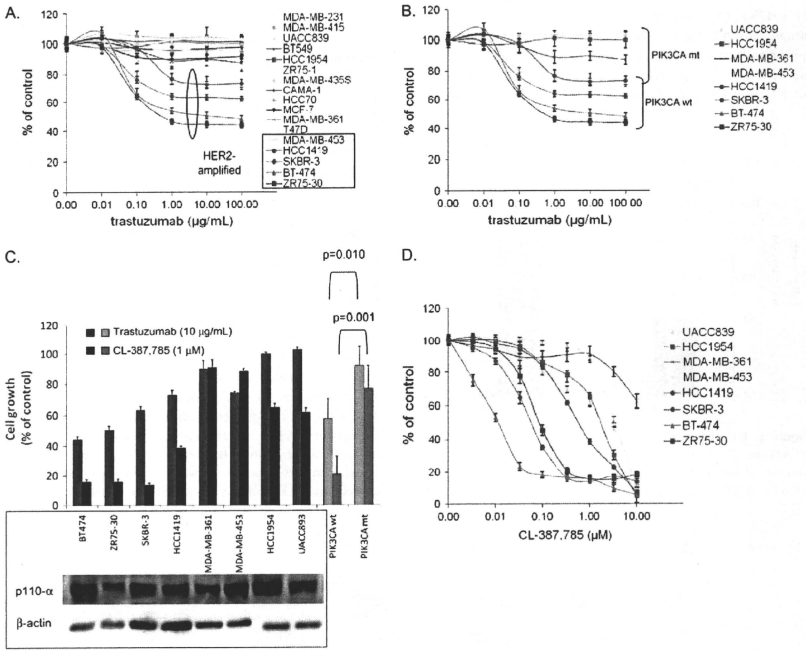

Figure 2. Effect of trastuzumab and CL-387,785 on growth inhibition in breast cancer cells *in vitro* [(**A**) trastuzumab on 17 breast cancer cell lines; (**B**) and (**D**) trastuzumab and CL-387,785 on eight *HER2*-amplified cell lines, respectively]. Breast cancer cells were grown in 10% serum-containing media for 5 days in the presence of various concentrations of trastuzumab (**A** and **B**) or CL-387,785 (**D**). The percentage of viable cells is shown relative to that of the untreated control and plotted on the *y*-axis, whereas trastuzumab and CL-387,785 concentrations are plotted on the *x*-axis. Each data point represents the mean value and standard deviation of 6–12 replicate wells. (**C**, top) Mean percentage of control and standard deviation of 6–12 replicate wells treated with 10 μg/ml trastuzumab and 1 μM CL-387,785, as well as those of *PIK3CA*-wild-type and -mutant cell lines (bottom), were plotted. (**C**, bottom) Protein expression of p110-α in *HER2*-amplified breast cancer cells. Blots were stripped and re-probed for β-actin as loading control.

Phosphorylation signals were then quantified and correlated with growth inhibition caused by trastuzumab and CL-387,785. As shown in Figure 4B, the closest association was observed between phospho-S6K changes and growth inhibition caused by trastuzumab and CL-387,785 [correlation coefficient (r), 0.811]. Further, close associations between phospho-S6K and cell growth were consistent when analyzed for trastuzumab and CL-387,785 separately (r for phospho-S6K versus growth: 0.8487 and 0.6970 for trastuzumab and CL-387,785, respectively).

dependency of *HER2*-amplified breast cancer cells on PI3K pathway

Given that inhibition of the PI3K pathway is critical in distinguishing cells sensitive from resistant to HER2-targeted agents (Figure 4B), we evaluated cell lines for the effects of LY294002, a PI3K inhibitor. As shown in Figure 5A, with the exception of ZR75-30, LY294002 induced a >30% growth inhibition compared with control in all cell lines. No significant difference in LY294002 sensitivity was observed between *PIK3CA*-mutant and -wild-type cell lines (Figure 5; $P = 0.655$). These results indicate that most *HER2*-amplified cells at least partly depend on the PI3K pathway regardless of the presence or absence of *PIK3CA* hotspot mutations.

To further gain insight into this concept, we evaluated phosphorylation levels of Akt and ERK1/2 in protein extracts obtained from cells under serum-starved conditions for 24 h. As shown in Figure 5B, despite the absence of serum factors, all *HER2*-amplified breast cancer cells showed a high level of phospho-Akt, regardless of *PIK3CA* genotype. High levels of phospho-Akt were also observed in MDA-MB-468, which lacks PTEN [30], and T47D, which harbors a *PIK3CA* mutation

(H1047R) [14]. These two cell lines do not show *HER2* amplification [23]. In contrast, no significant levels of phospho-Akt were observed in MDA-MB-231 and MDA-MB-435S, which show no *HER2* amplification, *PIK3CA* mutation, or PTEN loss [14, 23]. Further, with the exception of MDA-MB-231, all cell lines showed very low levels of phospho-ERK1/2 under serum-starved conditions. MDA-MB-231, in particular, was reported to contain double activating mutations in KRAS (G13D) and BRAF (G464V), whereas MDA-MB-435S showed an activating mutation in BRAF alone (V600E) [31]. These findings further support the concept that *HER2*-amplified cells tend to have HER2-PI3K signaling axis and they are thus dependent on the PI3K pathway rather than on extracellular signal-regulated kinase pathway.

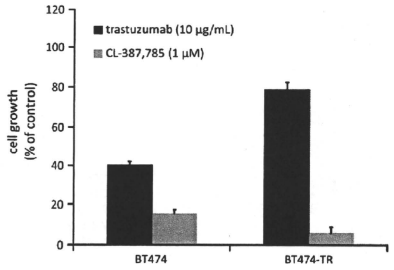

Figure 3. Effect of trastuzumab and CL-387,785 on growth inhibition in BT-474 and BT474-TR cells. Mean percentage of control and standard deviation of 6–12 replicate wells treated with 10 μg/ml trastuzumab and 1 μM CL-387,785 were plotted. BT474-TR remains sensitive to CL-387,785.

discussion

In this study, we show that gain-of-function mutations in *PIK3CA* genes are associated with trastuzumab resistance in naturally derived breast cancer cell lines showing *HER2* amplification. This finding is consistent with a recent study by Berns et al. [19] reporting trastuzumab resistance in SKBR-3 cells transfected with mutant *PIK3CA* (H1047R) compared with GFP control. Transfection of wild-type *PIK3CA*, however, appeared to equally cause trastuzumab resistance [19]. This observation does not identify either quantitative or qualitative changes in *PIK3CA* mutation as the major factor in developing trastuzumab resistance. In the present study, no clear association was observed between *PIK3CA* protein (p110-α) expression levels and *in vitro* sensitivity to trastuzumab

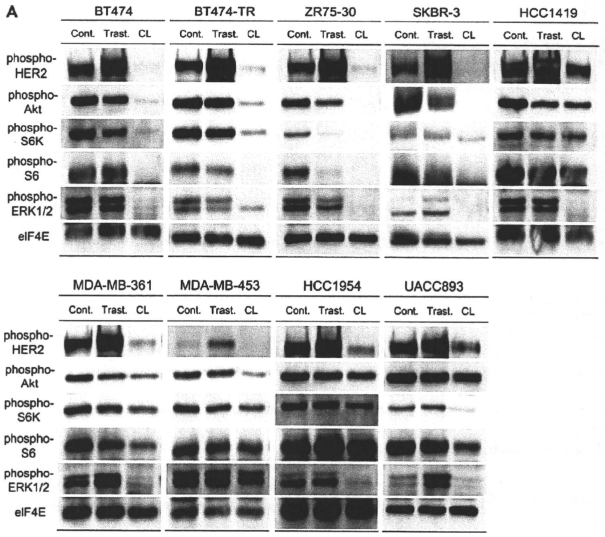

Figure 4. (A) Expression of phosphorylated-HER2, -Akt, -S6K, -S6 and -ERK1/2 in HER2-amplified breast cell lines with and without treatment with trastuzumab (10 μg/ml) and CL-387,785 (1 μM). Breast cell lines grown in 10% serum-containing media were lysed and immunoblotted for each protein. Blots were stripped and re-probed for eIF4E as loading control.

doi:10.1093/annonc/mdp304

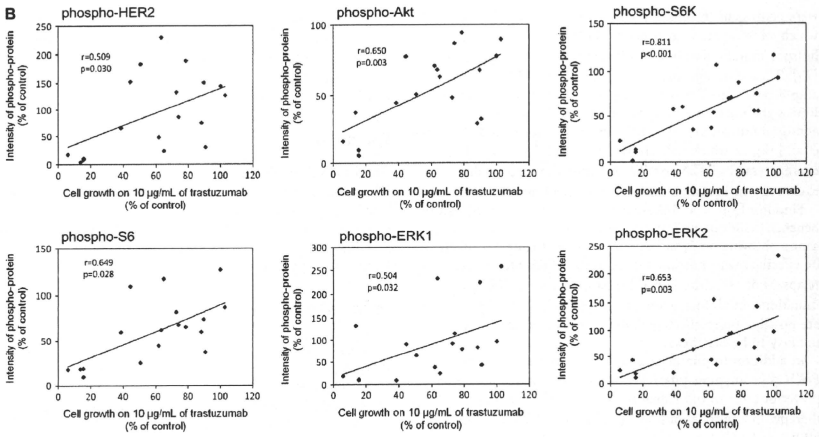

Figure 4. (Continued) (**B**) Correlation between changes in phosphorylation of HER2 signaling molecules and cell growth. Immunoblot quantification was carried out by densitometry using ImageJ software. Correlations were analyzed by calculating Pearson's correlation coefficient.

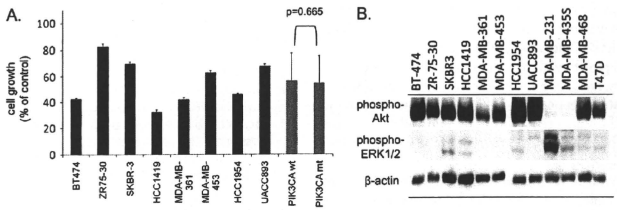

Figure 5. (**A**) Effect of LY294002 on growth inhibition in HER2-amplified breast cancer cell lines. Mean percentage of control and standard deviation of 6–12 replicate wells treated with 10 μM LY294002 were plotted. (**B**) Protein expression of phospho-Akt and phospho-ERK1/2 in HER2-amplified breast cancer cells under serum-starved condition. Blots were stripped and re-probed for β-actin as loading control.

(Figure 2C). A study by Haverty et al. [32], which analyzed copy number alterations in 51 breast tumors using a high-resolution single nucleotide polymorphism array, showed no gain in copy number on chromosome 3p, the location of the *PIK3CA* gene. These results indicate that qualitative changes in the *PIK3CA* gene itself may cause trastuzumab resistance in naturally derived breast cancer cells.

The CL-387,785 HER2-TKI was first evaluated to identify groups of compounds which may overcome trastuzumab resistance. Of note, results show an association between *PIK3CA* hotspot mutations and CL-387,785 resistance. Further, the difference in sensitivity between *PIK3CA*-wild-type and -mutant cell lines was more significant for CL-387,785 than for trastuzumab (Figure 2C). These results are consistent with a recent study by Eichhorn et al. [22], which showed that transfection of mutant *PIK3CA* (H1047R) in BT474 cells, which are sensitive to lapatinib, results in drug resistance. In contrast, the results of the present study show that BT474-TR cells remain highly sensitive to CL-387,785, which is consistent with a previous study by Konecny et al. [20] which reported that lapatinib remains active against cell lines selected by long-term exposure to trastuzumab. Although the study did not show the effect of lapatinib on cell signaling in secondary resistant cells, our present findings indicate that BT474-TR remains dependent on HER2/PI3K signaling and sensitive to HER2-TKI (Figure 4A).

We then evaluated LY294002 as a model PI3K inhibitor. Results show that *HER2*-amplified cells are generally sensitive to LY294002 regardless of *PIK3CA* genotype (Figure 5A), which indicates that *HER2* amplification is associated with dependency on PI3K pathway. Supporting this notion, all *HER2*-amplified breast cancer cells have high level of phosphorylation of Akt even in serum-starved condition. The Akt phosphorylation levels observed in *HER2*-amplified cells

were equivalent to those in MDA-MB-468 and T47D cells, which were reported to contain PTEN loss and a *PIK3CA* hotspot mutation without *HER2* amplification, respectively [23]. These findings therefore indicated that *HER2* amplification itself may have equivalent biological effect on PI3K signaling with PTEN loss or *PIK3CA* hotspot mutation. In addition, our results are consistent with a recent study by Oda et al. [33], in which they showed that HER2 and/or HER3 overexpression, PTEN, or *PIK3CA* mutations occur almost exclusively in breast and other cancer cell lines.

Findings in past and present studies may potentially lead to beneficial clinical applications. For *HER2*-amplified breast cancer showing no *PIK3CA* mutations, trastuzumab is likely to be effective, with possible rescue using HER2-TKIs in cases of relapse. For *HER2*-amplified breast cancer with *PIK3CA* mutations, inhibitors against molecules of the PI3K pathway are possibly more effective than anti-HER2 agents, which are unlikely to be beneficial.

In addition to pharmacogenetic approaches, including *PIK3CA* genotyping, pharmacodynamic markers are potentially powerful tools in individualized use of molecularly targeted therapy. In a number of previous pharmacodynamic studies on HER2- or EGFR-targeted therapy, phospho-Akt was used as a surrogate marker for PI3K pathway activity [34, 35]. In the present study, however, growth inhibition is more closely associated with changes in phospho-S6K than that in phospho-Akt. These findings indicate that the prediction of tumor response to trastuzumab may strongly benefit from measurements of S6K phosphorylation levels. The cause of the discrepancy between the association of cell growth with phospho-Akt and that with phospho-S6K, however, remains unclear. It may be due to the difference in sensitivity of phospho-specific antibodies used in the present study or the higher sensitivity of phospho-Akt to positive feedback signals following initial inhibition of the PI3K pathway compared with phospho-S6K.

The present study shows several limitations. First, although a relatively large panel of *HER2*-amplified breast cancer cell lines ($N = 8$) were used, the properties of all HER2-overexpressing breast tumors are not necessarily represented. Despite HER2 amplification being retained, particular tumor subtypes may have been selected in the establishment of cell lines. Secondly, in addition to inhibition of HER2 signaling, a few studies have indicated the contribution of antigen-dependent cellular cytotoxicity (ADCC) in the antitumor effect of trastuzumab. Because ADCC only works in *in vivo* conditions, our current data do not necessarily deny the potential effect of trastuzumab on tumors showing *PIK3CA* mutations [36]. Thirdly, although wild-type *PIK3CA* appeared necessary for trastuzumab sensitivity *in vitro*, other factors may be involved, as shown by results showing moderate resistance of HCC1419 to trastuzumab (Figure 2C). The mechanisms of *PIK3CA*-unrelated resistance remain unknown but are under current investigation in our laboratory.

In conclusion, our findings show an association between the presence of *PIK3CA* hotspot mutations and resistance to not only trastuzumab but also HER2-TKI in naturally derived *HER2*-amplified breast cancer cell lines. Further, PI3K inhibitors are potentially effective in overcoming trastuzumab resistance caused by *PIK3CA* mutations. Assessment of S6K phosphorylation levels may be a useful pharmacodynamic marker correlated to the antitumor effect of HER2-targeted therapy. A better understanding of these findings, however, may require further investigation in clinical trials and concomitant translational studies.

funding

Grant-in-Aid for Young Scientists (B) from Ministry of Education, Culture, Sports, Science and Technology of Japan to T.M.; AstraZeneca Research Grant 2007 to T.M.; Kobe University Medical School Research Grant for Young Scientists to T.M.; Grants-in-Aid for Cancer Research from Ministry of Health, Labor and Welfare of Japan to H.M.

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Annals of Oncology Advance Access published November 25, 2010

original article

Annals of Oncology
doi:10.1093/annonc/mdq585

FcγR2A and 3A polymorphisms predict clinical outcome of trastuzumab in both neoadjuvant and metastatic settings in patients with HER2-positive breast cancer

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Received 27 April 2010; revised 2 August 2010; accepted 24 August 2010

Background: Antibody-dependent-mediated cytotoxicity (ADCC) is one of the modes of action for trastuzumab. Recent data have suggested that fragment C γ receptor (FcγR) polymorphisms have an effect on ADCC. This prospective phase II trial aimed to evaluate whether these polymorphisms are associated with clinical efficacies in patients who received trastuzumab.

Patients and methods: Patients in a neoadjuvant (N) setting received Adriamycin and cyclophosphamide followed by weekly paclitaxel/trastuzumab. Patients in a metastatic (M) setting received single trastuzumab until progression. In total, 384 distinct single nucleotide polymorphisms of different FcγR, HER2, and fucosyltransferase loci were assessed.

Results: Fifteen operable and 35 metastatic HER2-positive breast cancer patients were enrolled in each of the N and M settings, respectively. The FcγR2A-131 H/H genotype was significantly correlated with the pathologically documented response (pathological response) ($P = 0.015$) and the objective response ($P = 0.043$). The FcγR3A-158 V/V genotype was not correlated with the pathological response, but exhibited a tendency to be correlated with the objective response. Patients with the FcγR2A-131 H/H genotype had significantly longer progression-free survival in the M setting ($P = 0.034$).

Conclusion: The FcγR2A-131 H/H polymorphism predicted the pathological response to trastuzumab-based neoadjuvant chemotherapy in early-stage breast cancer, and the objective response to trastuzumab in metastatic breast cancer.

Key words: ADCC, FcγR, trastuzumab

introduction

The humanized HER-2/*neu* immunoglobulin G (IgG) 1 monoclonal antibody (mAb) trastuzumab is an effective treatment of HER-2/*neu*-positive breast cancer. However, large differences in clinical outcome remain among patients treated with trastuzumab. Identifying molecular markers that can select patients who are to benefit from trastuzumab treatment is crucial for avoiding chemotherapy toxicity and reducing treatment costs.

Antibody-dependent cytotoxicity (ADCC) mediated by fragment C γ receptor (FcγR) on immune cells such as macrophages and natural killer cells plays an important role in the antitumor effect of IgG1 antibodies [1]. Genetic polymorphisms have been identified in genes encoding the activating receptors FcγR2A and 3A. A histidine (H)/arginine (R) polymorphism at position 131 for FcγR2A and a valine (V)/phenylalanine (F) polymorphism at position 158 for FcγR3A are two polymorphisms that affect the affinity of the receptors to human IgG [2–4]. Clinical studies have shown that FcγR2A-131 H/H and FcγR3A-158 V/V genotypes are associated with better clinical outcomes following the administration of rituximab as a first-line treatment of follicular lyoma [5, 6] and diffuse large lyoma [7] and cetuximab as a first-line treatment of metastatic colorectal cancer [8].

FcγR-deficient mice show a significantly reduced antitumor effect after trastuzumab treatment, with wild-type mice [9]. HER-2/*neu*-positive breast cancer cell lines are susceptible to ADCC in the presence of trastuzumab [10–12]. The activity of trastuzumab *in vivo* has also been correlated with a significant increase in the numbers of peritumoral lyomonocytes and *in vitro* ADCC [13]. In a clinical trial, Musolino et al. [14]

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demonstrated that a better response to trastuzumab-based therapy in metastatic breast cancer (MBC) was associated with the above two genotypes. In contrast, a recent large prospective trial (BCIRG006) [15] showed that the two FcγR single nucleotide polymorphisms (SNPs) did not predict disease-free survival in early breast cancer or progression-free survival (PFS) in MBC after trastuzumab-based therapy. Most of the previous studies reporting that FcγR SNPs are correlated with outcome [5–8, 14] have been under-powered, with the exception of BCIRG006. However, this inconsistency might have been influenced by the different modalities of therapeutic use [i.e. adjuvant and metastatic (M) settings] or the combinations of cytotoxic agents.

The goal of our prospective study was to determine the predictive values of these two SNPs as biomarkers in predicting the objective response to the single use of trastuzumab and to these predictive values with other SNPs in FcγR, HER2 or fucosyltransferase in MBC patients. We also analyzed their potential as a predictive marker of pathological complete response (pCR) in a neoadjuvant (N) setting with trastuzumab-based chemotherapy.

materials and methods

eligibility criteria

Eligible patients had histologically confirmed breast cancer, operable stage II–IIIA disease (tumor size > 3 cm) in an N setting or stage IV disease in an M setting (recurrent disease after curative surgery was also eligible), HER2-positive (IHC 3+ or FISH positive), chemotherapy, measurable disease, age ≥ 20 years, Eastern Cooperative Oncology Group performance status of 0–2, and adequate organ function (white blood cell count ≥ 4000/μl, platelet count ≥ 100 000/μl, hemoglobin concentration ≥ 9.0 g/dl, serum bilirubin ≤ 2.0 mg/dl, aspartate aminotransferase and alanine aminotransferase ≤ 100 IU/l, serum creatinine ≤ institutional upper limit of normal range, PaO_2 ≥ 60 mmHg, baseline left ventricular ejection fraction >50%). The main exclusion criteria were active concomitant malignancy, congestive heart failure, uncontrolled angina pectoris, arrhythmia, symptomatic infectious disease, severe bleeding, pulmonary fibrosis, obstructive bowel disease or severe diarrhea, symptomatic peripheral or cardiac effusion, and symptomatic brain metastasis. This study was conducted according to a protocol approved by the institutional review board/independent ethics committee, and informed consent was obtained from all patients for the use of blood samples and the analysis of clinical information.

analysis of FcγR, HER2, and fucosyltransferase polymorphisms

In a previous study [14], the authors focused only on the hot spot of SNPs at FcγR2A-131 and FcγR3A-158. However, other loci of FcγR, including 2B-232 I/T, have already been reported [16] as potential markers for predicting the response to trastuzumab. Additionally, FUT8 is known to transfer a fucose residue to N-linked oligosaccharides on glycoproteins [17], and we reported that FUT8 plays an important role in ADCC activity [18]. Goldgate Genotyping is a novel technique that can be used to determine 384 SNPs quickly and simultaneously. Based on these backgrounds, 384 SNPs harboring FcγR1, R2, R3, HER2, and fucosyltransferase (FUT8) loci were custom designed using Goldgate Genotyping [19] (Illumina Co., CA) in this study. Among them, 67 SNPs were designed in exons. Genomic DNA was purified from peripheral blood using QIAamp Micro kits (QIAGEN K.K., Tokyo, Japan). Genomic DNA was isolated from specimens using QIAamp Micro kits. Genomic DNA (250 ng) was hybridized using a bead array and the Goldgate Genotyping Assay manual [20]. The presence of SNPs was analyzed using a bead array reader. Gene clustering was carried out automatically using a software algorithm (Beadstudio) several times, and all the spots were confirmed by visual inspection. When the separation of the clustering was poor or when some samples provided inconsistent data, the assay was repeated to confirm the results. We also combined standard DNA (HapMap) with the assay as a control. Molecular data were independently interpreted by two biologists (FK and KN) who were blinded to the clinical outcomes of the study participants.

treatment and assessment

Treatment in the N setting consisted of Adriamycin and cyclophosphamide (60/600 mg/m^2) × 4 i.v. every 3 weeks followed by paclitaxel (80 mg/m^2) with trastuzumab (4 mg/kg followed by 2 mg/kg) × 12 i.v. every week. Treatment of MBC consisted of trastuzumab (8 mg/kg, followed by 6 mg/kg) every 3 weeks until disease progression. Routine clinical and laboratory assessments were carried out every 3 weeks, and a CT or echo examination of the target lesion was carried out every 2 months. The pathologically documented response (pathological response) after N therapy was assessed using the histopathological criteria of the Japanese Breast Cancer Society [21]. The objective response was evaluated every month using the Response Evaluation Criteria in Solid Tumors guidelines [22]. All the adverse effects that occurred during treatment were reported, and the severity of each adverse effect was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events, Version 3.0.

statistical analysis

The association of each polymorphism with either the pathological response for the N setting or the objective tumor response for the M setting was the primary end point of the analysis. The association of each polymorphism with the PFS for the M setting and with a linkage analysis between FcγR2A and 3A were the secondary end points. First, all genotypes (wild, hetero and homo) of the 384 SNPs were assessed as to whether or not a difference in the primary end points was present with a statistical power <0.1. Second, the pathological responses and objective tumor responses of the patients were according to the selected FcγR polymorphisms using a two-tailed Fisher's exact test [23], chi-square test [23], linear correlation test [24], and analysis of variance (ANOVA) test [24]. Linkage disequilibrium was determined using a Fisher's exact test, chi-square test, and linear correlation test. The PFS was calculated as the length of time between the first day of trastuzumab treatment and the first observation of disease progression or death from any cause. If a patient had not progressed or died, the PFS was censored at the time of the last follow-up examination. The association of each polymorphism with PFS was analyzed using Kaplan–Meier curves [25] and the log-rank test [26]. All tests of statistical significance were two-tailed. The analyses were carried out using the SAS statistical package, version 9.0 (SAS Institute Inc., Cary, NC).

results

patient characteristics

Between December 2005 and August 2008, 40 and 36 patients were prospectively screened for N and M settings, respectively. Out of the 40 patients in the N setting, 15 (37.5%) patients were diagnosed as being HER2-positive using tissue samples obtained during a core needle biopsy. One patient in an M setting was ineligible because of an incorrect diagnosis of breast cancer. The clinical and pathologic features of the patients are presented in Table 1.

genotypic frequencies of the polymorphisms

A total of 384 SNPs harboring FcγRI, RII, RIII, HER2 and FUT8 loci were custom designed and analyzed in all 50 patients (15 in an N setting and 35 in an M setting). After a study to establish the correlations between the genotypes of these SNPs and the clinical outcome, we found that only two hot spots, FCγR2A-131 H/H and FCγR3A-158 V/V, among the 384 loci were predictive markers of the response to trastuzumab-based therapy. Forty-four percent (22 of 50) of the patients were homozygous for the FCγR2A-131 H allele, 48% (24 of 50) were heterozygous (H/R), and 8% (4 of 50) were homozygous(R/R) for the 131R allele (Tables 3 and 4). Forty-four percent (22 of 50) of the patients were homozygous for the FCγR3A-158 F allele, 46% (23 of 50) were heterozygous carriers (F/V), and 10% (5 of 50) were homozygous for the 158V allele. The distribution of genotypes between the N and M settings was similar and was not significantly different from that would be expected if each group was in Hardy–Weinberg equilibrium.

Table 1. Patient characteristics

Characteristics	N setting		M setting	
	No.	%	No.	%
No. of patients	15	100	35	100
Median age, years	44		58	
Range	23–66		28–76	
Menopausal status				
Pre	9	60	14	40
Post	6	40	21	60
Eastern Cooperative Oncology Group performance status				
0	12	80	18	51
1	3	20	16	46
2	0	0	1	3
Stage				
II	11	73	0	0
III	4	27	0	0
IV (+recurrence)	0	0	35	100
Histological grade				
1	3	20	2	6
2	5	33	9	26
3	7	47	24	68
Estrogen receptor status				
Positive	6	40	12	34
Negative	9	60	23	66
Progesterone receptor status				
Positive	5	33	7	20
Negative	10	67	28	80
Number of axillary lymph node				
1	10	67	–	–
1–3	5	33	–	–
≧4	0	0	–	–
Number of metastatic sites				
1	–	–	16	46
2	–	–	12	34
≧3	–	–	7	20

N, neoadjuvant; M, metastatic.

clinical response to trastuzumab therapy and FcγR polymorphisms

The pCR rate for the N setting was 33% [95% confidence interval (CI), 11.6% to 61.6%] (Table 2). No significant difference in the pretreatment features was observed between the FCγR2A and R3A genotypes. The FcγR2A-131 H/H genotype was significantly correlated with the pathological response [71% (5/7) for H/H versus 0% (0/8) for H/R + R/R; $P = 0.015$, Fisher's exact test; $P = 0.007$, chi-square test; $P < 0.05$, both linear correlation test and ANOVA test; Table 3]. The FcγR3A-158 V/V genotype was not correlated with the pathological response.

The objective response rate for the M setting was 23% (95% CI, 10.4% to 40.1%), and the disease control rate was 66% (95% CI, 47.8% to 80.9%) (Table 2). The median duration time of stable disease ($n = 15$) was 9.5 (5.3–17.7) months. A significant difference in the objective response rate was observed between patients with FcγR2A-131 H/H and those with either the 131 H/R or the 131 R/R genotype ($P = 0.043$, Fisher's exact test; $P < 0.05$, both linear correlation test and ANOVA test; Table 4). Although this difference did not reach the level of statistical significance, patients with FcγR3A-158 V/V also showed an overall higher response rate than the other two FcγR3A-158 genotypes [40% (6/15) for V/V versus 10% (2/20) for F/V + F/F; $P = 0.053$, Fisher's exact test; $P = 0.051$, chi-square test].

PFS analysis according to FcγR polymorphisms

The median follow-up times for the N and M settings were 24.8 and 22.6 months, respectively. Six patients (four local, two distant) had already relapsed as of July 2010. The PFS was assessed at 1 year after the last patient's enrollment in the study.

Table 2. Responses of patients in M or N settings

Response	No.	%
Neoadjuvant setting ($n = 15$)		
Grade 3[a] (pathological CR)	5	33
Grade 2 (marked response)	5	33
Grade 1b (moderate response)	3	20
Grade 1a (mild response)	2	14
Grade 0 (no response)	0	0
Pathological CR rate	5	33
95% CI	11.6–61.6	
M setting ($n = 35$)		
Compete response	1	3
Partial response	7	20
Stable disease	15	43
Progressive disease	12	34
Objective response rate	8	23
95% CI	10.4–40.1	
Disease control rate	23	66
95% CI	47.8–80.9	

[a]Grade refers to the histopathological criteria for the assessment of therapeutic response [21].

N, neoadjuvant; M, metastatic; CR, complete response; CI, confidence interval.

Table 3. FcγR polymorphisms and pathological responses to trastuzumab in an N setting

Polymorphism	Patients	Pathological response (grade)							
		1a		1b		2		3 (pCR)	
		No.	%	No.	%	No.	%	No.	%
FcγR2A									
H/H	7	0	0	0	0	2	29	5	71
H/R	6	1	17	3	50	2	33	0	0
R/R	2	1	50	0	0	1	50	0	0
Fisher's exact test: *P*				*0.015*					
Chi-square test (pCR versus others): *P*[a]				*0.007*					
Linear correlation test: *P*				*0.0076*					
ANOVA test: *P*				*0.0088*					
FcγR3A									
V/V	7	0	0	1	14	2	29	4	57
F/V	6	1	17	2	33	2	33	1	17
F/F	2	1	1	0	0	1	1	0	0
Fisher's exact test: *P*				*0.45*					
Chi-square test (pCR versus others): *P*[a]				*0.12*					
Linear correlation test: *P*				*0.069*					
ANOVA test: *P*				*0.16*					

[a]Comparison of H/H versus R carrier (H/R + R/R) or V/V versus F carrier (F/V + F/F).

N, neoadjuvant; FcγR, fragment C γ receptor; pCR, pathological complete response; ANOVA, analysis of variance.

Table 4. FcγR polymorphisms and tumor responses to trastuzumab in an M setting

Polymorphism	Patients	Response					
		CR/PR		SD		PD	
		No.	%	No.	%	No.	%
FcγR2A							
H/H	15	6	40	7	47	2	13
H/R	18	2	12	8	44	8	44
R/R	2	0	0	0	0	2	100
Fisher's exact test: *P*				*0.043*			
Chi-square test (CR/PR versus SD/PD): *P*[a]				*0.051*			
Linear correlation test: *P*				*0.0077*			
ANOVA test: *P*				*0.029*			
FcγR3A							
V/V	15	6	40	5	33	4	27
F/V	17	1	6	10	59	6	35
F/F	3	1	33	0	0	2	67
Fisher's exact test: *P*				*0.053*			
Chi-square test (CR/PR versus SD/PD): *P*[a]				*0.051*			
Linear correlation test: *P*				*0.12*			
ANOVA test: *P*				*0.16*			

[a]Comparison between H/H versus R carrier (H/R + R/R) or V/V versus F carrier (F/V + F/F).

M, metastatic; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; FcγR, fragment C γ receptor; ANOVA, analysis of variance.

The median PFS time was 6.4 months (95% CI, 3.9–8.6 months). The PFS of patients with FcγR2A-131 H/H was significantly longer than that of patients with 131 H/R or R/R. (Figure 1A: 9.2 versus 3.5 months, $P = 0.034$). In contrast, no statistical difference in the PFS of patients with FcγR3A-158 V/V and that of patients with 158 F/V or F/F was observed (Figure 1B: 8.5 versus 5.3 months, $P = 0.37$). Linkage disequilibrium analyses were conducted among the two FcγR polymorphisms (Table 5). The incidence of the FcγR2A-131 genotype was associated with that of the FcγR3A-158 genotype according to a Fisher's exact test, a chi-square test, and a linear correlation test.

discussion

The overexpression of HER2 protein is observed in ~20–30% of patients with breast cancer and is correlated with a poor clinical outcome. Trastuzumab is an IgG1-type humanized HER2 mAb that has been shown to exhibit significant clinical efficacy as a treatment of MBC [27] and as an adjuvant treatment of operable breast cancer [28]. However, the clinical effectiveness of trastuzumab is somewhat limited: the response rate to single-agent trastuzumab as a first-line treatment is 20–30%; the pCR rate to neoadjuvant therapy including trastuzumab is ~30%. A substantial numbers of HER2-positive tumors exhibit *de novo* resistance to trastuzumab; therefore, the development of biomarkers to select patients who might benefit from trastuzumab is warranted, as a way of decreasing toxicity and reducing unnecessary cost.

The principal mechanism of action of trastuzumab is HER2 blockade with the inactivation of the signal transduction pathway, leading to apoptosis. ADCC is another not insignificant and generally accepted mechanism of trastuzumab action. In ADCC, the cytotoxicity of mAbs that target tumor cells, is mediated by immune effector cells that express FcγR. Recently, two FcγR gene polymorphisms have been identified that affect the binding affinity of IgG, thus changing the effectiveness of ADCC and affecting tumor response. FcγR3A-158 V/V, either alone or in combination with the FcγR2A-131 H/H genotype, was significantly associated with a better response and PFS among patients with follicular lyoma [5, 6] and among MBC patients [14] treated with rituximab- or trastuzumab-based therapy, respectively. Inconsistent data have been reported in metastatic colorectal cancer patients who had not responded to previous irinotecan- or oxaliplatin-based therapy and were subsequently treated with single-agent cetuximab [29]. The

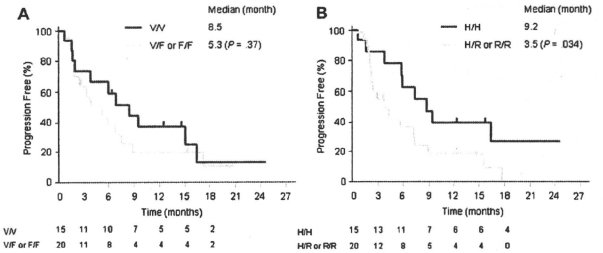

Figure 1. Progression-free survival for patients with metastatic breast cancer receiving single-agent trastuzumab categorized according to fragment C γ receptor (FcγR) polymorphisms. (A) Progression-free survival (PFS) curves were plotted for FcγR2A-131 H/H and H/R or R/R carriers. (B) PFS curves were plotted for FcγR3A-158 V/V and F/V or F/F carriers. V, valine allele; F, phenylalanine allele; H, histidine allele; R, arginine allele.

Table 5. Linkage analysis between FcγR2A and 3A alleles

	Patients	FcγR3A					
		V/V		F/V		F/F	
		No.	%	No.	%	No.	%
FcγR2A							
H/H	22	13	59	9	41	0	0
H/R	24	8	33	13	54	3	13
R/R	4	1	25	1	25	2	50
Total	50	22	44	23	46	5	10
Fisher's exact test: *P*				*0.030*			
Chi-square test (V/V versus F carrier) *P*[a]				*0.020*			
Linear correlation test: *P*				*0.0067*			

[a]Comparison between H/H versus R carrier.
FcγR, fragment C γ receptor.

influence of cytotoxic agents in combination with antibody therapy or the retrospective natures of these analyses might explain the previous inconsistencies. In the present prospective study, the FcγR2A-131 H/H genotype was significantly associated with a stronger tumor response and a longer PFS, and the FcγR3A-158 V/V genotype tended to be correlated with the tumor response after single-agent trastuzumab therapy.

Metastatic cancer patients mostly have suppressed immune function. Thus, early-stage breast patients treated with trastuzumab might be more sensitive to ADCC activity. In the current study, we have demonstrated for the first time that the FCγR2A-131 H/H genotype was significantly correlated with the pathological response after neoadjuvant trastuzumab-based treatment. Our data suggest that this genotype was correlated with not only the pCR rate but also the gradation of the response based on a precise assessment of pathological responses using established histopathological criteria (grade 1a–3; Table 3). A recent large adjuvant trial with trastuzumab-based therapy [15] has raised questions regarding the usefulness of the two FcγR SNPs as predictive biomarkers for recurrence. The sample size of this trial was relatively large; thus, the results seemed to be confirmatory. However, one possible explanation for this difference is that ADCC might be influenced by the existence of a target tumor volume. Another possible explanation is that the cytotoxic agents might influence outcome. Theoretically, the clinical efficacy of trastuzumab is based on both the direct blockade of signal transduction and its indirect effect, ADCC. On the other hand, the efficacy of cytotoxic agents is based on the direct DNA damaging effect and not on ADCC. Thus, the change in ADCC induced by different SNPs might be diluted in cases where trastuzumab and cytotoxic agents are combined, with cases in trastuzumab is used singly.

In this study, we examined 384 SNPs at FcγRI, RII, RIII, HER2 and FUT8 loci. Our findings demonstrated that only two SNP hot spots were correlated with the clinical efficacy of trastuzumab, indicating a high specificity. Our finding that the incidences of the two FCγR2A-131 H/H and FcγR3A-158 V/V genotypes were moderately linked with each other is inconsistent with a previous report [14]. One possible explanation for this discrepancy might be ethnical differences in SNP frequency. Zhang et al. [29] showed that the FCγR2A-131 H/H and FcγR3A-158 V/V genotypes were more frequent among Asian populations than among Western populations. Statistical approaches, including a linear correlation or ANOVA test, suggested that heterozygosity for the two SNPs might have a minimal effect on ADCC activity. Additional studies evaluating the relationship between ADCC activity and the SNP status are needed.

In conclusion, this study supports the hypothesis that FcγR polymorphisms play a role in trastuzumab-mediated ADCC and can predict the clinical outcome of patients with both early and MBC in Asian populations.

funding

Health and Labor Scientific Research Grants, Research on Advanced Medical Technology (H17-Parmaco-006).

acknowledgements

We wish to thank Dr Masaru Sekijima, PhD (Director, Research Division for Advanced Technology Kashima

Laboratory, Mitsubishi Chemical Institute Ltd.) for providing technical support. We also thank Seiichiro Yamamoto for support with the statistical analysis.

disclosure

The authors declare no conflict of interest.

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Published OnlineFirst October 19, 2010; DOI:10.1158/0008-5472.CAN-10-0043

Therapeutics, Targets, and Chemical Biology

Cancer Research

Loss of PTEN Expression by Blocking Nuclear Translocation of EGR1 in Gefitinib-Resistant Lung Cancer Cells Harboring Epidermal Growth Factor Receptor–Activating Mutations

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Abstract

Gefitinib (Iressa) and erlotinib (Tarceva), which target the epidermal growth factor receptor (EGFR), are approved for treatment of patients with advanced non–small cell lung cancer (NSCLC). Patients whose tumors harbor mutations in the *EGFR* gene, including delE746-A750 in exon 19 and L858R in exon 21, may benefit in particular from gefitinib treatment. However, acquired resistance to gefitinib has been a serious clinical problem, and further optimization is needed for application of EGFR-targeted drugs in lung cancer patients. In this study, we established gefitinib-resistant NSCLC cells from PC-9 cell line, which harbors the delE746-A750 mutation, by exposing the cell line to gefitinib for over 7 months. Gefitinib-resistant PC-9/GEFs cell lines showed a marked downregulation of PTEN expression and increased Akt phosphorylation. In revertant, gefitinib-sensitive clones (PC-9/Rev) derived from PC-9/GEF1-1 and PC-9/GEF2-1, PTEN expression, as well as sensitivity to gefitinib and erlotinib, was restored. Knockdown of PTEN expression using small interfering RNA specific for PTEN in PC-9 cells resulted in drug resistance to gefitinib and erlotinib. Nuclear translocation of the EGR1 transcription factor, which regulates PTEN expression, was shown to be suppressed in resistant clones and restored in their revertant clones. Reduced PTEN expression was also seen in tumor samples from a patient with gefitinib-refractory NSCLC. This study thus strongly suggests that loss of PTEN expression contributes to gefitinib and erlotinib resistance in NSCLC. Our findings reinforce the therapeutic importance of PTEN expression in the treatment of NSCLC with EGFR-targeted drugs. *Cancer Res; 70(21); 8715–25. ©2010 AACR.*

Introduction

Lung cancer is the leading cause of death worldwide (1, 2), and non–small cell lung cancer (NSCLC) is the most common type of this disease. The development of drugs that target specific molecules, such as gefitinib and erlotinib, which target the epidermal growth factor receptor (EGFR), has improved the efficacy of therapy for NSCLC. The presence of activating EGFR mutations within the kinase domain of the *EGFR* gene in NSCLC adenocarcinomas has a key role in determining the therapeutic efficacy of EGFR-targeted drugs (3–5). We previously reported that activating EGFR mutations in lung cancer cells selectively enhanced their EGF/transforming growth factor α–independent growth capacity as well as their susceptibility to the cytotoxic effects of gefitinib (6). Of the EGFR-driven signaling pathways that specifically mediate the cytotoxicity and therapeutic efficacy of EGFR-targeted drugs, Akt signaling is rather more selectively activated than extracellular signal-regulated kinase (ERK) signaling in cells harboring mutant EGFRs to promote cell survival in lung cancer cells (7). About 80% of tumors with EGFR-activating mutations respond to EGFR tyrosine kinase inhibitors (TKI). In NSCLC tumors, >90% of EGFR mutations are located in exon 19 (delE746-A750) or 21 (L858R point mutation). These activating EGFR mutations sensitize the tumors to EGFR-targeted drugs and are often significantly associated with an adenocarcinoma-associated histology, smoking status, gender, and ethnicity (8, 9). Mok and colleagues (10) recently reported that gefitinib is superior to carboplatin-paclitaxel as an initial treatment for NSCLC adenocarcinomas among nonsmokers or former light smokers in eastern Asia.

In addition to these mutations in EGFR itself, various other molecular mechanisms are involved in EGFR activation and responses to gefitinib, erlotinib, and other EGFR-targeted drugs (11, 12). Of the EGFR family of proteins, the expression of HER3 and/or HER2 has been shown to affect

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-10-0043

Published OnlineFirst October 19, 2010; DOI:10.1158/0008-5472.CAN-10-0043

responses to EGFR-targeted drugs both *in vitro* and *in vivo* (13–15). A secondary EGFR mutation, T790M, has been identified that emerged after EGFR-TKI treatment (16–18), whereas K-ras mutations have been reported to be linked to primary resistance to EGFR-TKIs (19, 20). Amplification of the *Met* gene and overexpression of hepatocyte growth factor (HGF) have been reported as mechanisms for the acquisition of resistance to EGFR-TKIs (21–23). Sos and colleagues (24) recently reported that loss of PTEN expression contributed to erlotinib resistance by the activation of downstream signaling pathways of EGFR in lung cancer, suggesting a possible involvement of PTEN downregulation and Akt activation in the altered therapeutic efficacy of EGFR-targeted drugs. In addition to the mechanisms identified to date, including activating EGFR mutations, activation of the HGF/Met pathway, Ras mutations, and HER2/HER3 expression, further understanding of novel mechanisms that control the sensitivity to and therapeutic efficacy of EGFR-targeted drugs is required for the development of a more complete evidence base for therapeutics that are effective in lung cancer patients.

In this study, we identified a novel mechanism for drug resistance to gefitinib and erlotinib in NSCLC cell lines and the relevant molecules involved. We established gefitinib resistant cell lines from the lung cancer–derived PC-9 cell line harboring EGFR-activating mutations, and also their spontaneous revertants. We found that PTEN expression was specifically lost in resistant cell lines and restored in their drug-sensitive revertants. PTEN loss was observed in recurring NSCLC tumors harboring EGFR deletions after monotherapy with gefitinib. We investigated the possible mechanism by which PTEN expression was lost in resistant cell lines.

Materials and Methods

Cell culture and reagents

PC-9, QG56, and 11-18 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum in an atmosphere of 5% CO_2 (6, 14). Gefitinib was provided by AstraZeneca, Inc., and erlotinib was provided by F. Hoffmann–La Roche Ltd. SU11274 and anti–phospho-EGFR (pEGFR) antibodies were purchased from Calbiochem. Anti-HER2 and anti–phospho-HER2 (pHER2) were from Upstate Biotechnology. Anti-EGFR, anti–phospho-Met (pMet), anti–phospho-HER3 (pHER3), anti-ERK1/2, anti–phospho-ERK1/2 (pERK1/2), anti-Akt, anti–phospho-Akt (pAkt), anti-PTEN, and anti-CREB antibodies were purchased from Cell Signaling Technology. Mutation-specific antibodies (delE746-A750 in exon 19, L858R in exon 21) were also purchased from Cell Signaling Technology. Anti-Met, anti-HER3, anti-EGR1, and anti-HSP90 antibodies were purchased from Santa Cruz Biotechnology. Anti–β-actin antibody was purchased from Abcam. The small interfering RNAs (siRNA) corresponding to PTEN (5′-AUUGUCAUCUUCACUUAGCCAUUGG-3′) and EGR1 (5′-UCCAGCUUAGGGUAGUUGUCCAUGG-3′) were purchased from Invitrogen. Cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX and Opti-MEM (Invitrogen) according to the manufacturer's recommendations.

Plasmid construction

To obtain full-length cDNA sequences for human EGR1, PCR was carried out with a SuperScript cDNA library (Invitrogen) using the following primer pairs (single underlining indicates the start and stop codon): 5′-ATGGCCGCG-GCCAAGGCC-GAGATG-3′ and 5′-TTAGCAAATTTCAAT-TGTCCTGGGA-GAAA-3′. This PCR product was cloned into the pGEM-T easy vector (Promega). To construct a plasmid expressing HA-tagged EGR1, NH_2-terminal HA-tagged EGR1 cDNA was ligated into pcDNA3 vector (Invitrogen).

Cell proliferation assay

Exponentially growing cell suspensions (2–4 × 10^3/100 μL) were seeded into 96-well microtiter plates per well. The following day, various concentrations of gefitinib, erlotinib, cisplatin, or SU11274 were added. After incubation for 72 hours at 37°C, 20 μL of Cell Count Reagent SF (Nacalai Tesque) were added to each well and the plates were incubated for a further 1 to 2 hours at 37°C. Absorbance was measured at 450 nm with a 96-well plate reader. Triplicate wells were tested at each drug concentration. The IC_{50} value, defined as the concentration giving a 50% reduction in absorbance, was calculated from the survival curves.

Western blot analysis

Cells were rinsed with ice-cold PBS and lysed in Triton X-100 buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 50 mmol/L NaF, 1% Triton X-100, and 10% glycerol containing 5 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L sodium orthovanadate). Cell lysates were separated by SDS-PAGE and transferred to Immobilon membranes (Millipore Corp.). After transfer, membranes were incubated in blocking solution, probed with various antibodies, washed, and visualized using horseradish peroxidase (HRP)–conjugated secondary antibodies (GE Healthcare) and enhanced chemiluminescence reagents (Amersham).

Quantitative real-time PCR

RNA was reverse transcribed from random hexamers using avian myeloblastosis virus reverse transcriptase (Promega). Quantitative real-time PCR was performed using the Real-Time PCR System 7300 (Applied Biosystems). In brief, 20 μL of PCR amplification reaction mixtures contained cDNA, primer pairs (Applied Biosystems), a dual-labeled fluorogenic probe (Applied Biosystems), and Taqman Universal PCR Master Mix (Applied Biosystems). The thermal cycle conditions included maintaining the reactions at 50°C for 2 minutes and 95°C for 10 minutes, and then 40 cycles alternating between 95°C for 15 seconds and 60°C for 1 minute. The relative gene expression for each sample was determined using the formula $2^{(-\Delta Ct)} = 2^{[Ct\ (GAPDH)\ -\ Ct\ (target)]}$, which reflected the target gene expression normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

Published OnlineFirst October 19, 2010; DOI:10.1158/0008-5472.CAN-10-0043

Table 1. Cytotoxicity of gefitinib, erlotinib, cisplatin, or SU11274 in PC-9 and gefitinib-resistant cell lines

Cell lines	IC_{50} (μmol/L)			
	Gefitinib	Erlotinib	Cisplatin	SU11274
PC-9	0.059 (1.0)	0.095 (1.0)	1.6 (1.0)	5.5 (1.0)
PC-9/GEF1-1	12.8 (217)	7.1 (75)	1.2 (0.8)	6.7 (1.2)
PC-9/GEF2-1	13.9 (236)	23.7 (249)	2.2 (1.4)	6.3 (1.1)
PC-9/GEF2-2	18.0 (305)	29.0 (305)	1.4 (0.9)	5.7 (1.0)

NOTE: IC_{50} values, causing 50% growth inhibition, were calculated from log-logit regression lines. The assays were carried out in triplicate. The relative resistance, defined as the IC_{50} value divided by the IC_{50} value of the parental PC-9 cells, is shown in parentheses.

Peptide nucleic acid–locked nucleic acid PCR clamp method and direct DNA sequencing

Activating mutations of the *EGFR* gene in exons 19 (delE746-A750) and 21 (L858R) were examined by peptide nucleic acid–locked nucleic acid (PNA-LNA) PCR clamp as described previously (25, 26). In brief, genomic DNA was purified from paraffin-embedded tissue using a QIAamp DNA Micro kit (Qiagen). The PCR primers used were synthesized by Invitrogen. PNA clamp primers and LNA mutant probes were purchased from FASMEC and IDT, respectively. The PNA-LNA PCR clamp method used a SDS-7500 System (Applied Biosystems). To analyze the T790M mutation, exon 20 of the *EGFR* gene was amplified using the PCR primer set forward (5′-CATTCATGCGTCTTCACCTG-3′) and reverse (5′-CATATCCCCATGGCAAACTC-3′) and TaKaRa Ex Taq polymerase (TaKaRa BIO, Inc.). PCR products were directly used as templates for cycle sequencing reactions using the BigDye Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems). The forward or reverse primers were used for cycle sequencing reactions, which were carried out in an ABI PRISM 310 Genetic Analyzer.

Immunohistochemistry

Informed consent for the use of tumor tissue specimens was obtained from all patients or from their legal guardians, as approved by the institution's ethics review board. Eight patients had an acquired resistance after receiving gefitinib therapy at University Hospital, University of Occupational and Environmental Health, Japan, between 1995 and 2007 (27). Three gefitinib-refractory tumors were obtained from pulmonary metastases in a patient who did not harbor the T790M mutation in the *EGFR* gene. In this patient, three separated metastatic pulmonary tumors were obtained by a video-assisted thoracoscopic surgery, which were located in segments 5, 6, and 8 of the left lung. Paraffin-embedded tissue samples were sectioned at a thickness of 4 μm, mounted on coated glass slides, and labeled with anti-PTEN antibody (DakoCytomation), pAkt antibody (Cell Signaling Technology), EGR1 antibody (Cell Signaling Technology), and anti-Met antibody (Immuno-Biological Laboratories) using the ChemMate ENVISION method (DakoCytomation). The intensity of PTEN, pAkt, EGR1, and Met labeling was assessed using the following scale: none (0), weak (1+), moderate (2+), and strong (3+; ref. 26). All slides were evaluated by two experienced observers who were blinded to the condition of the patients (A.K. and M.Ka.).

Mutation-specific EGFR antibodies recently developed by Yu and colleagues (28) were used to specifically identify L858R and delE746-A750 mutations (Cell Signaling Technology) and to analyze the expression of mutant EGFR in our cell lines and clinical specimens (26). Cells were rinsed with ice-cold PBS and fixed in 4% paraformaldehyde in PBS for 30 minutes. After fixation, slides were briefly washed in water. Antigen retrieval was carried out by boiling for 30 minutes in 1 mmol/L EDTA (pH 9.0) in a microwave. Intrinsic peroxidase activity was blocked using Peroxidase Blocking Reagent (Dako) for 5 minutes. After washing in TBS for 5 minutes, the slides were incubated with the primary antibodies diluted 1:100 at room temperature for 30 minutes. After washing in TBS for 5 minutes, slides were incubated for 30 minutes with polymer HRP-labeled secondary antibody (ChemMate ENVISION kit, Dako) at room temperature, washed in TBS for 10 minutes, and then visualized using 3,3′-diaminobenzidine (Dako). Sections from clinical specimens were incubated with the primary antibodies at 4°C overnight.

Results

Establishment and characterization of gefitinib-resistant sublines

To isolate gefitinib-resistant sublines from PC-9 cells harboring the activating EGFR mutation delE746-A750, we cultured the cells in increasing doses of drug from 0.05 to 15 μmol/L, which were increased stepwise over 7 months. We independently selected gefitinib-resistant cell lines from two plastic culture flasks, which had been exposed to various doses of the drug. We cloned three gefitinib-resistant sublines: PC-9/GEF1-1 from one flask, and PC-9/GEF2-1 and PC-9/GEF2-2 from another flask. These three clones all showed 70- to 300-fold higher resistance to gefitinib and erlotinib than the parental PC-9 cell line (Table 1). By contrast, these resistant cells were as sensitive as PC-9 cells to the non-EGFR–targeted drug, cisplatin, and the Met TKI, SU11274 (Table 1).

Loss of PTEN expression and increased expression of activated Akt in gefitinib-resistant cell lines

We compared the expression level and phosphorylation status of EGFR, HER2, HER3, Met, PTEN, Akt, and ERK in the gefitinib-resistant cell lines (PC-9/GEF cell lines) and the parental PC-9 line by Western blot analysis (Fig. 1A). Compared with PC-9 cells, the drug-resistant cell lines expressed 30% to 50% lower levels of Met. However, the most striking differences between the sensitive and resistant lines were in PTEN protein expression and Akt phosphorylation

Published OnlineFirst October 19, 2010; DOI:10.1158/0008-5472.CAN-10-0043

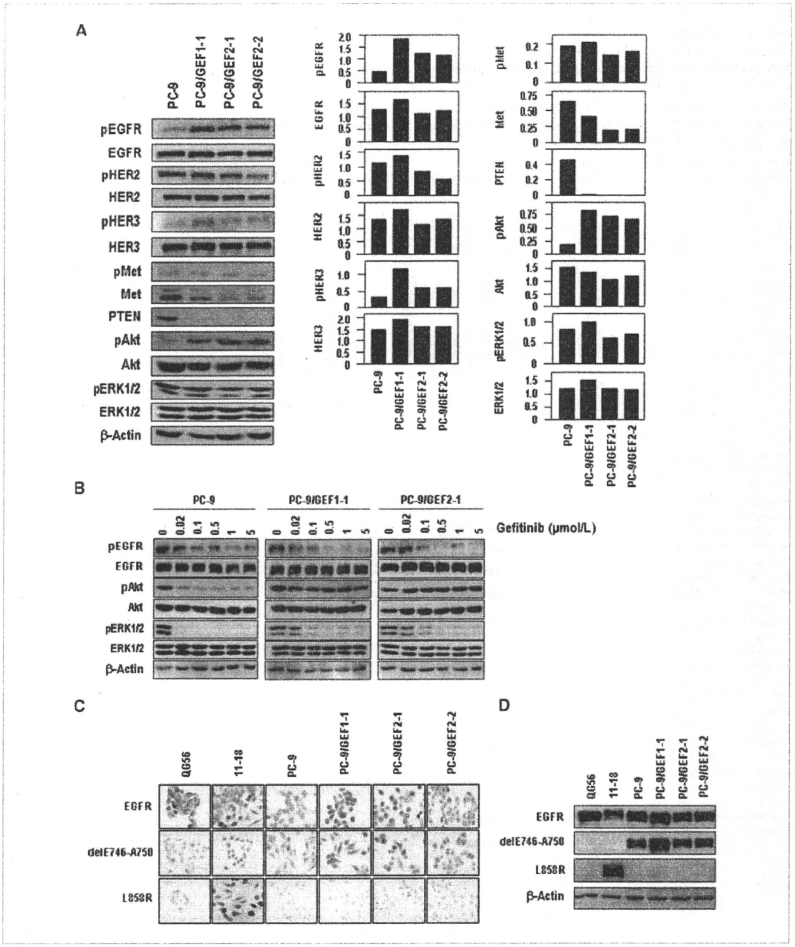

Figure 1. Activation or expression of EGFR, the EGFR family proteins, and downstream signaling molecules. A, Western blots showing the expression of pEGFR, EGFR, pHER2, HER2, pHER3, HER3, pMet, Met, PTEN, pAkt, Akt, pERK1/2, and ERK1/2 proteins and β-actin, as a loading control, in cell lysates (left) and their expression levels relative to β-actin (right). B, effect of gefitinib on expression of pEGFR, pAkt, and pERK1/2. Cells were treated with gefitinib for 3 h at the concentrations indicated. C, immunohistochemical staining of QG56, 11-18, PC-9, and PC-9/GEF0 cell lines. The QG56 cell line is a negative control for both anti–delE746-A750 and anti-L858R antibodies. Top, the anti-EGFR antibody stained all six cell lines; middle, the delE746-A750–specific antibody stained PC-9 and PC-9/GEF cells; bottom, the L858R-specific antibody stained only 11-18 cells. D, Western blots showing the presence or absence of the EGFR mutation delE746-A750 or L858R in different cell lines.

Published OnlineFirst October 19, 2010; DOI:10.1158/0008-5472.CAN-10-0043

(Fig. 1A). There was almost no expression of PTEN protein and markedly increased phosphorylation of Akt in the drug-resistant sublines compared with PC-9 cells. We further examined if phosphorylation of EGFR, ERK1/2, and Akt was susceptible to the inhibitory effect of gefitinib in the drug-resistant cell lines. As shown in Fig. 1B, only phosphorylation of Akt was resistant to gefitinib in resistant clones. By contrast, phosphorylation of EGFR and ERK1/2 remained sensitive to gefitinib-resistant cell lines as PC-9. PC-9/GEF2-2 cell lines also showed the same results (data not shown). Downregulation of PTEN is thus mainly responsible for gefitinib resistance through altered phosphorylation status of Akt.

We analyzed the DNA sequences of these three resistant cell lines for the T790M mutation, known to be associated with secondarily acquired resistance, but this mutation was not present (data not shown). Based on the recently developed antibodies specific for the delE746-A750 and L858R mutations (26, 28), we next asked whether the delE746-A750 mutation was conserved in the three drug-resistant sublines by both immunocytochemical and Western blot analysis

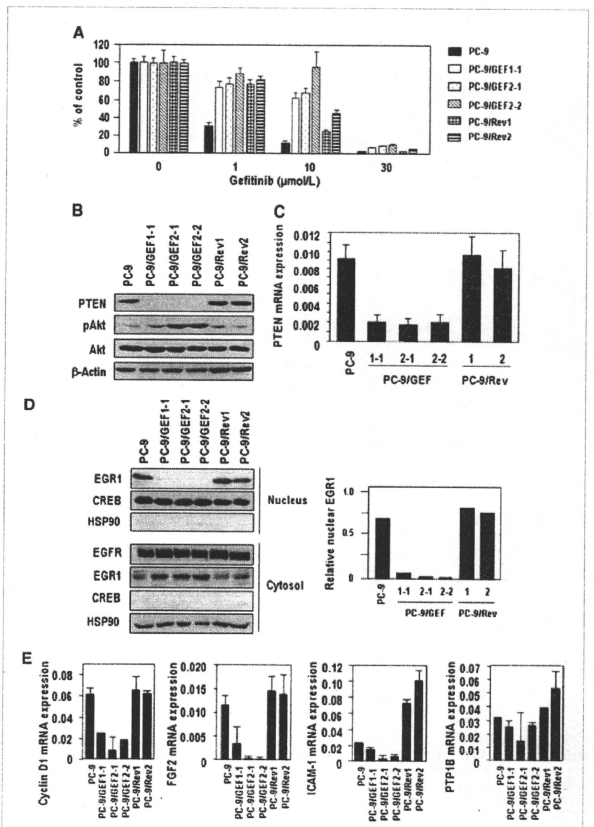

Figure 2. Restoration of PTEN expression in drug-sensitive revertant cell lines derived from gefitinib-resistant cells. A, results of proliferation assays showing the sensitivity of revertant cell lines PC-9/Rev1 and PC-9/Rev2 to gefitinib. B, Western blots showing the expression of PTEN, pAkt, Akt, and β-actin. C, expression of PTEN mRNA in PC-9, gefitinib-resistant PC-9/GEF, and drug-sensitive revertant PC-9/Rev cell lines. D, Western blots showing expression of EGR1 in nuclear and cytosolic fractions of resistant and revertant cell lines. CREB and HSP90 acted as loading controls for nuclear and cytosolic fractions, respectively. E, expression of cyclin D1, FGF2, ICAM-1, and PTP1B mRNA in PC-9, gefitinib-resistant (PC-9/GEF1-1, PC-9/GEF2-1, and PC-9/GEF2-2), and their drug-sensitive revertant cell lines (PC-9/Rev1 and PC-9/Rev2).

Published OnlineFirst October 19, 2010; DOI:10.1158/0008-5472.CAN-10-0043

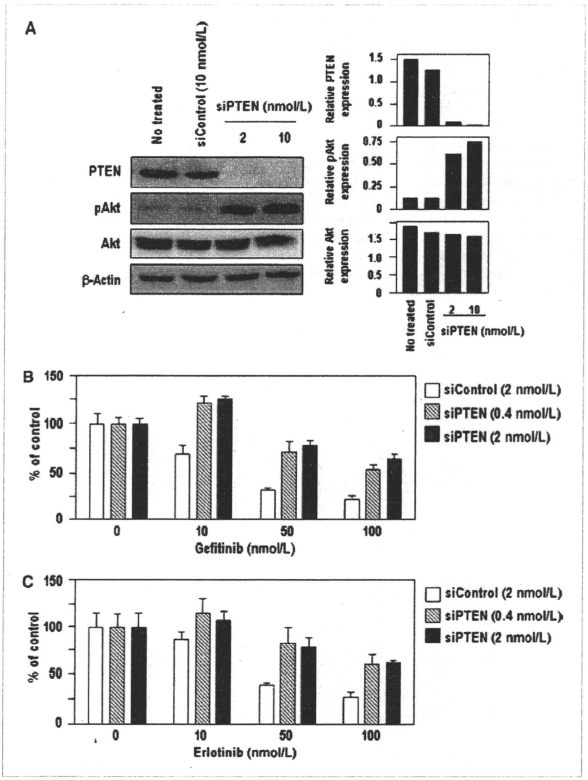

Figure 3. Effect of PTEN knockdown on the sensitivity of PC-9 cells to EGFR-targeted drugs. A, Western blots showing the knockdown of PTEN expression by treatment with PTEN siRNA. Cells were incubated with siRNA for 96 h, and lysates were then prepared. B and C, effects of knockdown of PTEN expression on sensitivity of PC-9 cells to gefitinib (B) and erlotinib (C). Cells were treated with siRNA for 24 h, and then gefitinib or erlotinib was added to the wells for a further 72 h.

(Fig. 1C and D). Both methodologies showed that the gefitinib-resistant cell lines retained the delE746-A750 mutation, characteristic of the PC-9 cell line. We also confirmed this by direct DNA sequencing of all three resistant lines (data not shown).

Isolation of drug-sensitive revertants from gefitinib-resistant cell lines and PTEN expression

We continuously cultured two of the resistant cell lines, PC-9/GEF1-1 and PC-9/GEF2-1, for ~9 months in the absence of gefitinib. Cell lines PC-9/Rev1, derived from PC-9/GEF1-1, and PC-9/Rev2, derived from PC-9/GEF2-1, were established and cloned. We compared their drug sensitivity with that of their parental drug-resistant cell lines. A higher sensitivity to gefitinib was restored in the PC-9/Rev1 and PC-9/Rev2 cell lines compared with their drug-resistant counterparts (Fig. 2A), with IC_{50} values of 3.5 and 5.1 μmol/L for PC-9/Rev1 and PC-9/Rev2, respectively, compared with 12.8 to 18.0 μmol/L for the PC-9/GEF cell lines (see Table 1). These gefitinib-sensitive revertants and their parental drug-resistant lines showed almost identical sensitivity to cisplatin and SU11274: IC_{50}/cisplatin values of 2.7 and 1.6 μmol/L for PC-9/Rev1 and PC-9/Rev2, respectively; IC_{50}/SU11274 values of 4.8 and 4.2 μmol/L for PC-9/Rev1 and PC-9/Rev2, respectively. The sensitivity of these sublines to gefitinib and erlotinib was not completely restored to the level seen for PC-9 cells. However, Western blot analysis showed that PTEN expression was completely restored in the revertant cell lines and also Akt activation was reduced (Fig. 2B).

Published OnlineFirst October 19, 2010; DOI:10.1158/0008-5472.CAN-10-0043

Nuclear translocation of EGR1 is required for PTEN expression

We next investigated the mechanism by which PTEN protein expression was downregulated in resistant cell lines. Quantitative reverse transcription-PCR analysis showed markedly decreased expression of PTEN mRNA in all three resistant sublines, PC-9/GEF1-1, PC-9/GEF2-1, and PC-9/GEF2-2, and restored expression of PTEN mRNA in revertant cell lines, PC-9/Rev1 and PC-9/Rev2, compared with PC-9 cells (Fig. 2C). As PTEN expression has been shown to be induced by the EGR1 transcription factor (29, 30), we analyzed the possible role of EGR1 in the reduced expression of PTEN in these cell lines. Compared with parental PC-9 cells, the three resistant PC-9/GEF cell lines showed EGR1 expression in the cytosolic fraction but lower in the nuclear fraction (Fig. 2D). By contrast, the expression of nuclear EGR1 was restored in the drug-sensitive revertant cell lines (Fig. 2D). We further examined whether expression of EGR1 and PTEN was affected by exposure to low dose of gefitinib in PC-9 cells (Supplementary Fig. S1). Phosphorylation of EGFR, ERK1/2, and Akt was markedly blocked when PC-9 cells were exposed to low doses of the drug for 1, 3, and 5 days. Under this condition, however, expression of PTEN was not changed by treatment with gefitinib.

PTEN gene is transcriptionally regulated by Akt-EGR1-PTEN axis (29), but EGR1 is also responsible for expression of various other genes including cyclin D1, intercellular adhesion molecule-1 (ICAM-1), fibroblast growth factor 2 (FGF2), and protein tyrosine phosphatase-1B (PTP1B; refs. 31–36). We examined whether expression of cyclin D1, ICAM-1,

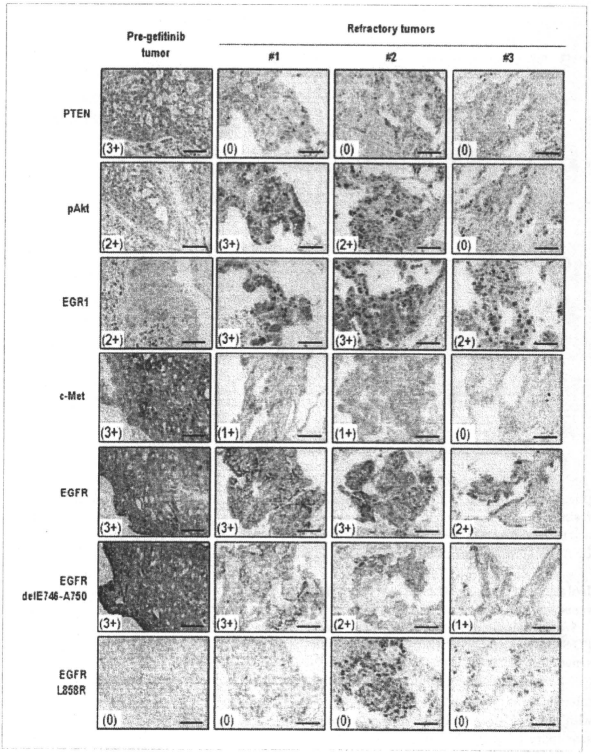

Figure 4. Immunohistochemical analysis of PTEN, pAkt, EGR1, Met, EGFR (control), EGFR (delE746-A750), and EGFR (L858R) in a patient who had received monotherapy with gefitinib. Expression of PTEN was reduced in secondary, gefitinib-refractory tumors compared with the primary tumor. Three gefitinib-refractory tumors (1–3) from pulmonary metastasis after the monotherapy and pretreatment tumor were analyzed. The intensity of immunohistochemical labeling is assessed by none (0), weak (1+), moderate (2+) and strong (3+). Scale bars, 100 μm. Magnification, ×200.

Published OnlineFirst October 19, 2010; DOI:10.1158/0008-5472.CAN-10-0043

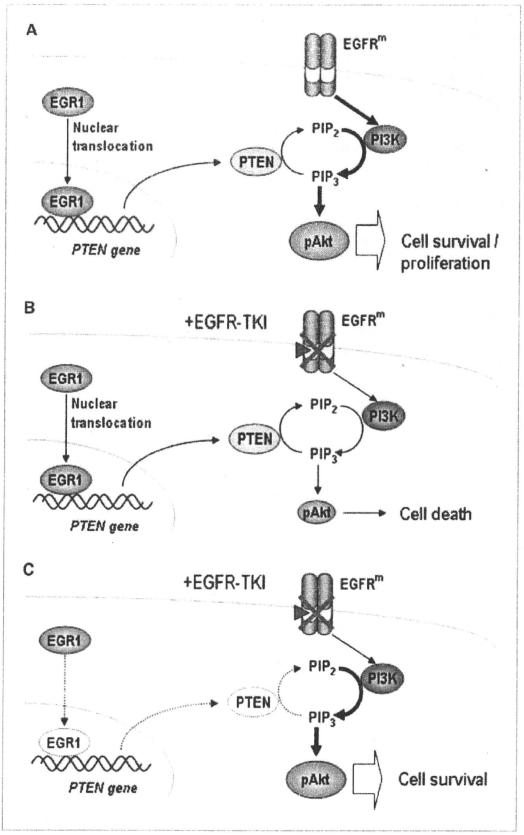

Figure 5. A model showing how resistance to EGFR-targeted drugs is acquired as a result of loss of PTEN expression through impaired nuclear translocation of EGR1 in lung cancer cells. A, continuous activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway in cells harboring EGFR-activating mutations (EGFR^m) promotes cell survival. PTEN is a negative regulator of PI3K/Akt pathway, dephosphorylating PIP_3, but the PI3K/Akt pathway is constitutively activated by these EGFR mutations, resulting in cell survival and proliferation. B, EGFR-TKIs inhibit the activating EGFR tyrosine kinase, which is accompanied by a block of PI3K/Akt signaling, and results in cell death when lung cancer cells harboring EGFR^m are treated with EGFR-TKI. C, loss of PTEN expression further promotes the activation of PI3K/Akt signaling, resulting in the acquisition of resistance to EGFR-TKI and thus in cell survival of drug-resistant lung cancer cell lines such as PC-9/GEFs. In drug-resistant cell lines, PTEN expression is downregulated through impaired EGR1 translocation into the nucleus.

FGF2, and PTP1B was differentially altered between PC-9 and drug-resistant cell lines. Of the four genes examined, mRNA expression of cyclin D1, ICAM-1, and FGF2 was downregulated in gefitinib-resistant cell lines and restored in these drug-sensitive revertants to similar levels as PC-9 (Fig. 2E). Some EGR1 target genes in addition to PTEN are regulated under the control by impaired nuclear translocation of EGR1. To further determine if EGR1 directly regulates PTEN expression, we examined the effect of EGR1 knockdown on PTEN expression in PC-9 cells. Treatment with siRNA specific for EGR1 induced a reduced level of PTEN expression (Supplementary Fig. S2A), suggesting a close association of EGR1 with PTEN. We also examined whether expression of PTEN could be restored by EGR1 overexpression in the PC-9/GEF cells. The overexpression of EGR1, however, could not restore PTEN expression in PC-9/GEF1-1 cells when transfected with EGR1 cDNA (Supplementary Fig. S2B).

PTEN knockdown reduced sensitivity to EGFR-targeted drugs

Because PTEN expression was downregulated and Akt phosphorylation increased in gefitinib-resistant lines, we also

Published OnlineFirst October 19, 2010; DOI:10.1158/0008-5472.CAN-10-0043

assessed the effect of PTEN knockdown on sensitivity to gefitinib and erlotinib. Treatment with siRNA specific for PTEN induced a reduced level of PTEN expression and increased Akt phosphorylation in PC-9 cells (Fig. 3A). This knockdown of PTEN expression also conferred resistance to gefitinib compared with a control siRNA used in the same cell line (Fig. 3B). The IC_{50} values for gefitinib were 0.025 μmol/L in cells treated with 2 nmol/L control siRNA, 0.29 μmol/L in cells treated with 0.4 nmol/L PTEN siRNA, and 1.1 μmol/L in cells treated with 2 nmol/L PTEN siRNA. Similar results were obtained in PC-9 cells with erlotinib (Fig. 3C), which showed IC_{50} values of 0.037 μmol/L in cells treated with 2 nmol/L control siRNA, 0.18 μmol/L in cells treated with 0.4 nmol/L PTEN siRNA, and 0.72 μmol/L in cells treated with 2 nmol/L PTEN siRNA.

Loss of PTEN expression in patients with gefitinib-refractory NSCLC

We finally examined whether such PTEN loss was observed in NSCLC patients who are refractory to monotherapy by gefitinib. We screened expression of PTEN in tumors from patients showing acquired resistance to gefitinib (27) by immunohistochemical analysis. During screening of 10 refractory tumors without T790M mutation, *Met* gene amplification, HGF overexpression, and K-ras mutations, we found one refractory tumor showing low expression of PTEN. As shown in Fig. 4, three gefitinib-refractory tumors were obtained from three different pulmonary metastatic foci in a patient who had received monotherapy with gefitinib. Both the patient's primary tumor, before treatment, and the three refractory tumors harbored the EGFR delE746-A750 mutation. However, PTEN expression was markedly lower in these three refractory tumors compared with the tumor before gefitinib treatment, and Akt phosphorylation was apparently enhanced in tumors 1 and 2, but was not apparent in tumor 3. The expression levels of Met were lower in three refractory tumors (Fig. 4).

Immunohistochemical analysis with anti-EGR1 antibody showed that EGR1 was expressed in both nucleus and cytoplasm of many cancer cells in both primary tumor and refractory tumors (Fig. 4). EGR1 was also expressed in other cell types of tumor stroma. However, there was no apparent difference in expression levels of EGR1 between the primary tumor and refractory tumors. It remains to be further studied whether EGR1 expression in the nucleus and/or cytoplasm is correlated with PTEN expression in clinical tumor samples.

Discussion

The deletion or inactivation of PTEN is often seen in a variety of tumor types, including lung, bladder, renal, breast, endometrial, melanoma, and thyroid, and there are no other family members that can replace PTEN function (37). Furthermore, the role of PTEN is an important one because its loss results in the misregulation of multiple Akt-dependent and Akt-independent pathways critical for the progression of malignant cancers (37). In this study, we have shown that the loss of PTEN expression was critical for the acquisition of resistance to EGFR-targeted drugs, such as gefitinib and erlotinib, in lung cancer cells (Fig. 5) based on the following evidence: (*a*) the gefitinib-resistant PC-9/GEF sublines, derived from the NSCLC PC-9 cell line harboring the drug-sensitive EGFR delE746-A750 mutation, showed much lower PTEN expression than the parental PC-9 cells; (*b*) PTEN expression was restored in gefitinib-sensitive PC-9/Rev revertants, derived from the resistant sublines, to levels comparable with those seen in the parental PC-9 cells; (*c*) nuclear translocation of EGR1, a critical transcription factor in PTEN expression, was markedly inhibited in drug-resistant cell lines and was restored in revertant cells; (*d*) knockdown of PTEN conferred resistance to gefitinib and erlotinib in lung cancer cells; and (*e*) secondary gefitinib-refractory metastases from a patient with NSCLC who had been treated with gefitinib showed lower expression of PTEN than the same patient's primary tumor, removed before treatment.

In our gefitinib-resistant PC-9/GEF sublines and gefitinib-sensitive PC-9/Rev revertants, the cellular levels of functional PTEN expression and Akt activation were closely correlated. This close coupling of PTEN-Akt signaling confers prolonged cell survival through constitutive Akt activation and acquisition of resistance to EGFR-targeted drugs (Fig. 5). PTEN expression itself is important for predicting the therapeutic efficacy of gefitinib in NSCLC patients (38). The acquisition of gefitinib resistance as a result of PTEN loss can be overcome by restoring PTEN function or by inhibiting the constitutive activation of Akt (39). Sos and colleagues (24) have recently reported the critical role played by both PTEN loss and Akt activation in resistance to erlotinib in lung cancer cells. Taken together, these results show that the Akt signaling pathway is critical in the effects that PTEN loss has on cell survival and sensitivity to EGFR-targeted drugs.

Revertant drug-sensitive sublines were isolated after the prolonged culture of gefitinib-resistant cells in the absence of the drug, and showed a 3- to 4-fold higher sensitivity to gefitinib than the drug-resistant cell lines, indicating that drug sensitivity in these sublines could be partially recovered. However, expression of PTEN was almost completely restored to the levels seen in the parental PC-9 cells. During the acquisition of 200- to 300-fold higher drug resistance, after long exposure to the drug, changes in the expression of multiple factors, including PTEN, and other growth factor receptors might be expected to be modulated. PTEN loss thus might be one of several altered pathways that could be involved in the acquisition of drug resistance to EGFR-TKIs.

The loss of PTEN expression has been reported to be in part due to the methylation of the *PTEN* gene promoter in some tumors (40–42). However, treating the gefitinib-resistant cell lines with a DNA methyltransferase inhibitor or histone deacetylase inhibitors did not affect the expression of PTEN,[7] suggesting that the methylation status of the PTEN promoter is not likely to be associated with PTEN

[7] Unpublished data.