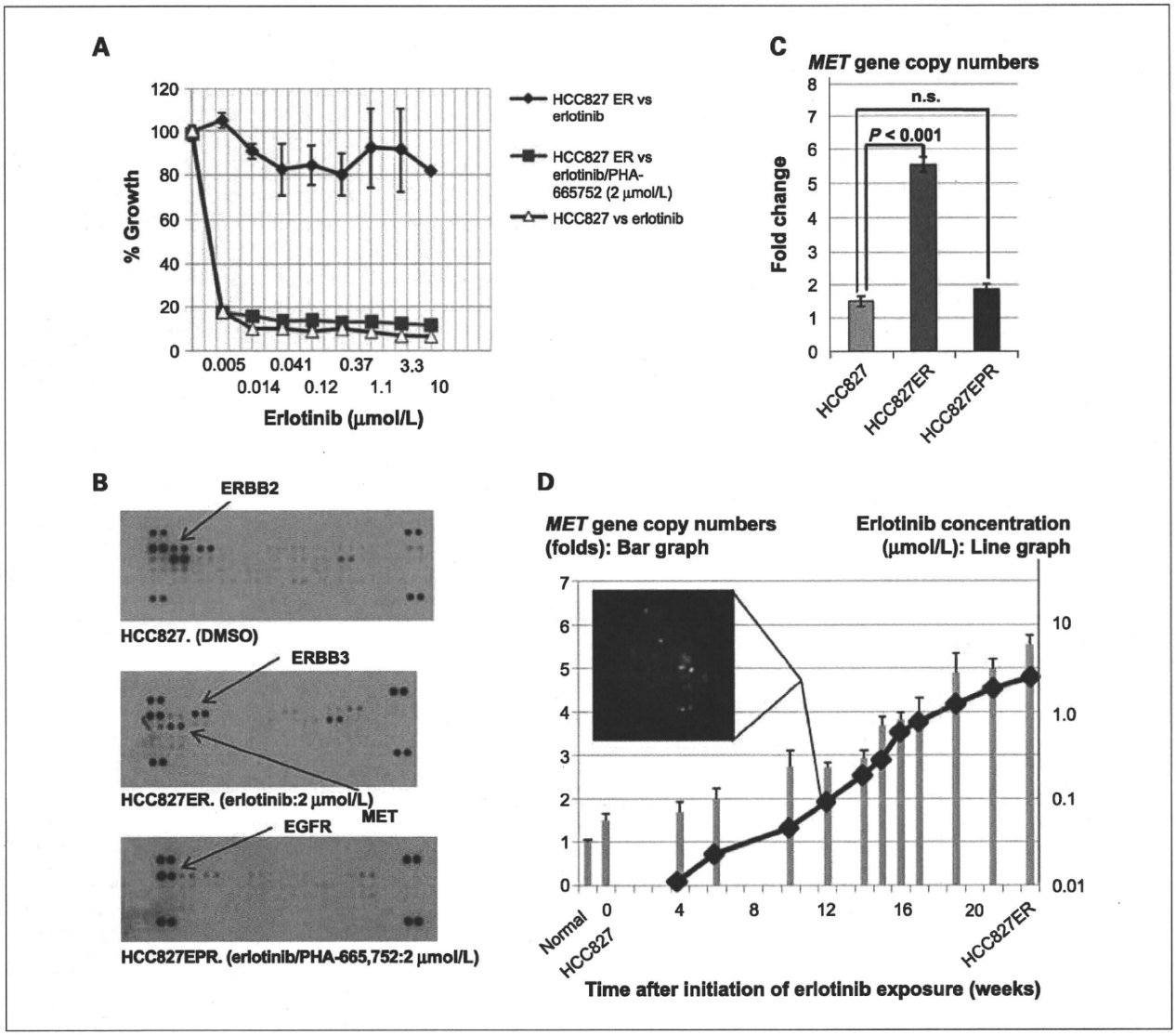

Fig. 1. Amplified *MET* gene caused erlotinib resistance in HCC827ER cells but not in HCC827EPR cells. A, HCC827ER cells were resistant to erlotinib, and PHA-665,752 restored erlotinib sensitivity. HCC827 or HCC827ER cells were incubated for 24 hours and for an additional 72 hours with the indicated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

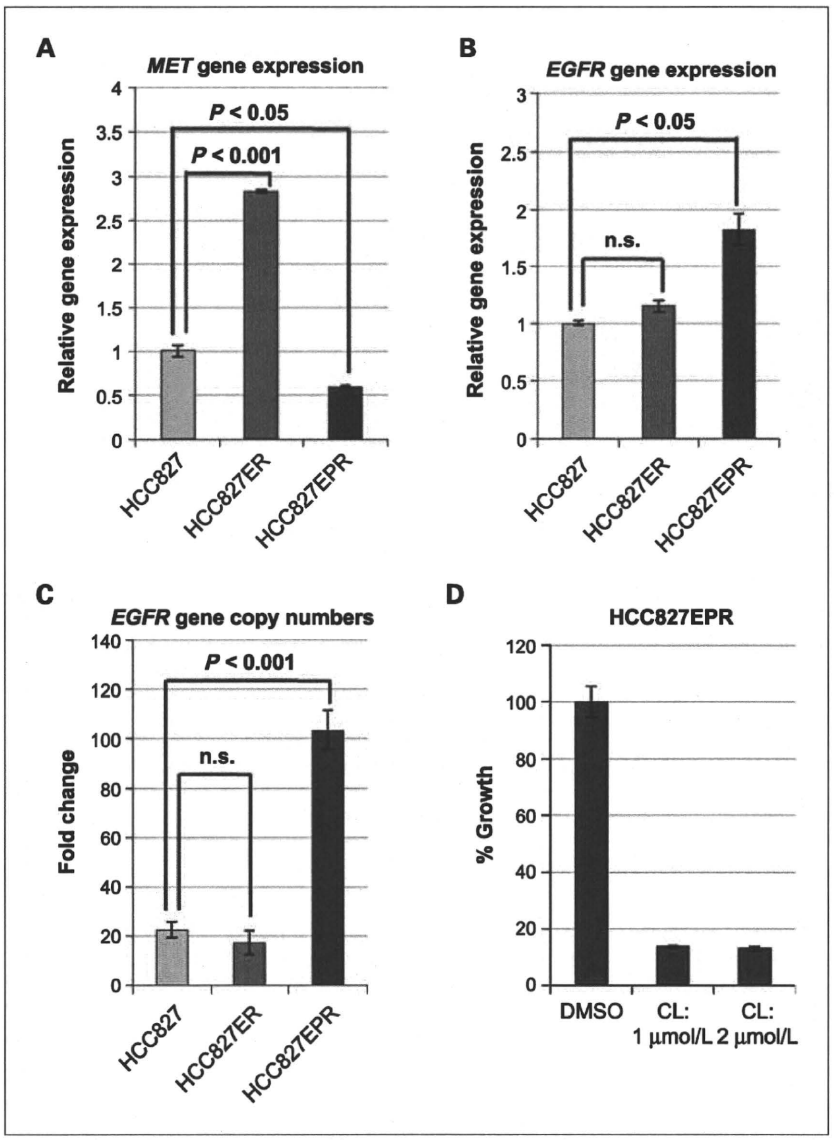

Fig. 2. Increased dependency on EGFR in HCC827EPR cells. A, *MET* gene expression increased in HCC827ER cells but decreased in HCC827EPR cells. B, *EGFR* gene expression increased in HCC827EPR cells. Quantitative real-time RT-PCR was done using validated TaqMan probes. The assays were done in triplicate,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

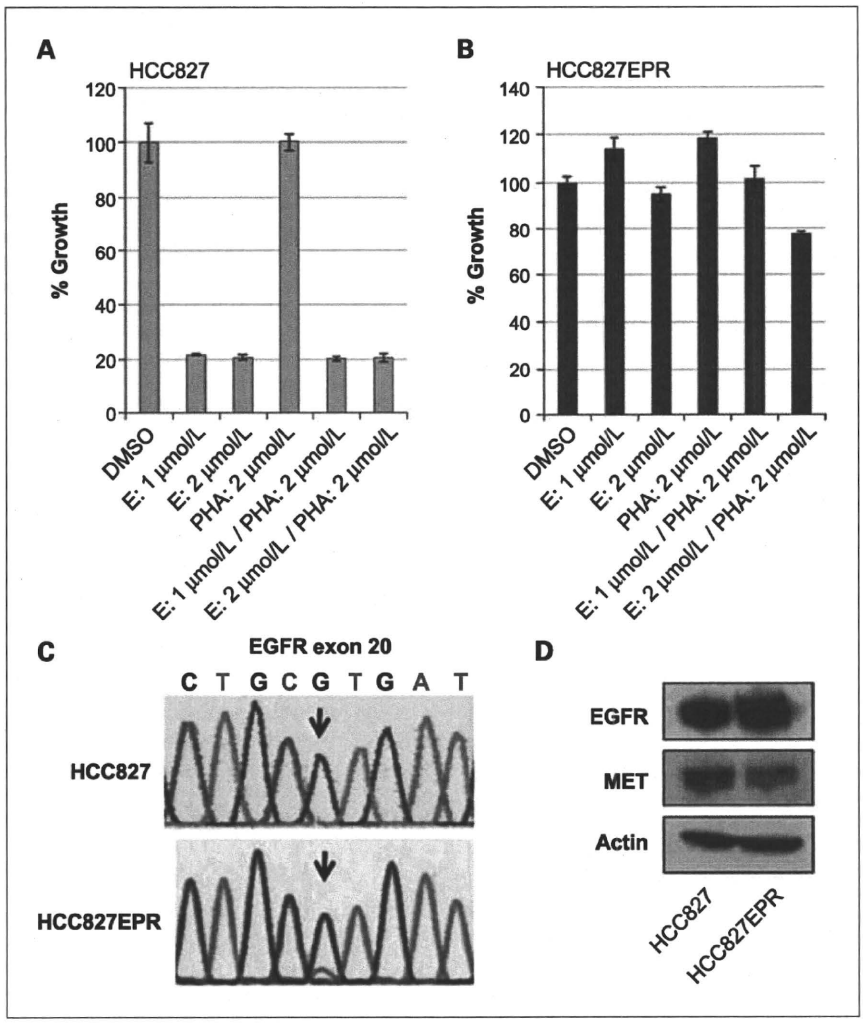

Fig. 3. HCC827EPR cells were resistant to erlotinib and/or PHA-665,752 and harbored the T790M mutation. A, HCC827 cells were sensitive to erlotinib (E) but not to PHA-665,752 (PHA). B, HCC827EPR cells were resistant to erlotinib and to the combination of erlotinib and PHA-665,752. HCC827 and HCC827EPR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

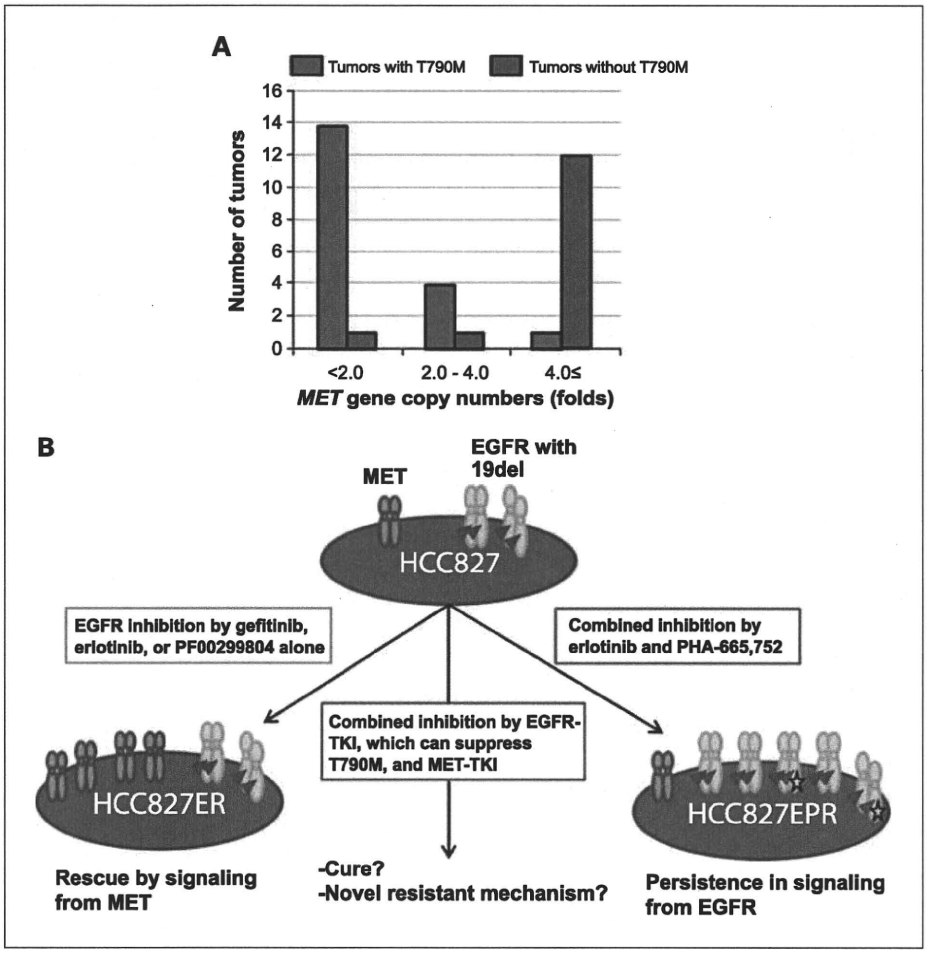

Fig. 4. Reciprocal relationship between *MET* amplification and T790M mutation. A, tumor numbers with or without T790M mutation by *MET* gene copy numbers in gefitinib-refractory tumors obtained from autopsy. B, schema of the difference of escape hatches of HCC827 cells depending on the selection pressure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

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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References

1. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
2. Paez JG, Janne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004; 304:1497–500.
3. Mitsudomi T, Yatabe Y. Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensitivity in lung cancer. Cancer Sci 2007;98:1817–24.
4. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361: 947–57.
5. Mitsudomi T, Morita S, Yatabe Y, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. Lancet Oncol 2010;11:121–8.
6. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A 2004;101:13306–11.
7. Rosell R, Moran T, Queralt C, et al. Screening for epidermal growth factor receptor mutations in lung cancer. N Engl J Med 2009;361: 958–67.
8. Morita S, Hirashima T, Hagiwara K, et al. Gefitinib combined survival analysis of the mutation positives from the prospective phase II trials (I-CAMP). J Clin Oncol 2008;26:absr 8101.
9. Kobayashi S, Boggon TJ, Dayaram T, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352:786–92.
10. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med 2005;2:e73.
11. Engelman JA, Zejnullahu K, Mitsudomi T, et al. MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 2007;316:1039–43.
12. Bean J, Brennan C, Shih JY, et al. MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. Proc Natl Acad Sci U S A 2007; 104:20932–7.
13. Inukai M, Toyooka S, Ito S, et al. Presence of epidermal growth factor receptor gene T790M mutation as a minor clone in non-small cell lung cancer. Cancer Res 2006;66:7854–8.
14. Turke AB, Zejnullahu K, Wu YL, et al. Preexistence and clonal selection of MET amplification in EGFR mutant NSCLC. Cancer Cell 2010; 17:77–88.
15. Toyooka S, Kiura K, Mitsudomi T. EGFR mutation and response of lung cancer to gefitinib. N Engl J Med 2005;352:2136, author reply.
16. Kubo T, Yamamoto H, Lockwood WW, et al. MET gene amplification or EGFR mutation activate MET in lung cancers untreated with EGFR tyrosine kinase inhibitors. Int J Cancer 2009;124:1778–84.
17. Onozato R, Kosaka T, Kuwano H, Sekido Y, Yatabe Y, Mitsudomi T. Activation of MET by gene amplification or by splice mutations deleting the juxtamembrane domain in primary resected lung cancers. J Thorac Oncol 2009;4:5–11.
18. Ogino A, Kitao H, Hirano S, et al. Emergence of epidermal growth factor receptor T790M mutation during chronic exposure to gefitinib in a non small cell lung cancer cell line. Cancer Res 2007;67:7807–14.
19. Yoshida T, Okamoto I, Okamoto W, et al. Effects of Src inhibitors on cell growth and epidermal growth factor receptor and MET signaling

in gefitinib-resistant non-small cell lung cancer cells with acquired MET amplification. Cancer Sci 2010;101:167–72.
20. Ercan D, Zejnullahu K, Yonesaka K, et al. Amplification of EGFR T790M causes resistance to an irreversible EGFR inhibitor. Oncogene 2010;29:2346–56.
21. Kosaka T, Yatabe Y, Endoh H, et al. Analysis of epidermal growth factor receptor gene mutation in patients with non-small cell lung cancer and acquired resistance to gefitinib. Clin Cancer Res 2006; 12:5764–9.
22. Yatabe Y, Hida T, Horio Y, Kosaka T, Takahashi T, Mitsudomi T. A rapid, sensitive assay to detect EGFR mutation in small biopsy specimens from lung cancer. J Mol Diagn 2006;8:335–41.
23. Zhao X, Weir BA, LaFramboise T, et al. Homozygous deletions and chromosome amplifications in human lung carcinomas revealed by single nucleotide polymorphism array analysis. Cancer Res 2005;65: 5561–70.
24. Usami N, Fukui T, Kondo M, et al. Establishment and characterization of four malignant pleural mesothelioma cell lines from Japanese patients. Cancer Sci 2006;97:387–94.
25. Jackman D, Pao W, Riely GJ, et al. Clinical definition of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. J Clin Oncol 2010;28:357–60.
26. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000;92:205–16.
27. Martha Guix, Anthony C. Faber, Shizhen Emily Wang, et al. Acquired resistence to EGFR tyrosine kinase inhibitors in cancer cells is mediated by loss of IGF-binding proteins. J Clin Invest 2008;118: 2609–19.
28. Ranson M, Turke JAE, Ferry D, et al. ZD1839, a selective oral epidermal growth factor receptor-tyrosine kinase inhibitor, is well tolerated and active in patients with solid, malignant tumors: results of a phase I trial. J Clin Oncol 2002;20:2240–50.
29. Hidalgo M, Siu LL, Nemunaitis J, et al. Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. J Clin Oncol 2001;19:3267–79.
30. Gandhi J, Zhang J, Xie Y, et al. Alterations in genes of the EGFR signaling pathway and their relationship to EGFR tyrosine kinase inhibitor sensitivity in lung cancer cell lines. PloS One 2009;4:e4576.
31. Engelman JA, Mukohara T, Zejnullahu K, et al. Allelic dilution obscures detection of a biologically significant resistance mutation in EGFR-amplified lung cancer. J Clin Invest 2006;116:2695–706.
32. Maheswaran S, Sequist LV, Nagrath S, et al. Detection of mutations in EGFR in circulating lung-cancer cells. N Engl J Med 2008;359:366–77.
33. Suda K, Onozato R, Yatabe Y, Mitsudomi T. EGFR T790M mutation: a double role in lung cancer cell survival? J Thorac Oncol 2009;4:1–4.
34. Yano S, Wang W, Li Q, et al. Hepatocyte growth factor induces gefitinib resistance of lung adenocarcinoma with epidermal growth factor receptor-activating mutations. Cancer Res 2008;68:9479–87.
35. Yamada T, Matsumoto K, Wang W, et al. Hepatocyte growth factor reduces susceptibility to an irreversible epidermal growth factor receptor inhibitor in EGFR-T790M mutant lung cancer. Clin Cancer Res 2010;16:174–83.
36. Shattuck DL, Miller JK, Laederich M, et al. LRIG1 is a novel negative regulator of the Met receptor and opposes Met and Her2 synergy. Mol Cell Biol 2007;27:1934–46.
37. Cipres A, Abassi YA, Vuori K. Abl functions as a negative regulator of Met-induced cell motility via phosphorylation of the adapter protein CrkII. Cell Signal 2007;19:1662–70.
38. Takano T, Fukui T, Ohe Y, et al. EGFR mutations predict survival benefit from gefitinib in patients with advanced lung adenocarcinoma: a historical comparison of patients treated before and after gefitinib approval in Japan. J Clin Oncol 2008;26:5589–95.

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MINIREVIEW

Epidermal growth factor receptor in relation to tumor development: *EGFR* gene and cancer

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Keywords
cancer; epidermal growth factor receptor (EGFR); gefitinib; non-small cell lung carcinoma (NSCLC); tyrosine kinase inhibitor (TKI)

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Epidermal growth factor receptor (EGFR) and its three related proteins (the ERBB family) are receptor tyrosine kinases that play essential roles in both normal physiological conditions and cancerous conditions. Upon binding its ligands, dynamic conformational changes occur in both extracellular and intracellular domains of the receptor tyrosine kinases, resulting in the transphosphorylation of tyrosine residues in the C-terminal regulatory domain. These provide docking sites for downstream molecules and lead to the evasion of apoptosis, to proliferation, to invasion and to metastases, all of which are important for the cancer phenotype. Mutation in the tyrosine kinase domain of the *EGFR* gene was found in a subset of lung cancers in 2002. Lung cancers with an *EGFR* mutation are highly sensitive to EGFR tyrosine kinase inhibitors, such as gefitinib and erlotinib. Here, we review the discovery of EGFR, the EGFR signal transduction pathway and mutations of the *EGFR* gene in lung cancers and glioblastomas. The biological significance of such mutations and their relationship with other activated genes in lung cancers are also discussed.

Identification of epidermal growth factor, epidermal growth factor receptor and ERBB family proteins

Epidermal growth factor (EGF) was originally isolated by Stanley Cohen in 1962 as a protein extracted from the mouse submaxillary gland that accelerated incisor eruption and eyelid opening in the newborn animal [1]. Therefore, it was originally termed 'tooth-lid factor', but was later renamed EGF because it stimulated the proliferation of epithelial cells [1]. In 1972, the amino acid sequence of the EGF was determined. The presence of a specific binding site for EGF, the EGF receptor (EGFR), was confirmed in 1975 by showing that ^{125}I-labeled EGF binds specifically to the surface of fibroblasts [1].

In 1978, EGFR was identified as a 170kDa protein that showed increased phosphorylation when bound to EGF in the A431 squamous cell carcinoma cell line that had an amplified *EGFR* gene. The discovery (in 1980) that the transforming protein of Rous sarcoma virus, v-src, has tyrosine-phosphorylation activity led to the discovery that EGFR is a tyrosine kinase activated by binding EGF [1]. In 1984, the cDNA of human *EGFR* was isolated and characterized. A high degree of similarity was found between the amino acid sequence of *EGFR* and that of v-erbB, an oncogene of the avian erythroblastosis virus [1].

Abbreviations
ALK, anaplastic lymphoma kinase; BAC, bronchioloalveolar cell carcinoma; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EML4, echinoderm microtubule-associated protein-like 4; NRG, neuregulin; STAT, signal transducer and activator of transcription; TKI, tyrosine kinase inhibitor; TRU, terminal respiratory unit.

Screening of cDNA libraries using an EGFR probe identified a family of proteins closely related to EGFR. This family consists of EGFR (also known as ERBB1/HER1), ERBB2/HER2/NEU, ERBB3/HER3 and ERBB4/HER4. ERBB2, ERBB3 and ERBB4 show extracellular homologies, relative to the EGFR, of 44, 36 and 48%, respectively, while those for the tyrosine kinase domain are 82, 59 and 79%, respectively. The degrees of homology in the C-terminal regulatory domain are relatively low, being 33, 24 and 28%, respectively.

Structure of the ERBB proteins and diversity of their ligands

The EGFR gene is located on chromosome 7p12-13 and codes for a 170kDa receptor tyrosine kinase. All ERBB proteins have four functional domains: an extracellular ligand-binding domain; a transmembrane domain; an intracellular tyrosine kinase domain; and a C-terminal regulatory domain [2]. The extracellular domain is subdivided further into four domains. The tyrosine kinase domain consists of an N-lobe and a C-lobe, and ATP binds to the cleft formed between these two lobes. The C-terminal regulatory domain has several tyrosine residues that are phosphorylated specifically upon ligand binding, as described below (Fig. 1A).

Eleven ligands are known to bind to the ERBB family of receptors [3]. These can be classified into three groups (a) ligands that specifically bind to EGFR (including EGF, transforming growth factor-a, amphiregulin and epigen); (b) those that bind to EGFR and ERBB4 (including betacellulin, heparin-binding EGF and epiregulin); and (c) neuregulin (NRG) (also known as heregulin) that binds to ERBB3 and ERBB4. NRG1 and NRG2 bind to both ERBB3 and ERBB4, whereas NRG3 and NRG4 only bind to ERBB4 [3]. Although these ligands show redundancy, heparin-binding-EGF is the only ligand whose absence in knockout mice results in postnatal lethality as a result of heart and lung problems, while mice lacking other EGF ligands, or even triple null mice deficient for amphiregulin, EGF and transforming growth factor-a are viable [4]. These ligands are synthesized as transmembrane proteins, and soluble ligands (growth factors) are released into the extracellular environment via proteolytic processing. This shedding is mediated by ADAM (a disintegrin and metalloprotease) proteins that are membrane-anchored metalloproteases [4].

Signal transduction by ERBB proteins

Binding of a family of specific ligands to the extracellular domain of ERBB (except for ERBB2, see below) leads to the formation of homodimers and heterodimers. This process is mediated by rotation of domains I and II, leading to promotion from a tethered configuration to an extended configuration (Fig. 1B) [2]. This exposes the dimerization domain. ERBB2 does not have corresponding ligands but is expressed constitutively in the extended configuration. ERBB2 is a preferred dimerization partner, and heterodimers containing ERBB2 mediate stronger signals

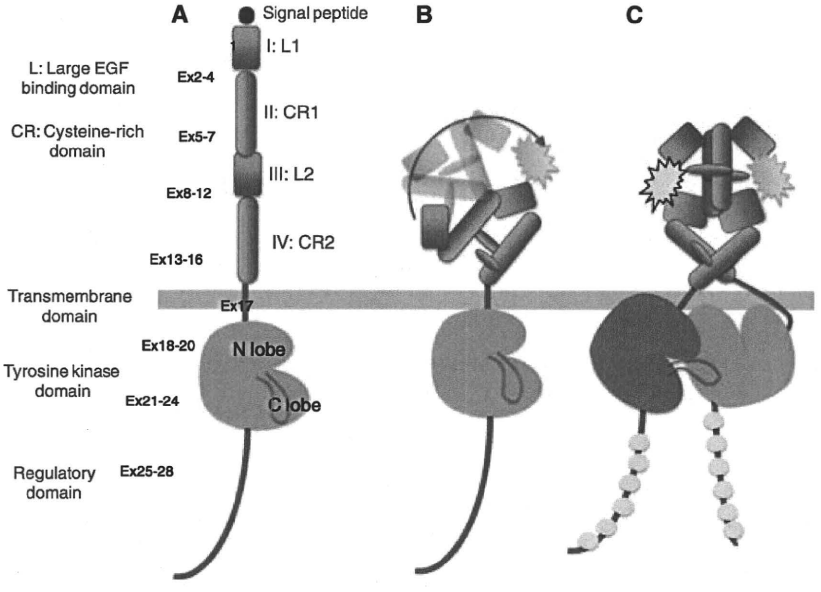

Fig. 1. Structure of the EGFR protein (A), activation (B) and dimerization by ligand binding (C).

than other dimers. In the cytoplasm, the kinase domain dimerizes asymmetrically in a tail-to-head orientation (Fig 1C) [5]. In this manner, tyrosine kinase becomes activated, as in the case of activation of cyclin-dependent kinases by cylclins. 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 phospholipase Cg, CBL, GRB2, SHC and p85. For example, tyrosine-X-X-methionine (where X is any amino acid) is a motif for the p85 binding site. Several signal transducers then bind to these adaptors to initiate multiple signalling pathways, including mitogen-activated protein kinase, phosphatidylinositol 3-kinase/AKT and the signal transducer and activator of transcription (STAT)3 and STAT5 pathways (Fig. 2) [3]. These eventually result in cell proliferation, migration and metastasis, evasion from apoptosis, or in angiogenesis, all of which are associated with cancer phenotypes. ERBB3 lacks tyrosine kinase activity because of substitutions in crucial residues in the tyrosine kinase domain. However, it has many binding sites for p85, a regulatory subunit of phosphatidylinositol 3-kinase, and thus is a preferred dimerization partner.

EGFR overexpression and cancer

EGFR is expressed in a variety of human tumors, including those in the lung, head and neck, colon, pancreas, breast, ovary, bladder and kidney, and in gliomas. EGFR expression and cancer prognosis have been investigated in many human cancers. Although there some discrepancies have been reported, patients with tumors that show high expression of EGFR tend to have a poorer prognosis in general. However, it was not possible to predict super-responder of gefitinib degree of EGFR expression, as determined by immunohistochemistry or immunoblotting.

Mutations of the extracellular domain are frequent in glioblastomas

Three different types of deletion mutations (categorized according to the extent of deletion, and termed *EGFR vI, EGFR vII* and *EGFR vIII*) have been reported in the extracellular domain of the *EGFR* gene [6]. In the *EGFR vI* mutation, the extracellular domain has been totally deleted and resembles the v-erbB oncoprotein. In the *EGFR vII* mutation, 83 amino acids in domain IV of the extracellular domain have been deleted; however, this mutation does not appear to contribute to a malignant phenotype. The most

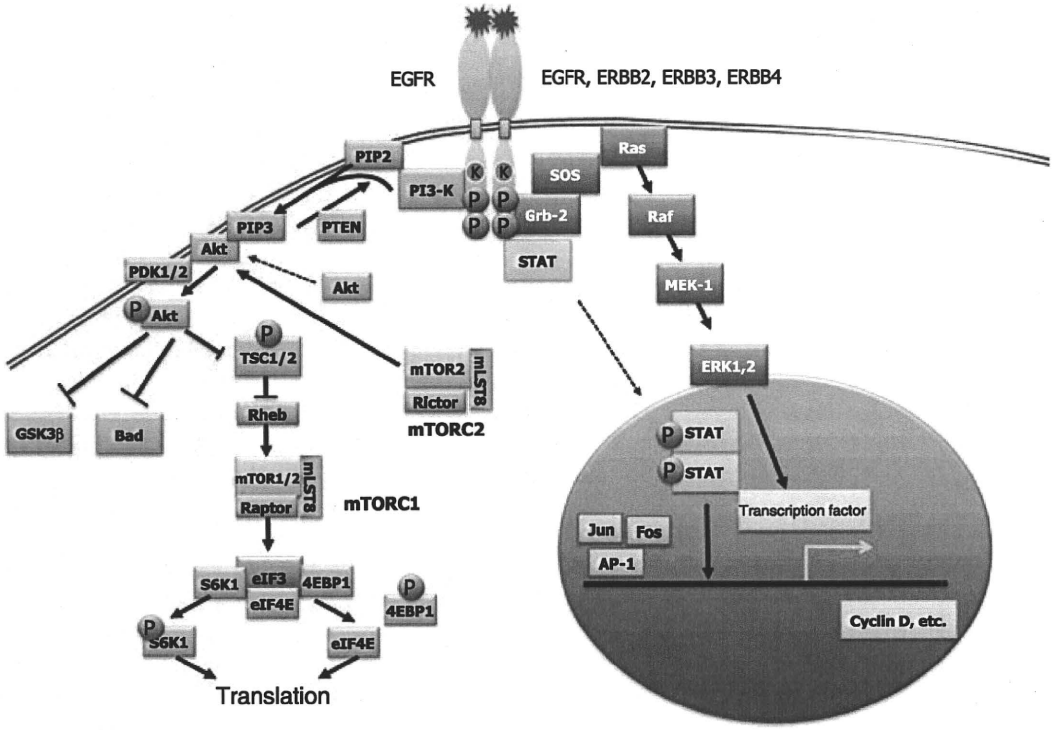

Fig. 2. EGFR and ERBB proteins and their downstream pathways.

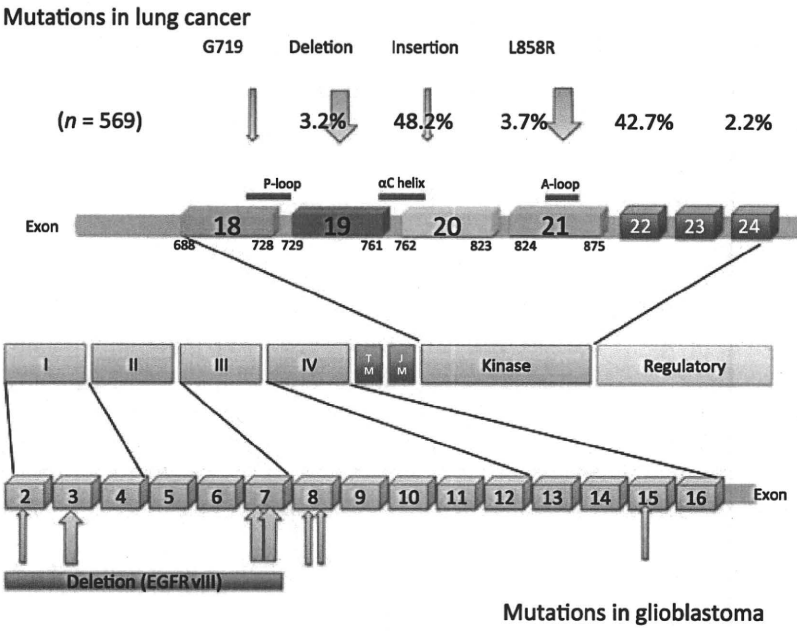

Fig. 3. Distribution and frequency of *EGFR* mutations occurring in the kinase domain in lung cancer (upper part of the figure) [12] and in the extracellular domain in glioblastoma (lower part of the figure) [8].

common of the three types of deletion mutations is *EGFR vIII*. This mutation often accompanies gene amplification, resulting in the overexpression of EGFR lacking amino acids 30–297, corresponding to domains I and II. In this case, the EGFR tyrosine kinase is activated constitutively without ligand binding, as in the case of *EGFR vI*. *EGFR vIII* is reported to occur in 30–50% of glioblastomas [6]. In lung cancers, *EGFR vIII* is found in 5% of squamous cell carcinomas, while none of 123 adenocarcinomas were found to harbor this mutation [7]. It is also known that tissue-specific expression of *EGFR vIII* leads to the development of lung cancer [7]. There is also a suggestion that lung tumors with *EGFR vIII* are sensitive to the irreversible EGFR tyrosine kinase inhibitor (TKI), HKI272, despite the fact these tumors are relatively resistant to the reversible inhibitors, gefitinib and erlotinib [7].

Recently, novel missense mutations in the extracellular domain of the *EGFR* gene have been identified in 13.6% (18/132) of glioblastomas and in 12.5% (1/8) of glioblastoma cell lines [8] (Fig. 3). There appear to be several hot spots: five R108K mutations were found in domain I, three T263P mutations and five A289V/D/T mutations were found in domain II, and two G598V mutations were found in domain IV. These *EGFR* mutations occur independently of *EGFR vIII* and provide an alternative mechanism for *EGFR* activation in glioblastomas [8]. Furthermore, these mutations are associated with increased *EGFR* gene dosage and confer anchorage-independent growth and tumorigenicity to NIH-3T3 cells. Cells transformed by expression of these *EGFR* mutants are sensitive to small-molecule EGFR kinase inhibitors [8]. In contrast, none of 119 primary lung tumors was found to harbor these ectodomain mutations [8].

EGFR mutations in the tyrosine kinase domain

In April 2004, two groups of researchers in Boston [9,10], and subsequently a group in New York [11], reported that activating mutations of the *EGFR* gene are present in a subset of non-small cell lung cancer and that tumors with *EGFR* mutations are highly sensitive to EGFR-TKIs. This discovery solved the enigma of why female, nonsmoking, adenocarcinoma patients of East Asian origin with lung cancers had a higher response to EGFR-TKIs, because patients with these characteristics have a higher incidence of *EGFR* mutations. Figure 4 shows the incidence of *EGFR* mutations found in 559 mutations in 2880 lung cancer patients in the literature [12]. It is also intriguing that *EGFR* mutations in the tyrosine kinase domain are almost exclusively seen in lung cancers and not in other types of tumor.

It is of particular interest that *EGFR* mutations are the first molecular aberrations found in lung cancer that are more frequent among patients without a smoking history than among those with one. Furthermore, the *EGFR* mutation frequency is inversely associated with the total amount of tobacco smoked [13]. However, it should be noted that *EGFR* mutations

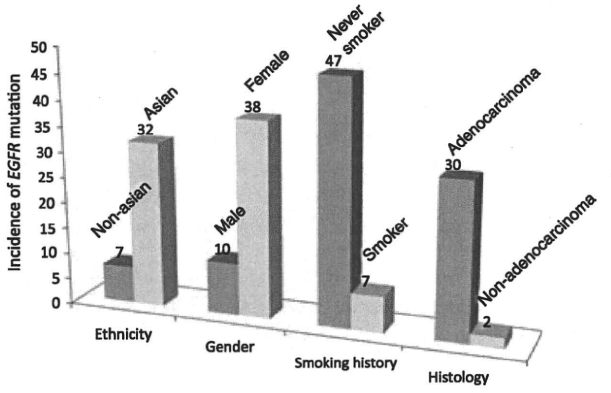

Fig. 4. Incidences of *EGFR* mutations in lung cancer in various different clinical backgrounds [12]. Hx, history; adeno, adenocarcinoma.

have been detected in more than 20% of patients with a history of heavy smoking [13]. These findings do not necessarily 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 indicate that the apparent negative correlation with smoking dose occurs as a result of diluting the number of tumors containing *EGFR* mutations with an increased number of tumors containing wild-type *EGFR* as the smoking dose increases. Indeed, this was shown in our case–control study [14].

Pathology of lung cancers with *EGFR* gene mutations

Bronchioloalveolar cell carcinoma (BAC) is defined as a carcinoma *in situ* without stromal, vascular or pleural invasion, showing growth of neoplastic cells along pre-existing alveolar structures (lepidic growth). Although it is relatively rare to present with pure BAC, invasive adenocarcinomas with areas exhibiting lepidic growth are frequently seen. This type of adenocarcinoma is sometimes referred to as an adenocarcinoma with BAC features. Such tumors respond more to gefitinib than do other types of adenocarcinoma [15] and thus have a higher incidence of *EGFR* mutations. As expected, adenocarcinomas with BAC features are more common in adenocarcinomas of never-smoking patients (13%) than in smokers (5%).

We proposed a terminal respiratory unit (TRU)-type of adenocarcinoma [16]. This type of cancer is characterized by distinct cellular features (expression of thyroid transcription factor 1 and surfactant proteins, and lepidic growth in the periphery), and it resembles adenocarcinomas with nonmucinous BAC features. Although, according to the World Health Organization classification, mucinous BACs form a subset of BACs, this type of BAC does not express thyroid transcription factor 1 or surfactant apoprotein, and is thus not a TRU-type adenocarcinoma. It is also known that *KRAS* mutations are more frequent in mucinous BAC than in nonmucinous BAC.

In our series of 195 adenocarcinomas, 149 were of the TRU type and 46 were of other types [17]. TRU-type adenocarcinomas are associated with a significantly higher incidence of female patients, never-smokers and *EGFR* mutations, but with fewer *KRAS* and *TP53* mutations than other types of adenocarcinoma [17]. An *EGFR* mutation was detected in 97/195 adenocarcinomas, in 91/149 TRU-type adenocarcinomas and in 6/46 tumors of other types. Conversely, 91/97 *EGFR*-mutated adenocarcinomas were categorized as TRU-type adenocarcinomas [17]. In addition, *EGFR* mutations were detected in some cases of atypical adenomatous hyperplasias known to be precursor lesions for BAC [17]. These findings further confirm that the TRU-type adenocarcinoma is a distinct adenocarcinoma subset involving a particular molecular pathway. It is of note that *EGFR* mutations can also occur in poorly differentiated adenocarcinomas, as long as the tumor belongs to the TRU cellular lineage.

Types of *EGFR* mutations

EGFR mutations are mainly present in the first four exons of the gene encoding the tyrosine kinase domain (Fig. 3) [12]. About 90% of the *EGFR* mutations are either small deletions encompassing five amino acids from codons 746–750 (ELREA) or missense mutations resulting in a substitution of leucine with arginine at codon 858 (L858R). There are more than 20 variant types of deletion, including larger deletions, deletions plus point mutations and deletions plus insertions. About 3% of the mutations occur at codon 719, resulting in the substitution of glycine with cysteine, alanine or serine (G719X). In addition, about 3% are in-frame insertion mutations in exon 20. These four types of mutations seldom occur simultaneously. There are many rare point mutations, some of which occur together with L858R [12].

Exon 19 deletional mutation and L858R result in increased and sustained phosphorylation of EGFR and other ERBB family proteins without ligand stimulation. It has been shown that mutant EGFR selectively activates the AKT and STAT signaling pathways that promote cell survival, but has no effect on the mitogen-activated protein kinase pathway that induces cell proliferation [18]. EGFR mutants in the

kinase domain are oncogenic [19]. The mutant EGFR protein can transform both fibroblasts and lung epithelial cells in the absence of exogenous EGFR, as evidenced by anchorage-independent growth, focus formation and tumor formation in immunocompromised mice [19]. Transformation is associated with constitutive autophosphorylation of EGFR, SHC phosphorylation and STAT pathway activation [19]. Whereas transformation by most EGFR mutants confers cell sensitivity to erlotinib and gefitinib, transformation by an exon 20 insertion (D770insNPG) makes cells resistant to these inhibitors but more sensitive to the irreversible inhibitor CL-387,785 [19]. In that study, the G719S mutation of exon 18 showed intermediate sensitivity *in vitro* [19]. However, the authors did not observe any difference between the exon 19 deletion and L858R in their cell-based assay. However, biochemical analysis of the kinetics of purified wild-type and mutant kinases revealed that mutant kinases have a higher K_m for ATP (wild-type, 5 $\mu mol \cdot L^{-1}$; L858R, 10.9 $\mu mol \cdot L^{-1}$; deletion, 129.0 $\mu mol \cdot L^{-1}$) and a lower K_i for erlotinib (wild-type, 17.5 $\mu mol \cdot L^{-1}$; L858R, 6.25 $\mu mol \cdot L^{-1}$; deletion, 3.3 $\mu mol \cdot L^{-1}$;) [20]. Mulloy *et al.* [21] showed that the Del747–753 kinase had a higher autophosphorylation rate and higher sensitivity to erlotinib than L858R kinase. These data reflect differences in the clinical response rate between the exon 19 deletion and L858R.

Oncogenic activity of *EGFR* mutants has also been shown *in vivo*. Two groups of researchers have developed transgenic mice that express either the exon 19 deletion mutant or the L858R mutant in type II pneumocytes under the control of doxycyclin [22,23]. Expression of either *EGFR* mutant led to the development of adenocarcinomas similar to human BACs, and the withdrawal of doxycycline to reduce expression of the transgene, or erlotinib treatment, resulted in tumor regression. These experiments show that persistent EGFR signaling is required for tumor maintenance in human lung adenocarcinomas expressing *EGFR* mutants.

EGFR gene copy numbers

EGFR amplification is detectable in 40% of human gliomas and is often associated with deletion mutations, as discussed below. When the topographical distribution of *EGFR* amplification in lung cancers with confirmed mutations was examined, gene amplification was found in 11 of 48 specimens [24]. Nine of the cancers showed heterogeneous distribution, and amplification was associated with higher histological tumor grades or invasive growth [24]. However, the amplification status of the metastatic lymph node was not always associated with gene amplification of the primary tumors [24]. Only one of 21 carcinomas *in situ*, and none of 17 precursor lesions, harbored gene amplifications [24]. These results suggest that mutations occur early in the development of lung adenocarcinomas and that amplification might be acquired in association with tumor progression.

Relationship between EGFR and mutations of the related genes

The activating mutation of the *KRAS* gene was one of the earliest discoveries of genetic alterations in lung cancer, and has been known as a poor prognostic indicator since 1990 [25]. We were the first group to report that the occurrence of *EGFR* and *KRAS* mutations are strictly mutually exclusive [13]. One explanation is that the KRAS–mitogen-activated protein kinase pathway is one of the downstream signaling pathways of EGFR. Interestingly, *KRAS* mutations predominantly occur in White people with a history of smoking. Mutations of the *ERBB2* gene are present in a very small fraction ($\sim$ 3%) of adenocarcinomas and they appear to target the same population targeted by *EGFR* mutations: never-smokers and female patients [26]. Most of the *ERBB2* mutations are insertion mutations in exon 20 [26]. As anticipated, tumors with *ERBB2* mutations are resistant to treatment with EGFR-TKIs [27] because constitutively activated ERBB2 kinase will phosphorylate other ERBB family proteins, resulting in the activation of downstream molecules even when the EGFR tyrosine kinase is blocked. Mutation of the *BRAF* gene occurs in about 1–3% of lung adenocarcinomas.

By retrieving transforming genes from mouse 3T3 fibroblasts transfected with a cDNA expression library constructed from a lung adenocarcinoma arising in a male smoker, Soda *et al.* [28] identified the gene resulting from the fusion of that for transforming echinoderm microtubule-associated protein-like 4 (*EML4*) and the gene for anaplastic lymphoma kinase (*ALK*). This *EML4–ALK* fusion gene resulted from a small inversion within chromosome 2p. The *EML4–ALK* fusion transcript is detected in about 5% of non-small cell lung cancers. *ALK* translocation was associated with patients being never-smokers of a younger age and acinar-type adenocarcinomas, in a larger study [29]. It is also noteworthy that *EGFR*, *ERBB2*, *BRAF*, *KRAS* and *ALK* mutations almost never occur simultaneously in individual patients, suggesting a complementary role of these mutations in lung carcinogenesis.

Conclusions

In this minireview, we have described how Cohen's discovery of the 'tooth-lid factor' led to the identification of the genetic causes of certain types of human cancers, and to the genetic classification of a variety of tumors of apparently the same phenotype that has significant therapeutic implications.

References

1 Gschwind A, Fischer OM & Ullrich A (2004) The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nat Rev* **4**, 361–370.

2 Burgess AW, Cho H-S, Elgenblot C, Ferguson KM, Garrett TPJ, Leahy DJ, Lemmon MA, Siwkowski MX, Ward CW & Yokoyama S (2003) An open-and -shut case? Recent insights into the activation of EGF/ErbB receptors *Mol Cell* **12**, 541–552.

3 Hynes NE & Lane HA (2005) ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev* **5**, 341–354.

4 Schneider MR & Wolf E (2009) The epidermal growth factor receptor ligands at a glance. *J Cell Physiol* **218**, 460–466.

5 Zhang X, Gureasko J, Shen K, Cole PA & Kuriyan J (2006) An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* **125**, 1137–1149.

6 Voldborg BR, Damstrup L, Spang-Thomsen M & Poulsen HS (1997) Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials. *Ann Oncol* **8**, 1197–1206.

7 Ji H, Zhao X, Yuza Y, Shimamura T, Li D, Protopopov A, Jung BL, McNamara K, Xia H, Glatt KA *et al.* (2006) Epidermal growth factor receptor variant III mutations in lung tumorigenesis and sensitivity to tyrosine kinase inhibitors. *Proc Natl Acad Sci USA* **103**, 7817–7822.

8 Lee JC, Vivanco I, Beroukhim R, Huang JH, Feng WL, DeBiasi RM, Yoshimoto K, King JC, Nghiemphu P, Yuza Y *et al.* (2006) Epidermal growth factor receptor activation in glioblastoma through novel missense mutations in the extracellular domain. *PLoS Med* **3**, e485.

9 Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG *et al.* (2004) Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N Engl J Med* **350**, 2129–2139.

10 Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ *et al.* (2004) EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science (New York, NY)* **304**, 1497–1500.

11 Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, Singh B, Heelan R, Rusch V, Fulton L *et al.* (2004) EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci USA* **101**, 13306–13311.

12 Mitsudomi T & Yatabe Y (2007) Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensitivity in lung cancer. *Cancer Sci* **98**, 1817–1824.

13 Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T & Mitsudomi T (2004) Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. *Cancer Res* **64**, 8919–8923.

14 Matsuo K, Ito H, Yatabe Y, Hiraki A, Hirose K, Wakai K, Kosaka T, Suzuki T, Tajima K & Mitsudomi T (2006) Risk factors differ for non-small-cell lung cancers with and without EGFR mutation: assessment of smoking and sex by a case-control study in Japanese. *Cancer Sci* **98**, 96–101.

15 Miller VA, Kris MG, Shah N, Patel J, Azzoli C, Gomez J, Krug LM, Pao W, Rizvi N, Pizzo B *et al.* (2004) Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. *J Clin Oncol* **22**, 1103–1109.

16 Yatabe Y, Mitsudomi T & Takahashi T (2002) TTF-1 expression in pulmonary adenocarcinomas. *Am J Surg Pathol* **26**, 767–773.

17 Yatabe Y, Kosaka T, Takahashi T & Mitsudomi T (2005) EGFR mutation is specific for terminal respiratory unit type adenocarcinoma. *Am J Surg Pathol* **29**, 633–639.

18 Sordella R, Bell DW, Haber DA & Settleman J (2004) Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science (New York, NY)* **305**, 1163–1167.

19 Greulich H, Chen T-H, Feng W, Janne PA, Alvarez JV, Bulmer SE, Zappaterra M, Frank DA, Hahn WC, Sellers WR *et al.* (2005) Oncogenic transformation by inhibitor-sensitive and resistant EGFR mutations. *PLoS Med* **2**, e313.

20 Carey KD, Garton AJ, Romero MS, Kahler J, Thomson S, Ross S, Park F, Haley JD, Gibson N & Sliwkowski MX (2006) Kinetic analysis of epidermal growth factor receptor somatic mutant proteins shows increased sensitivity to the epidermal growth factor receptor tyrosine kinase inhibitor, erlotinib. *Cancer Res* **66**, 8163–8171.

21 Mulloy R, Ferrand A, Kim Y, Sordella R, Bell DW, Haber DA, Anderson KS & Settleman J (2007) Epidermal growth factor receptor mutants from human lung cancers exhibit enhanced catalytic activity and increased sensitivity to gefitinib. *Cancer Res* **67**, 2325–2330.

22 Ji H, Li D, Chen L, Shimamura T, Kobayashi S, McNamara K, Mahmood U, Mitchell A, Sun Y, Al-Hashem R *et al.* (2006) The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. *Cancer Cell* **9**, 485–495.
23 Politi K, Zakowski MF, Fan PD, Schonfeld EA, Pao W & Varmus HE (2006) Lung adenocarcinomas induced in mice by mutant EGF receptors found in human lung cancers respond to a tyrosine kinase inhibitor or to down-regulation of the receptors. *Genes Dev* **20**, 1496–1510.
24 Yatabe Y, Takahashi T & Mitsudomi T (2008) Epidermal growth factor receptor gene amplification is acquired in association with tumor progression of EGFR-mutated lung cancer. *Cancer Res* **68**, 2106–2111.
25 Slebos RJ, Kibbelaar RE, Dalesio O, Kooistra A, Stam J, Meijer CJ, Wagenaar SS, Vanderschueren RG, van Zandwijk N, Mooi WJ *et al.* (1990) K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. *N Engl J Med* **323**, 561–565.
26 Shigematsu H, Takahashi T, Nomura M, Majmudar K, Suzuki M, Lee H, Wistuba II, Fong KM, Toyooka S, Shimizu N *et al.* (2005) Somatic mutations of the HER2 kinase domain in lung adenocarcinomas. *Cancer Res* **65**, 1642–1646.
27 Han SW, Kim TY, Jeon YK, Hwang PG, Im SA, Lee KH, Kim JH, Kim DW, Heo DS, Kim NK *et al.* (2006) Optimization of patient selection for gefitinib in non-small cell lung cancer by combined analysis of epidermal growth factor receptor mutation, K-ras mutation, and Akt phosphorylation. *Clin Cancer Res* **12**, 2538–2544.
28 Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, Fujiwara SI, Watanabe H, Kurashina K, Hatanaka H *et al.* (2007) Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. *Nature* **448**, 561–566.
29 Inamura K, Takeuchi K, Togashi Y, Hatano S, Ninomiya H, Motoi N, Mun MY, Sakao Y, Okumura S, Nakagawa K *et al.* (2009) EML4-ALK lung cancers are characterized by rare other mutations, a TTF-1 cell lineage, an acinar histology, and young onset. *Mod Pathol* **22**, 508–515.

Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial

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Summary

Background Patients with non-small-cell lung cancer harbouring mutations in the epidermal growth factor receptor (*EGFR*) gene respond well to the EGFR-specific tyrosine kinase inhibitor gefitinib. However, whether gefitinib is better than standard platinum doublet chemotherapy in patients selected by EGFR mutation is uncertain.

Methods We did an open label, phase 3 study (WJTOG3405) with recruitment between March 31, 2006, and June 22, 2009, at 36 centres in Japan. 177 chemotherapy-naive patients aged 75 years or younger and diagnosed with stage IIIB/IV non-small-cell lung cancer or postoperative recurrence harbouring *EGFR* mutations (either the exon 19 deletion or L858R point mutation) were randomly assigned, using a minimisation technique, to receive either gefitinib (250 mg/day orally; n=88) or cisplatin (80 mg/m², intravenously) plus docetaxel (60 mg/m², intravenously; n=89), administered every 21 days for three to six cycles. The primary endpoint was progression-free survival. Survival analysis was done with the modified intention-to-treat population. This study is registered with UMIN (University Hospital Medical Information Network in Japan), number 000000539.

Findings Five patients were excluded (two patients were found to have thyroid and colon cancer after randomisation, one patient had an exon 18 mutation, one patient had insufficient consent, and one patient showed acute allergic reaction to docetaxel). Thus, 172 patients (86 in each group) were included in the survival analyses. The gefitinib group had significantly longer progression-free survival compared with the cisplatin plus docetaxel goup, with a median progression-free survival time of 9·2 months (95% CI 8·0–13·9) versus 6·3 months (5·8–7·8; HR 0·489, 95% CI 0·336–0·710, log-rank p<0·0001). Myelosuppression, alopecia, and fatigue were more frequent in the cisplatin plus docetaxel group, but skin toxicity, liver dysfunction, and diarrhoea were more frequent in the gefitinib group. Two patients in the gefitinib group developed interstitial lung disease (incidence 2·3%), one of whom died.

Interpretation Patients with lung cancer who are selected by EGFR mutations have longer progression-free survival if they are treated with gefitinib than if they are treated with cisplatin plus docetaxel.

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Introduction

Lung cancer is a major cause of cancer-related mortality worldwide.[1] However, current standard platinum doublet therapy seems to have reached a therapeutic plateau,[2] although it has recently been shown that patients with non-squamous histology who are treated with pemetrexed disodium have better survival than if they are treated with older drugs.[3]

Targeted therapies are actively being developed to improve efficacy in selected patient populations.[4] Small-molecule tyrosine kinase inhibitors (TKIs) that target the epidermal growth factor receptor (EGFR), such as gefitinib and erlotinib, are the first targeted drugs to enter clinical use for the treatment of lung cancer. Subgroups of patients of east-Asian origin, female sex, adenocarcinoma, and no history of smoking have been shown to be significantly associated with a favourable response to EGFR TKIs.[5,6] In 2004, researchers noted that activating mutations of the *EGFR* gene present predominantly in patients with the above-mentioned clinical characteristics, and determine sensitivity to EGFR TKIs.[7,8] *EGFR* mutations are present in the first four exons of the tyrosine kinase domain of the *EGFR* gene, and about 90% of these *EGFR* mutations are either short in-frame deletions in exon 19, or point mutations that result in a substitution of arginine for leucine at aminoacid 858 (L858R).[7–9] Subsequent retrospective and prospective trials confirmed that the response rate to gefitinib or erlotinib in patients with *EGFR* mutations is about 70–80%.[10–13] Furthermore, patients with *EGFR* mutations have a significantly longer survival than those with wild-type *EGFR* when treated

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with EGFR TKIs.[14,15] We proposed that the absence of any survival advantage conferred by gefitinib monotherapy in previous studies[16–18] is due at least in part to a lack of patient selection, and that gefitinib would confer a survival advantage compared with platinum doublet chemotherapy in a first-line setting if eligible patients were selected on the basis of *EGFR* mutation status. To address this issue, we did a phase 3 trial that compared gefitinib with cisplatin plus docetaxel in patients with an *EGFR* mutation.

Methods

Patients

This study (WJTOG 3405) was a multicentre, randomised, open-label, phase 3, trial of first-line treatment with gefitinib versus cisplatin plus docetaxel for patients with advanced or recurrent non-small-cell lung cancer (NSCLC) harbouring an activating mutation of the *EGFR* gene. We recruited patients between March 31, 2006, and June 22, 2009, at 36 centres in Japan. All centres were members of the West Japan Oncology Group (WJOG), which is a Japanese non-profit organisation for oncological clinical trials (formerly the West Japan Thoracic Oncology Group, or WJTOG).

Initially, only patients with postoperative recurrence were eligible, because these surgical specimens were expected to ensure good sample quality. However, because of the initial slow accrual, the protocol was amended on July 10, 2006, to include patients with stage IIIB/IV disease. Patients were eligible if they had histologically or cytologically confirmed NSCLC, harbouring activating *EGFR* mutations (either exon 19 deletion or L858R in exon 21), were aged 75 years or younger, had WHO performance status 0–1, had measurable or non-measurable disease according to the Response Evaluation Criteria in Solid Tumours (RECIST), and had adequate organ function. Patients with postoperative recurrence, treated with adjuvant therapy other than cisplatin plus docetaxel, were included when the interval between the end of adjuvant chemotherapy and registration exceeded 6 months for platinum-doublet

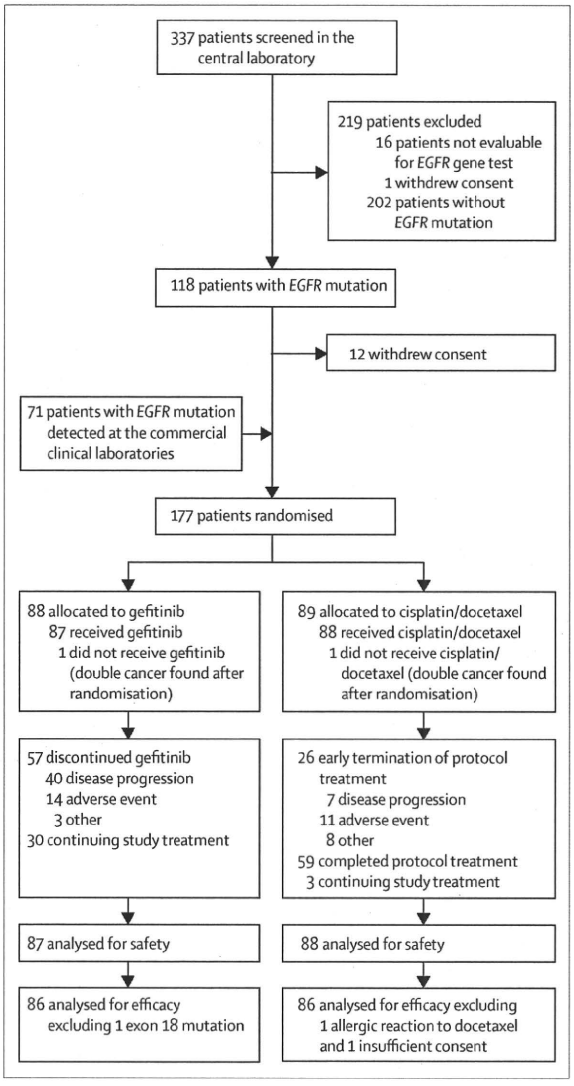

Figure 1: **Trial profile**

	Gefitinib (N=86)	Cisplatin plus docetaxel (N=86)
Sex		
Male	27	26
Female	59	60
Age (years; median; range)	64·0 (34–74)	64·0 (41–75)
Histological type		
Adenocarcinoma	83	84
Adenosquamous carcinoma	0	1
Squamous-cell carcinoma	1	0
Non-small-cell lung cancer; not otherwise specified	2	1
Smoking history		
Never	61	57
Former/current	25	29
Performance status		
0	56	52
1	30	34
Stage		
Postoperative recurrence	35	36
With postoperative adjuvant chemotherapy	19	23
Without postoperative adjuvant chemotherapy	16	13
IIIB	10	9
IV	41	41
EGFR mutation		
Exon 19 deletion	50	37
L858R	36	49

Table 1: **Demographic and baseline characteristics of the modified intention-to-treat population**

therapy and more than 1 month for oral tegafur plus uracil therapy. Patients were not eligible if they had received previous drug therapy that had targeted EGFR, had a history of interstitial lung disease, severe drug allergy, active infection or other serious disease condition, symptomatic brain metastases, poorly controlled pleural effusion, pericardial effusion or ascites necessitating drainage, active double cancer, or severe hypersensitivity to drugs containing polysolvate 80. Patients in pregnancy or lactation, or whose participation in the trial was judged to be inappropriate by the attending doctor, were not eligible. All patients provided written informed consent. Study approval was obtained from independent ethics committees at every institution. The study was undertaken in accordance with the Declaration of Helsinki.

Procedures

Patients were randomly assigned in a 1:1 ratio to receive gefitinib (250 mg/day, administered orally), or docetaxel (60 mg/m², administered intravenously over a 1 h period) followed by cisplatin (80 mg/m², administered intravenously over a 90-min period), with adequate hydration, in cycles of once every 21 days for three to six cycles.Treatment continued until progression of the disease, development of unacceptable toxic effects, a request by the patient to discontinue treatment, serious non-compliance with the protocol, or completion of three to six chemotherapy cycles. Further therapy after progression of the disease was at the physician's discretion. The primary endpoint was progression-free survival. Secondary endpoints included overall survival and response rate. Tertiary endpoints were disease control rate, safety, and mutation-type-specific survival.

Initially, patients were screened for *EGFR* mutation in a central laboratory at the Department of Molecular Diagnostics, Aichi Cancer Centre Hospital, Nagoya, Japan. The exon 19 deletion mutation was screened by fragment analysis and the L858R point mutation was screened by the Cycleave method, as described previously,[19] followed by confirmation by direct sequencing. On Feb 16, 2008, the protocol was amended to allow outsourcing of *EGFR* genetic testing from each institution to commercial clinical laboratories, either at SRL in Tokyo (direct sequencing), Mitsubishi Chemical Medience in Tokyo (peptide nucleic acid-locked nucleic acid PCR clamp[20]), or BML in Tokyo (PCR invader[21]), as this amendment would further facilitate patient accrual. The sensitivity of direct sequencing was anticipated to be less than that of other methods; however, false negativity was not a problem in this trial, since patients judged to lack EGFR mutations were not randomly allocated to a treatment.

Progression-free survival was assessed from the date of randomisation to the earliest sign of disease progression as determined by CT or MRI imaging using RECIST criteria, or death from any cause. Overall survival was assessed from the date of randomisation until death from any cause. Tumour response was assessed every 2 months

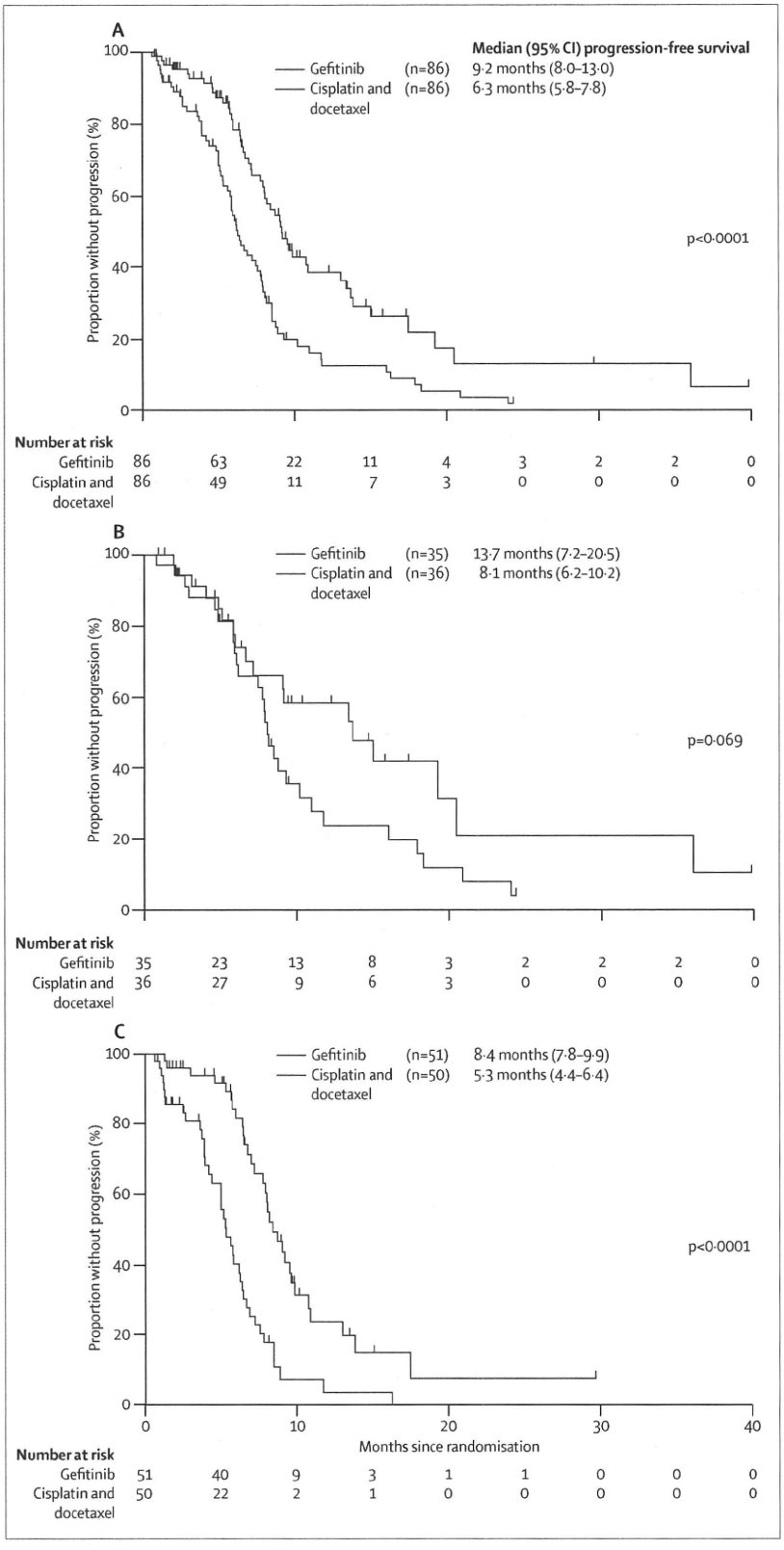

Figure 2: **Progression-free survival in the overall population (A), in patients with postoperative recurrence (B), and in patients with stage IIIB/IV disease (C)**

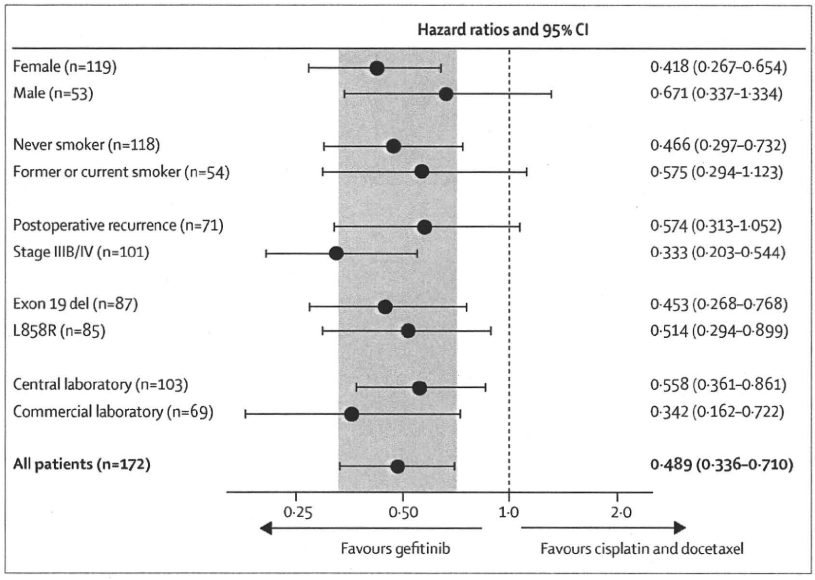

Figure 3: **Hazard ratios for progression-free survival using subgroup analysis in the overall population**
The shaded band represents the 95% CI of the hazard ratio for the overall population of patients.

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p	HR (95% CI)	p
Group (gefitinib/cisplatin plus docetaxel)	0·489 (0·336–0·710)	0·0002	0·258 (0·385–0·575)	<0·0001
Sex (male/female)	0·935 (0·625–1·398)	0·742	0·628 (0·361–1·092)	0·099
Age (<65 years /≥65 years)	1·091 (0·757–1·572)	0·641	1·183 (0·813–1·721)	0·380
Smoking history (never/former or current)	0·801 (0·541–1·186)	0·268	0·646 (0·378–1·105)	0·111
Stage (recurrence/IIIB–IV)	0·463 (0·220–0·976)	0·043	0·433 (0·290–0·649)	<0·0001
Mutation (exon 19 del/L858R)	1·001 (0·694–1·444)	0·996	1·135 (0·777–1·658)	0·514

Table 2: **Univariate and multivariate analysis of progression-free survival**

during the first year after randomisation, every 3 months between 12 and 18 months, and thereafter the interval of assessment was at the physician's discretion. Safety and tolerability were assessed according to National Cancer Institute Common Terminology Criteria (CTC) for Adverse Events, version 3.0. All events were confirmed via source-document verification at site visits to each participating institution by members of the WJOG data centre and the investigators.

Randomisation and masking

The investigator provided the necessary information to personnel at the WJOG data centre by fax. After an eligibility check, patients were allocated at the WJOG data centre to each treatment group using a desktop computer programmed for the minimisation method.[22] In this way, patient allocation was concealed from the investigator.

Because of the nature of treatment in each group, the study was open label. Stratification factors were: institution; postoperative adjuvant chemotherapy (presence *vs* absence); interval between surgery and recurrence (≥1 *vs* <1 year) for patients with postoperative recurrent disease; and institution; stage (IIIB *vs* IV); and sex (male *vs* female) for patients with stage IIIB/IV disease.

Statistical analysis

In previous studies the progression-free survival of patients harbouring EGFR mutations and treated with gefitinib was reported as 12·6 months,[15] compared with 6·6 months for patients harbouring EGFR mutations treated with carboplatin plus paclitaxel.[23] Assuming a progression-free survival for gefitinib and platinum doublet chemotherapy of 12·5 and 7 months, respectively, would yield a hazard ratio (HR) of 0·56. Taking this HR into consideration, 146 patients would be required to achieve 90% power to show superiority with α=0·05 (two-sided). Therefore, sample size was initially set at 200 patients. While this trial was ongoing, the results of the Iressa Pan-Asia Study (IPASS) were presented at the annual meeting of the European Society for Medical Oncology (Stockholm, Sweden, Sept 12–16, 2008), and were later published.[24] Subgroup analysis of patients with EGFR mutations using about a third of the patients showed that the HR of gefitinib compared with carboplatin plus paclitaxel for progression-free survival was 0·48. Similarly, the HR of gefitinib compared with carboplatin plus paclitaxel for progression-free survival in patients with EGFR mutations was 0·36 in the study done by the North East Japan (NEJ) 002 Gefitinib Study Group, which was presented at the annual meeting of the American Society of Clinical Oncology (Orlando, FL, USA, May 29–June 2, 2009).[25] NEJ 002 was a phase 3 trial that analysed 198 patients with EGFR mutation randomised either to gefitinib or carboplatin plus paclitaxel. 177 patients had been randomised in our trial as of June 13, 2009, and 79 events had been noted during the regular monitoring done in March, 2009. The number of events needed to detect a conservative HR of 0·48 was calculated to be 78, based on normal approximation of the logarithm of the hazard ratio under α=0·05 (two-sided) and 90% power. Therefore, further accrual of patients was considered to be futile and potentially unethical. Although interim analysis was originally planned to analyse progression-free survival, this analysis was not done. Instead, the steering committee held on June 13, 2009, proposed the amendment of the sample size and the final analyses be done using available data. This proposal was approved by the independent data and safety monitoring committee on Aug 28, 2009. The data were locked on June 30, 2009. Patient follow-up for safety and survival will continue until 1·5 years after the last patient entry, as originally described in the study protocol.

Progression-free and overall survival were analysed for the modified intention-to-treat population as defined previously.[26] They were analysed using the Kaplan-Meier method, and were compared using the log-rank test. Hazard ratios in the overall population and in patient