-smoking patients of East Asian origin with adenocarcinoma. Data on 2,880 patients compiled from the literature show that the presence of *EGFR* mutations is highly dependent on ethnicity (East Asians, 32% compared with

Caucasians, 7%), sex (male, 10% compared with female, 38%), smoking history (never smoked, 47% compared with ever smoked, 7%), and histological type (adenocarcinoma, 30% compared with other types of lung cancer, 2%) [54]. A recent report also showed that African Americans are significantly less likely to harbor *EGFR* mutations (2%), whereas the frequency of *KRAS* mutations (23%) does not differ from that in Caucasians [55].

Although *EGFR* and *KRAS* are both proto-oncogenes associated with lung adenocarcinoma, lung cancer harboring *EGFR* mutations and those harboring *KRAS* mutations differ markedly (Table 1 and Fig. 2). Our group and others have reported that the occurrence of *EGFR* and *KRAS* mutations is strictly mutually exclusive [33, 35]. This finding may be relevant to the fact that the KRAS–MAPK pathway is an important downstream signaling pathway of EGFR.

It is of particular interest that *EGFR* mutations are the first molecular aberrations found predominantly in lung cancers in never-smoking patients than those in smoking patients. In contrast to *KRAS* mutations, *EGFR* mutations are frequently inversely associated with cumulative smoking dosage. Our analysis, mentioned earlier, showed that the incidence of *EGFR* mutations decreases as smoking dose increases (68%, 55%, 27%, and 22%, for 0, <20, 20–50, and >50 PY, respectively) [35]. These findings should not be construed to mean that smoking has a preventive effect on *EGFR* mutations; rather, they suggest that *EGFR* mutations are caused by carcinogen(s) other than those contained in tobacco smoke and that the apparent negative correlation with smoking dose results from diluting the number of tumors with *EGFR* mutations with an increasing number of tumors without these mutations as smoking dose increases. This idea is supported by our case–control study [56], in which we enrolled 152 NSCLC patients harboring *EGFR* mutations, 283 NSCLC patients without *EGFR* mutations, and 2,175 age- and sex-matched controls. When cumulative smoking exposure was classified into three groups, the cumulative amount showed a linear increased risk for lung cancer only in patients without *EGFR* mutations (trend $P<0.001$). The odds ratios (OR) for 1–40 PY and >40 PY were 2.72 (1.79–4.14; $P<0.001$) and 10.0 (6.33–15.8; $P<0.001$), respectively [56]. By contrast, the risk did not increase in patients harboring *EGFR*

Table 1 Summary of recent studies comparing gefitinib with chemotherapy in patients according to *EGFR* mutational status

Study	Design	Line	*EGFR* mutated				*EGFR* wild-type			
			PFS		OS		PFS		OS	
			G	CTx	G	CTx	G	CTx	G	CTx
Takano (64)	Retrospective	All	–	–	27.2	13.6	–	–	13.2	10.4
	Japanese, observational, no crossover									
I-CAMP[a] (92)	P II	1st	10.7	6.0	27.7	25.7	–	–	–	–
	Nonrandomized, pooled analysis, crossover CTx→G 100%, G→CTx?									
INTEREST[b] (93)	P III	2nd	7.0	4.1	14.2	16.6	1.7	2.6	6.4	6.0
	Global, gefitinib vs. docetaxel, crossover CTx→G 37%, G→CTx 46%									
V15-32[b] (94)	P III	2nd	~8	~9	–	–	~3	~3	–	–
	Japanese, gefitinib vs. docetaxel, crossover CTx→G 53%, G→CTx 60%									
IPASS (66)	P III	1st	9.5	6.3	~20	~20	~1.5	~5.5	~13	~13
	Asians, gefitinib vs. carboplatin-paclitaxel, crossover CTx→G 39%, G→CTx 49%									
NEJ002 (67)	P III	1st	10.4	5.5	28.0	23.6				
	Japanese, gefitinib vs. carboplatin-paclitaxel, patients selected by activating mutation of *EGFR*, crossover CTx→G 94%, G→CTx 73%									
WJTOG3405 (68)	P III	1st	9.2	6.3	N/A	N/A				
	Japanese, gefitinib vs. cisplatin-docetaxel, activating mutation of *EGFR*									

G Gefitinib, *CTx* chemotherapy, *P II* phase II, *P III* phase III.

[a] I-CAMP is the pooled analyses from seven Japanese phase II studies that prospectively evaluated the efficacy of gefitinib for patients with *EGFR* mutations. Gefitinib was administered to 87 patients as the first-line therapy and to 61 patients as the second-line or later therapy [92].

[b] The INTEREST and V15-32 studies are comparative studies of gefitinib versus docetaxel as second-line or later treatment for advanced or recurrent non-small cell lung cancer. The non-inferiority of gefitinib was not demonstrated in the V15-32 study conducted in Japan [93], but was demonstrated in the INTEREST study conducted overseas [94]. The subset analysis of the INTEREST study showed that *EGFR* mutation-positive patients had longer PFS with gefitinib than with chemotherapy, but no difference in OS was observed [94]. By contrast, in the V15-32 study, patients with *EGFR* mutations had a favorable prognosis overall, but no differences were demonstrated between docetaxel and gefitinib [93]. Interpretation of these analyses is difficult because only a portion of the patients were analyzed retrospectively.

mutations (OR, 0.68 (0.42–1.12, P=0.134) for 1–40 PY; OR, 0.79 (0.42–1.46, P=0.45) for > 40 PY) [56].

5 Clinical significance of *KRAS* and *EGFR* mutations in lung cancer

5.1 Prognostic implications

In 1990, Slebos et al. reported that the presence of a *KRAS* mutation is a significant poor prognostic marker in patients with adenocarcinoma of the lung who underwent potential curative surgery [57]. Mascaux et al. performed a meta-analysis of 53 published studies that assessed the prognostic value of mutations in the *KRAS* gene. They identified *KRAS* mutations as a negative prognostic factor with a hazard ratio for death of 1.50 (95% confidence interval (CI), 1.26–1.80) in lung adenocarcinoma [58]. In our recent analysis, *KRAS* mutations were associated with poor prognosis, whereas *EGFR* mutations were associated with good prognosis by univariate analysis. However, multivariate analysis revealed that smoking status and stage were significant predictors, whereas none of the mutations, *EGFR*, *KRAS*, or *TP53*, was an independent prognostic factor [59].

On the other hand, Soh et al. found that gene dosage is associated with prognostic impact [43]. In the paper mentioned earlier, *KRAS* mutations (P=0.2) or CNG (P=0.1) alone showed a non-significant trend for poor prognosis. However, of 237 lung adenocarcinoma patients, six patients who harbored both a *KRAS* mutation and CNG had significantly shortened survival (P=0.04) [43].

5.2 Predictive implications

Small molecules with an anilino-quinazoline structure that specifically inhibit the tyrosine kinase activity of the EGFR, such as gefitinib and erlotinib, are the first molecular targeted drugs for lung cancer. It became evident that non-smoking Asian women with adenocarcinoma are highly likely to respond to EGFR-TKIs during early clinical development of the drugs [60, 61]. In 2004, somatic mutations of the *EGFR* gene were found in a subset of patients with lung cancer predominantly with the above-mentioned clinical backgrounds; it was also found that tumors with *EGFR* mutations are highly sensitive to EGFR-TKIs [51–53], providing a molecular basis for the earlier clinical observations. Activating mutations of the *EGFR* gene increase and sustain the phosphorylation of EGFR and other human EGF receptor (HER) family proteins without ligand stimulation [62]. Mutant EGFR selectively activates the AKT- and signal-transducer and activator of transcription protein (STAT)-signaling pathways, which promote cell survival [62]. These cells become highly dependent on these aberrant signals, and when the oncogenic activated protein is inhibited by short hairpin RNA (shRNA) or EGFR-TKIs, they undergo extensive apoptosis [62].

About 70–80% of patients harboring *EGFR* mutations respond to EGFR-TKIs, whereas 10% of patients without *EGFR* mutations do so [54]. Patients with *EGFR* mutations have a significantly longer survival than those without *EGFR* mutations when treated with EGFR-TKIs [63, 64]. However, the clinical significance of *EGFR* mutation as a predictor of survival in patients treated with EGFR-TKIs was initially controversial. Some investigators claimed that *EGFR* mutations are not predictive of better survival upon EGFR-TKI treatment but are prognostic only (i.e. *EGFR* mutation defines a subset of patients with good prognosis irrespective of the treatment) [65]. Instead, it was thought that the *EGFR* gene copy number is more important [65]. This controversy continued until the molecular subset analysis of the IPASS study was reported [66].

IPASS is a phase III trial that compared gefitinib with standard chemotherapy as a first-line treatment for Asian patients with lung adenocarcinoma and with no smoking history or only light use [66]. The progression-free survival (PFS) was significantly better in patients in the gefitinib group, although the PFS curves crossed at six months (chemotherapy was better initially but later the gefitinib therapy was better), suggesting the presence of two or more qualitatively different subgroups. Molecular subset analysis showed that the benefit was limited to the patients with *EGFR* mutations and that gefitinib therapy was even detrimental in those without mutations [66]. Two recent Japanese phase III trials (NEJ002 and WJTOG3405) that compared gefitinib with platinum-doublet chemotherapy for patients selected according to the presence of *EGFR* mutations confirmed the predictive impact of these mutations in patients treated with EGFR-TKIs[67, 68].

However, the overall survival in the gefitinib group and in the chemotherapy group did not differ significantly in the IPASS trial or in NEJ002, although the final analyses have not been reported yet. This lack of effect is probably related to a high rate of crossover of the treatment; i.e. a considerable fraction of patients in the chemotherapy arm had been given gefitinib after disease progression and vice versa. Takano et al. classified lung cancer patients into those treated from 1999 to 2001, which was before approval of gefitinib in Japan, and those treated from 2002 to 2004. Gefitinib was administered to only 15% of the former group, but to 91% of the latter group. When the prognosis was examined according to the presence or absence of *EGFR* mutations, the median survival time was 27.2 months in the 2002–2004 cohort with *EGFR* mutations but only about 1 year in the other three groups [64] . Although this is not a randomized trial, it indicates

clearly that gefitinib treatment doubled the survival of lung cancer patients harboring *EGFR* mutations. Table 1 summarizes the recent clinical studies that compared gefitinib with standard chemotherapy in patients with lung cancer according to *EGFR* mutational status.

In addition, for patients treated with carboplatin/paclitaxel chemotherapy in the IPASS trial, the response rate was higher (47.3% vs. 23.5%) and PFS was longer (6.3 vs. 5.5 months) in patients with *EGFR* mutations than in those without *EGFR* mutations. This result suggests that *EGFR* mutations may also be predictive for the response to cytotoxic chemotherapy [66].

By contrast, lung cancers harboring *KRAS* mutations are resistant to EGFR-TKIs. The response rate for patients with *KRAS* mutations to EGFR-TKIs is virtually zero [69]. A recent meta-analysis of data on 165 patients with *KRAS* mutations from 17 studies confirmed that the presence of *KRAS* mutations is significantly associated with an absence of response to TKIs [70]. In the analysis, the pooled estimate of the specificity (number of KRAS wild-type patients / number of patients with objective response by RECIST) was 0.94 (95% Cl, 0.89–0.97). However, because an *EGFR* mutation is a strong predictor of the response to EGFR-TKI therapy and because *EGFR* mutations and *KRAS* mutations have a mutual exclusionary relationship, it is not clear whether the response to EGFR-TKIs differs between tumors harboring *KRAS* mutations and those harboring neither *KRAS* nor *EGFR* mutations. In an analysis of 223 patients from five studies who were treated with EGFR-TKIs as a first-line therapy, Jackman et al. found no impact of *KRAS* mutations on the overall survival in patients without *EGFR* mutations [71]. The presence of a *KRAS* mutation in a given tumor may merely indicate that the tumor does not have an *EGFR* mutation. In addition, the role of *KRAS* mutations as a negative predictor of the response of lung cancer to treatment with anti-EGFR antibody, cetuximab, is not clear [72, 73], in contrast to the established role of anti-EGFR antibody in the treatment of colorectal cancer [74].

6 Novel strategies to circumvent *KRAS*-mutated tumors

Because *RAS* genes are the most frequently mutated oncogenes in various types of human cancers, researchers and clinicians are interested in developing efficient treatment strategies for tumors harboring mutant *RAS*. The first strategy was to inhibit farnesylation of RAS, which is essential for membrane localization and thus for RAS function. Some of these farnesyltransferase inhibitors (FTIs) are active *in vitro* or in animal models; however, all clinical trials failed mainly because alternative geranylgeranylation of the KRAS protein occurs, which can also support the biological activity of KRAS [75]. The concurrent inhibition of farnesyltransferase and geranylgeranyltransferase was too toxic [75].

Cancer cells usually contain multiple genetic and epigenetic abnormalities. For example, about 40% of adenocarcinomas harboring an *EGFR* mutation also have a *TP53* mutation [35]. Despite this complexity, their growth and survival can often be impaired by the inactivation of a single oncogene, the phenomenon known as "oncogene addiction" [76]. A typical example is the effect of EGFR-TKIs, as described above. However, not all cancers with *RAS* mutations are addicted to mutant RAS. Upon treatment of shRNAs to deplete KRAS in lung cancer cell lines harboring *KRAS* mutations, half of the cell lines maintained viability without expressing KRAS, whereas the cell density of the other half was reduced to one-fourth at most [77]. Even this sole observation illustrates the difficulties of RAS-targeted therapy. Comparing KRAS-independent and KRAS-dependent cancer cells reveals that KRAS-dependency is correlated with *KRAS* CNG and KRAS protein overexpression [77]. In addition, a gene-expression signature reveals that well-differentiated epithelial phenotype is also correlated with KRAS-dependency [77], showing that epithelial–mesenchymal transition (EMT) is associated with RAS independence. In this respect, it is noteworthy that RAS and transforming growth factor (TGF) β-Smad signaling cooperate selectively in the induction of Snail, a transcription factor that represses the expression of E-cadherin and induces EMT when overexpressed [78].

Because inhibition of KRAS by itself was insufficient to kill cancer cells with a *KRAS* mutation in the experiments mentioned above, one alternative approach might be to use the combined inhibition of two main downstream pathways of the RAS-RAF-MAPK/ERK kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway and the phosphatidylinositol 3 kinase (PI3K)–AKT pathway. When an artificial mutation was introduced to the RAS-binding domain of the *PIK3CA* gene, tumor formation was inhibited in a Kras 12D mouse model [79], showing the importance of PI3K in the RAS-signaling pathway. Another study also showed that the PI3K inhibitor, PX866, blocks both the development of new Kras G12D-induced tumors and the growth of Kras G12D-driven AAHs and early adenomas [80]. However, NVP-BEZ235, a potent dual pan-PI3K–mammalian target of rapamycin (mTOR) inhibitor, could not shrink established Kras G12D-driven tumors, suggesting that suppression of PI3K signaling is insufficient [81]. Although treatment of Kras G12D mice with the MEK inhibitor, ARRY-142886, alone led to only modest tumor regression, the combination of MEK and PI3K inhibitors led to marked synergistic tumor regression, and pathological analysis at the completion of treatment revealed only scant remnants of tumor nodules [81]. A recent cell line-based study revealed that *KRAS* gene-mutant cancers

exhibit variable responses to MEK inhibition and that PI3K pathway activation strongly influences the sensitivity of *KRAS* gene-mutant cells to MEK inhibitors. Activating mutations in the *PIK3CA* gene reduces the sensitivity to MEK inhibition, whereas *PTEN* mutations cause complete resistance [82]. In this study, the dual inhibition of the PI3K and MEK pathways was required for complete inhibition of the downstream mTOR and induction of cell death [82].

The second approach is the systematic prediction of drug activity using a genomically validated large cohort of cell lines [83]. In that study, 84 genomically validated cell lines were treated with erlotinib and with 11 other inhibitors that were under either clinical or preclinical evaluation [83], and they found that *KRAS* mutations conferred enhanced heat shock protein (HSP) 90 dependency. This finding was validated in mice with Kras-driven lung adenocarcinoma, showing these mice exhibited dramatic tumor regression when treated with an HSP90 inhibitor, 17-(dimethylamino-ethylamino)-17-demethoxygeldanamycin (17-DMAG).

The most recent approach is to identify synthetic lethal interactions in cancer cells harboring *KRAS* mutations using genome-wide RNA interference screening. In other words, this approach investigates which genes that, when silenced, kill cells harboring mutant *RAS* genes but not cells without this mutation. Using this approach, Scholl et al. identified serine threonine kinase, STK33 [84]. On the other hand, Luo et al. identified a diverse set of proteins whose depletion selectively impaired the viability of *RAS* mutant cells. In particular, they observed a strong enrichment of genes with mitotic functions including a mitotic kinase, polo-like kinase 1 [85]. More recently, Barbie et al. showed that TBK1, an upstream regulator of the nuclear factor-κB (NF-κB) pathway, was most effective in selectively killing *RAS* gene mutant cells [86]. TBK1 activates NF-κB by inhibiting IκB, an inhibitor of NF-κB. TBK1 also plays an important role downstream of RAS (Fig. 1b) [87]. In addition, inhibition of NF-κB by treatment with a non-phosphorylatable, dominant-negative form of IκB (IκB-DR) markedly decreased both the number and the size of lung tumors induced by concomitant loss of *TP53* and expression of oncogenic Kras (G12D) [88]. These findings provide support for the development of novel strategies to explore targeted therapies for the treatment of patients with mutated *RAS* gene.

7 Conclusion

We have described the clinical and biological significance of *KRAS* and *EGFR* mutations in lung cancer. The two mutant proto-oncogenes contrast sharply in terms of their epidemiological, pathological, biological, and clinical aspects in lung cancer (Table 2). Although *EGFR* mutation testing almost becomes a must for selecting patients who would most benefit from treatment with EGFR-TKIs, the role of *KRAS* mutations and their clinical applications are unclear. Specific treatment strategies that target activated KRAS are awaited eagerly. In addition to KRAS and EGFR, recent evidence suggests that some adenocarcinomas of the lung are dependent on or addicted to the HER2 [89] or anaplastic lymphoma kinase pathway [90, 91] although the incidence is low.

Table 2 Comparison of *KRAS* with *EGFR* mutations in human lung cancer

	KRAS	*EGFR*
Discovery of mutation	1982	2004
Biochemical function	Small GTP-binding protein	Receptor tyrosine kinase
Common mutation	Missense mutation at codons 12, 13 or 61	Exon 19 deletion, missense mutation at codon 858 (L858R) in tyrosine kinase domain
Allele-specific imbalance	Present (uniparental disomy common)	Present (copy number gain common)
Mutation in tumors other than lung cancer	Common (pancreas, colon, bile duct, etc.)	Absent[a]
Smoking status	Smokers	Non-smokers
Ethnicity	Caucasians>East Asians	East Asians>Caucasians
Sex	Male>female	Female>male
Histology	Adenocarcinoma (mucinous BAC[b])	Adenocarcinoma (non-mucinous BAC[b])
Prognostic impact	Poorer	Better
Response rate for EGFR-TKI therapy	0%	70–80%

[a] Mutations in the extracellular domain are reported in glioblastomas [95]

[b] Peripheral arising adenocarcinomas with partial or complete bronchiolealveolar growth component

We have treated NSCLC as one disease, although it is actually an aggregate of many genetically different diseases. Development of translational science involving close cooperation among clinicians, researchers, and pharmaceutical companies should make it possible to personalize lung cancer treatment, to turn this fatal disease into a chronic disorder and, eventually, to cure it.

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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

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.

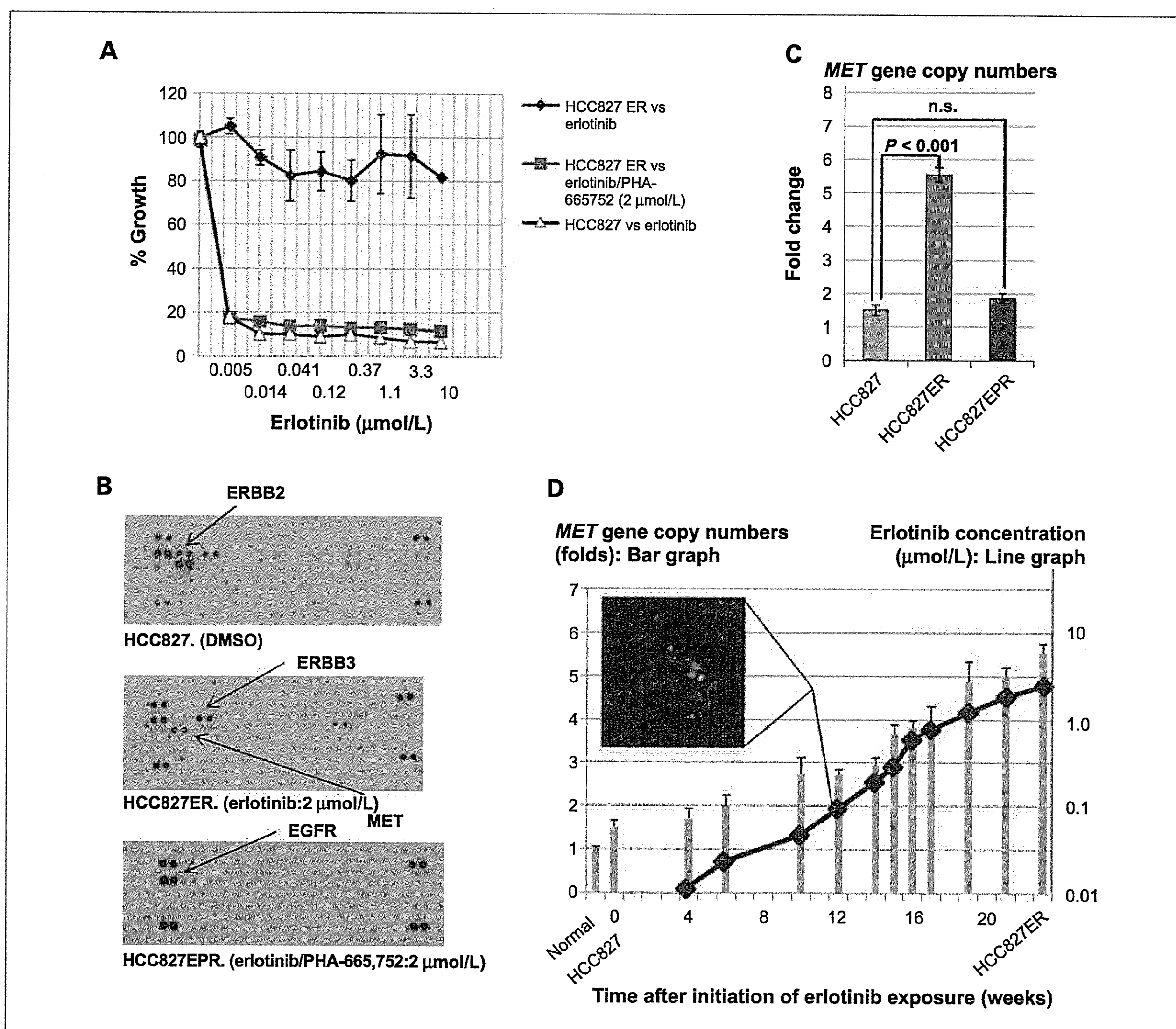

Fig. 1. Amplified *MET* gene caused erlotinib resistance in HCC827ER cells but not in HCC827EPR cells. A, HCC827ER cells were resistant to erlotinib, and PHA-665,752 restored erlotinib sensitivity. HCC827 or HCC827ER cells were incubated for 24 hours and for an additional 72 hours with the indicated 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

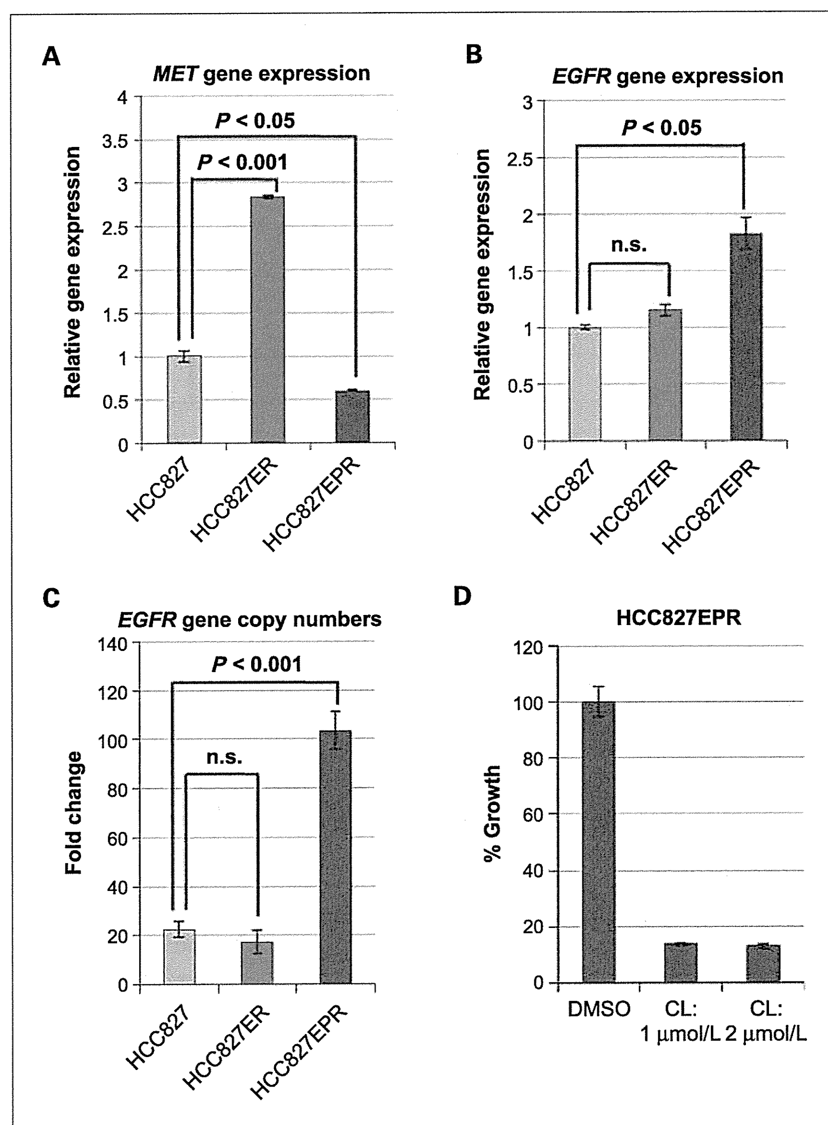

Fig. 2. Increased dependency on EGFR in HCC827EPR cells. A, *MET* gene expression increased in HCC827ER cells but decreased in HCC827EPR cells. B, *EGFR* gene expression increased in HCC827EPR cells. Quantitative real-time RT-PCR was done using validated TaqMan probes. The assays were done in triplicate, 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.

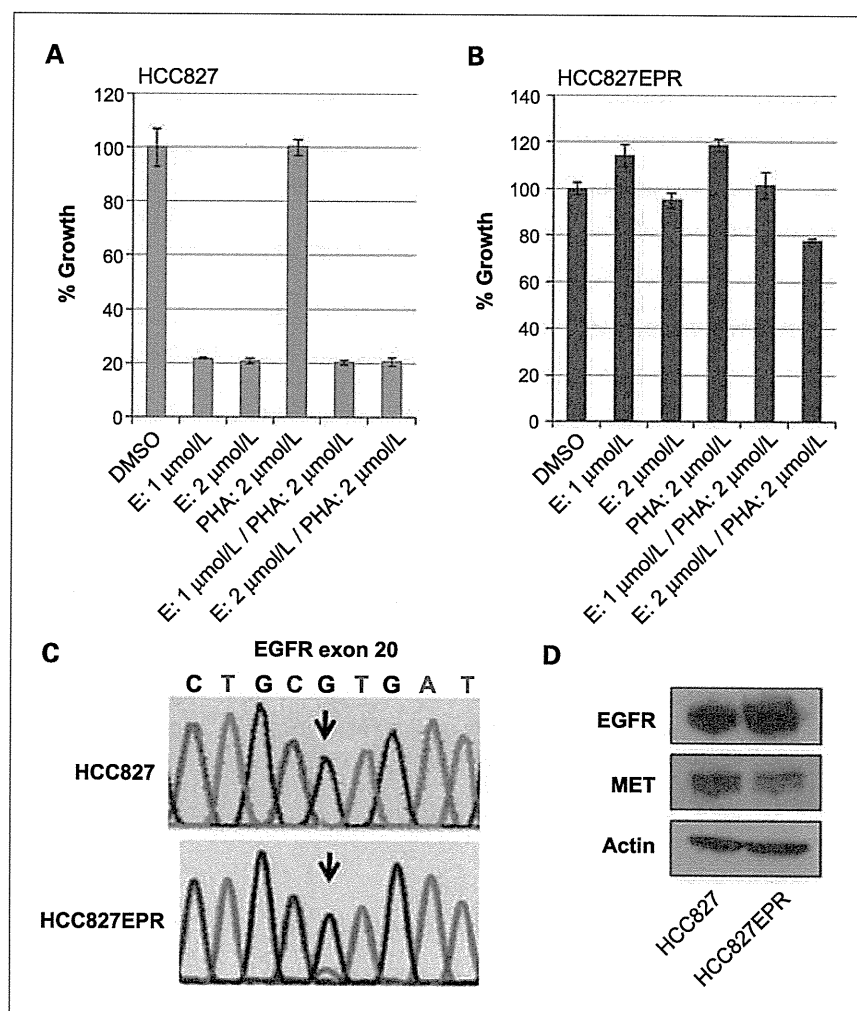

Fig. 3. HCC827EPR cells were resistant to erlotinib and/or PHA-665,752 and harbored the T790M mutation. A, HCC827 cells were sensitive to erlotinib (E) but not to PHA-665,752 (PHA). B, HCC827EPR cells were resistant to erlotinib and to the combination of erlotinib and PHA-665,752. HCC827 and HCC827EPR 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

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

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†	—	—	—	—

(*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