Double *EGFR* mutants containing rare *EGFR* mutant types show reduced *in vitro* response to gefitinib compared with common activating missense mutations

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Abstract

Epidermal growth factor receptor (*EGFR*) mutations are common in lung adenocarcinomas, especially from nonsmoking women of Asian descent. We have previously shown *EGFR* mutations occur in >70% of lung adenocarcinoma from nonsmokers in our population with a complex mutational profile, including 13% of *EGFR* double mutations. In this study, we investigated the *in vitro* gefitinib response of four *EGFR* double mutants identified in untreated patients, including Q787R+L858R, E709A+G719C, T790M+L858R, and H870R+L858R. The phosphorylation profiles of EGFR and downstream effectors AKT, STAT3/5, and ERK1/2 were compared by immunoblot analyses among the single and double mutants transfected into H358 cells. Results showed that mutants responded to *in vitro* gefitinib treatment with different sensitivities. The G719C and L858R single mutants showed the highest gefitinib sensitivity compared with the corresponding coexisting single mutants E709A, Q787R, H870R, and T790M. The double mutants E709A+G719C, Q787R+L858R, and H870R+L858R showed attenuated responses to gefitinib in the EGFR and downstream effector phosphorylation profiles compared with G719C or L858R alone. T790M+L858R showed strong resistance to gefitinib. Clinically, the patient whose tumor contained H870R+L858R showed tumor stabilization by 250 mg oral gefitinib daily but cerebral metastasis developed 6 months later. Correlation with the *in vitro* phosphorylation profile of H870R +L858R suggested that treatment failure was probably due to inadequate suppression of EGFR signaling by the drug level attainable in the cerebrospinal fluid at the given oral dosage. Overall, the findings suggested that rare types of *EGFR* substitution mutations could confer relative gefitinib resistance when combined with the common activating mutants. [Mol Cancer Ther 2009;8(8):2142–51]

Introduction

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that transmits extracellular signals through ligand binding, receptor dimerization, and triggering of phosphorylation events, resulting in the activation of multiple downstream signaling pathways that control cell proliferation, differentiation, and survival (1, 2). Clinical studies on monotherapy of tyrosine kinase inhibitors targeting EGFR as first-line regimen in chemonaive non–small cell lung carcinoma (NSCLC) patients showed that preselection of patients based on *EGFR* mutations in the TK domain is a reliable predictor for tyrosine kinase inhibitor response (3). Small in-frame deletions in exon 19 and L858R in exon 21 of the *EGFR* gene together comprise 80% to 90% of all *EGFR* mutations and are shown to confer tyrosine kinase inhibitor sensitivity *in vitro* (4). On the other hand, the coexistence of D761Y and T790M with L858R are associated with tyrosine kinase inhibitor resistance (5–7). Different *EGFR* mutations could serve as positive or negative predictors of tyrosine kinase inhibitor responsiveness.

We and others have reported that lung cancers from ethnic Chinese patients encompass a complex *EGFR* mutational profile with many uncommon mutation types that have not yet been characterized. In this study, we investigated the *in vitro* tyrosine kinase inhibitor response patterns of novel *EGFR* double mutants that were identified in our population. Three of these were in-*cis* double mutations, including Q787R+L858R, E709A+G719C, and T790M+L858R, found *de novo* in patients without previous drug treatment (8). Another double mutant consisting of H870R+L858R was identified from the adenocarcinoma of a chronic smoker who initially responded to 250 mg daily gefitinib but who developed leptomeningeal tumor metastasis 6 months later. The effects of these mutations in EGFR activation, as well as the effects of gefitinib administration on tyrosine

phosphorylation in the mutant EGFR and downstream effectors, were investigated *in vitro*.

Materials and Methods

Sample Collection

DNA of cases E109, E120, and E297 were extracted from fresh-frozen tumors resected for curative purposes from previously untreated patients in the Grantham Hospital, Hong Kong, in 2002 to 2006. DNA of case E1283 was extracted from formalin-fixed, paraffin-embedded tumor tissue microdissected from the pleural biopsy. The study had been approved by the Institutional Review Board jointly administered by the Hospital Authority, Hong Kong West Cluster, and Faculty of Medicine, The University of Hong Kong. An informed written consent was obtained from each recruited patient.

Bidirectional Sequencing

Sequencing of the clinical samples was done using *EGFR* intron-based primers previously described by Tam et al. (8). Mutant constructs were sequenced using primers shown in Supplementary Table S1 to ensure there was no mutation in the vector region. Briefly, after PCR, products were treated with exonuclease I and shrimp alkaline phosphatase at 37°C for 15 min, followed by heating at 80°C for 15 min to stop the enzymatic reaction. After treatment, PCR products were sequenced using Big Dye Terminator v3.1 sequencing kit (Applied Biosystems). PCR and sequencing reaction were done twice to confirm the *EGFR* mutations. Previous subcloning experiments of the cDNA showed that the double mutations occurred on the same allele for cases E109, E120, and E297 (in *cis*; ref. 8). Tumor cDNA was unavailable for E1283 for subcloning studies.

Site-Directed Mutagenesis

Full-length cDNA constructs for *EGFR* mutants E709A, G719C, E709A+G719C, Q787R, L858R, Q787R+L858R, T790M, T790M+L858R, H870R, and H870R+L858R were generated by site-directed mutagenesis using the Quik-Change II XL Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's recommendations and the commercially available vector pUSEamp with full-length *EGFR* cDNA (Upstate) as template. Briefly, mutant strand synthesis reaction was done by initial denaturation at 95°C for 1 min, followed by 18 thermal cycles at 95°C for 50 s, 60°C for 50 s, and 68°C for 9 min 24 s, with a final extension at 68°C for 7 min. *EGFR* mutant constructs created by site-directed mutagenesis were fully sequenced for verification. The mutagenic oligo sequences for the mutant constructs were presented in Supplementary Table S2.

Cell Culture, Reagents, and Transfection

The human lung carcinoma cell line H358 was maintained in RPMI 1860 with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C, 5% CO_2. Serum starvation referred to incubating the cells in serum-free media with penicillin and streptomycin for 24 h. Epidermal growth factor (EGF) stimulation referred to introducing 100 ng/mL EGF to serum starved cells for 30 min at 37°C, 5% CO_2. Gefitinib (ZD1839, Iressa) was a gift from AstraZeneca. The vectors were transfected into H358 cells transiently by GeneJuice (Novagen). Cells were seeded at 5×10^5 cells/well in a 6-well plate with 3 mL RPMI full medium and were allowed to attach for 24 h. At room temperature, 100 μL RPMI medium and 3 μL GeneJuice were mixed and allowed to settle for 5 min; then, 1 μg pUSEamp with full-length *EGFR* cDNA or *EGFR* mutant constructs were mixed with the transfection reagent for another 10 min. Transfection mixture was added to wells and was removed 24 h posttransfection. Cells were then treated according to experimental purposes and collected for Western blotting analysis.

Antibodies

To ensure clarity in the numbering system for EGFR amino acids used in this manuscript, naming of the antibodies against specific phosphorylated tyrosine sites included two numbers used in different conventions. For example, for p-Y869 (Y845), the first number denoted the amino acid position according to National Center for Biotechnology Information accession number (NP_005219) with M (start codon 'ATG') as +1, and the bracketed number denoted the shortened mature receptor used by the antibody manufacturers. Antibodies used in this study included rabbit anti-total EGFR, p-Y1197 (Y1173), p-Y1016 (Y992), and p-Y869 (Y845; Cell Signaling Technology; 1:2000); rabbit anti–p-Y1110 (Y1086; Zymed; 1:10,000), rabbit antiactin (Sigma; 1:5,000), and horseradish peroxidase–conjugated goat anti-rabbit IgG (Bio-Rad; 1:2,000); and EGFR downstream effectors antibodies rabbit anti–p-STAT3, total STAT3, p-STAT5, total STAT5, p-ERK1/2, total ERK, p-AKT, total AKT, p-SRC, and total SRC (Cell Signaling Technology). For the downstream effectors, all antibodies were used at 1:1,000 except rabbit anti–p-AKT, which was used at 1:500 dilution.

Western Blotting

Harvested cells were washed with 1X phosphate-buffered saline and lysed on ice with Lysis buffer (10 mmol/L Tris; 150 mmol/L NaCl; 1 mmol/L ethylenediaminetetraacetic acid; 1% Triton X-100; 0.5% NP-40, pH 7.4; freshly added 0.2 mmol/L phenylmethylsulfonylfluoride in isopropanol; 1:50 phosphatase inhibitor cocktail 2; 1:50 protease inhibitor cocktail) for 30 min. The cell lysate was then centrifuged at 13,000 rpm for 20 min at 4°C to remove cell debris. The protein amount in the lysate was quantified with the Dc Protein Assay (Bio-Rad). For each lysate, 15 μg protein was loaded on SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Amersham). The membranes were blocked by incubation with shaking in 1% bovine serum albumin blocking buffer at room temperature for 1 h. Primary antibodies were diluted in TBS/Tween 20 with 5% bovine serum albumin. Secondary antibody was diluted in 1% bovine serum albumin blocking buffer. Target proteins on the membrane were visualized on X-ray films by using the ECL Plus Western Blotting Detection Reagents (Amersham). Results were obtained from at least three independent experiments, and representative results were shown. Signal intensities were determined densitometrically using the NIH ImageJ version 1.36b software.[4] The expression levels

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[4] http://rsb.info.nih.gov/ij

of the phosphorylated proteins were normalized by the corresponding total protein and actin levels and then compared as ratios to the levels of the unstimulated cells or before drug treatment.

Results

Novel EGFR Double Mutants in Chinese NSCLC Patients

Tumors containing the E709A+G719C, Q787R+L858R, and T790M+L858R mutations have been previously reported, and details of their mutational profiles and clinicopathologic features were listed in Table 1. Their sequencing chromatograms were shown in Fig. 1A to C. The double mutant H870R+L858R (case E1283) was found in a 56-year-old male smoker who presented with a lung cancer with widespread metastasis to mediastinal lymph nodes, liver, and pleura. Sequencing analysis for *EGFR* on microdissected tumor cells from the pleural biopsy revealed H870R and L858R mutations in addition to wild-type (WT) sequences (Fig. 1D). After a single course of conventional gemcitabine and carboplatin first-line chemotherapy terminated because of persistent thrombocytopenia, the patient was treated with 250 mg/d of gefitinib orally. Tumor reduction of >50% by volume was observed in the lung and liver within 1 week with symptomatic improvement, and the disease remained stable for 6 months, after which leptomeningeal and brain metastasis developed. Brain biopsy showed metastatic adenocarcinoma, which was verified by the phenotypic expression of the pulmonary marker TTF-1. Mutational analysis of the metastatic brain tumor showed dominant L858R and H870R double mutations with undetectable WT alleles (Fig. 1D). The patient did not respond to erlotinib or further gefitinib therapy and died after 3 months.

Baseline Phosphorylation Profile of WT and Mutant EGFR Transfectants

The human NSCLC cell line H358, which carries native WT and weak endogenous expression of EGFR, was used as the transfection host. A time-course study on EGF stimulation on empty vector showed that endogenous EGFR was induced at 5 min and subsided by 30 min (Fig. 2A), indicating that, in subsequent experiments, the observed phosphorylation profiles of the transfectants after 30 min stimulation were not confounded by H358 endogenous EGFR activation. Figure 2B shows that transfection of WT EGFR in H358 did not lead to constitutive activation of EGFR under serum starvation. Stimulation with 100 ng/mL EGF for 30 minutes led to EGFR activation with increased phosphorylated Y869 (Y845), Y1016 (Y992), Y1110 (Y1086), and Y1197 (Y1173). Transfectants containing the single EGFR mutants E709A, G719C, Q787R, T790M, H870R, and L858R, and the double mutants E709A+G719C, Q787R+L858R, T790M+L858R, and H870R+L858R all showed constitutive activation with different phosphorylation profiles. L858R and G719C showed maximum autophosphorylation without further increase despite EGF stimulation. Phosphorylation of the other mutants was enhanced to different extents after EGF treatment.

Tyrosine Kinase Inhibitor Dosage Response of WT, L858R, and T790M Transfectants

The transfectants were serum starved and treated with or without gefitinib in 2-fold serial dilutions from 2,048 to 2 nmol/L for 3 hours, followed by EGF stimulation at 100 ng/mL for 30 minutes. Using p-Y1110 (Y1086) level as an indicator of gefitinib response, phosphorylation of WT transfectants were markedly suppressed by 512 nmol/L gefitinib. L858R, which is known to be clinically gefitinib sensitive, responded to 32 nmol/L gefitinib with greatly reduced p-Y1110 (Y1086) to barely detectable levels. T790M, which is known to be clinically gefitinib resistant, did not show significant suppression even by 2,048 nmol/L gefitinib (Fig. 3). In subsequent experiments involving tyrosine kinase inhibitor treatments, 8, 32, and 512 nmol/L gefitinib were used as standard dosages.

Effects of Tyrosine Kinase Inhibitor Treatment on EGFR Phosphorylation Profiles in EGFR Mutants

Figure 4A shows that G719C transfectants were sensitive to gefitinib treatment, with complete suppression of phosphorylation of Y1110 (Y1086) and Y1197 (Y1173) at 32

Table 1. *EGFR* mutation patterns and clinicopathologic features of studied cases

No.	Sex	Age	SS	Histology	Df	T size (cm)	T stage	N stage	M stage	Path stage	*EGFR*	*KRAS*	Exon	Nucleotide alteration	AA change	Exon	Nucleotide alteration	AA change	Remarks
E109	M	53	Ex	AD	WD	3	1	0	0	1A	Q787R+ L858R	WT	20	2,606A>G	Q787R	21	2,819T>G	L858R	Novel
E120	F	63	NS	AD	MD	2.5	1	0	0	1A	E709A+ G719C	WT	18	2,372A>C	E709A	18	2,401G>T	G719C	Novel
E297	F	71	PS	AD	WD	3	1	0	0	1A	T790M+ L858R	WT	20	2,615C>T	T790M	21	2,819T>G	L858R	
E1283	M	56	SM	AD	NA	NA	4	3	1	4	H870R+ L858R	WT	21	2,819T>G	L858R	21	2,855A>G	H870R	

Abbreviations: F, female; M, male; SS, smoking status as defined in the previous study (8); Ex, exsmoker; NS, nonsmoker; PS, passive smoker; SM, current smoker; AD, adenocarcinoma; Df, differentiation; WD, well differentiated; MD, moderately differentiated; T, tumor; N, lymph node; M, metastasis; Path stage, pathologic stage; AA, amino acid; NA, not available.
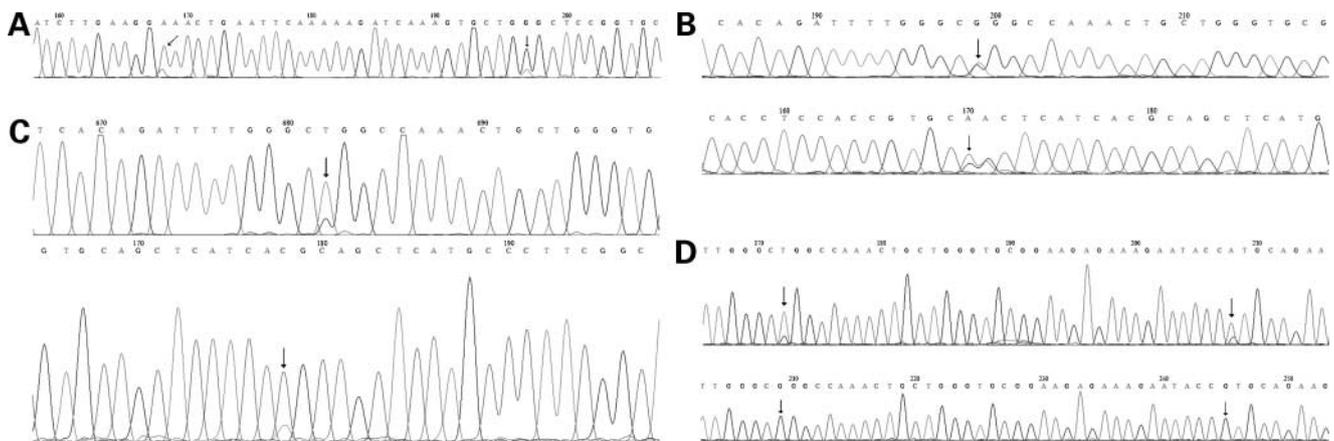
Figure 1. Chromatograms of DNA sequencing of the double mutant cases with either sense or antisense strand sequences shown. **A,** case E120 (E709A + G719C). **B,** case E109 (Q787R + L858R). **C,** case E297 (T790M + L858R). **D,** case E1283 (H870R + L858R). *Top,* primary lung tumor; *bottom,* brain metastasis. *Arrows,* the mutant base positions.

nmol/L gefitinib and Y869 (Y845) at 512 nmol/L. E709A was less sensitive, and suppression of p-Y869 (Y845), p-Y1110 (Y1086), and p-Y1197 (Y1173) was achieved by 512 nmol/L. Phosphorylation profile of the double mutant E709A+G719C showed a similar pattern as that induced by gefitinib treatment of G719C, with complete suppression of p-Y869 (Y845) by 512 nmol/L and p-Y1110 (Y1086) and p-Y1197 (Y1173) by 32 nmol/L.

Figure 4B shows that L858R was highly sensitive to gefitinib, as complete inhibition of phosphorylation at Y869 (Y845), Y1110 (Y1086), and Y1197 (Y1173) were observed at 32 nmol/L. Q787R showed a lower sensitivity, with retention of >90% of tyrosine phosphorylation at 32 nmol/L. Q787R+L858R, on the other hand, showed intermediate response with partial suppression of p-Y869 (Y845) by 32 nmol/L and complete suppression by 512 nmol/L,
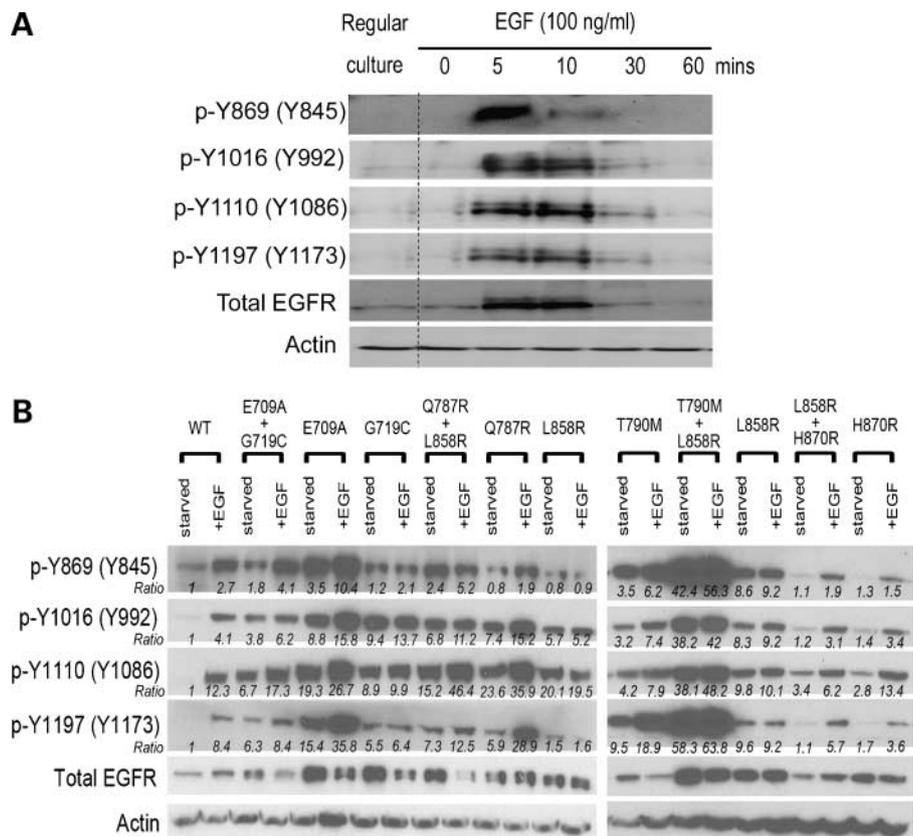

Figure 2. Western blot analysis of constitutive and EGF-induced EGFR expression and phosphorylation. **A,** endogenous EGFR expression and phosphorylation levels in host H358 cells transfected with empty vector. Cells were serum starved for 24 h, followed by EGF stimulation (100 ng/mL) for 0, 5, 10, 30, and 60 min. Transient increase in EGFR levels was observed at 5 to 10 min, but by 30 min, total EGFR decreased to similar or lower level than prestimulation. H358 regular culture in medium with 10% fetal bovine serum was also included for comparison. **B,** EGFR expression and phosphorylation levels in H358 cells transfected with EGFR WT or mutant constructs. Cells were serum starved for 24 h, followed by EGF stimulation for 30 min. The expression levels of the phosphorylated EGFR were normalized to the total EGFR and actin levels and then expressed as a ratio compared with the corresponding reading of the WT construct.
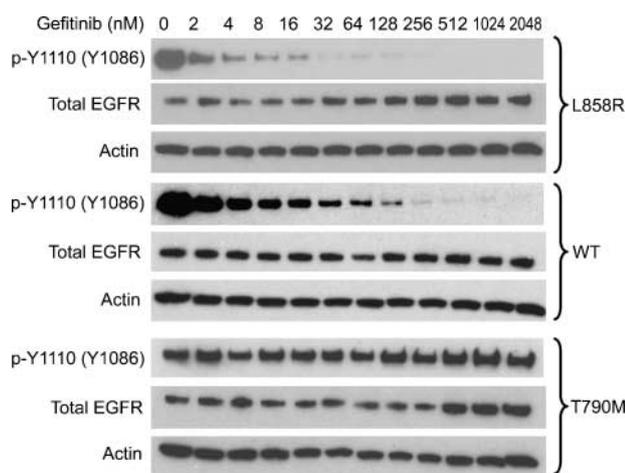
Figure 3. Western blot analysis of EGFR p-Y1110 (Y1086) of a tyrosine kinase inhibitor–sensitive construct (L858R), a WT construct and a tyrosine kinase inhibitor–resistant construct (T790M) treated by a wide range of gefitinib concentrations. Cells were serum starved for 24 h, treated with the indicated gefitinib dosage for 3 h, followed by EGF simulation for 30 min. Normalization was according to total EGFR and actin levels.

whereas p-Y1110 (Y1086) and p-Y1197 (Y1173) were suppressed by 32 nmol/L.

H870R showed persistent phosphorylation of Y1110 (Y1086) and Y1197 (Y1173), although Y869 (Y845) was inhibited at high dose by 512 nmol/L gefitinib, indicating that H870R was more resistant than L858R to gefitinib treatment. The response of the double mutant L858R+H870R was more similar to H870R, being sensitive at Y869 (Y845) but resistant at Y1197 (Y1173) and Y1110 (Y1086) at 32 nmol/L (Fig. 4C).

T790M single and T790M+L858R double mutants showed strong resistance to gefitinib treatment. T790M+L858R showed persistently high phosphorylation levels of Y869 (845) and Y1110 (1086), even under 512 nmol/L gefitinib, whereas Y1197 (1173) showed slight inhibition at 32 nmol/L (Fig. 4D). The findings supported that coexistent T790M and L858R mutations conferred strong gefitinib resistance.

Effects of Tyrosine Kinase Inhibitor Treatment on EGFR Downstream Effectors in Different EGFR Mutants

The EGFR downstream signaling molecules analyzed included known regulators of apoptosis STAT3/5 [downstream of Y869 (Y845)] and AKT [downstream of Y1110 (Y1086)] and the proliferation regulator ERK1/2 [downstream of Y1197 (Y1173); refs. 9–11]. SRC, another molecule downstream of Y869 (Y845) regulating apoptosis, cell adhesion, invasion, and migration, was also studied (11, 12).

In G719C, the phosphorylation of STAT3/5 was totally inhibited by 512 nmol/L gefitinib, whereas phosphorylation of AKT, ERK1/2, and SRC was suppressed at an even lower dose of 8 to 32 nmol/L (Fig. 5A). For E709A, p-ERK1/2 and p-AKT were suppressed by 512 nmol/L gefitinib, whereas p-STAT3, p-STAT5, and p-SRC showed incomplete response, indicating relative resistance of this mutant to gefitinib compared with G719C. For the double E709A+G719C

mutant, p-AKT was markedly suppressed at 32 nmol/L gefitinib. With 512 nmol/L treatment, p-STAT3, p-STAT5, and p-SRC were only slightly suppressed, which resembled the response pattern of E709A single mutant. Overall, the phosphorylation profile of the double mutant showed changes that were intermediate between those of the component single mutants.

Similar effects were observed for the set of L858R, Q787R, and L858R+Q787R mutants (Fig. 5B). L858R showed the highest gefitinib sensitivity with great reduction of phosphorylation of all tested EGFR downstream molecules by 8 to 32 nmol/L gefitinib, whereas Q787R showed strong resistance, with only slight inhibition of p-STAT5 by 512 nmol/L gefitinib. Q787R+L858R, on the other hand, showed intermediate changes, with suppression of p-ERK1/2 and p-AKT by 512 nmol/L and little change of p-STAT3, p-STAT5, and p-SRC.

For H870R, 512 nmol/L gefitinib was required to achieve marked suppression of p-SRC, p-STAT3, and p-STAT5, whereas ERK1/2 and AKT were resistant to treatment and showed persistent phosphorylation. H870R+L858R was also less sensitive to gefitinib compared with L858R and overall resembled the response pattern of H870R single mutant, although there was slight inhibition of p-ERK1/2 and p-SRC at 512 nmol/L (Fig. 5C).

Discussion

Complex EGFR mutations are more common in Asian lung cancer patients, including various forms of double mutations (13, 14). The reported double mutations (13–18) generally involve a common activating mutation such as L858R and a rare mutation such as T790M. Concomitant T790M and L858R mutations confer gefitinib resistance in NSCLC patients (17, 18). This combination has been identified under different situations such as familial occurrence (15), induction after gefitinib treatment in *trans* to each other (6), or *de novo* in-*cis* somatic mutations in untreated patients such as those found in our population (8) and included in this study. Limited information about the significance of double mutations is available in the literature. In this study, we investigated the effects of gefitinib treatment on three EGFR double mutants found in our population. Two of them, E709A+G719C and Q787R+L858R, have been described in our previous report but not in any other study (8). The third, H870R+L858R, was encountered during clinical investigation of the metastatic brain tumor of a patient whose primary lung tumor had been controlled by gefitinib for 6 months. This double mutant has also been identified in a 70-year-old Japanese female nonsmoker patient with bronchioloalveolar-type carcinoma, but the response to tyrosine kinase inhibitor treatment was not reported (19).

Reports in the literature have shown that different forms of *EGFR* mutations may lead to different levels of tyrosine activation and associated with different oncogenic potentials (20). Common *EGFR* mutations that are found at high frequencies in clinical tumors such as L858R and exon 19 deletions might be more oncogenic and thus highly selected
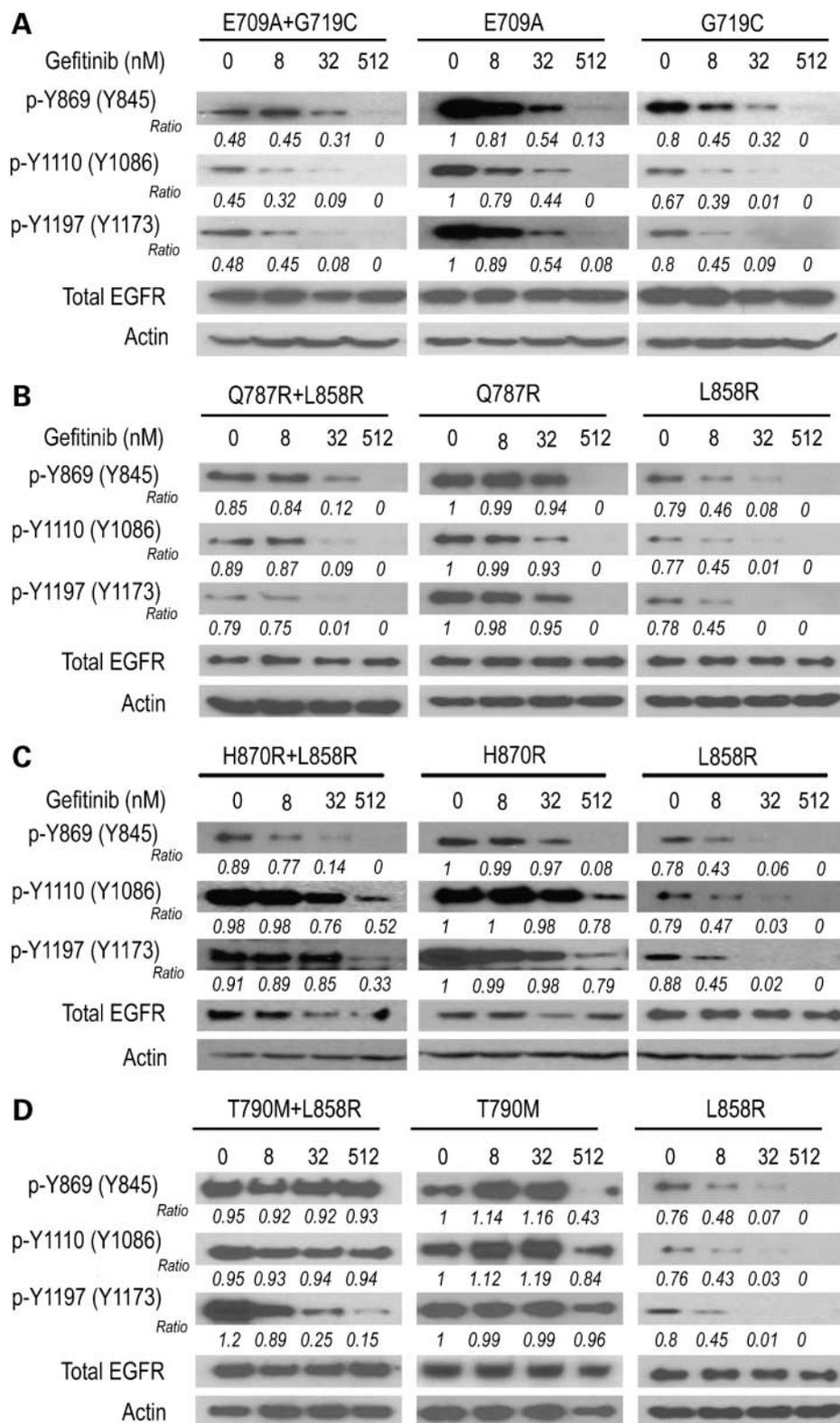
Figure 4.   Western blot analysis of EGFR phosphorylation profile at Y869 (Y845), Y1110 (Y1086), and Y1197 (Y1173) in *EGFR* single or double mutant constructs treated by gefitinib for 3 h. **A,** E709A+G719C group. **B,** Q787R+L858R group. **C,** H870R+L858R group. **D,** T790M+L858R group. Signal intensities were determined quantitatively by densitometric measurements. Phosphorylated EGFR signal intensities were normalized to total EGFR and actin levels and represented as ratios compared with the untreated constructs.
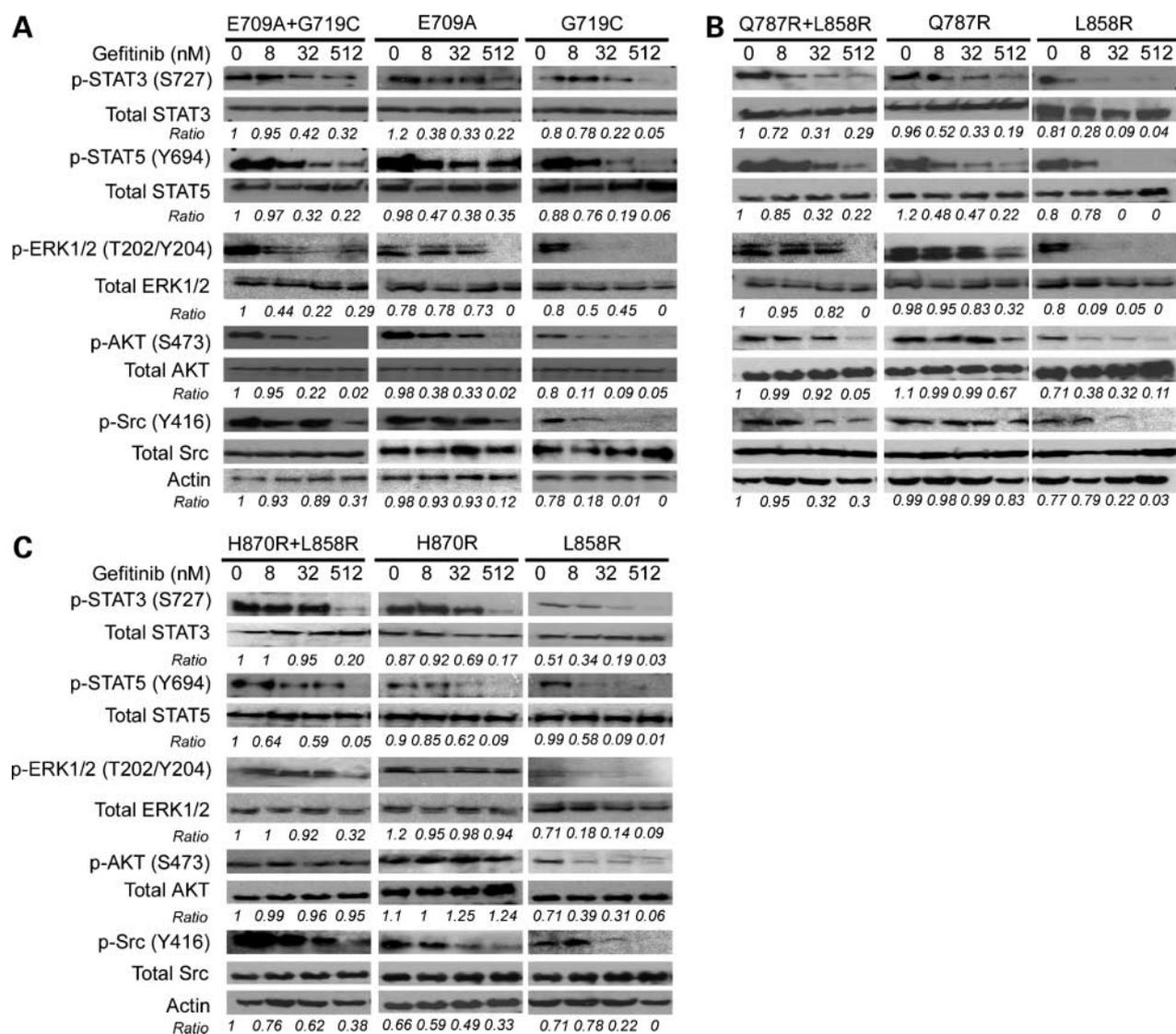
Figure 5. Western blot analysis of EGFR downstream effectors phosphorylation profiles, including STAT3, STAT5, ERK1/2, AKT, and SRC, in *EGFR* single or double mutant constructs after gefitinib treatment for 3 h. **A,** E709A + G719C group. **B,** Q787R + L858R group. **C,** H870R + L858R group. Signal intensities were determined quantitatively by densitometric measurements. The signal intensities of phosphorylated EGFR effectors were normalized to total EGFR and actin levels and represented as ratios compared with the untreated double mutant constructs.

for. The oncogenic potentials of less common mutations, and even more so for double mutants comprising a common and a rare mutation, are largely unknown. Their functional capacity might be indirectly inferred from their autoactivation statuses, response to ligand stimulation and sensitivity to inhibition by tyrosine kinase inhibitor. In Fig. 2A, we showed that there was an increase of endogenous EGFR protein expression for 5 to 10 minutes when H358 was serum starved for 24 hours, followed by 100 ng/mL EGF stimulation, but the increase was transient and the expression returned to prestimulation level after around 30 minutes. Thus, phosphorylated EGFR levels of transfected WT or mutant constructs shown in Fig. 2B reflected solely the expression changes of the construct. Using

this system, we have found that L858R and G719C showed maximum autoactivation at starvation with no further increase despite EGF stimulation, whereas the less common single mutants T790M, E709A, Q787R, and H870R showed mild increase in phosphorylation levels.

Tyrosine phosphorylation of the E709A+G719C double mutant and the corresponding single mutants, E709A and G719C, were suppressed to different extents by tyrosine kinase inhibitor treatment (Fig. 4A), with corroborative changes in the phosphorylation profiles of the downstream signaling molecules (Fig. 5A). In the tyrosine kinase inhibitor–sensitive G719C mutant, phosphorylation of the Y869 (Y845) downstream molecules STAT3/5 were almost completely inhibited by 512 nmol/L, whereas SRC was

inhibited at 32 nmol/L. p-AKT [downstream of Y1110 (Y1086)] as well as p-ERK1/2 [downstream of Y1197 (Y1173)] showed even higher sensitivity, being almost totally suppressed by 8 nmol/L gefitinib. In the E709A resistant mutant, phosphorylation of all EGFR downstream effectors was markedly suppressed only by at least 512 nmol/L gefitinib. The corresponding double mutant, E709A+G719C, showed a response profile that was intermediate between those of E709A and G719C, suggesting that the presence of the uncommon E709A, in addition to the activating G719C, would confer relative resistance to gefitinib treatment.

Similarly, for both the sets of Q787R, L858R, and Q787R +L858R, and H870R, L858R, and H870R+L858R mutations, gefitinib caused intermediate inhibitory effects on the combined mutants compared with the single mutants separately, but fine differences were observed. For example, 32 nmol/L of gefitinib caused almost total suppression of p-Y1110 (Y1086) and p-AKT in E709A+G719C, whereas the same dosage caused less precipitous suppression for Q787R+L858R and almost no effect for H870R+L858R.

In the H870R and H870R+L858R double mutant, p-Y869 (Y845), p-Y1197 (Y1173), p–signal transducers and activators of transcription 3/5 (STAT3/5), p-SRC, and p-ERK1/2 were markedly inhibited by 512 nmol/L gefitinib, but p-Y1110 (Y1086) and p-AKT were resistant at this dosage compared with the L858R single mutant (Figs. 4C and 5C). The expected concentration of plasma gefitinib is reported to be 0.5 to 1 µmol/L, following 250 or 500 mg/d taken orally (21). According to our results, this drug level could be marginally sufficient for controlling tumor cells in the lung. However, the gefitinib drug concentration attainable in the cerebrospinal fluid could be as low as 18 nmol/L for an oral dose of 500 mg/d and 42 nmol/L for 1,000 mg/d (22). This drug level would be grossly insufficient to inhibit the phosphorylation and activation of H870R+L858R double mutant as shown in this study. Although some clinical reports have shown that small molecule EGFR tyrosine kinase inhibitor can pass through the blood-brain barrier and cause shrinkage of brain metastasis (23–26), other reports have suggested that tyrosine kinase inhibitor penetration into the brain could be incomplete (5, 22), and brain metastasis was strongly associated with the emergence of acquired resistance in NSCLC patients treated with gefitinib (27). As shown in Fig. 1, WT alleles are not detectable in the metastatic brain tumor of the patient. It is also possible that the first round of gefitinib treatment has facilitated the preferential selection of metastatic tumor cells that harbor amplified mutant alleles, leading to reduced sensitivity to gefitinib treatment.

To the best of our knowledge, only three other studies have investigated the functional roles of *EGFR* double mutations in cell models. Chen et al. (28) have studied the activation and response profile of EGFR double mutants identified in patients from Taiwan. One of them comprised E709A+G719S instead of G719C, as in this study. They showed that gefitinib induced suppression of total phosphorylated tyrosines in E709A and G719S mutants separately transfected into H1299 cells, with a calculated IC$_{50}$ of

<20 nmol/L. However, the combined mutant E709A +G719S was not investigated. In another study on *in vitro* kinase assay, the catalytic activity of G719S mutant was found to be 10-fold more active than WT EGFR (29). As amino acid glycine at position 719 is part of the highly conserved glycine-rich GXGXΦG motif (719GSGAFG724) present in the ATP-binding loop of protein kinases, it is suggested that it plays a crucial role in WT EGFR function (30). From our finding of higher EGFR and effector phosphorylation levels of G719C (Figs. 4A and 5A), it is likely that this mutant could also induce increased EGFR kinase activity. Choong et al. (31) reported a patient with stage IV lung carcinoma containing L858R+E884K double mutation who initially responded to first-line carboplatin/paclitaxel concurrent with erlotinib therapy but who developed new brain tumors and leptomeningeal metastases after being on erlotinib maintenance for 11 months. The new lesions responded to 250 mg/d gefitinib. *In vitro* transfection study showed that the in-*cis* L858R+E884K double mutation result in disruption of the conserved E884-R958 ion pair in the C-lobe of the EGFR catalytic domain, and the double mutant act in a dominant manner to cause enhanced signaling of the mitogen-activated protein kinase pathway with increased tumor cell proliferation and altered sensitivity to tyrosine kinase inhibitor differentially (32).

In a recent review by Chen et al. (28), 66 publications describing *EGFR* mutational patterns of 6,874 patients from the literature were compared. Mutations of five amino acids at E709, G719, T790, S768, and L861 were found to be commonly involved in doublet but uncommon in singlet forms, and the doublet mutants are somatic and involve the same allele. For E709 and G719, multiple amino acid substitutions are observed (33). The variations of the component mutants could lead to differences in biochemical properties. For example, T790M not only confers strong tyrosine kinase inhibitor resistance (Figs. 3 and 4D) but also enhances the basal and EGF-induced phosphorylation signals in its double mutants (Fig. 2B). Such enhancement of EGFR phosphorylation and activity by T790M in double mutants has also been showed by other researchers (34, 35). However, detailed clinical studies are necessary to ascertain whether the *in vitro* differences would translate into clinically observable differences in the patients' responses.

When transfected as a single mutant, E709A, Q878R, and H870R showed a more activated phosphorylation profile compared with WT EGFR. However, as double mutants, they do not confer a greater level of activation than the most activating mutations L858R or G719S alone. These observations lead to the interesting question of what selective advantage is conferred by the rare mutations and which mutation came first whether as a somatic or germline event. Notably, we have not found these rare mutations in peripheral blood leukocytes of our previous cohort or in additional 155 lung cancer patients (data not shown). Although the numbers analyzed are small, there is no evidence that germline *EGFR* mutations predispose to lung cancers in our population. Furthermore, from our own study as well as reported clinical series, it is clear that these rare mutants

are infrequent as single mutants in primary lung carcinomas and so it is not likely that they alone would provide adequate oncogenic drive for development of tumors with full malignant potentials. It is possible that they confer slight selective advantage to premalignant somatic cells, expanding the pool of susceptible cells for further clonal selection by mitogenic forces acting on the *EGFR* genomic locus of nonsmokers, eventually leading to the most activating common mutations observed in clinical tumors.

In summary, our results showed that the various mutations identified in the complex oriental *EGFR* mutational profile respond to *in vitro* tyrosine kinase inhibitor at different concentrations. The G719C and L858R single mutants showed ligand-independent activation and the highest gefitinib sensitivity compared with the corresponding coexisting single mutants E709A, Q787R, H870R, and T790M. The combined double mutants E709A+G719C, Q787R +L858R, and H870R+L858R showed intermediate responses to gefitinib in both the phosphorylation profiles of EGFR tyrosine sites and downstream effectors when compared with their respective single mutants. The findings suggest that, although the response of double mutants cannot be predicted from the response of the individual single mutant partners, rare types of *EGFR* substitution mutations could induce relative gefitinib resistance compared with the common activating mutant alone. Multiple factors are likely to affect the clinical response of NSCLC cancer patients to tyrosine kinase inhibitor treatment, and information of the mutation types and signaling changes are important factors that need to be considered when treatment protocols are planned.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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