72.6 months, $P = 0.039$) (Figure 3). Univariate analysis revealed that, among the characteristics examined, only the expression score for PD-L1 ($P = 0.039$) and p stage ($P < 0.001$) were significantly associated with OS (Table 3). Cox regression analysis also revealed that high PD-L1 expression ($P = 0.020$) and p stage II or III ($P < 0.001$) were significantly associated with a shorter OS independently of other factors (Table 3).

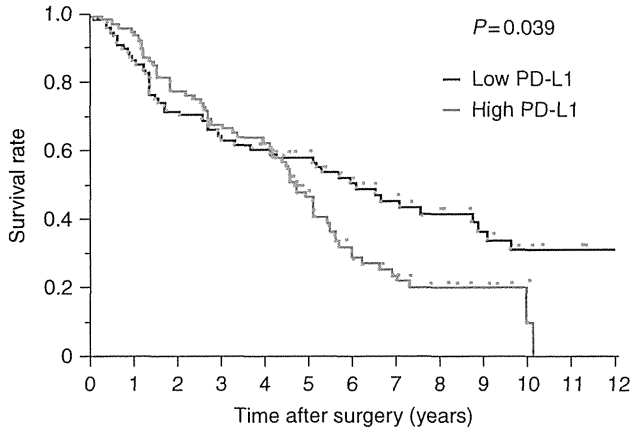

Figure 3. Kaplan–Meier analysis of OS according to PD-L1 expression score in NSCLC patients. The median value (30) of all *H* scores was a priori chosen as the cutoff point for separating tumors with high or low PD-L1 expression. The *P* value for the difference between the two curves was determined by the log-rank test.

discussion

Therapeutic strategies that target the PD1–PD-L1 axis have yielded objective responses in a subset of individuals with advanced NSCLC in early-phase clinical trials. Preliminary results suggest that PD-L1 expression in tumors is correlated with a higher likelihood of response to PD1-targeted antibodies [3]. Although several studies have examined PD-L1 expression in NSCLC [4, 5], the clinicopathologic characteristics associated with PD-L1 expression in such tumors have remained largely unknown. Expression of PD-L1 was recently found to be increased in EGFR-driven murine lung tumors, with this increased expression contributing to escape from the antitumor immune response [10]. We have now examined PD-L1 expression by immunohistochemistry in 164 surgically resected specimens of NSCLC. The data from our Japanese cohort revealed higher PD-L1 expression in women than in men, in never smokers than in smokers, and in individuals with adenocarcinoma than in those with squamous cell carcinoma, which also correspond to the subsets of patients who are more likely to harbor *EGFR* mutations. Multivariate analysis revealed that the presence of *EGFR* mutations and adenocarcinoma histology were also significantly associated with increased PD-L1 expression independently of other factors. The relatively high prevalence of *EGFR* mutations in the Japanese population may have facilitated the detection of this association [2, 9].

Consistent with the positive association between *EGFR* mutations and increased PD-L1 expression in completely resected NSCLC tissue, we also found that the expression level of PD-L1

Factor	No. of patients	Median OS (months)	*P* value[a]	Coefficient (95% CI)[b]	*P* value[b]
Age (years)					
>66	84	55.9	0.395		
≤66	80	64.5			
Sex					
Female	73	64.5	0.482		
Male	91	56.1			
Smoking status					
Never smoker	95	64.5	0.624		
Smoker	69	54.3			
Histology					
Adenocarcinoma	114	61.1	0.893		
SCC	50	55.9			
EGFR mutation status					
Negative	107	61.0	0.594		
Positive	57	61.0			
p Stage					
I	67	78.1	<0.001	0.463 (0.309–0.695)	<0.001
II or III	97	48.8			
PD-L1 expression (*H* score)					
High (>30)	82	55.9	0.039	1.602 (1.078–2.380)	0.020
Low (≤30)	82	72.6			

[a]*P* values for univariate analysis and the log-rank test.
[b]Multivariate analysis by Cox proportional-hazards model.
SCC, squamous cell carcinoma.

was significantly higher in NSCLC cell lines positive for *EGFR* mutations that in those with wild-type *EGFR*. Similar to previous findings [10], inhibition of EGFR signaling with the EGFR-TKI erlotinib resulted in downregulation of the surface expression of PD-L1 in *EGFR* mutation-positive NSCLC cells but not in those with wild-type *EGFR*, indicating that the expression of PD-L1 is likely dependent on EGFR signaling conferred by activating *EGFR* mutations. Treatment with a PD1-specific antibody was also found to inhibit tumor growth and to improve survival in mice bearing EGFR-dependent lung tumors [10]. These data suggest that therapeutic blockade of the PD1–PD-L1 pathway may enhance the efficacy of treatment of *EGFR* mutation-positive NSCLC. Furthermore, in addition to inhibition of tumor growth and tumor survival dependent on EGFR signaling, downregulation of PD-L1 expression and consequent activation of an antitumor immune response also may contribute to the durable therapeutic response of *EGFR* mutation-positive NSCLC patients to EGFR-TKIs. The secondary mutation T790M of *EGFR* has been detected in up to 60% of NSCLC tumors with acquired resistance to EGFR-TKIs [6]. We have now found that EGFR-TKI-resistant H1975 cells, which harbor both an activating mutation and T790M in *EGFR*, continue to express PD-L1 at a high level even in the presence of erlotinib, suggesting that blockade of PD1–PD-L1 signaling is a potential approach to help overcome acquired resistance to EGFR-TKIs in *EGFR* mutation-positive NSCLC.

We found that patients with a high expression score for PD-L1 had a significantly poorer survival outcome compared with those with a low expression score. Multivariate analysis revealed that high expression of PD-L1 and advanced stage were significantly associated with poor prognosis independently of the other factors examined. These results are consistent with previous studies showing that high expression of PD-L1 is associated with poor prognosis in several human malignancies [3, 11–13], suggesting that high expression of PD-L1 on tumor cells promotes tumor recurrence by interrupting antitumor immunity [3]. Indeed, we observed that patients with a high expression score for PD-L1 had a significantly higher rate of recurrent disease than did those with a low expression score (79% versus 50%, $P < 0.0001$). We also analyzed survival rate and recurrence rate at each year after surgery for the NSCLC patients in the present study (supplementary Tables S1 and S2, available at *Annals of Oncology* online). The survival rates according to PD-L1 expression score differed significantly at ≥6 years after tumor resection but not before. This delayed separation of the survival curves according to PD-L1 expression is consistent with the late curve divergence repeatedly observed in successful immunotherapy trials [14]. This long latent period may reflect the time required for tumor cells with a high level of PD-L1 expression to relapse through escape from antitumor immunity. Although our study is retrospective in nature and has a relatively small sample size, our results provide a rationale for future clinical investigations of the PD1–PD-L1 axis as a target for adjuvant chemotherapy in individuals with NSCLC whose resected tumors are found to have a high expression score for PD-L1.

Given that we measured PD-L1 expression in operable NSCLC patients at p stages I–III, it remains unclear whether our findings will also be applicable to patients with stage IV disease. Furthermore, although our semiquantitative immunohistochemical method for analysis of PD-L1 expression is widely accessible, PD-L1 expression in tumor cells has been evaluated with various antibodies and conditions [11–13]. For future clinical applications, further efforts to standardize a quantitative assay for PD-L1 expression are warranted.

In conclusion, high expression of PD-L1 in resected tumors was found to be positively associated with the presence of *EGFR* mutations and to be an independent negative prognostic factor in NSCLC patients. Further studies are warranted to clarify the molecular mechanisms responsible for regulation of PD-L1 expression in *EGFR* mutation-positive NSCLC.

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disclosure

The authors have declared no conflicts of interest.

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Humoral Immune Responses to EGFR-Derived Peptides Predict Progression-Free and Overall Survival of Non-Small Cell Lung Cancer Patients Receiving Gefitinib

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Abstract

Somatic mutations in the epidermal growth factor receptor (EGFR) gene are associated with clinical response to EGFR tyrosine kinase inhibitors (TKIs), such as gefitinib, in patients with non-small cell lung cancer (NSCLC). However, humoral immune responses to EGFR in NSCLC patients have not been well studied. In this study, we investigated the clinical significance of immunoglobulin G (IgG) responses to EGFR-derived peptides in NSCLC patients receiving gefitinib. Plasma IgG titers to each of 60 different EGFR-derived 20-mer peptides were measured by the Luminex system in 42 NSCLC patients receiving gefitinib therapy. The relationships between the peptide-specific IgG titers and presence of EGFR mutations or patient survival were evaluated statistically. IgG titers against the egfr_481–500, egfr_721–740, and egfr_741–760 peptides were significantly higher in patients with exon 21 mutation than in those without it. On the other hand, IgG titers against the egfr_841–860 and egfr_1001–1020 peptides were significantly lower and higher, respectively, in patients with deletion in exon 19. Multivariate Cox regression analysis showed that IgG responses to egfr_41_ 60, egfr_61_80 and egfr_481_500 were significantly prognostic for progression-free survival independent of other clinicopathological characteristics, whereas those to the egfr_41_60 and egfr_481_500 peptides were significantly prognostic for overall survival. Detection of IgG responses to EGFR-derived peptides may be a promising method for prognostication of NSCLC patients receiving gefitinib. Our results may provide new insight for better understanding of humoral responses to EGFR in NSCLC patients.

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Introduction

Lung cancer is the leading cause of cancer death worldwide [1]. The epidermal growth factor receptor (EGFR), one of the most studied tyrosine kinase receptors, is a prototypic cell-surface receptor that can be targeted by drugs against lung cancer. The EGFR family is known to play an important role in the regulation of cell proliferation, differentiation, and migration [2]. Somatic mutations in the EGFR gene have been identified as a major determinant of the clinical response to treatment with EGFR tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, in patients with non-small cell lung cancer (NSCLC). Most of the EGFR mutations occur in exons 19 to 21, which encode the tyrosine kinase domain of the receptor. Deletions in exon 19 (such as delE746-A750) and the L858R point mutation in exon 21 are the commonest mutations found in NSCLC, accounting for about 90% of all EGFR mutations. These mutations are found more frequently in female patients, in individuals who have never smoked, and in patients of East Asian ethnicity [3–5]. Prospective clinical trials of EGFR-TKI treatment in NSCLC patients with *EGFR* mutations have demonstrated remarkable response rates in the order of 80% [6–8].

Previously, we have developed personalized peptide vaccination (PPV) as a novel modality for cancer therapy, in which vaccine antigens are selected on the basis of pre-existing immune responses against tumor-associated antigens (TAA) [9–13]. We reported that immunoglobulin G (IgG) responses to TAA-derived CTL epitope peptides were well correlated with overall survival (OS) in patients with advanced cancer undergoing PPV [14,15]. These results suggested that humoral immune responses against TAA-derived peptides might significantly impact the clinical course of cancer patients. However, there is little information regarding the clinical significance of humoral immune responses to EGFR-derived peptides in NSCLC patients.

Recently, novel high-throughput technologies have been developed for discovering biomarkers that clearly reflect clinical outcomes and/or responses to treatment in patients with cancer [16–21]. In the present study, we employed the high-throughput Luminex suspension array system to measure IgG responses to EGFR-derived peptides in patients with NSCLC. Here we report

for the first time that IgG responses to some EGFR-derived peptides are detectable in NSCLC patients, and that they could be potentially useful predictors of progression-free (PFS) and OS in NSCLC patients receiving gefitinib.

Materials and Methods

Patients, treatments, and sample collection

We enrolled 42 NSCLC patients treated with gefitinib between 2006 January and 2008 December at a single institution (Kurume University Hospital, Kurume, Japan). Details of the patients' clinical characteristics, including age, sex, histology, smoking status, performance status (PS), stage, treatment response, and type of *EGFR* mutations were obtained from chart reviews by an independent reviewer who was unaware of the clinical courses (Table 1). All of the patients had advanced NSCLC and received gefitinib (250 mg) orally once a day. Tumor response was examined by computed tomography (CT) and was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST). Response was confirmed at least 4 weeks (for a complete or partial response) or 6 weeks (for stable disease) after it was first documented. Plasma samples were collected from the patients before gefitinib treatment and frozen at −80°C until use. Plasma was also collected from healthy donors (HD) (n = 20, 59+/−11years, Male = 8, Female n = 12). The present study complied with the provisions of the Declaration of Helsinki, and was approved by the Institutional Review Board of Kurume University. Written Informed consent was obtained from all subjects.

EGFR mutation analysis

Mutations of the EGFR gene were examined in exons 19 (E746-A750del) and 21 (L858R) by peptide nucleic acid-locked nucleic acid (PNA-LNA) PCR clamp, as described previously [22]. In brief, genomic DNA was purified from paraffin-embedded tissues using a QIAamp DNA Micro kit (Qiagen, Inc., Valencia, CA). The PCR primers employed were synthesized by Invitrogen (Carlsbad, CA). PNA clamp primers and LNA mutant probes were purchased from FASMEC (Kanagawa, Japan) and IDT (Coralville, IA), respectively. PNA-LNA PCR clamp was performed using a SDS-7500 System (Applied Biosystems, Foster City, CA).

Peptides and measurement of IgG titers against peptides derived from EGFR

Sixty different non-overlapping 20-mer peptides were designed from the sequence of the EGFR protein and synthesized by Sigma Aldrich (St. Louis, MO), as shown in Figure 1E. The peptides were dissolved in DMSO as reported previously [23]. The IgG titers specific to each of the peptides were measured using a multiplex bead suspension array on the Luminex system, as reported previously [24]. In brief, 100 µl of diluted plasma was incubated with xMAP beads (Luminex Corp., Austin, TX), which were coated with the EGFR-derived peptides, in a 96-well filter plate (MABVN1250; Millipore Corp., Bedford, MA) for 2 h at room temperature on a plate shaker. The plate was then washed and incubated with 100 µl of biotin-conjugated goat anti-human IgG (BA-3080; Vector Laboratories, Burlingame, CA) for 1 h at room temperature on a plate shaker. After washing, 100 µl of streptavidin-PE was added to the wells, and the plate was incubated for 30 min at room temperature on a plate shaker. The beads were washed three times, followed by addition of 100 µl of PBS to each well. The fluorescence intensity in 50 µl of each sample was examined using the Luminex system. The peptide-specific IgG titers were estimated in terms of fluorescence intensity and expressed as fluorescence intensity units (FIU), as reported previously [24]. The cut-off level was set at 10 FIU because the FIU curves obtained from the sample dilution assays were linear from 10 to 10,000 FIU (data not shown).

Table 1 Patients' characteristics.

Characteristics		Number
Age (years)		
	Median	63.5
	Range	38–82
Gender		
	Male	17
	Female	25
Histology		
	Adenocarcinoma	38
	Squamous cell carcinoma	4
Smoking status		
	Never smoker	24
	Smoker	18
Perfomance status		
	0	34
	1	4
	2	4
c-Stage		
	StageIIIB	4
	StageIV or reccurent	38
EGFR mutation		
	746DEL	8
	L858R	13
	Negative	21
Treatment response		
	Partial response (PR)	19
	Stable disease (SD)	14
	Progressive disease (PD)	9

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Statistical analysis

To examine whether IgG titers against each of the 60 different peptides were associated with EGFR mutation status, their median values were compared among patients with EGFR mutations (delE746-A750 and L858R) and the wild-type EGFR using the Wilcoxon rank-sum test. PFS was calculated from the date of initiation of gefitinib treatment until either the date of disease progression or the date of last contact. OS was defined as the period from the date of initiation of gefitinib treatment until the date of death due to any cause, or to the date of last contact. To examine whether IgG titers against each of the 60 different peptides were associated with PFS or OS, we applied the Cox proportional hazards model with anti-peptide IgG titers, mutation status, smoking status, gender, and PS as explanatory variables. We also examined whether they were associated with the tumor response using logistic regression, where CR and PR were regarded as responses. Since 60 different peptides were examined, a severe multiplicity issue existed in this study. Therefore, we

identified peptides that were significantly associated with PFS, OS, or tumor response by controlling the false discovery rate (FDR) at the 5% level.

In this study, it was considerably challenging to identify the anti-peptide IgG responses that would be useful for prognostication than simply using clinicopathological characteristics alone. Since the number of peptides exceeded the number of patients, the standard multivariate Cox regression (multiple regression) could not be employed. To avoid influential observations, anti-peptide IgG was log-transformed (anti-peptide IgG +1), and also standardized with zero-mean and unit standard deviation. We applied the Cox regression with a lasso-type penalty [25,26], which has recently been reported to be useful for analysis of high-dimensional data. Since a notable feature of the lasso method is its sparsity, regression coefficients for anti-peptide IgG responses not associated with PFS (OS) could be estimated as zero. Based on this feature, we identified a few peptides that might be useful for prognostication. To examine whether or not IgG responses to the identified peptides were, in fact, useful for prognostication, we applied Cox regression analysis and time-dependent ROC analysis [27]. Areas under the ROC curve (AUCs) were estimated for risk scores by Cox regression with clinicopathological characteristics alone and also with both anti-peptide IgG responses and clinicopathological characteristics. They were compared by testing the equality of AUCs by calculating a bootstrap p-value for 1000 replicates. Statistical analysis was performed with R version 2.13 and SAS version 9.3 software (SAS Institute, Cary, NC).

Results

Patient characteristics and survival analysis

The clinical characteristics of the 42 patients are shown in Table 1. Twenty-five patients (60%) were female and 24 (57%) had never smoked; the median age of the patients overall was 63.5 years (range, 38 to 82 years). Thirty-eight patients (90%) had adenocarcinoma, 34 (81%) had a good performance status (Eastern Cooperative Oncology Group 0), and 15 (36%) received gefitinib as first-line chemotherapy. With regard to the type of *EGFR* mutation, 8 patients (19%) had deletions in exon 19, 13 (31%) had the L858R missense mutation in exon 21, and 21 (50%) had the wild-type *EGFR*.

At the time of analysis, the median follow-up period was 418 days (range, 16 to 1532 days). The median PFS was 201 days (range, 11 to 1379 days), and the median OS was 418 days (range, 16 to 1532 days). Kaplan-Meier analysis of PFS and OS after the start of gefitinib treatment is shown in Figure 1. The log-rank test revealed that gefitinib treatment resulted in a significantly longer PFS in patients with EGFR mutations than in those without them (median of 347 versus 54 days, $P = 0.0029$) (Fig. 1A), whereas there was no significant difference in OS between the two groups of patients (median of 575 versus 368 days, respectively, $P = 0.1095$) (Fig. 1B). The differences in PFS, but not OS, between patients with mutations and those with the wild-type EGFR were apparent for both types of EGFR mutation (Fig. 1C and 1D).

Correlation between IgG titers against EGFR-derived peptides and EGFR mutations in NSCLC patients treated with gefitinib

We examined IgG titers against each of 60 different peptides in plasma samples from NSCLC patients using the Luminex system. We analyzed whether the peptide-specific IgG titers were correlated with the presence of EGFR mutations, and found that IgG titers specific to the egfr_481–500, egfr_721–740, and egfr_741–760 peptides were significantly higher in patients with exon21 mutation than in those without it (P = 0.017 for egfr_481–500; P = 0.036 for egfr_721–740; P = 0.007 for egfr_741–760) (Table S1). For these three peptides, the median values of peptide-specific IgG titers in patients with exon 21 mutation were about double those in patients without exon 21 mutation (Table S1). On the other hand, the titer of IgG specific to the egfr_841–860 peptide was significantly lower in patients with deletion in exon 19 than in those without it (P = 0.047), whereas the titer of IgG specific to the egfr_1001–1020 peptide was significantly higher in those with deletion in exon 19 (Table S1). IgG responses to other peptides showed no significant correlation with EGFR mutations.

Relationship between titers of IgG against EGFR-derived peptides and survival in NSCLC patients treated with gefitinib

We further investigated whether the peptide-specific IgG titers were well correlated with PFS or OS in NSCLC patients receiving gefitinib treatment. In the Cox regression, IgG responses against 38 and 32 EGFR-derived peptides showed p-values of less than 5% for PFS and OS, respectively. When FDR was controlled at the 5% level, IgG responses against 35 and 20 peptides were identified as significant for PFS and OS, respectively (Table S2). We also examined whether the IgG titers against each peptide were associated with tumor response (CR or PR). Logistic regression analysis indicated that there were no peptide-specific IgG responses associated with tumor response (data not shown).

Identification of peptide-specific IgG responses useful for prognostication

As shown above, IgG responses to many of the EGFR-derived peptides were significantly associated with PFS and/or OS. Since many pairs of peptides were moderately or strongly correlated (data not shown), it was suggested that measurement of IgG titers against relatively small numbers of peptides might be sufficiently prognostic. By Cox regression with the lasso penalty, IgG titers against the egfr_41_60, egfr_61_80, and egfr_481_500 peptides had relatively large effects on PFS (Figure S1A). We employed the IgG titers against these three peptides for constructing a prediction rule for PFS. As shown in Table 2A, Cox regression adjusting for possible confounding factors, including PS, age, gender and smoking status, demonstrated that all of the IgG responses against the egfr_41_60, egfr_61_80, and egfr_481_500 peptides were significantly prognostic and independent of any clinicopathological characteristics (P = 0.001, P = 0.020, and P = 0.028, respectively).

By Cox regression with the lasso penalty, IgG titers against the egfr_41_60, egfr_481_500, and egfr_881_900 peptides were shown to have relatively large effects on OS (Figure S1B). Since IgG titers against the egfr_41_60 and egfr_881_900 peptides were strongly associated (Spearman's rank correlation coefficient 0.71; P<0.001), we employed only the titers of IgG against egfr_41_60 and egfr_481_500 for constructing a prediction rule for OS. As shown in Table 2B, Cox regression showed that the IgG responses to both peptides were significantly prognostic, independent of any clinicopathological characteristics (P = 0.018 for egfr_41_60 and P = 0.027 for egfr_481_500).

Kaplan-Meier plots of PFS and OS by stratification with IgG titers to the selected peptides are shown in Figure 2A and Figure 2B, in order to grasp their marginal effects without adjusting for clinicopathological characteristics. Using time-dependent ROC analysis, we also examined whether or not adding peptide-specific IgG titers to clinicopathological characteristics improved the accuracy of prognostication. Figures 3A and 3B show the ROC curves for 1 year and 2 years of the risk score estimated by the Cox

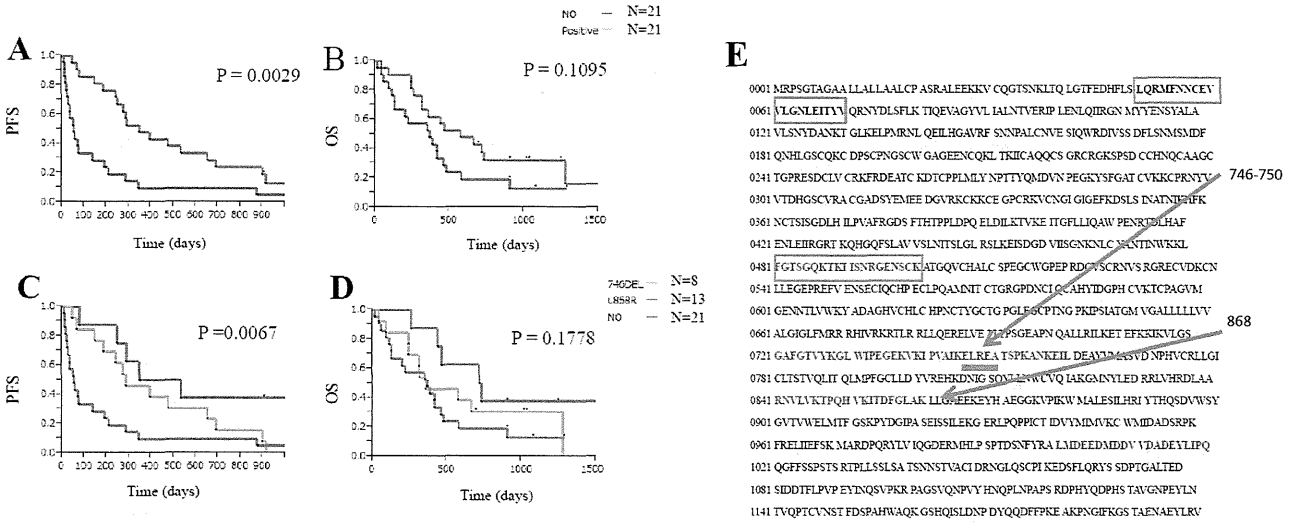

Figure 1. Kaplan-Meier analysis of PFS and OS in NSCLC patients receiving gefitinib treatment. Log-rank test revealed that gefitinib treatment significantly prolonged PFS (A), but not OS (B), in NSCLC patients with EGFR mutations. Significant differences in PFS (C), but not in OS (D), between patients with and without EGFR mutations were also apparent for mutations in both EGFR exon 19 (E746-A750del) and exon 21 (L858R). (E) Sixty different 20-mer peptides were designed from the amino acid sequence of EGFR protein. The sequences shown in red (egfr_481–500, egfr_721–740, egfr_741–760,egfr_841–860, and egfr_1001–1020) represent the peptides exhibiting specific IgG responses that were correlated with EGFR mutations. The sequences shown in blue (egfr_41_ 60, egfr_61_80, and egfr_481_500) represent the peptides exhibiting specific IgG responses that were correlated with PFS.
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regression given in Table 2A (for PFS) and Table 2B (for OS) with peptide-specific IgG titers and clinicopathological characteristcs and those with the latter alone. The ROC curves indicated that addition of peptide-specific IgG titers to the clinicopathological characteristics led to substantial improvement in the ability to predict PFS at 1 year and 2 years ($P<0.001$ by comparison of AUCs). AUCs of the time-dependent ROC for the 1-year and 2-year OS were also significantly increased by adding the peptide-specific IgG titers in comparison with clinicopathological characteristics alone ($P<0.001$) (Fig.3C and 3D). These findings suggested that adding peptide-specific IgG titers to the clinicopathological characteristics might lead to more accurate prognostication of both PFS and OS.

Table 2 Cox regression analysis of PFS for NSCLC patients.

factor	P-value	HR	95%CI	
mutation (Mutant/Wild-type)	0.000	0.17	0.07	0.43
egfr_481_500	0.028	0.59	0.37	0.94
egfr_61_80	0.020	0.54	0.32	0.91
egfr_41_60	0.001	0.24	0.10	0.56
sex (F/M)	0.929	1.11	0.11	11.17
ps (1–3/0)	0.105	2.18	0.85	5.60
smoke (Smoker/Never)	0.913	1.13	0.12	10.83
age	0.101	1.03	0.99	1.08
factor	P-value	HR	95%CI	
mutation (Mutant/Wild-type)	0.331	0.67	0.30	1.50
egfr_481_500	0.027	0.63	0.42	0.95
egfr_41_60	0.018	0.39	0.18	0.85
sex (F/M)	0.472	0.43	0.04	4.35
ps (1–3/0)	0.199	1.82	0.73	4.54
smoke (Smoker/Never)	0.632	0.58	0.06	5.47
age	0.540	1.01	0.97	1.05

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Discussion

Recent advances in molecular oncology have dramatically improved our understanding of the growth and survival pathways of NSCLC. For example, EGFR, a member of the HER or Erb-B family of receptor tyrosine kinases, is implicated in the development and progression of NSCLC. EGFR consists of an extracellular ligand-binding domain, a transmembrane region, and a multifunctional cytoplasmic tail with integral kinase activity [2,28–30]. EGF is a secreted growth factor whose binding to EGFR induces structural changes, leading to receptor homodimer formation, followed by an increase of EGFR kinase activity and subsequent phosphorylation of the intracellular domain [2,28–30]. The most frequently observed mutation in EGFR is the substitution L858R in the activating loop (A-loop), or deletion of eight residues in the region spanning residues 746–759, extending from the beta3 strand to the alphaC helix in the N-lobe of the kinase domain [2–5,28–30]. In this study, we found that the IgG responses to the peptides egfr_481–500, egfr_721–740, and egfr_741–760 were significantly higher in patients with exon 21 mutation. On the other hand, the IgG responses to the egfr_841–860 and egfr_1001–1020 peptides were significantly lower and higher, respectively, in patients with deletion in exon 19. Interestingly, egfr_721–740, egfr_741–760, and egfr_841–860 are located in the ATP-binding domain, which encodes the tyrosine kinase domain of the receptor, and humoral immune responses to these sequences were correlated with the presence of activating EGFR mutations, such as L858R or 746DEL. On the other hand, egfr_1001–1020 is located in the regulatory domain in the C-terminal tail, which can increase autophosphorylation of EGFR [31]. Previous studies have reported that this domain interacts extensively with both the C-lobe and N-lobe of the kinase domain

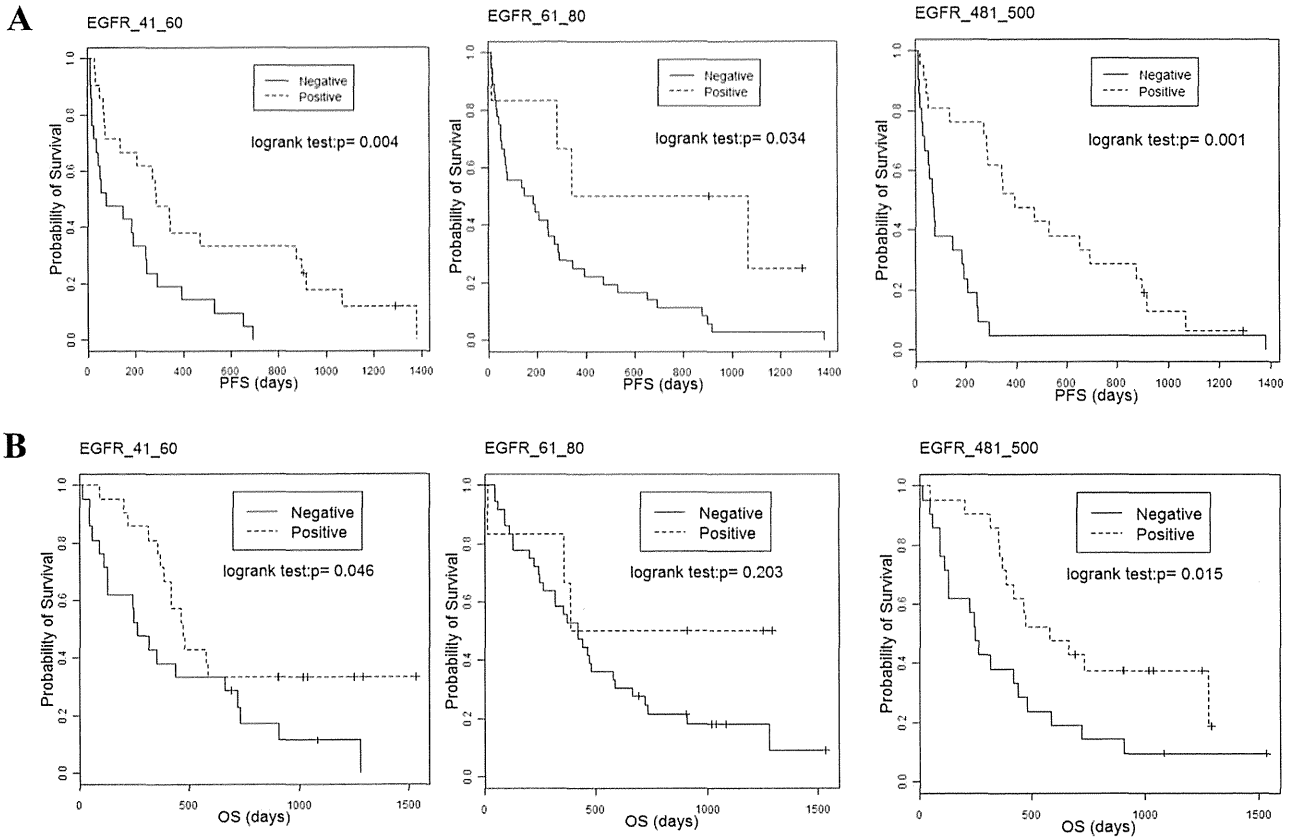

Figure 2. Kaplan-Meier analysis of PFS and OS after stratification by IgG titer against selected EGFR-derived peptides in NSCLC patients receiving gefitinib treatment. Kaplan-Meier plots of PFS (A) and OS (B) in patients showing higher and lower IgG titers against the selected peptides, egfr_41_ 60, egfr_61_80, and egfr_481_500, are shown. Lower and higher IgG titers were defined by their median values.
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[32–33]. These findings suggest that the sequences related to tyrosine kinase activity in EGFR might be immunogenic in patients with EGFR mutations, although further study is needed to clarify the mechanisms of the increased IgG responses to these sequences.

The development of rapid and precise diagnostic techniques for detecting EGFR mutations is particularly important for devising personalized therapeutics for NSCLC patients with activating EGFR mutations. Several highly sensitive methods for detection of EGFR mutations in tissue specimens have been reported [22], [37], but sample collection for these methods requires invasive procedures, such as transbronchial biopsy or pleural puncture. In contrast, the present results suggest that screening of the IgG responses to EGFR-derived peptides in peripheral blood might be feasible for detecting EGFR mutations. Detection of humoral responses against EGFR-derived peptides using the Luminex suspension array system is simple and non-invasive. In particular, this method may be useful for patients with NSCLC, whose tumor tissues are difficult to obtain for detailed pathological and molecular characterization.

We further investigated whether IgG responses against EGFR-derived peptides could be predictive of PFS and OS in NSCLC patients receiving gefitinib. We found that the IgG responses against the peptides egfr_41_ 60, egfr_61_80 and egfr_481_500 had large effects on PFS, and that those against egfr_41_60 and egfr_481_500 had large effects on OS. Interestingly, all of these sequences are located in the extracellular domain. Binding of EGF occurs within the amino-terminal 622-amino-acid extracellular domain, which consists of four domains, I-IV, of EGFR. Recently, structural data have demonstrated how anti-EGFR antibodies inhibit signal transduction from EGFR. For example, the egfr_481_500 sequence belongs to extracellular domain III, where anti-EGFR antibodies, such as cetuximab, nimotuzumab, and matuzumab, are known to bind and block the binding of EGF to EGFR [34-36]. Although the reasons why IgG responses to these peptides might impact on survival are not fully understood, one possible explanation is that IgG against the extracellular domain might affect signal transduction from EGFR.

We examined plasma from 20 healthy donors (HD) using the Luminex system to detect the antibodies against egfr_41_ 60, egfr_61_80 and egfr_481_500 peptides. We found that the titers of antibodies against egfr_41_60, egfr_61_80, and egfr_481_500 in plasma were not significantly different between NSCLC patients and HD (data not shown). This result is consistent with our previous finding that antibodies against cytotoxic T lymphocytes (CTL) epitope peptides derived from many of tumor-associated antigens were detected as positive in HD, and that their titers were not significantly different between cancer patients and HD [38]. This finding suggested that humoral immune responses to EGFR could be widely detectable not only in cancer patients but also in HD, since this molecule is ubiquitously expressed not only cancer tissues but also in normal tissues.

In conclusion, the present study has demonstrated that detection of humoral immune responses to EGFR-derived peptides in plasma using the Luminex suspension array system may be a

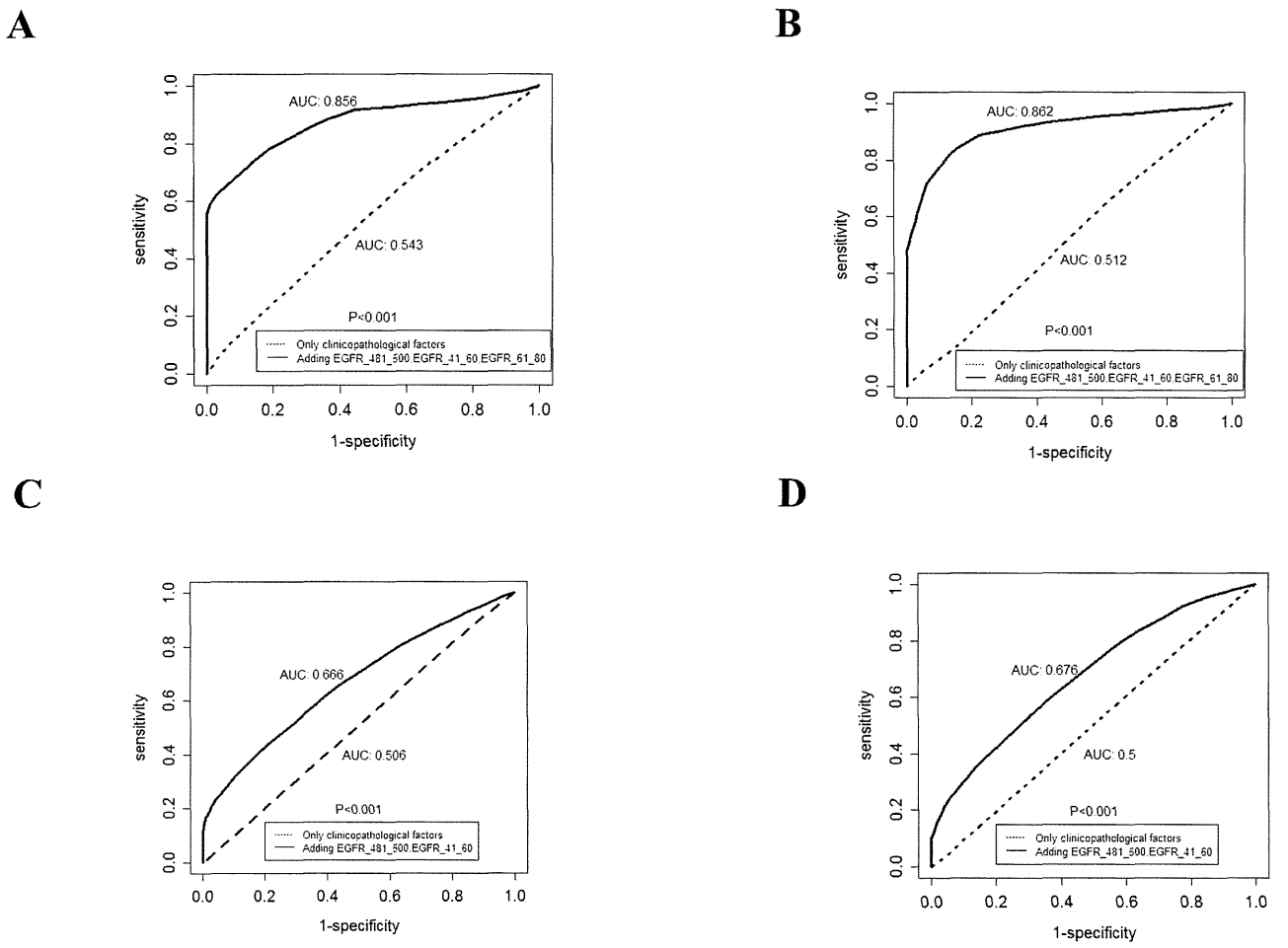

Figure 3. ROC curves for PFS and OS. The ROC curves for 1-year (A) and 2-year (B) PFS of the risk score estimated by Cox regression indicate a substantially improved correlation with 1-year and 2-year PFS. The ROC curves for 1-year (C) and 2-year (D) OS are also shown.
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promising method for not only detecting the presence of EGFR mutations but also the prognostication of NSCLC patients receiving EGFR-TKIs. These results may provide new insight for better understanding of the humoral immune responses to EGFR in NSCLC patients. Since the main drawback of this study was its small sample size, a further prospective study is now underway to confirm the findings in larger cohorts. In addition, it will be necessary to clarify the clinical applicability of our findings to personalized treatment for NSCLC patients.

Supporting Information

Figure S1 Solution path of the Cox regresson with a lasso penalty for PFS (A) and OS(B). (A)By Cox regression with the lasso penalty, IgG titers against the egfr_41_60, egfr_61_80, and egfr_481_500 peptides had relatively large effects on PFS. (**B**) By Cox regression with the lasso penalty, IgG titers against the egfr_41_60, egfr_481_500, and egfr_881_900 peptides were shown to have relatively large effects on OS.
(TIF)

Table S1 Correlation between EGFR mutation and expression of peptide in NSCLC patients. We examined IgG titers against each of 60 different peptides in plasma samples from NSCLC patients using the Luminex system. We found that IgG titers specific to the egfr_481–500, egfr_721–740, and egfr_741–760 peptides were significantly higher in patients with exon21 mutation than in those without it. On the other hand, the titer of IgG specific to the egfr_841–860 peptide was significantly lower in patients with deletion in exon 19 than in those without it ($P = 0.047$), whereas the titer of IgG specific to the egfr_1001–1020 peptide was significantly higher in those with deletion in exon 19.
(XLSX)

Table S2 Cox regression analysis of PFS and OS for NSCLC patients. In the Cox regression, IgG responses against 38 and 32 EGFR-derived peptides showed p-values of less than 5% for PFS and OS, respectively. When FDR was controlled at the 5% level, IgG responses against 35 and 20 peptides were identified as significant for PFS and OS, respectively.
(XLSX)

Author Contributions

Conceived and designed the experiments: KA NK KI. Performed the experiments: KN SM AK. Analyzed the data: SH. Contributed reagents/materials/analysis tools: SH TH. Wrote the paper: KA TS TH. NO.

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FGFR1 activation is an escape mechanism in human lung cancer cells resistant to afatinib, a pan-EGFR family kinase inhibitor

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ABSTRACT:

Most NSCLC patients with *EGFR* mutations benefit from treatment with EGFR-TKIs, but the clinical efficacy of EGFR-TKIs is limited by the appearance of drug resistance. Multiple kinase inhibitors of EGFR family proteins such as afatinib have been newly developed to overcome such drug resistance. We established afatinib-resistant cell lines after chronic exposure of activating EGFR mutation-positive PC9 cells to afatinib. Afatinib-resistant cells showed following specific characteristics as compared to PC9: [1] Expression of EGFR family proteins and their phosphorylated molecules was markedly downregulated by selection of afatinib resistance; [2] Expression of FGFR1 and its ligand FGF2 was alternatively upregulated; [3] Treatment with anti-FGF2 neutralizing antibody blocked enhanced phosphorylation of FGFR in resistant clone; [4] Both resistant clones showed collateral sensitivity to PD173074, a small-molecule FGFR-TKIs, and treatment with either PD173074 or FGFR siRNA exacerbated suppression of both afatinib-resistant Akt and Erk phosphorylation when combined with afatinib; [5] Expression of twist was markedly augmented in resistant sublines, and twist knockdown specifically suppressed FGFR expression and cell survival. Together, enhanced expression of FGFR1 and FGF2 thus plays as an escape mechanism for cell survival of afatinib-resistant cancer cells, that may compensate the loss of EGFR-driven signaling pathway.

INTRODUCTION

Lung cancer is the leading cause of cancer death worldwide (1). Somatic mutations in the epidermal growth factor receptor (EGFR) gene have been identified as a major determinant of the clinical efficacy of treatment with EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib in patients with non-small cell lung cancer (NSCLC). Prospective clinical trials of EGFR-TKI treatment in NSCLC patients with *EGFR* mutations have demonstrated remarkable response rates of approximately 80% (2-8).Whereas most NSCLC patients with *EGFR* mutations benefit from treatment with EGFR-TKIs. However, almost all the individuals eventually develop resistance to these drugs.

Acquired resistance to EGFR-targeted drugs is one of the major obstacles to further improve clinical outcomes in this field. Further intensive research efforts

have been focused on clarifying the mechanisms by which cancer cells acquire resistance to EGFR-targeted drugs (9, 10). T790M mutation, *Met* amplification, loss of PTEN, IGF-IR overexpression, and the AXL and Slug are reported to be the underlying mechanisms responsible for the EGFR-TKI resistance phenotype (11-16). The T790M mutation of *EGFR* has often been associated with acquired resistance to EGFR-TKIs in *EGFR* mutation-positive NSCLC. However, this mutation is present even in 31.5% of NSCLC patients pretreated with EGFR-TKIs, indicating that T790M is **associated** with de novo resistance (17, 18). Activation of alternative pathways, such as *Met* amplification or IGF-IR overexpression, has also been implicated in resistance to EGFR-TKIs in cells harboring activated *EGFR* mutation (12, 14). Furthermore, loss of PTEN and increased overexpression of MAPK, ABCG2, IGF1R, AXL, and BCL-2 have been reported as mechanisms of acquired resistance to EGFR-TKIs (9, 10). We have also reported that loss of PTEN expression and loss of activating EGFR gene allele results in acquisition of resistance to EGFR-TKIs in lung cancer cells harboring activated EGFR mutations (13, 19). However, the underlying mechanisms of resistance to EGFR-TKIs in patients with *EGFR* mutations have not been fully elucidated. The appearance of drug resistance in tumors during treatment of NSCLC patients with EGFR-TKIs has been a persistent obstacle.

In order to overcome drug resistance in relapsed NSCLC, multiple kinase-targeted drugs such as afatinib and ARQ197 have been further developed, and these are now being investigated in clinical trials (20, 21). Afatinib is an irreversible HER2/ErbB-family blocker that shows high affinity for EGFR T790M mutation. In phase III trials comparing afatinib with cisplatin and pemetrexed as first-line therapy, NSCLC patients with EGFR mutation had a higher response rate than patients without EGFR mutations when they received afatinib (22). In the present study, we invstigated how afatinib resistance was acquired in lung cancer cells, and also which oncogenic signaling pathway could be activated as a compensatory mechanism for cell survival. Here we report bypass activation of FGFR, and discuss the use of afatinib in combination with FGFR inhibitors for reversal strategy.

RESULTS

Establishment of afatinib-resistant lung cancer cells

The PC9 cells were grown initially in medium containing 0.01 μM afatinib, and the concentration of afatinib was gradually increased up to 1 μM over the following 11 months to establish the afatinib-resistant cell lines PC9 BR(3Mo), PC9BR(10Mo), and PC9BR(11Mo). We also established a revertant cell line, PC9 BR (21Mo), by culturing PC9 BR (11Mo) under drug free condition for 10 months. Dose response curves for PC9 and drug-resistant PC9 BR, PC9BR (3Mo), (10Mo), (11Mo) and (21Mo) cells to various doses of afatinib were determined by WST assay (Figure 1A). PC9BR (3Mo) cells that were selected after continuous exposure to the drug for 3 months already showed higher resistance, similar to that of PC9BR (10Mo) and PC9BR(11Mo). The IC_{50} values for each cell line were determined from the dose response curves for gefitinib and afatinib (Supplementary Table 1). PC9BR (3Mo), PC9BR (10Mo) and PC9BR (11Mo) cells were 3370-12900 times and 1170-135400 times more resistant to afatinib and gefinitib, respectively, than PC9 cells. By contrast, PC9BR (21Mo) cells showed similar sensitivity to both drugs as their parental PC9 cells (Supplementary Table 1), indicating that PC9 BR (21Mo) cells lost its drug resistant characteristic.

We then performed Western blotting analysis for biochemical profiling of these cells in the absence or presence of afatinib (Figure 1B). Drug-resistant PC9BR (10Mo) and PC9BR (11Mo) cells showed markedly decreased expression of pEGFR, HER2/pHER2, and HER3/pHER3 compared with PC9 and PC9BR (21Mo). By contrast, we observed increased expression of FGFR1 and pFGFR in the PC9BR (10Mo) and PC9BR (11Mo) cells relative to PC9 and PC9BR (21Mo) cells. Selection for afatinib resistance did not affect expression of EGFR expression. Phosphorylation of EGFR was susceptible to affatinib at 100 nM and 1000 nM in all of PC9 BR (10Mo), PC9BR (11Mo), PC9 and PC9BR (21Mo) cell lines. Afatinib markedly suppressed phosphorylation of Akt and Erk in PC9 and PC9BR (21M0) cells but not in PC9BR (10Mo) and PC9BR (11Mo) cells without affecting Akt and Erk expression (Figure 1B).

All of these cell lines did not harbor T790M mutation in the EGFR gene.

Enhanced expression of FGFR1 by selection of afatinib resistance

To further characterize afatinib-resistant cells, we cloned three sublclones, PC9/B3 (B3), PC9/B19 (B19) and PC9/B20 (B20), from PC9BR (11Mo) cells, and Rev1 from PC9BR (21Mo) cells. Dose response curves for afatinib were obtained for PC9 and their three drug-resistant subclones in the presence of various doses of afatinib (Figure 1C). From the dose response curves, IC_{50} values were determined, and all resistant clones showed 750- to 880-fold higher resistance to afatinib than PC9(Table1). We also determined the dose response curves of PC9, B19 and B20 to various drugs (Supplementary Figure S1), and the IC_{50} values of these three cell lines for each drug were calculated(Table 1). Both afatinib-resistant sublclones showed more than 900-fold higher resistance to gefitinib, about 50-fold higher resistance to lapatinib,

and 2-fold higher resistance to foretinib, respectively, than their parental PC9 cells. By contrast, B19 and B20 showed 2- to 5-fold higher collateral sensitivity to PD173074 (Figure 1D), an inhibitor of FGFR 1 and 3 tyrosine kinase (Table 1). The sensitivities of B19 and B20 cells to axitinib, dasatinib, cisplatin and paclitaxel were found to be similar to those of PC9 (Table 1).

Therefore, we next compared expression levels of various growth factor receptors and their downstream regulatory molecules between PC9 and its resistant subclones (Figure 1D). Both resistant clones showed markedly decreased expression of pEGFR, and activated mutant EGFR (746del), HER2/pHER2, and HER3/pHER3 in comparison with PC9 cells. By contrast, there was no apparent change in the expression levels of IGF1R/p-IGF1R between the resistant subclones and PC9. We observed increased expression of FGFR1 and pFGFR in the resistant subclones relative to their parental counterpart (Figure 1E). Expression levels of unphosphorylated and phosphorylated Akt and Erk in PC9 and its drug-resistant subclones were similar.

Microarray analysis revealed that expression of FGFR2, FGFR3, and FGFR4 was only slightly or negligibly expressed in the resistant clones (unpublished data), suggesting that other FGFR family proteins except FGFR1 are unlikely to be involved in acquisition of drug resistance in B19 and B20 cells.

Constitutive activation of FGFR through increased expression of both FGF2 and FGFR1 by acquisition of afatinib resistance

Since FGFR1 was constitutively phosphorylated in drug resistant clones, we examined whether FGFR was phosphorylated through an autocrine loop by its own FGF2 in resistant subclones. Using ELISA assay, we next compared the protein expression levels of FGF2 in serum-free conditioned medium among PC9, B19, B20, and Rev1 clones (Figure 2A). Both resistant sublclones produced more than 30-fold higher levels of FGF2 of about 50 pg/ml than PC9 and Rev1.

As shown in Figure 2B, we next compared the effect of afatinib on phosphorylation of EGFR family proteins, and their downstream signaling molecules and also the expression levels of FGFR1 among PC9, Rev1, and drug-resistant sublclones. Phosphorylation of EGFR, HER2 and HER3 was almost completely blocked in PC9, B19, B20, and Rev1 upon treatment with afatinib at 100 and 1000 nM (Figure 2B). By contrast, phosphorylation of Akt and Erk in both resistant subclones was not at all affected by afatinib. Expression of FGFR1 was also markedly upregulated in resistant sublclones relative to PC9, but its phosphorylation was not blocked by afatinib (Figure 2B). Furthermore, Rev1 showed similar expression levels of pEGFR to that of PC9, and EGFR phosphorylation was highly susceptible to afatinib as in PC9. Expression of FGFR1 was found to be markedly downregulated, as in PC9, and phosphorylation of Akt and Erk was also similarly susceptible to afatinib in Rev1. The restored sensitivity to afatinib in Rev1 was accompanied by both activation of EGFR and decreased activation of FGFR1.

We next compared the effect of exogenous addition of FGF2 on FGFR phosphorylation in PC9 and its drug-resistant subclones. Expression level of pFGFR were already higher in both resistant clones than in PC9 in the absence of FGF2. The time kinetics for treatment with FGF2 showed time-dependent enhancement of FGFR phosphorylation in both B19 and B20, accompanied by enhanced activation of Akt and Erk (Figure 2C). By contrast, no apparent phosphorylation of FGFR was observed in the parental PC9 cells. Figure 2D shows dose-dependent increased activation of FGFR and Akt and Erk in B19 and B20 when treated with various doses of FGF2. However, FGFR phosphorylation in PC9 was not augumented by FGF2. FGFR in both resistant sublclones thus seemed to be constitutively phosphorylated, and further phosphorylated in the presence of exogenous FGF2 (Figure 2C and 2D). We then investigated whether autocrine stimulation of B19 by secreted endogenous growth factor was responsible for activation of FGFR phosphorylation and was thus responsible for weaken of pFGFR and its downstream signaling (Figure 2E).

FGFR activation is closely correlated with acquired resistance to afatinib

We finally investigated whether FGFR was closely correlated with afatinib resistance in B19 and B20. Both resistant subclones were collaterally sensitive to an inhibitor of FGFR-TKI, PD173074 (Table1), and their FGFR was constitutively activated through an autocrine loop by FGF2. We first examined whether FGFR-TKI was able to block constitutive activation of Akt and Erk, which was not susceptible to the inhibitory effect of afatinib. The phosphorylation of FGFR was almost completely blocked by PD173074 alone and afatinib augmented this inhibitory effect in resistant subclones (Figure 3A). Apoptosis was also induced in two resistant clones by treatment with PD173074 alone or with both PD17074 and afatinib when assayed by PARP band cleavage.

We next examined whether the cell growth of drug-resistant clones was inhibited by FGFR-TKI. Cell growth of PC9 was blocked by afatinib alone, but not by PD173074 (Figure 3B). By contrast, there was marked growth inhibition of both resistant sublclones upon treatment with PD173074 alone or with both PD173074 and afatinib. We further examined whether FGFR1 knockdown by its cognate siRNA also exacerbated the inhibitory effect of afatinib on apoptosis and Akt/Erk phosphorylation in drug-resistant sublclones (Figure 3C).

Table 1: Comparison of sensitivity to various drugs between afatinib-resistant sublclones and their parental PC9 cells

		Relative drug resistance (IC50)		
Drugs	Targets	PC9	B19	B20
Afatinib	EGFR, HER2, HER3	1 (10 nM)	750 (7.5 μM)	880 (8.8 μM)
Gefitinib	EGFR	1 (10 nM)	2600 (260 μM)	960 (96 μM)
Lapatinib	EGFR, HER2	1 (0.27 μM)	52 (14.3 μM)	50 (13.5 μM)
Foretinib	Met	1 (0.7 μM)	2.7 (1.9 μM)	1.85 (1.3 μM)
PD173074	FGFR1,3	1 (15 μM)	0.5 (7.5 μM)	0.20 (2.9 μM)
Axitinib	PDGFR,VEGFR,	1 (2.4 μM)	1.3 (3.3 μM)	0.7 (1.7 μM)
Dasatinib	Src	1 (10 nM)	1 (10 μM)	1 (10 nM)
Cisplatin	DNA	1 (5.4 μM)	1.2 (6.9 μM)	1.5 (8.2 μM)
Paclitaxel	tubulin	1 (10 nM)	1 (10 nM)	1 (10 nM)

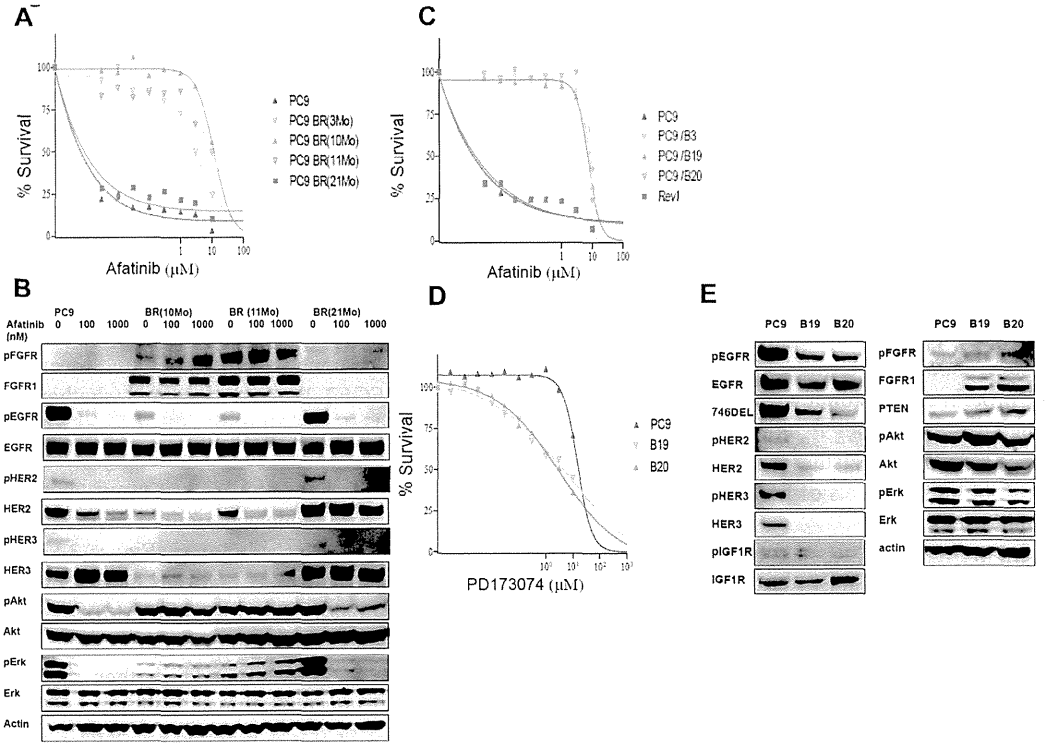

Figure 1:Establishment of afatinib-resistant lung cancer cells. (A) Dose response curves for PC9, and drug-resistant PC9BR, PC9BR (3Mo), (10Mo), (11Mo), and (21Mo) cells to various doses of afatinib were determined by WST assay. (B) Western blotting analysis was performed for biochemical profiling of these cells in the absence or presence of afatinib for 6 h. Expression of pEGFR, HER2/ pHER2, and HER3/pHER3 were markedly downregulated by resistance to afatinib, and activation of downstream regulating molecules for cell growth and survival was found to be highly resistant to the drugs. Downregulation of EGFR family proteins and upregulation of FGFR1 by selecting for afatinib resistance. (C) Dose response curves for afatinib were acquired for PC9 and its drug-resistant subclones, B3, B19, B20 and Rev1, with various doses of afatinib. (D) B19 and B20 showed 2- to 5-fold higher collateral sensitivity to PD173074.(E) Increasing expression of FGFR1 and pFGFR in resistant sublclones relative to their parental cells

Silencing of FGFR1 reduced the expression of FGFR1, accompanied by inhibition of Erk phosphorylation but not Akt phospholylation in B19 and B20 cells (Figure 3C). Treatment with both of FGFR1-siRNA and afatinib further suppressed the phosphorylation of Akt and Erk. Cleaved PARP was also induced when resistant sublclones were treated with FGFR1 siRNA in the absence and presence of afatinib. Together, these findings suggest that the growth and survival of afatinib-resistant B19 and B20 cells become selectively addicted to the FGFR1 pathway during the selection of afatinib-resistant cells.

Twist knockdown specifically blocked FGFR1 expression and Akt phospholylation in afatinib resistant cell lines

We finally asked how FGFR1 expression was specifically augmented in resistant cells. Microarray analysis showed decreased expression of other FGFR family proteins, FGFR2, FGFR3 and FGFR4 in afatinib-resistant cell line when expression of FGFR1 was enhanced (Figure 4A). Figure 4A also showed increasing expression of Twist and Snail that are closely involved in transcription of EMT-related genes in resistant cells. Figure 4B also shows that expression of Twist, Snail, Slug, and ZEB1 was increased in resistant cells, accompanied by a decrease in the expression of E-cadherin and an increase in that of vimentin. We also observed morphological changes of fibroblast-line cell by selection of afatinib-resistant cells, accompanying by decreasing expression of E-cadherin with increasing expression of vimentin (data not shown).

We examined whether Snail and other related transcription factors were responsible for the enhanced expression of FGFR1 in drug resistant cell lines. Expression of ZEB1, Snail and Slug proteins was relatively much higher in B19 than PC9 (Figure 4C), and expression of Twist mRNA was also much higher in B19 and B20 than PC9 (Figure 4D). We confirmed that

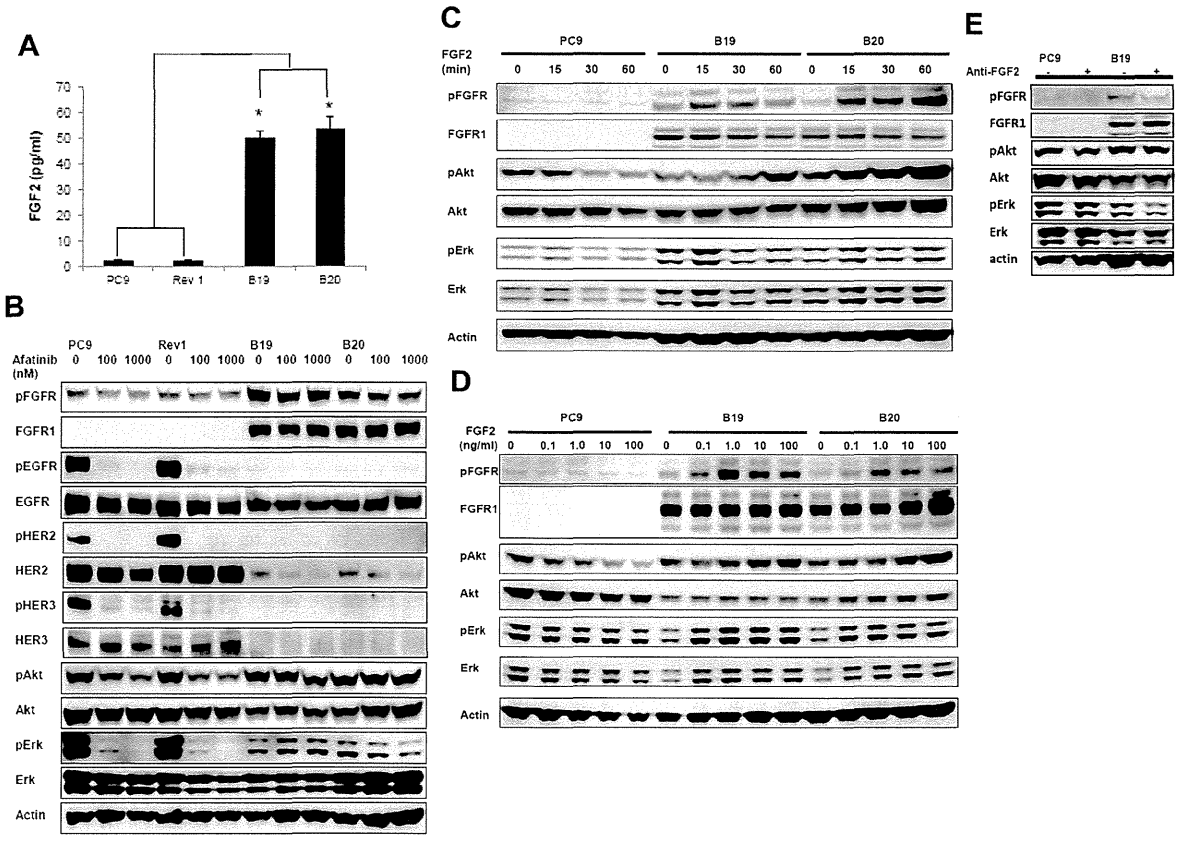

Figure 2:Increased expression of FGF2 and FGFR1 upon acquisition of afatinib resistance. (A) Both resistant sublclones produced more than 30-fold higher levels of FGF2 than PC9 and Rev1. (B) Phosphorylation of EGFR, Akt and Erk in Rev1 was similarly susceptible to the inhibitory effect of afatinib (6 h) in PC9 when phosphorylation of Akt and Erk was resistant to the inhibitory effect of the drug in both resistant subclones. (C) Time kinetics for treatment with FGF showed enhanced phosphorylation of FGFR in both B19 and B20, accompanying by enhanced activation of Akt and Erk. (D) Increasing dose-dependent activation of FGFR, Akt and Erk in B19 and B20 upon treatment with various doses of FGF. All experiments were performed under serum free condition.(E) Autocrine stimulation of B19 by secreted endogenous growth factor was responsible for activation of FGFR phosphorylation

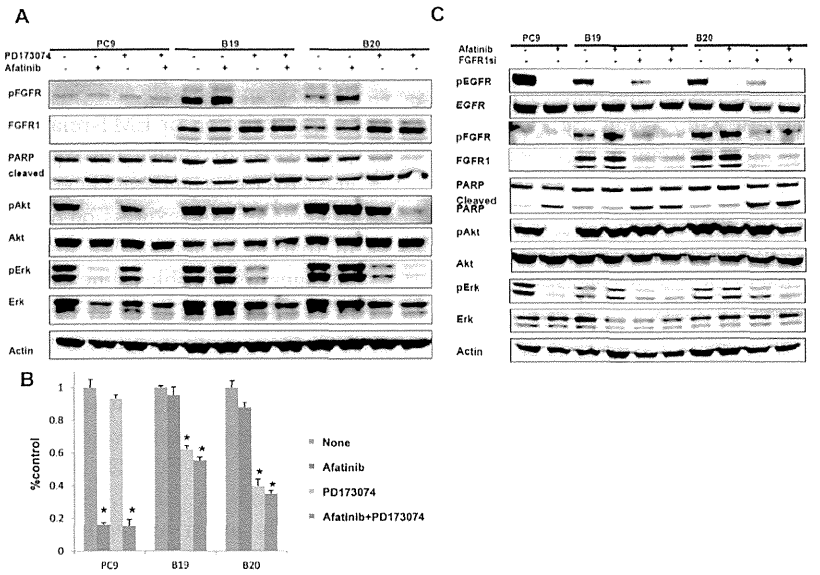

Figure 3:The close association of FGFR activation with acquired resistance to afatinib. (A) Effect of FGFR-TKI against afatinib-resistant cells. The phosphorylation of FGFR was blocked upon treatment with either PD173074 (1 μM) alone or with both PD173074 (1 μM) and afatinib (1 μM) for 24 h. (B) Growth of both resistant sublclones was blocked upon treatent with PD173074 (1 μM) alone or with PD173074 (1 μM) and afatinib (1 μM). (C) Treatment with FGFR1 siRNA reduced the expression of FGFR1, accompanied by inhibition of both Akt and Erk phosphorylation in B19 and B20 cells. Cleaved PARP was also induced when resistant sublclones were treated with siRNA FGFR1 in the absence or presence of afatinib (1 μM) for 24 h.

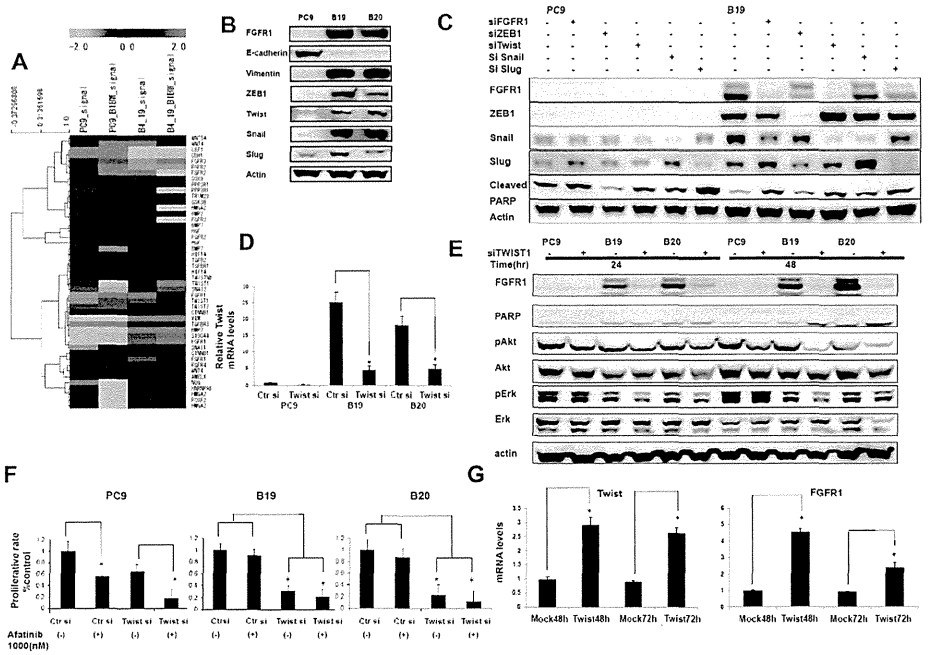

Figure 4: Twist knockdown specifically blocked FGFR1 expression and Akt phospholylation in afatinib resistant cell lines. (A) Microarray analysis showed that the resistant subclones B19 acquired typical EMT characteristics relative to their drug-sensitive parental PC9. (B) Expression of Twist, Snail, Slug, and ZEB1 was increased in resistant cells, accompanied by a decrease in the expression of E-cadherin and an increase in that of vimentin. (C) Western blot analyses showed that expression of all three transcription factors was downregulated by their cognate siRNA. Phosphorylation of Akt and Erk was decreased when expression of twist was knockedowned. (D) Real-time PCR analysis revealed that expression of Twist mRNA was downregulated by its cognate siRNA by RT-PCR (E) Expression of FGFR1 was almost completely blocked accompanying by decreased phosphorylation of Akt and ERK when B19 or B20 cells were treated with Twist siRNA for 24hr and 48hr. (F) Cell growth inhibition of B19 and B20 when treated with afatinib and Twist siRNA. (G) FGFR1 mRNA levels in PC9cells were also increased to 2.5-4 folds of the control when twist was overexpressed.

expression of Twist mRNA was downregulated by its cognate siRNA. Treatment with siRNAs for ZEB1, Twist, Snail and Slug resulted in markedly decreased expression of ZEB1, Snail and Slug proteins, and also Twist mRNA (Figure 4C and 4D).

As seen in Figure 4C, treatment with Twist siRNA, but not with ZEB1, Snail and Slug siRNAs, specifically suppressed expression of FGFR1 in resistant clones. Expression of FGFR1 was almost completely blocked, accompanying by decreased phosphorylation of Akt and ERK when B19 or B20 cells were treated with Twist siRNA for 24hr and 48hr (Figure 4E). We also observed cell growth inhibition of B19 and B20 when treated with Twist siRNA alone or with afatinib (Figure 4F). We next examined whether Twist overexpression might promote FGFR1 expression. FGFR1 mRNA levels in PC9 were found to be increased about 3 fold over the control when twist was overexpressed by transfection of Twist cDNA (Figure4G).Expression of FGFR1 thus seems to be specifically promoted by Twist than other transcription factors in afatinib-resistant clones.

DISCUSSION

Our present study revealed novel characteristics of afatinib-resistant sublclones established from the drug-sensitive lung cancer cell line PC9 harboring the activated deletion E746-A750 mutant EGFR. In these afatinib-resistant sublclones,[1] expression of most of the EGFR protein family, including pEGFR, mutant EGFR, HER2 and HER3, and Met, was markedly downregulated;[2] they showed collateral sensitivity to PD173074 (FGFR-TKI) ; [3] there was alternatively enhanced expression of FGFR1 and its ligand FGF2, and phosphorylation of Akt and Erk was resistant to the inhibitory effect of afatinib ; [4] of EMT-related transcriptional factors, Twist knockdown specifically reduced expression of FGFR1; and [5] afatinib together with either FGFR-TKI or FGFR1 knockdown markedly suppressed Akt and Erk phosphorylation, and cell growth and survival. Together, impaired expression of EGFR family proteins thus seems to compensatorily activate FGFR1-driven signaling pathway by acquired drug resistance to afatinib.

Acquisition of afatinib resistance resulted in markedly decreased expression of EGFR family proteins including activated EGFR, HER2 and HER3, which are targets for afatinib. This decreased expression of these EGFR family proteins might be mostly involved in acquisition of afatinib resistance. Our relevant study has recently demonstrated that loss of the activated mutant EGFR gene copy is closely associated with resistance to erlotinib and gefitinib, suggesting that expression levels of activated mutant EGFR can limit cellular sensitivity to such EGFR-TKIs (19). Furthermore, afatinib-resistant sublclones are also cross-resistant to gefitinib and also lapatinib (Table 1). EGFR forms a duplex with HER2 or HER3 (31), and sensitivity to lapatinib is controlled through HER2 and/or EGFR (32, 33). The cross-resistance to lapatinib in afatinib-resistant sublclones might be due to marked downregulation of HER2/pHER2 and pEGFR/activated mutant EGFR. With regard to the pleiotropic mechanisms involved in acquisition of resistance to EGFR-TKIs and other kinase inhibitors, the alternative pathway is one mechanism of escape from the cytotoxic or therapeutic effects of EGFR-targeted drugs (10). Activation of alternative pathways, such as *Met* amplification and IGF1R overexpression, has been implicated in resistance to EGFR-TKIs in non-small cell lung cancer cells bearing *EGFR* mutation (12, 14), and

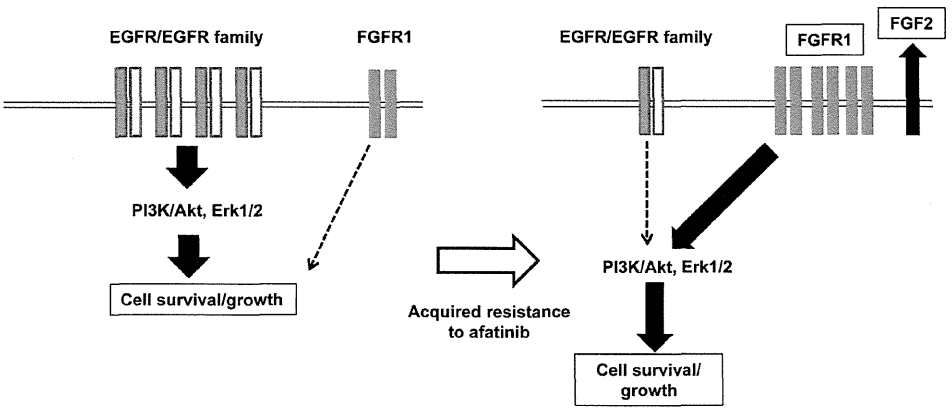

Figure5:Our hypothetic model shows how afatinib resistance is acquired in lung cancer cells. In drug sensitive cell line, the cell survival and growth of human lung cancer cells harboring activating EGFR depends upon the EGFR/EGFR family driven PI3K/Akt and Erk pathways, and this cell survival and growth is highly susceptible to afatinib and other EGFR-TKIs. By contrast, afatinib-resistant subclones express elevated levels of FGFR1 together with FGF2, resulting in activation of Akt and Erk, when EGFR/EGFR family-driven cell growth/survival signaling pathways are mostly attenuated. Of EMT-related transcription factors, Twist seems to specifically responsible for elevated expression of FGFR1 in afatinib resistant cell lines.

these molecules bypass the original oncogenic pathway to confer resistance to previously effective therapy. In afatinib-resistant sublclones, however, there was no altered expression of IGF1R (Figure 1D). and no phosphorylation of Met (date not shown), suggesting that the alternative pathway involving IGF1R and Met is unlikely to be involved in afatinib resistance.

The FGFR tyrosine kinase family is consisted of 4 receptors and 23 ligands and activation of FGFRs is common oncogenic event (34). Recent study by Herrera-Abrea et al has demonstrated that EGFR limits drug sensitivity to FGFR tyrosine kinase inhibitor in FGFR3-mutant cell lines, and also that combination of FGFR and EGFR tyrosine kinase inhibitors overcome drug resistance to FGFR inhibitors, suggesting the close interaction of EGFR-and FGFR-driving cell growth or signaling pathways (35). In our present study using lung cancer cell lines, FGFR1 is most abundant receptor of the four family proteins in afatinib-resistant clones of PC9, and there was no enhancement in expression of other FGFR family proteins FGFR2, FGFR3 and FGFR4 (see Figure 4A). Ligand binding leads to FGFR1 dimerization, autophosphorylation, and activation of signaling components including Akt and Erk kinases, further affecting malignant transformation of cancer cells. We observed that the growth factor receptor-driven downstream molecules, Akt and Erk, were still highly phosphorylated in the presence of afatinib in resistant sublclones when expression of most of the EGFR family proteins was downregulated (Figure 1B, Figure 1E and Figure 2D). A possible mechanism underlying such activation of Akt and Erk in drug-resistant subclones treated with high doses of afatinib is that they induce increased expression of FGFR1 and pFGFR together with increased expression of FGF2 (see Figure5). Mark et al. have demonstrated various levels of expression of the FGF family proteins, FGFR1 and FGFR2, in NSCLC cell lines, and also shown that FGF2/FGFR1 autocrine signaling affects their sensitivities to gefitinib and FGFR-TKI (36). Both resistant sublclones, B19 and B20, showed more than 20-fold higher expression with 50 ng/ml FGF2 than their drug-sensitive counterpart cell lines, PC9 and Rev1 (Figure 2A). Both B19 and B20 already showed FGFR phosphorylation in the absence of exogenous FGF, suggesting an autocrine activation loop for FGF2-FGFR1 by afatinib resistance (Figure 2B and 2C). Exogenous addition of FGF further augmented FGFR phosphorylation and activation of both Akt and Erk in both resistant sublclones, but not at all in their parental counterpart PC9 cells (Figure 2B and 2C), suggesting the absence of FGF2-FGFR1 autocrine activation loop in PC9, possibly due to loss of active FGFR1 and FGF2 expression in the parental drug sensitive counterpart.

Concerning the possible link between FGF/FGFR and drug resistance to EGFR-TKIs, we have previously demonstrated amplification of the *FGFR2* gene in lapatinib-resistant breast cancer cells (37). Furthermore, Ware et al. (30) have reported that gefitinib-resistant cells after chronic exposure of several NSCLC cell lines to gefitinib showed increased expression of both mRNA and protein for FGFR1 and FGF2. A relevant study by Terai et al. has demonstrated that gefitinib-resistant sublclones from PC9 had enhanced expression of FGFR1 and FGF2, and also that gefitinib sensitivity in drug-resistant cells was restored by a combination of FGFR-TKI and gefitinib (38). Treatment with FGFR-TKI or FGFR knockdown also induced marked reduction of Akt and Erk activation in afatinib-resistant sublclones (Figure 3A and C). Co-adminstration of afatinib and FGFR-TKI also reduced apoptosis and suppression of cell growth in drug-resistant cells (Figure 3A , 3B, and 3C). These results strongly suggest that acquisition of afatinib resistance is due to oncogenic switch from activated EGFR family proteins to the FGF/FGFR signaling pathway (Figure5). FGFR1 may thus function as a survival factor for afatinib-resistant cancer cells, and activation of the FGFR-driven bypass signaling pathway confer resistance to previously effective therapy.

FGFR1 expression is often upregulated when epithelial cells are transformed into mesenchymal cells (39, 40).Microarray analysis demonstrated enhanced expression of EMT-related transcription factors such as Snail and Twist in afatinib-resistant clone (Figure 4A). Furthermore, we screened whether knockdown of these four EMT-related transcription factors could suppress phosphorylation of Akt and Erk in resistant clones in the presence of afatinib, and Twist knockdown specifically blocked Akt phosphorylation (unpublished data). Our present studies, clearly showed that increased expression of FGFR1 in afatinib-resistant clones was almost completely blocked only when treated with Twist siRNA (Figure 4B and 4D). Drug resistance to afatinib was also overcome by Twist knockdown in both resistant clone, B19 and B20, accompanying by suppression of both Akt and Erk activation (Figure 4D and E). Furthermore, transfection of Twist cDNA resulted in restored expression of FGFR1 in drug-resistant clone. It thus seems likely that Twist plays a pivotal role in enhanced expression of FGFR1 in resistant clones. Further study should be also required whether Twist alone plays a major role in overexpression of FGF2.

In conclusion, we have clarified one of the mechanism by which how PC9 cells acquired resistance to afatinib in vitro. Selection by afatinib resistance induced marked loss of the EGFR family proteins, EGFR, HER2 and HER3, together with inactivated EGFR family proteins, and simultaneously induced marked increases in the expression of FGF2 and activation of FGFR1. Such activation of the FGF/FGFR autocrine loop may have a compensatory role in promoting the survival and growth of afatinib-resistant cells. Whether this mechanism operates in patients with tumors refractory to EGFR-TKIs

and multikinase inhibitors remains to be further studied.

MATERIALS AND METHODS

Cell culture and reagents

The human lung cancer cell line PC9 harboring del E746-A750 activating mutation in EGFR was maintained in RPMI1640 supplemented with 10% fetal bovine serum (FBS) and incubated in a humidified atmosphere of 5% CO_2 at 37°C. The PC9 cells were kindly provided by Dr. Mayumi Ono (Kyushu University, Fukuoka, Japan) (13, 19, 23). Cells were routinely confirmed to be free of mycoplasma contamination using mycosensor QPCR Assay kits (Agilent Technologies).Afatinib, lapatinib, foretinib, gefitinib, and dasatinib were purchased from Selleck (Houston, USA). PD173074, cisplatin, paclitaxel and axitinib were from Sigma Aldrich (St. Louis, MO). The construction of pcDNA3-Twist has previously been described (24). The small interfering RNAs (siRNA) corresponding to FGFR1, Twist1, ZEB1, Snail, and Slug, mRNA and a non-specific siRNA (control) were purchased from Nippon Gene (Tokyo, Japan). Cells were transfected with siRNA duplexes using Lipofectamine RNAiMAX and Opti-MEM (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

Western blot analysis

Western blot analysis was done as previously described (36) with antibodies for phosphorylated FGFR (pFGFR), FGFR1, p EGFR(Y1086), EGFR, pHER2(Y1221/1222), HER2, pHER3, HER3, pAkt, Akt, Erk, cleaved PARP, PARP, Vimentin, E-cadherin, Snail, Slug, ZEB1 (Cell Signaling Technology, Danvers, MA), Twist (Sigma,St. Louis, MO), and pERK (Santa Cruz Biotechnology, CA) or β-actin (Sigma,St. Louis, MO).

Isolation of afatinib-resistant PC9 cells

To isolate afatinib-resistant cell lines, we cultured in increasing, step-wise doses of afatinib up to 1 μM over the following 11 months, and PC9 BR(3Mo), PC9BR(10Mo), and PC9BR(11Mo) were established (13, 19). We also established the revertant cells, PC9BR (21Mo), by culturing PC9BR (11Mo) cells under drug-free condition for 10 months and generated the subclones Rev1 from PC9BR (21Mo). Using limiting dilution, we further generated the clones B3, B19 and B20 from PC9 BR (11Mo). The identity of these clones was confirmed by analyzing their short tandem repeat profile using the Cell ID System (Promega, Madison, WI).

Cell growth assay in vitro

Cells were plated in 96-well flat-bottomed plates and cultured for 24 h before exposure to various concentrations of drugs for 72 h. Cell counting kit 8 (WST-8 Doujindo, Kumamoto, Japan) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 450 nm with a Multilabel counter ARVO MX (PerkinElmer, USA). Absorbance values were expressed as a percentage of that for untreated cells, and the concentration of tested drugs resulting in 50% growth inhibition (IC_{50}) was calculated using the Prism program (GraphPad, San Diego, CA). Triplicate wells were tested at each drug concentration.

Quantitative real-time polymerase chain reaction and EGFR mutation analysis

Quantitative real-time PCR and EGFR mutation analysis was done as previously described (13, 25). All experiments were performed in a triplicate assays. To analyze the T790M mutation, exon 20 of the *EGFR* gene was amplified using the PCR primer set and TaKaRa Ex Taq polymerase (TaKaRa BIO, Inc). PCR products were directly used as templates for cycle sequencing reactions using the BigDye Terminator v1.1 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.

Gene expression microarrays

The cRNA was amplified, labeled, and hybridized to a 44K Agilent 60-mer oligomicroarray according to the manufacturer's instructions. All hybridized microarray slides were scanned by an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using the Agilent Feature Extraction Software program (9.5.1.1).

Data analysis and filter criteria

Raw signal intensities and flags for each probe were calculated from the hybridization intensities, and spot information, according to the procedures recommended by Agilent. And the raw signal intensities of two samples were log2-transformed and normalized by a quantile algorithm (27) on the Bioconductor (28, 29). We selected probes that called the 'P' flag in both control and experimental samples. To identify up or down-regulated genes, we calculated Z-scores (29) and ratios (non-log scaled fold-change) from the normalized signal intensities of each probe. We thereafter established the criteria for the regulated genes: (up-regulated genes) Z-score $\geq$ 2.0 and

ratio ≥ 1.5-fold, (down-regulated genes) Z-score ≤ -2.0 and ratio ≤ 0.66.

Determination of FGF 2by ELISA

The concentrations of FGF2 in the conditioned medium were measured using commercially available ELISA kits (R&D Systems, Minneapolis, MN). Cells were plated in 24-well dishes in medium containing 10% FBS. When the cells reached subconfluence, the medium was replaced with RPMI1640 medium without FBS, and then the cells were incubated for a further 24 hours. The concentrations of FGF2 in the supernatants were measured using an ELISA kit in accordance with the manufacturer's protocols.

Neutralizing FGF2 secretion

The autocrine role of FGF2 in cell proliferation was examined by adding an anti-FGF2 neutralizing monoclonal antibody (clone bFM-1: Millipore) at 5μg/ml for 12 hours. As a negative control, IgG was added.

Statistical analysis

All tests were two-sided, and differences at P <0.05 were considered statistically significant. Statistical analysis was performed with JMP version 10 software (SAS Institute, Cary, NC).

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Conflict of interest

The authors declare no conflict of interest.

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