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Original Article

Clinicopathological characteristics of primary lung adenocarcinoma predominantly composed of goblet cells in surgically resected cases

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Primary lung adenocarcinomas predominantly composed of goblet cells (APGC) are relatively rare, and the clinicopathological characteristics have remained unclear. The aim of this study was to clarify the clinicopathological characteristics of APGC. We selected adenocarcinoma with a goblet cell-type component of ≥90% from 2228 cases of surgically resected primary lung adenocarcinoma. The clinicopathological characteristics of APGC (46 cases) were analyzed. APGC showed a significantly higher rate of tumor location on the left side, in the lower lobe and pathological stage I, when compared with the other types of adenocarcinoma. Furthermore, APGC displayed a lower frequency of central fibrosis, plural invasion, pulmonary metastasis, lymphatic permeation, and vascular invasion. APGC showed local recurrence in two of 46 cases (4.3%) and no incidents of distant metastasis. When compared with non-mucinous bronchioloalveolar adenocarcinomas (non-mucinous BAC) without central fibrosis, APGC without central fibrosis, corresponding to mucinous BAC, showed a significantly higher rate of tumor location on the left side and in the lower lobe. In conclusion, APGC formed a distinct subset and should be considered separately from lung adenocarcinoma based on frequent involvement of the left and lower lung and lack of central fibrosis.

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Pulmonary adenocarcinomas are classified according to their histological structure into bronchioloalveolar carcinoma (BAC), acinar, papillary, solid adenocarcinoma with mucin, and adenocarcinoma with mixed subtypes.[1] However, the cytological features of lung adenocarcinomas are highly heterogeneous from a histological perspective. With reference to the cytological features of carcinoma cells, Shimosato *et al.* subclassified primary lung adenocarcinoma cells into five subgroups, namely, goblet cells, bronchial cell surface-type cells, bronchial gland cells, Clara cells, and type II pneumocyte cells.[2,3] Goblet cell tumor cells are morphologically characterized by tall columnar cells with basal nuclei and pale cytoplasm, with varying amounts of cytoplasmic mucin. The apical site of each carcinoma cell is occupied by a large mass of mucin, which compresses the nucleus toward the basal end of the cell. Typically, carcinoma cells show mucin production with mucus pooling in the surrounding alveolar spaces. Cytological atypia is generally mild.[4–7]

To date, clinical research and genetic and phenotypical analyses of adenocarcinoma with a goblet cell component (AGC) have revealed that the biological characteristics of AGC differ from those of adenocarcinoma without a goblet cell component.[8–18] However, the proportion of goblet cell components in each AGC case varies, and whether such mixed cases should be regarded as belonging to the same category remains uncertain. Among the various AGC lesions, cases in which the goblet cell component is predominant (APGC) and pure lepidic growth without stromal invasion is present are defined as mucinous BAC.[1] However, the exact clinicopathological characteristics of APGC have not been fully analyzed.

We selected adenocarcinoma with a goblet cell component of ≥90% and analyzed their clinicopathological characteristics.

MATERIALS AND METHODS

Patients

Between August 1992 and September 2009, a total of 2228 patients with primary adenocarcinoma of the lung were treated surgically in the Division of Surgery of the Department of Thoracic Oncology, the National Cancer Center Hospital East, Chiba, Japan. A total of 46 cases (2.1%) of APGC (goblet cells ≥90%) were selected. Clinicopathological characteristics of the cases with APGC were examined. Cases with a history of other known malignancies were excluded.

Clinical information

All available clinical information was obtained from the clinical records and reports of the referring physicians. The records were reviewed for patient age, sex, smoking index, tumor size, location side, location lobe, stage, and duration of follow up (calculated from the date of surgery).

Six of the 46 patients underwent a wedge resection, 39 underwent a lobectomy, and one underwent a pneumonectomy (data not shown). The duration of patient follow up ranged from 1 to 132.3 months (mean, 58.5 months). The death of seven patients was confirmed; these patients had died of their disease between 37 days and 78.2 months (mean, 46.6 months) after surgery. The in-hospital mortality rate and 30-day mortality rate were 2.2% and 0%, respectively.

Pathological information

In each case, the resected tissue had been fixed in 10% formalin or absolute methyl alcohol and embedded in paraffin. The tumors had been cut into 5–10 mm slices, and serial 4-μm sections had been stained with HE; the Alcian blue-PAS method was used to visualize cytoplasmic mucin production and the Victoria van-Gieson (VVG) method was used to visualize elastic fibers. The median number of tissue blocks per resected specimen was 23. Two pathologists (H.I. and G.I.) reviewed all the slides through a multiheaded microscope. The tumors were classified according to the criteria of the current histological classification adopted by the World Health Organization. AGC is characterized by tall columnar cells with basal nuclei and pale cytoplasm with varying amounts of cytoplasmic mucin. The apical site of each carcinoma cell is occupied by a large mass of mucin which compresses the nucleus toward the basal end of the cell. Typically, carcinoma cells show mucin production with mucus pooling in the surrounding alveolar spaces. The cytological atypia is generally mild (Fig. 1a,b). Although we often found adenocarcinoma cases with tall columnar cells that did not fulfill the morphological criteria of goblet cells, we excluded these cases in the current study. We selected the AGC among the surgically resected lung carcinoma cases. We counted the proportion of the goblet cell component (GCC) in the maximum cut of the tumor and divided them according to the proportion of GCC (Fig. 1c). The number of cases of adenocarcinoma with GCC ≥ 90% and adenocarcinoma with 1% ≤ GCC ≤ 10% had been biased. Therefore we defined APGC as adenocarcinoma with a GCC ≥ 90%. Lymphatic permeation and pulmonary metastasis were evaluated using HE-stained sections. Vascular and pleural invasion were evaluated by examining the VVG-stained sections. Pathological staging was performed according to the classification of the Union Internationale Contre le Cancer.[19]

Statistical analysis

The Fisher's exact probability test was used to compare binomial proportions. A *P*-value of less than 0.05 was considered statistically significant. Patient survival time was calculated from the date of surgery until the date the first local or distant recurrence was diagnosed (disease-free survival) or until death (overall survival), and it was evaluated using the Kaplan-Meier method. The statistical significance of differences was estimated using the log–rank test. All statistical analyses were performed using SPSS version 11.0 commercial statistical software (SPSS Inc., Chicago, IL, USA). The data collection and analyses were approved by the Institutional Review Board, and the need to obtain informed consent from each patient was waived.

RESULTS

Clinicopathological findings of APGC

The clinicopathological data of APGC are summarized in Table 1. Of the 46 patients, 22 were male and 24 were female (male/female ratio: 1:1.1). Their ages at the time of diagnosis ranged from 40 to 84 years (median age 65 years; mean age 64 years). Nineteen (41%) of the patients were smokers (smoking index ≥200; 16 men, 3 women). The tumor arose in the right lung in 21 patients and in the left lung in 25 patients.

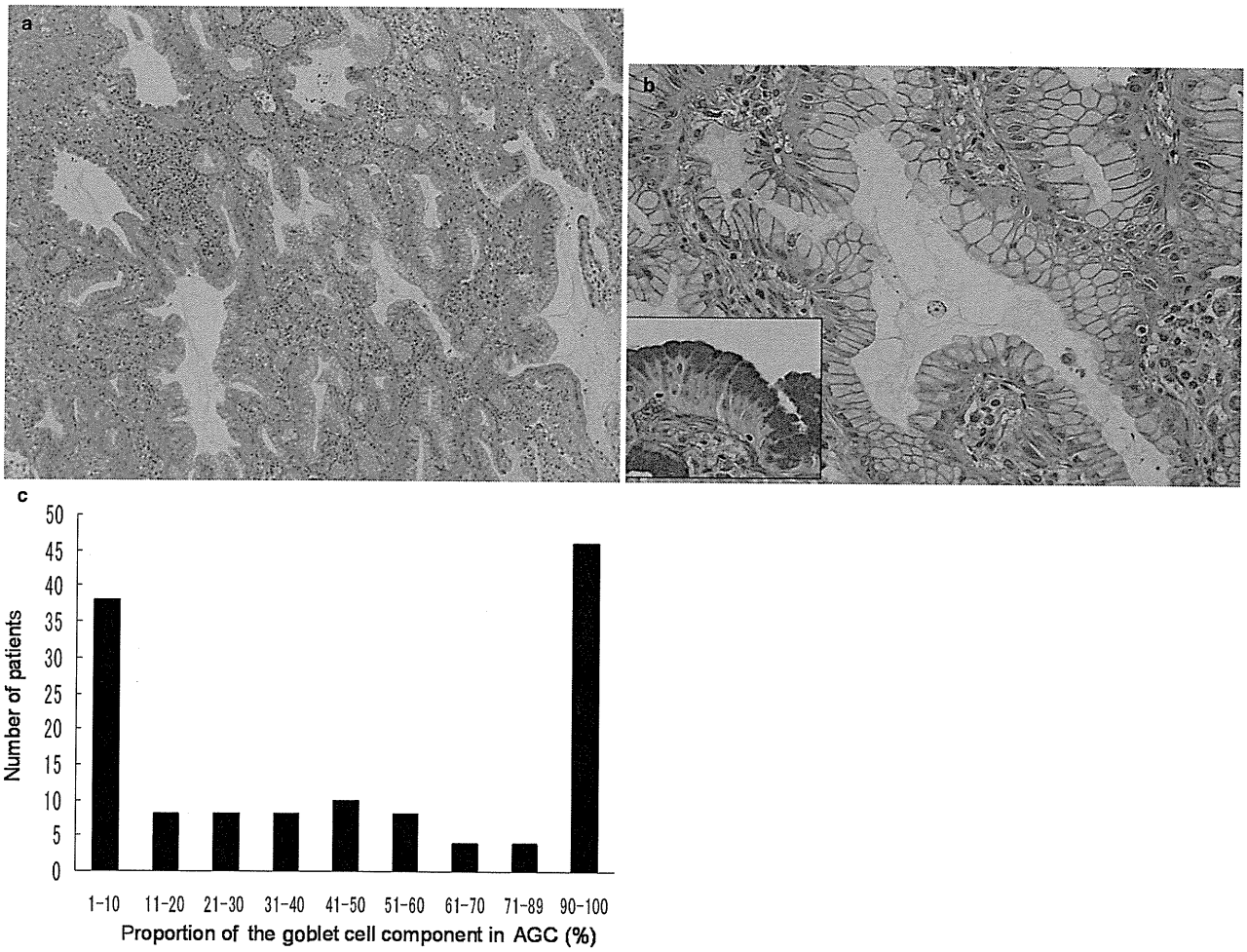

Figure 1 Typical morphology of adenocarcinoma with a goblet cell component (AGC). (**a**) The AGC shows a mainly lepidic growth pattern. Goblet cell-type adenocarcinoma is characterized by tall columnar cells with basal nuclei and pale cytoplasm and varying amounts of cytoplasmic mucin. (**b**) The apical site of each carcinoma cell is occupied by a large mass of mucus, which compresses the nucleus toward the basal end of the cell. Cytological atypia is generally mild. (**c**) The number of patients according to the proportion of goblet cell components.

The pathological stage was stage I in 45 cases (98%) (IA 27; IB 18), stage II in one case (2%) (IIB 1), and stage III and stage IV in no cases.

The maximum diameters of the tumors ranged from 0.7 cm to 19.5 cm (median 2.7 cm; mean 4.0 cm). Lymph node metastasis was present in one case (2%). Pleural invasion was observed in one case (2%). Lymphatic permeation was present in one case (2%). Pulmonary metastasis and vascular invasion was observed in no cases. Central fibrosis was observed in seven cases (15%).

We compared APGC ($n = 46$) and other types of adenocarcinoma ($n = 2182$) (Fig. 2). APGC showed significantly higher rates of tumor location on the left side ($P < 0.05$) and in the lower lobe ($P < 0.05$), N0-stage ($P < 0.05$), and stage I ($P < 0.05$). Furthermore, APGC had a lower frequency of central fibrosis ($P < 0.05$), pleural invasion ($P < 0.05$), lymphatic permeation ($P < 0.05$), and vascular invasion ($P < 0.05$). However, no significant differences in age, sex, smoking history or tumor size were noted between the two groups.

APGC has lower invasive capacity

We compared the frequency of invasive cases (positive for central fibrosis) in the two groups with respect to their size (tumor size; TS) (Fig. 3). In APGC, the frequency of invasive cases in TS ≤ 3 cm, 3 cm < TS ≤ 5 cm and TS > 5 cm was 3.8% (1/26), 8.3% (1/12), and 63% (5/8), respectively. In the other types of adenocarcinoma, the frequency of invasive case in TS ≤ 3 cm, 3 cm < TS ≤ 5 cm and TS > 5 cm was 91% (1544/1688), 99% (392/395), 100% (99/99), respectively. Therefore, even as TS became bigger, APGC displayed lower invasive capacity.

Table 1 Clinicopathological findings of adenocarcinoma with a goblet cell component ≥90%

	APGC $n = 46$ (%)	Other adenocarcinomas $n = 2182$ (%)	*P*
Age (years)			
<65	20 (43)	1021 (47)	
≥65	26 (57)	1161 (53)	0.66
Sex			
Male	22 (48)	1139 (52)	
Female	24 (52)	1043 (48)	0.56
Smoking history			
Smoking index <200	27 (59)	1101 (50)	
Smoking index ≥200	19 (41)	1081 (50)	0.27
Tumor size (cm)			
≤3	26 (57)	1419 (65)	
>3	20 (43)	763 (35)	0.23
Location side			
Right	21 (46)	1368 (63)	
Left	25 (54)	814 (37)	<0.05
Location lobe			
Upper and middle	8 (17)	1478 (68)	
Lower	38 (83)	704 (32)	<0.05
p-T			
1	27 (59)	1265 (58)	
2–4	19 (41)	917 (42)	0.92
p-N			
0	46 (100)	1539 (71)	
1–2	0 (0)	643 (29)	<0.05
p-ST			
1	45 (98)	1495 (69)	
2–4	1 (2)	687 (31)	<0.05
Pleural invasion	1 (2)	649 (30)	<0.05
Pulmonary metastasis	0 (0)	159 (7)	<0.05
Lymphatic permeation	1 (2)	608 (28)	<0.05
Vascular invasion	0 (0)	896 (28)	<0.05
Central fibrosis	7 (15)	2035 (93)	<0.05

Location lobe; upper and middle vs. lower.
p-T; T1 vs. T2, T3, T4. p-N; N0 vs. N1, N2. p-ST; ST1 vs. ST2, ST3, ST4.
APGC, adenocarcinoma predominantly composed of goblet cells; p-N, pathological nodes; p-ST, pathological staging; p-T, pathological tumor.

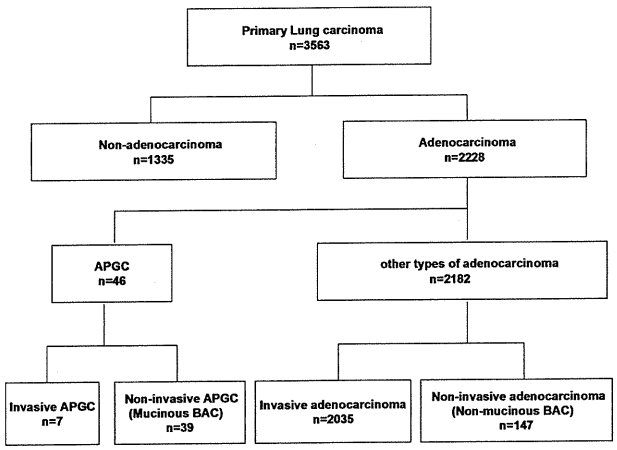

Figure 2 Classification design for the all surgical cases. APGC, adenocarcinoma predominantly composed of goblet cells; BAC, bronchioloalveolar carcinoma.

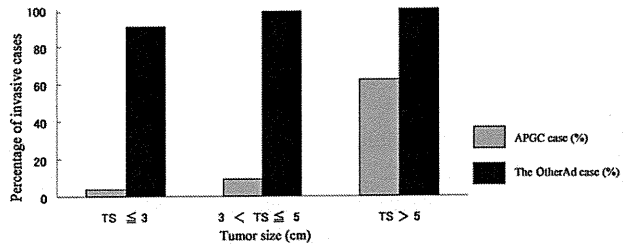

Figure 3 The frequency of invasive cases (positive for central fibrosis) between the adenocarcinoma predominantly composed of goblet cells (APGC) group and the adenocarcinoma (Ad) group with respect to tumor size (TS).

Follow up and clinical outcome of APGC

In APGC, only two local recurrences and one case of death as a result of lung cancer were observed. No distant recurrences were observed (Table 2).

The disease-free 5-year survival rate and the overall 5-year survival rate of the patients WITH APGC were 95.7% and 89.8%, respectively (data not shown).

Clinicopathological comparison of mucinous BAC and non-mucinous BAC

Among the 46 cases of APGC, 39 cases did not exhibit invasive foci; we considered these cases as corresponding to mucinous BAC without an invasive property. Furthermore we selected 147 cases of non-mucinous BAC (corresponding to Noguchi's type A or B) (Fig. 2) to compare them to.

Since non-invasive adenocarcinoma reflects a status of early stage adenocarcinoma and would be useful for considering the pathogenesis, we compared the clinicopathological factors of these 39 cases with those 147 cases of non-mucinous BAC (Table 3). Mucinous BAC cases showed significantly higher rates of more common tumors located on the left side ($P < 0.05$) or in the lower lobe ($P < 0.05$) than non-mucinous BAC cases. Mucinous BAC cases showed higher frequency in groups of the older aged than non-mucinous BAC, but the difference was marginally significant ($P = 0.054$).

Table 2 Recurrence and cause of death of adenocarcinoma predominantly composed of goblet cells

	$n = 46$ (%)
Recurrence	
Local	2 (4.3)
Distant	0 (0)
Cause of death	
Lung cancer	1 (2.2)
The others	6 (13)

DISCUSSION

APGC showed significantly higher rates of tumor location on the left side or in the lower lobe, pathological N0-stage, and p-stage of I, than the other types of adenocarcinoma. Moreover APGC displayed a lower frequency of central fibrosis, pleural invasion, pulmonary metastasis, lymphatic permeation, and vascular invasion. Even as tumor size became bigger, APGC displayed a lower invasive capacity. In addition, the prognosis of APGC was significantly better than that of the other groups.

Among the 46 cases of APGC, 39 did not exhibit central fibrosis or did not have small invasive foci; we considered these cases as corresponding to mucinous BAC. Recently Travis *et al.* proposed the new pathological classification of adenocarcinoma.[20] According to that study, the APGC in our study ($n = 46$) would be classified into three groups, mucinous adenocarcinoma *in situ* (AIS; size 3 cm or less; mucinous AIS $n = 25$), mucinous minimally invasive adenocarcinoma (MIA; size 3 cm or less; mucinous MIA $n = 1$), and invasive mucinous adenocarcinoma (size larger than 3 cm; $n = 20$; non-invasive or invasive cases $n = 14$; non-invasive cases $n = 6$). Since BAC reflects a status of early-stage adenocarcinoma, we compared these 39 cases with non-mucinous BAC. Mucinous BAC was significantly more common among tumors located on the left side or in the lower lobe than non-mucinous BAC. These findings suggest that the pathogenesis of APGC differs from that of adenocarcinoma without goblet cells.

Lantuejoul *et al.* reported that mucinous cells in type 1 congenital cystic adenomatoid malformation (CCAM) may represent mucinous BAC precursors.[21] In mucinous cell clusters associated with CCAM, KRAS mutations were detected in five out of six cases, while no epidermal growth factor

Table 3 Clinical findings of Mucinous BAC vs. Non-mucinous BAC

	Mucinous BAC $n = 39$ (%)	Non-mucinous BAC $n = 147$ (%)	P
Age (years)			
<65	16 (41)	82 (56)	
≥65	23 (59)	65 (44)	0.054
Sex			
Male	16 (41)	46 (31)	
Female	23 (59)	101 (69)	0.25
Smoking history			
Smoking index <200	24 (62)	105 (71)	
Smoking index ≥200	15 (38)	42 (29)	0.24
Location side			
Right	17 (44)	103 (70)	
Left	22 (56)	44 (30)	<0.05
Location lobe			
Upper and middle	7 (18)	94 (64)	
Lower	32 (82)	53 (36)	<0.05

Location lobe; upper and middle vs. lower.
BAC, bronchioloalveolar carcinoma.

receptor (EGFR) mutations were reported. Furthermore, all mucinous cell cluster lesions expressed MUC5AC but were negative for MUC2, CDX2, and TTF-1, similar to the staining pattern of mucinous BAC. These findings suggested that type 1 CCAM mucinous cells may represent mucinous BAC precursors. Actually, CCAM also exhibits a higher rate of location in the lower lobe,[22,23] similar to the feature of mucinous BAC revealed in the current study.

Vascular invasion, lymphatic permeation, and pleural invasion were not found in APGC except in one case. Only two cases of APGC developed local recurrence; however, the sizes of these primary tumors were 10 cm and 13.4 cm. Cases with distant metastasis were not found. Therefore, APGC can be treated as a local disease.

In the current study, we also selected adenocarcinoma with 1% ≤ GCC <90% (n = 88) and compared them with APGC (data not shown). APGC had a lower frequency of central fibrosis ($P < 0.05$), plural invasion ($P < 0.05$), pulmonary metastasis ($P < 0.05$), lymphatic permeation ($P < 0.05$), and vascular invasion ($P < 0.05$). However, no significant differences in age, sex, or smoking index were noted between the two groups. Taking this into consideration, the potential for the invasiveness of AGC cells would be lower than that of non-goblet cell type adenocarcinoma cells.

The clinical characteristics of mucinous BAC are aerogenous spread and satellite tumors surrounding the main mass. Extensive consolidation is common with a lobar and/or pneumonic pattern in inoperable cases. In the current study, all APGC cases displayed no pulmonary metastasis, which seems to be inconsistent with the clinical behavior of APGC. However, all APGC cases examined in our study were surgical cases and were thought to be in the early stage. In APGC cases with TS ≤ 3 cm (n = 27), the number of invasive APGC case was one (1/26, 3.8%). In contrast, in APGC cases with TS > 3 cm (n = 20), the number of invasive APGC was six (6/20, 30%). From this it would be possible to think that as tumor size becomes bigger the invasive component increases.

Wislez *et al.* reported that no response to gefitinib treatment was observed in patients with AGC, since AGC appeared to be resistant to EGFR- tyrosine kinase inhibitors.[24] Instead, AGC might be more sensitive to taxane therapy.[24–26] This suggest that the biological characteristics of AGC differ from those of adenocarcinoma without a goblet cell component with regard to EGFR signaling. However, the frequency of EGFR mutation is controversial (0–15%), and this might be because that the proportion of the goblet cell component in each AGC cases varies.[24–27] When considering the pathogenesis of and therapeutic strategies for AGC, we consider it might be important to examine the EGFR and KRAS mutation status in each AGC case.

In conclusion, we found that the pathogenesis of APGC differs from that of other types of adenocarcinoma based on the frequent involvement of the left and lower lung and lack of central fibrosis. Furthermore, APGC may be treated as a local disease. Although further studies with a larger number of cases are warranted, we believe that APGC should be classified as a special subtype of lung adenocarcinoma.

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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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Cancer Therapy: Preclinical

See commentary p. 5371

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.

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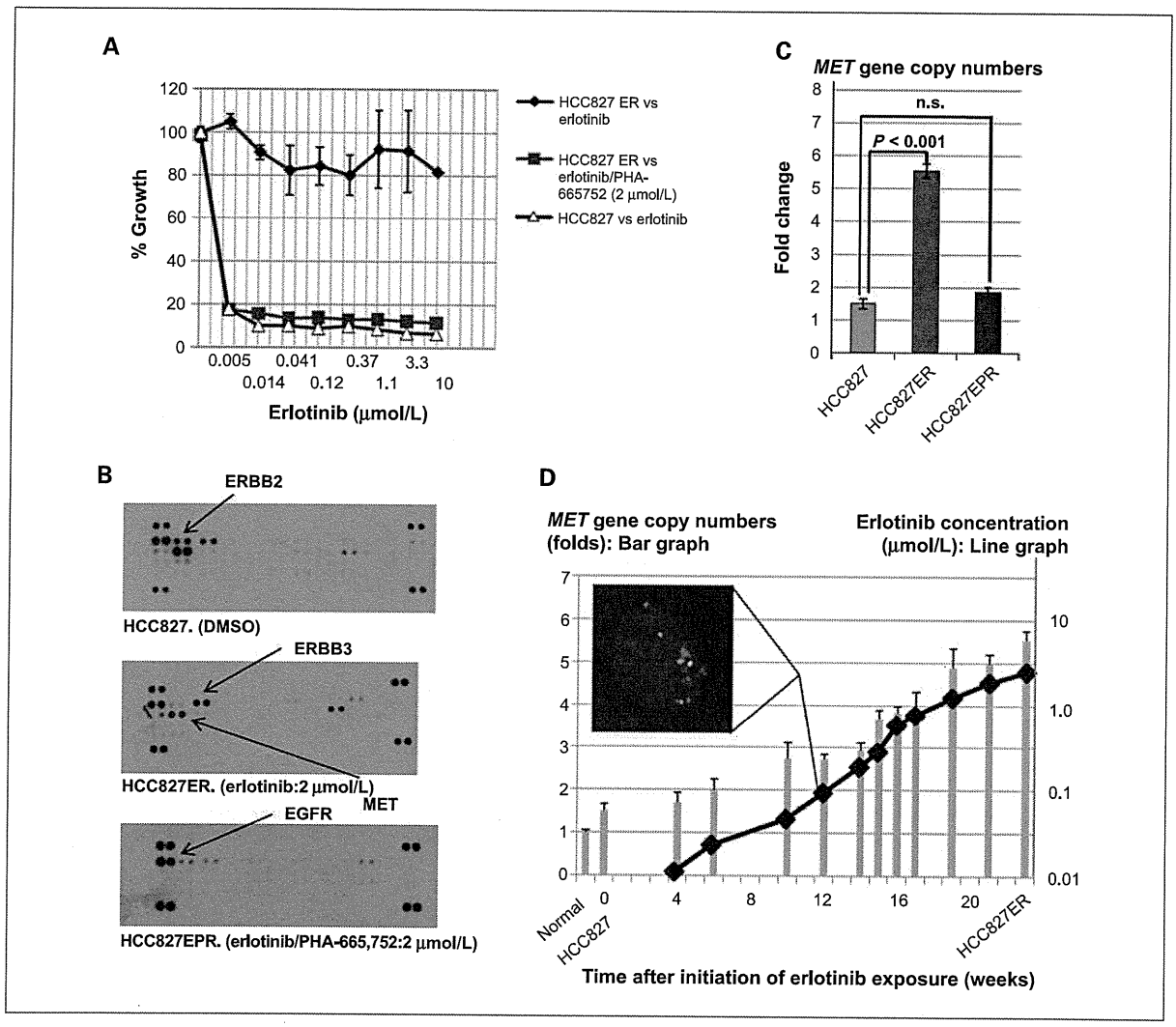

Fig. 1. Amplified *MET* gene caused erlotinib resistance in HCC827ER cells but not in HCC827EPR cells. A, HCC827ER cells were resistant to erlotinib, and PHA-665,752 restored erlotinib sensitivity. HCC827 or HCC827ER cells were incubated for 24 hours and for an additional 72 hours with the indicated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

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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

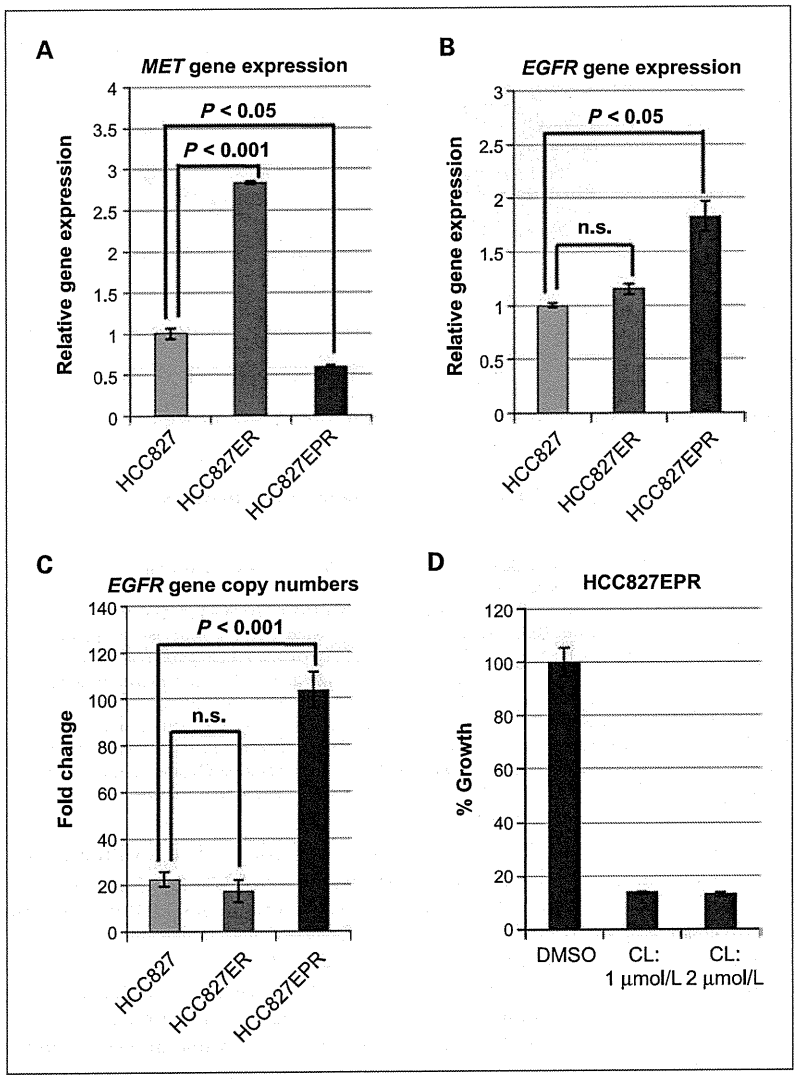

Fig. 2. Increased dependency on EGFR in HCC827EPR cells. A, *MET* gene expression increased in HCC827ER cells but decreased in HCC827EPR cells. B, *EGFR* gene expression increased in HCC827EPR cells. Quantitative real-time RT-PCR was done using validated TaqMan probes. The assays were done in triplicate,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

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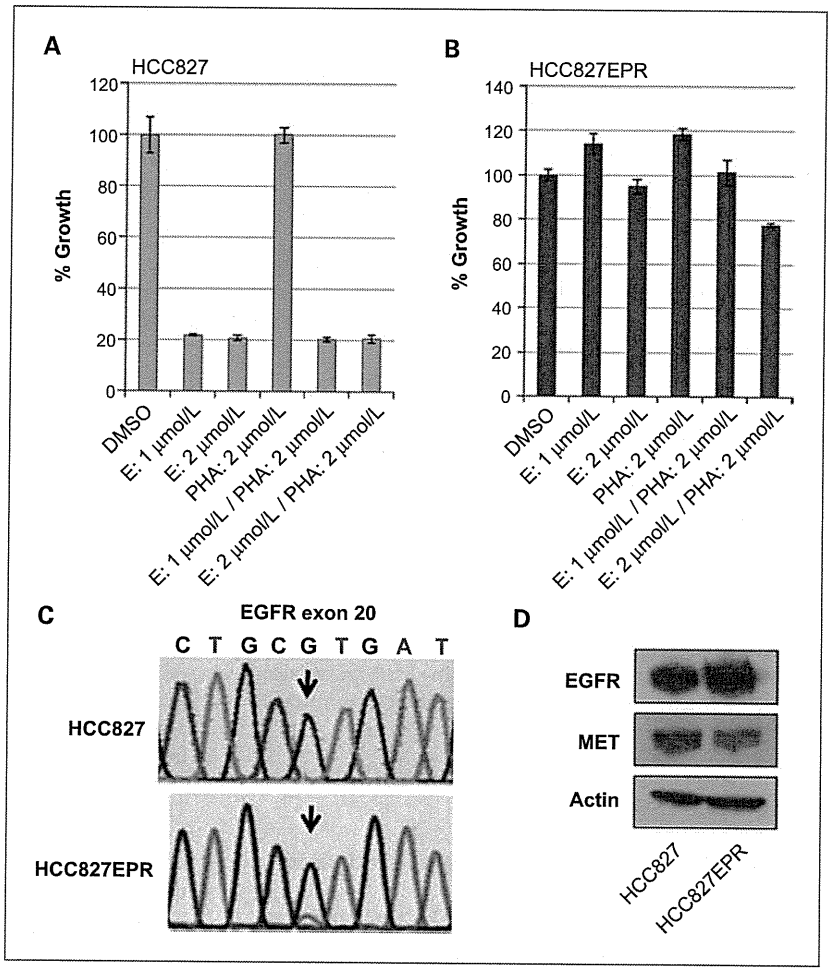

Fig. 3. HCC827EPR cells were resistant to erlotinib and/or PHA-665,752 and harbored the T790M mutation. A, HCC827 cells were sensitive to erlotinib (E) but not to PHA-665,752 (PHA). B, HCC827EPR cells were resistant to erlotinib and to the combination of erlotinib and PHA-665,752. HCC827 and HCC827EPR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

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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

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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

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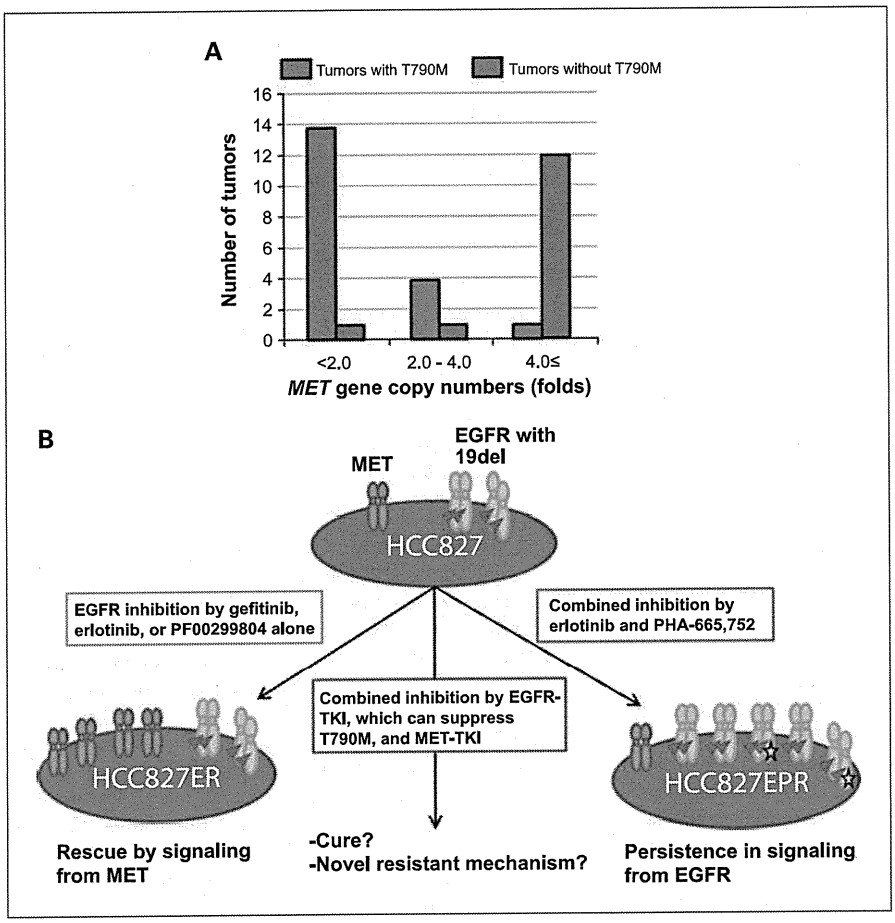

Fig. 4. Reciprocal relationship between *MET* amplification and T790M mutation. A, tumor numbers with or without T790M mutation by *MET* gene copy numbers in gefitinib-refractory tumors obtained from autopsy. B, schema of the difference of escape hatches of HCC827 cells depending on the selection pressure 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 interesting 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

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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

T. Mitsudomi has received lecture fees from AstraZeneca and Chugai. The other authors declare no conflict of interest.

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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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