Activation of the BMP-BMPR pathway conferred resistance to EGFR-TKIs in lung squamous cell carcinoma patients with EGFR mutations

Zhijie Wang[a,1], Zhirong Shen[b,c,1], Zhenxiang Li[a,d,1], Jianchun Duan[a,1], Shuai Fu[a], Zhentao Liu[a], Hua Bai[a], Zemin Zhang[e], Jun Zhao[a], Xiaodong Wang[b,c,2], and Jie Wang[a,2]

[a]Department of Thoracic Medical Oncology, Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Beijing Cancer Hospital & Beijing Institute for Cancer Research, Beijing 100142, China; [b]National Institute of Biological Sciences, Zhongguancun Life Science Park, Beijing 102206, China; [c]Collaborative Innovation Center of Systems Biomedicine, Shanghai Jiao Tong University School of Medicine, Shanghai 200240, China; [d]Department of Radiation Oncology, Shandong Cancer Hospital & Institute, Shandong Academy of Medical Sciences, Jinan 250117, China; and [e]Biodynamic Optical Imaging Center, Integrated Science Research Center, Peking University, Beijing 100871, China

Contributed by Xiaodong Wang, June 25, 2015 (sent for review April 3, 2015; reviewed by John D. Minna and Xiangwei Wu)

The empirical criteria for defining a clinical subtype of lung cancer are gradually transiting from histopathology to genetic variations in driver genes. Targeting these driver mutations, such as sensitizing epidermal growth factor receptor (EGFR) mutations, has dramatically improved the prognosis of advanced non–small cell lung cancer (NSCLC). However, the clinical benefit of molecularly targeted therapy on NSCLC appears to be different between lung adenocarcinomas and squamous cell carcinomas (SqCCs). We report here that the resistance of lung SqCC harboring EGFR mutations to EGFR tyrosine kinase inhibitors (EGFR-TKIs) was due to the activation of BMP-BMPR-Smad1/5-p70S6K. The combined treatment of these tumor cells with EGFR-TKI, together with inhibitors specific to BMPR or downstream mTOR, effectively reversed the resistance to EGFR-TKI. Moreover, blocking the whole PI3K-AKT-mTOR pathway with the PI3K/mTOR dual inhibitor BEZ235 also showed efficacy in treating this subtype of lung SqCC. This study details the empirical basis for a feasible clinical solution for squamous cell carcinomas with EGFR mutations.

epidermal growth factor receptor tyrosine kinase inhibitor | lung squamous cell carcinoma | bone morphogenetic proteins | drug resistance

Traditionally, the classification of lung cancer has been based primarily on histology and morphology (1, 2). With the identification of mutated driver oncogenes, methods from molecular pathology are gradually transforming the definition of the various types of lung cancers (3). Targeting gene aberrances such as epidermal growth factor receptor (EGFR) mutations (4–8) and anaplastic lymphoma kinase (ALK) fusion (9, 10) has significantly improved the prognosis of advanced non–small cell lung cancer (NSCLC). However, the clinical benefits brought by targeted therapies are mainly limited to nonsquamous NSCLC (11), while chemotherapy remains the major therapeutic choice for squamous cell carcinomas (SqCCs).

Recent studies assessing somatic mutations and copy number alteration (CNA) profiles in SqCC performed by the Cancer Genome Atlas project and other investigators have disclosed specific gene mutations, including GRM8, BAI3, ERBB4, RUNX1T1, KEAP1, and FBXW7, and CNAs in 3q26, 24, 27, 32–34, and 8p12.35 in lung SqCC (12, 13). About half of all patients with lung SqCC carry multiple gene aberrances, indicating that complex genomic characterizations are more common in lung SqCC than in adenocarcinoma (ADC). Thus, successful therapeutic strategies that target a single driver gene in lung ADC might not be feasible for lung SqCC patients.

Targeting EGFR mutations by EGFR tyrosine kinase inhibitors (TKIs) is one of the successful strategies in treating lung ADC. EGFR-TKIs obtained median progression-free survival (PFS) of 10–13 mo in EGFR-mutated lung ADC, but only ∼3 mo in lung SqCC with EGFR mutations (11, 14). Moreover, whether EGFR mutations exist in lung SqCC still remains controversial. There is a belief that EGFR mutations might not even occur in pure pulmonary SqCC, and that the occasional detection of these mutations in samples diagnosed as SqCC was due to mixed adenosquamous carcinoma and poorly differentiated ADC (15, 16). In current clinical practice, the utilization of EGFR-TKIs and the assessment of EGFR mutations are still routinely performed in lung SqCC, especially in nonsmokers. Therefore, it is critical to identify the subgroups of lung SqCC patients that are suitable for EGFR-TKI treatment. Exploring the mechanism of resistance to EGFR-TKIs in lung SqCC will deepen our understanding of the differences in tumorigenic profiling between lung SqCC and ADC, and should contribute to guiding clinical therapeutic decisions.

In the present study, we demonstrated that lung SqCC patients with EGFR mutations indeed represent a subset of NSCLC. We further showed that lung SqCC cell lines were resistant to EGFR-TKIs both in vitro and in vivo. We also investigated the mechanisms of resistance to EGFR-TKI in this subset of patients and provided potential strategies for overcoming TKI resistance in these patients.

Significance

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI) have demonstrated clinical benefits for patients suffering from non–small cell lung cancer of adenocarcinoma with activating mutation of EGFR. Same mutations observed in lung squamous cell carcinoma do not offer similar benefits. The underlining resistance mechanism revealed in this study turns out to be the concurrent activation of bone morphogenetic proteins (BMPs) signaling pathway in lung squamous cell carcinoma. The combination of EGFR-TKI with inhibitors of BMP receptors signaling pathway overcame the resistance. Such a finding provided a viable clinical strategy to treat those patients.

Author contributions: Z.W., Z.S., Z. Li, X.W., and J.W. designed research; Z.W., Z.S., Z. Li, J.D., S.F., Z. Liu, H.B., and J.Z. performed research; Z.W., Z.S., Z. Li, J.D., Z.Z., X.W., and J.W. analyzed data; and Z.W., Z.S., X.W., and J.W. wrote the paper.

Reviewers: J.D.M., University of Texas Southwestern Medical Center; and X.W., MD Anderson Cancer Center.

The authors declare no conflict of interest.

[1]Z.W., Z.S., Z.L., and J.D. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: wangxiaodong@nibs.ac.cn or wangjie_cc@yahoo.com.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1510837112/-/DCSupplemental.

Results

Lung Squamous Cell Carcinomas with EGFR Mutations Define a Unique Subtype of NSCLC. To determine the frequency of EGFR mutations in lung squamous cell carcinomas of Chinese patients, we first examined the percentage of EGFR mutations in Chinese lung cancer patients. We screened tumor tissue specimens from 2525 patients with advanced lung cancer for EGFR mutations (19del and 21L858R). There were 863 patients harboring EGFR mutations, among which lung SqCC contributed about 4.4% (38/863; Fig. S1*A*). In 396 lung SqCC cases, patients with EGFR mutations accounted for about 9.6% (38/396). In the 38 SqCC patients carrying EGFR mutations, the majority were male (81.6%), smokers (86.8%), and had the EGFR 19del mutation (57.9%) (Table S1). To confirm whether squamous cells were the pure component, we performed immunohistochemistry (IHC) biomarker analyses (P63 and TTF-1) in 18 patients with sufficient biopsied specimens. Most of these specimens (16/18) were positive for P63 and negative for TTF-1 (Table S2 and Fig. 1*A*).

To further confirm the existence of EGFR mutations in lung SqCC, we also checked IHC biomarkers in a cell line (SH416) derived from a patient diagnosed with advanced lung SqCC, who was initially identified as carrying EGFR 19del (17) (Fig. 1 *B* and *C*). immunocytochemistry (ICC) analysis of P63 (positive), P40 (positive), SCCA1 (partially positive), CK5/6 (negative), and TTF-1 (negative) confirmed that this cell line was a pure squamous cell line (Fig. 1*D*). To test for the existence of possible driver gene aberrances other than EGFR 19del that might affect the efficacy to EGFR-TKI, we detected PTEN loss, mutations in KRAS, mutations of BRAF V600E and PIK3CA (E545K, E542K, H1047R, and H1047L), and amplifications of FGFR1, PIK3CA, and c-MET; none of these aberrances were found (Fig. S1*B*).

To test whether EGFR mutations are also functional driver mutations in lung SqCC, we engineered cell lines using a human normal epithelial cell line (Beas/2b) in which mutant EGFR genes (EGFR 19 del or EGFR 21L858R) were stably expressed (Fig. S2). Beas/2b cell lines stably expressing mutant EGFR formed colonies at a much higher frequency in soft agar than did the control Beas/2b cell line. More dramatically, these EGFR-expressing mutant Beas/2b cell lines readily formed tumors in BALB/c nude mice, whereas no tumors were observed in mice inoculated with Beas/2b control cell lines (Fig. S2). ICC staining in these two cell lines (Beas/2b-19del cells and Beas/2b-21L858R cells) displayed strong and diffusive P40 positivity and partial P63 positivity, but displayed negative staining of TTF-1 (Fig. 1*E*), indicating the squamous cell histology of these two cell lines.

These results demonstrate that lung SqCC with EGFR mutations indeed exist as a significant fraction of the Chinese lung cancer population and that mutated EGFR can have oncogenic properties in the squamous cell background. Thus, these patients present a unique molecular and histological subtype.

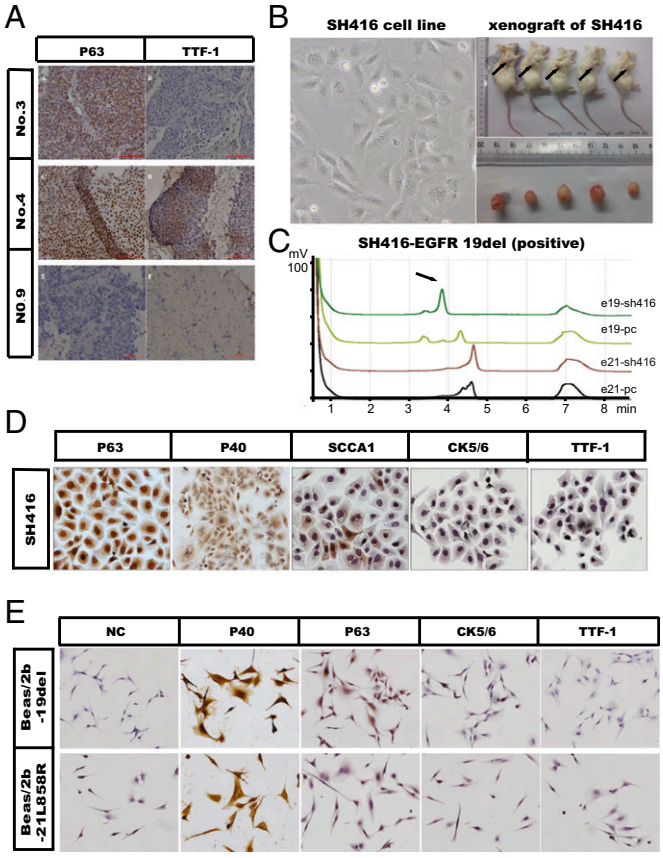

Fig. 1. SqCCs with EGFR mutations represent a subset of NSCLCs. (*A*) Representative IHC images of patients with lung SqCC. Formalin-fixed, paraffin-embedded sections (4 μm) were stained for P63 and TTF-1. (*B*) SH416 (*Left*), a stable cell line derived from a lung SqCC patient harboring EGFR 19del showed in vivo tumor formation (*Right*). (*C*) Denaturing high-performance liquid chromatography (DHPLC) analysis showing that SH416 cells had the EGFR 19del mutation. (*D*) SH416 cells showed a lung SqCC phenotype, with positivity for P63, P40, and SCCA1, and negativity for TTF-1 and CK5/6. Immunocytochemistry staining was used for these biomarkers. (*E*) Beas/2b-19del cells and Beas/2b-21L858R cells both showed a lung SqCC phenotype, with positivity for P40, and negativity for TTF-1, P63, and CK5/6. Immunocytochemistry staining was used for these biomarkers.

Lung SqCCs with EGFR Mutations Are Resistant to EGFR-TKIs Compared with Adenocarcinoma with EGFR Mutations. To compare the survival differences after EGFR-TKI treatment between patients with mutant and wild-type EGFRs, we screened 60 patients with advanced lung SqCC who meet following criteria: they received EGFR-TKI treatment and had EGFR mutations diagnosed in the period from June 2005 to June 2012. The median PFS after EGFR-TKI treatment was only 2.4 mo in patients with EGFR mutations, although this was slightly superior to those without EGFR mutations ($P = 0.064$; Fig. 2*A*). No significant difference in median overall survival (OS) was observed between patients with mutant and wild-type EGFR (Fig. 2*B*). The response data and multivariate analysis with COX proportional hazard ratios are presented in Tables S3 and S4. To exclude the possible interference of other common gene aberrances, we also tested for possible concurrent gene variations in PIK3CA, KRAS, DDR2, and FGFR1. Of 29 patients with EGFR mutations, there were nine cases with other key mutations, including two cases with FGFR1 amplification, one case with PIK3CA amplification, five cases with EGFR VIII mutation, and one case with KRAS mutation; there were no cases with PIK3CA mutation or DDR2 mutation. After removing the nine cases with other concurrent mutations, the median PFS and OS after EGFR-TKI treatment remained 2.3 and 17.5 mo, respectively.

We then tested the responses of lung SqCC cell lines to the EGFR inhibitor erlotinib. Cell viability tests showed that lung SqCC cell lines (SH416, Beas/2b-19del, and Beas/2b-21L858R) were more resistant to erlotinib than a lung ADC cell line with the EGFR 19del mutation (PC9 cell line; Fig. 2*C*). The IC_{50} values of the SH416, Beas/2b-19del, and Beas/2b-21L858R cell lines were 9.28, 3.43, and 11.9 μM, respectively. These concentrations were more than 100-fold higher than that of the PC9 cell line (34.36 nM). Further, we tested the response of lung SqCCs to erlotinib with in vivo xenograft experiments. Tumor shrinkage was not observed after erlotinib treatment in either the Beas/2b-19del xenografts or the Beas/2b-21L858R xenografts, although partial

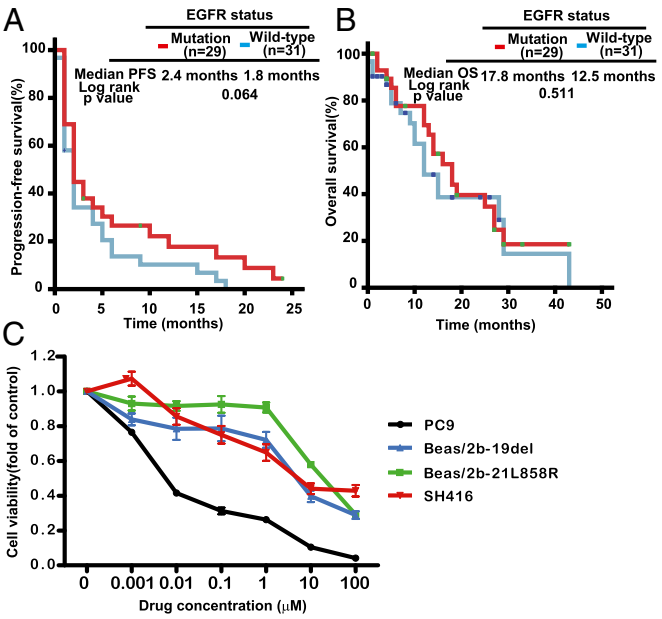

Fig. 2. SqCCs with EGFR mutation were resistant to EGFR-TKIs. (*A*) Kaplan–Meier curve shows that the median PFS after EGFR-TKI in lung SqCC patients carrying sensitizing EGFR mutation was 2.4 mo, similar to patients with wild-type EGFR (2.4 mo vs. 1.8 mo, $P = 0.064$). (*B*) Kaplan–Meier curve shows that the median overall survival after EGFR-TKI in lung SqCC patients carrying sensitizing EGFR mutation was 17.8 mo, similar to those with wild-type EGFR (17.8 mo vs. 12.5 mo, $P = 0.511$). (*C*) Cell viability analysis showed that lung SqCC cell lines (Beas/2b-19del, Beas/2b-21L858R, and SH416) presented stronger resistance to erlotinib than did a lung adenocarcinoma cell line with EGFR mutation (PC9). Viability at 72 h was calculated as the ratio of viable erlotinib-exposed cells to viable DMSO-treated cells.

suppressions were observed in the two xenograft models compared with the controls (Fig. S2 *B* and *C*).

Taken together, these results are consistent with the clinical observation that lung SqCCs with EGFR mutations are indeed resistant to EGFR-TKIs compared with lung ADC cells with the same EGFR mutations.

Activation of the BMP-BMPR-Smad1/5-70S6K Pathway Conferred Resistance to EGFR-TKIs in Lung SqCC Cells and Patients with EGFR Mutations. Activation of other signaling cascades has been demonstrated to be a mechanism of drug resistance (18). Therefore, to explore the mechanisms of erlotinib resistance in lung SqCC cells, we first examined the activation of several other intracellular pathways, including PI3K-AKT-mTOR and MAPK. As expected, erlotinib suppressed the activation of EGFR and AKT in all cell lines. Surprisingly, except in the PC9 cell (lung adenocarcinoma cell line), erlotinib did not affect the activation of p70S6 kinase in any of the squamous cell lines (SH416, Beas/2b-19del, and Beas/2b-21L858R cells) as tested by Western blot (Fig. 3*A* and Fig. S3*A*). The activation of p70S6K was not observed in the control Beas/2b cells. These results suggest that the activation of p70S6K may lead to the erlotinib resistance observed in these cell lines.

Multiple factors can lead to the activation of p70S6 kinase (19). We hypothesized that these factors were likely increased in the Beas/2b-19del and the Beas/2b-21L858R cells. To identify upstream factors that activate p70S6K, we used RNA sequencing to analyze global gene expression in these two cell lines with or without erlotinib treatment. We found 136 genes (with at least 2.5-fold changes) concurrently up-regulated both in the Beas/2b-19del and in the Beas/2b-21L858R cells after erlotinib treatment. By mapping all of these genes to the KEGG pathway database, we found that the TGF-β, PI3K-AKT-mTOR, and MAPK pathways were the dramatically changed pathways that were activated following erlotinib treatment (Fig. S4*A*). Because the activation status of MAPK differed between the SH416 and Beas/2b-19del/-21L858R cell lines, we hypothesized that mTOR-p70S6K might be activated by the TGF-β pathway and that this may induce the observed resistance to erlotinib.

The TGF-β superfamily includes two classes of members, classic TGF-β proteins and bone morphogenetic proteins (BMPs); these protein classes activate Smad2/3 and Smad1/5, respectively (20–22). In the present study, accumulation of pSmad1/5, but not pSmas2/3, was significantly increased in squamous cells (SH416, Beas/2b-19del, and Beas/2b-21L858R cells); this was not the case in ADC cells (PC9 and HCC827 cells; Fig. 3*B* and Fig. S3*B*). Consistently, the amount of BMPR-II protein, but not the amount of TGF-βR-II protein, was increased in squamous cells (SH416, Beas/2b-19del, and Beas2b/21L858R cells) following erlotinib treatment (Fig. 3*C* and Fig. S3*C*). To characterize the functional significance of the increase in BMPR-II levels in TKI resistance, we tested multiple TGF-β inhibitors, including LY2109761 (pan-inhibitor to TGF-βR and BMPR), GW788388 (inhibitor to TGF-βR), and LDN193189 (inhibitor to BMPR; Fig. S4*B*), for their ability to reverse TKI resistance. Both LDN193189 and LY2109761 improved the sensitivity of lung SqCC cells to erlotinib. LDN193189 in particular reversed erlotinib resistance even at 100-nM concentration relative to LY2109761 at 4 μM (Fig. S4*B*). Consistently, elimination of BMPR-II by shRNA knockdown also improved the sensitivity of lung SqCC cells to erlotinib, a phenomenon not observed in TGF-βR-I knockdown cells (Fig. S4*C*). In subsequent immunoblotting analyses, the combination of erlotinib and LDN193189 significantly suppressed pEGFR, pAKT, and p70S6K signals (Fig. S4*D*). We also observed the up-regulated transcription of BMP-related genes such as BMP4, BMP6, and ID2 in the RNA-seq results. These findings suggest that activation of the BMPR pathway resulted in phosphorylation of 70S6K and induced the observed resistance to erlotinib in lung SqCC cells.

To figure out which ligand activated the BMP pathway, we tested the effects of several TGF-β factors and BMPs on erlotinib resistance in lung SqCC cell lines. Cotreatment of BMP2 (10 ng/mL) or BMP4 (10 ng/mL) with erlotinib conferred more resistance to erlotinib compared with erlotinib only treatment in SH416, Beas/2b-19del, and Beas/2b-21L858R cells. This effect was not observed when cells were cotreated with TGF-β1 and erlotinib (Fig. 3*D* and Fig. S3*D*). We also detected significantly increased accumulation of BMP2, BMP4, and BMP7 in the conditional medium after erlotinib treatment, whereas the levels of TGF-β1, TGF-β2, and TGF-β3 remained unchanged (data not shown). These results suggest that BMPs such as BMP2 and BMP4 promoted the BMPR signaling pathway, including downstream mTOR-p70S6K signals.

To further confirm the results obtained in the in vitro experiments, we collected tumor tissue specimens from 15 patients with advanced lung SqCC and checked BMPR-II expression. Twelve such specimens were positive for BMPR-II in IHC staining experiments. Of the 15 total patients, 10 cases carried EGFR mutations and received EGFR-TKI treatment; 8 of these patients had PFS of less than 3 mo after EGFR-TKI treatment (group 1), and the other 2 patients had PFS more than 3 mo after EGFR-TKI treatment (group 2). Interestingly, all 8 patients in group 1 presented consistent positivity for staining for both BMPR-II and p70S6K, whereas the 2 patients of group 2 were negative for these signals (Fig. 3*E* and Fig. S3*E*). As a control, we performed IHC staining assays of BMPR-II and p70S6K expression in the 11 lung ADC patients with EGFR mutation and did not observe any differences between the changes in the levels of these two proteins with PFS following EGFR-TKI treatment (Fig. 3*F*). We retrospectively analyzed the expression status of

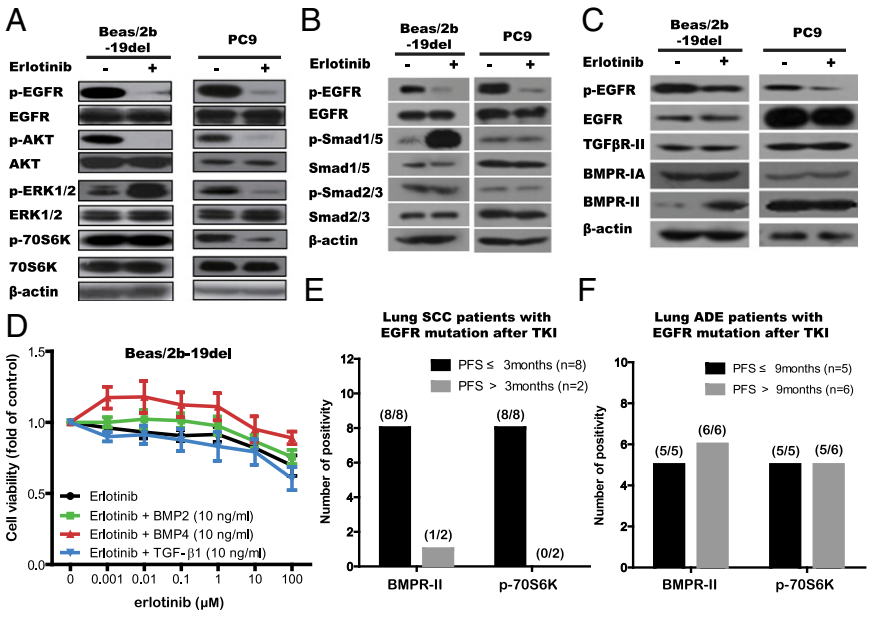

Fig. 3. Activation of the BMP-BMPR-Smad1/5-70S6K pathway conferred resistance to EGFR-TKIs in lung SqCC cells and patients with EGFR mutations. (*A–C*) The indicated cell lines were treated with erlotinib (1 μM) for 24 h. Cell extracts were immunoblotted to detect the indicated proteins. (*D*) Cell viability analysis showed that cotreatment with either BMP2 (10 ng/mL) or BMP4 (10 ng/mL) with erlotinib conferring stronger resistance to erlotinib in Beas/2b-19del cell line. Viability at 72 h was calculated as the ratio of drug-treated cells to viable DMSO-treated cells. (*E* and *F*) Formalin-fixed, paraffin-embedded sections (4 μm) were stained for BMPR-II and phos-70S6K. (*E*) Numbers of cases with positive proteins grouped by PFS after EGFR-TKI in lung SqCC patients ($n = 10$). (*F*) Numbers of cases with positive proteins grouped by PFS after EGFR-TKI in lung adenocarcinoma patients ($n = 11$).

BMPR-II and p70S6K protein in our lung SqCC cell lines and found that all of the cell lines (SH416 and Beas/2b-19del/-21L858R cells) harbored BMPR-II and p70S6K expression. These results indicated that the subset of EGFR mutation-carrying lung SqCC patients with BMPR-II expression and p70S6K activation were resistant to EGFR-TKI treatment (Fig. S4*E*).

Strategies to Overcome Resistance to EGFR-TKI in EGFR Mutant Lung SqCC Cells. Based on the aforementioned possible mechanisms of EGFR-TKI resistance in SqCC, we designed three strategies to counter such resistance; these included inhibition of mTOR (rapamycin), simultaneous inhibition of the EGFR and BMP pathways (erlotinib plus LDN193189), and blocking of the entire PI3K-AKT-mTOR pathway (erlotinib plus rapamycin or BEZ235, a dual inhibitor of PI3K and mTOR). Although we demonstrated that simultaneously inhibiting the EGFR and BMP pathways could reverse the sensitivity of SqCC cell lines to erlotinib, this regimen is not suitable for clinical use, owing to the lack of an FDA-approved BMPR-II inhibitor. We thus explored the antitumor activity of several other regimens that might be used in clinical practice that could yield similar activity.

In contrast to erlotinib, the use of rapamycin as single agent failed to exhibit effective antitumor activity in Beas/2b-19del and Beas/2b-21L858R cells. Surprisingly, BEZ235 displayed superior anti-tumor activity in SH416, Beas/2b-19del, and Beas/2b-21L858R cells, compared with erlotinib plus rapamycin or erlotinib plus LDN193189, although both such combination treatments also moderately suppressed the growth of cancer cells (Fig. 4 *A* and *B* and Fig. S5*A*). In the immunoblot, both BEZ235 and erlotinib plus rapamycin significantly suppressed the activation of pAKT and p70S6K in SH416, Beas/2b-19del, and Beas/2b-21L858R cells (Fig. 4*C* and Fig. S5*B*). Subsequently, we assessed the antitumor activity of BEZ235 and erlotinib plus rapamycin with in vivo models and observed effective control of tumor growth (Fig. 4*D* and Fig. S5*C*). These results suggest that combined treatment with inhibitors specific to BMPR or downstream p70S6K can effectively reverse erlotinib resistance. Moreover, blocking the whole PI3K-AKT-mTOR pathway with an agent such as BEZ235 is also a feasible therapy for this subtype of lung SqCC patients carrying EGFR mutations.

Discussion

Increasing knowledge about aberrances in driver genes is now promoting the understanding of tumorigenic profiles and is boosting the development of special molecular target agents that bring significant survival benefits in lung cancer treatment. However, lung cancers with different histology, such as squamous cancer and ADC, harbor distinct frequencies of driver genetic aberrances (10, 13, 23). It has been shown that cancers with the same driver mutation, but different histological subtypes, can have differential sensitivity to targeted therapies. In terms of EGFR mutations, lung SqCC patients harboring these gene variations showed inferior efficacy and survival rates compared with patients with lung ADCs when treated with TKIs (11, 14). The mechanism underlying this phenomenon remains unclear. Here, we systemically analyzed the in vitro and in vivo responses of lung SqCC with sensitizing EGFR mutations to EGFR-TKI, and experimentally evaluated both the mechanisms of resistance and strategies to overcome this resistance.

The discrepancies of morphology and function in histopathology between SqCC and ADC result from the different regions of airway tissue from which these cancer cells originate and may be associated with the variations of somatic mutations. In lung SqCCs, EGFR mutations are considered as a rare molecular event; they are reported in less than 5% of Caucasians and are reported at seemingly higher rates in Asians, ranging from 0% to 20.3% (10, 14, 24–30). In our study, the frequency of EGFR mutation in advanced lung SqCC patients was 9.6%. The divergence of these frequencies might be due to different detection methods, tumor heterogeneity, and/or ethnic differences in genetic background. Rekhtman et al. (15) postulated that EGFR mutation did not exist in pure SqCC and held that so-called "EGFR mutant lung SCC" was mixed in with ADC components (16). In the present study, we detected EGFR mutations in

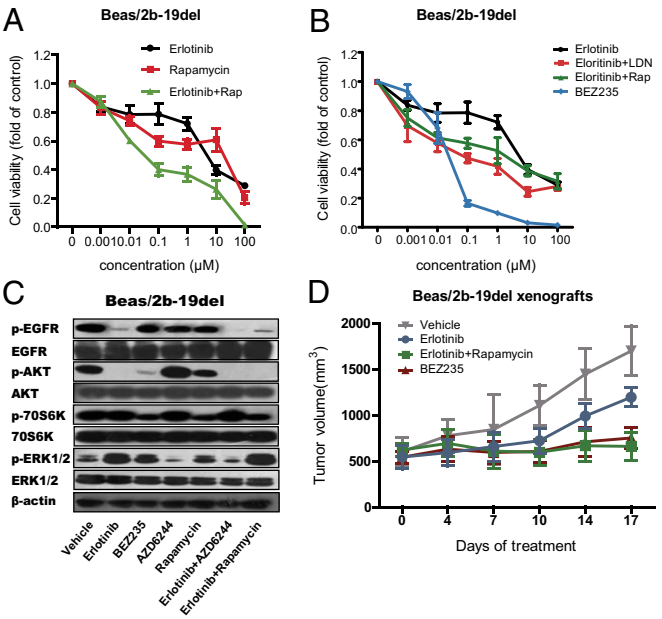

Fig. 4. Strategies for overcoming EGFR mutant lung SqCCs with BMPR-II and p70S6K expression. (*A*) Combinational treatment with erlotinib plus mTOR inhibitor [100 nM rapamycin (Rap)] overcame the EGFR-TKI resistance in lung Beas/2b-19del cell line. (*B*) Treatment with BEZ235 or combinational treatment with erlotinib plus BMPR inhibitor (100 nM LDN193189, LDN) overcame the EGFR-TKI resistance in lung Beas/2b-19del cell line. Viability at 72 h was calculated as the ratio of drug-exposed cells to viable DMSO-treated cells. (*C*) Beas/2b-19del cell lines were treated with the indicated drug combinations for 24 h. Cell extracts were immunoblotted to detect the indicated proteins. (*D*) Balb/C nude mice were s.c. engrafted with Beas/2b-19del cells, and tumors were treated daily with vehicle control, erlotinib, erlotinib plus rapamycin, or BEZ235 at the indicated dose. The tumor volumes (*y* axis) are plotted over time (*x* axis).

biopsied specimens and confirmed pure squamous components by IHC staining. Moreover, we confirmed that EGFR mutation is a functional driver mutation in lung SqCC using an EGFR mutant SqCC cell line and Beas/2b-based mutant EGFR cell lines. Because the success rate in establishing primary lung cancer cell line, especially SqCC cell line, still remains very low (2.3%), only one EGFR mutant SqCC cell line SH416 was used in this study. Although Beas/2b-based mutant EGFR cell lines formed colonies more rapidly in soft agar than did the parental Beas/2b cell line, they were still not as perfect for the in vitro cell model as patient-derived SqCC cells such as SH-416. Additional EGFR mutant SqCC cell lines were urgently needed for further studying function of mutant EGFR in SqCC cells and their responses to TKIs. Nevertheless, these results suggest that pure lung SqCC with EGFR mutations do exist and represent a unique subtype of NSCLC.

Targeting EGFR mutation by EGFR-TKI improves survival outcomes in lung ADCs, but does little with lung SqCCs. In this study, the median PFS after EGFR-TKI treatment in lung SqCC patients carrying EGFR mutations was less than 3 mo, although this was slightly superior to PFS in lung SqCC patients with wild-type EGFR. The survival time observed in present study was similar to previous reports, all of which show significantly shorter survival for SqCC than for lung ADC with EGFR mutations. Further, in vitro and in vivo experiments confirmed the relatively stronger resistance to erlotinib in lung SqCCs compared with ADCs harboring EGFR mutations. Our clinical and experimental results suggest that histopathology plays an important role in lung "EGFRoma."

With the exception of some identified mechanisms of resistance to EGFR-TKI such as EGFR T790M, c-MET amplification, KRAS mutation, epithelial–mesenchymal transitions, and transformation to SCLC (3), it is generally held that the activation of the classical TGF-β signaling pathway was related to secondary resistance to EGFR-TKI in lung ADC (31). Here, we observed the continuous phosphorylation of 70S6K due to the activation of another TGF-β superfamily BMP-BMPR protein that was significantly enhanced by erlotinib treatment. Although several studies have demonstrated that high expression of BMP2/4 is associated with poor prognosis and that inhibition of BMPR can decrease the invasiveness and proliferation of lung cancer cell lines (32), the current study provides, to our knowledge, the first clear association between BMPR activation and EGFR-TKI resistance. Importantly, we found that only lung SqCC patients with BMPR-II and p70S6K expression showed resistance to EGRR-TKI. Our results indicate that the mechanism of EGFR-TKI resistance differs between lung squamous and adenocarcinomas. This finding supports the potential importance of selecting EGFR mutant lung SqCC patients without BMPR-II and p70S6K expression as candidates for EGFR-TKI treatment.

Because the enhanced activation of the BMP-BMPR-p70S6K pathway confers EGFR-TKI resistance, erlotinib combined with a BMPR inhibitor (LDN193189) or an mTOR inhibitor (rapamycin) effectively reversed primary resistance. Similarly, BEZ235, a dual inhibitor of PI3K and mTOR could inhibit the proliferation of EGFR mutant lung SqCC cells. Taken together, these results all indicate that simultaneously blocking the activation of upstream and bypassing downstream pathways is an effective strategy for treating lung SqCCs with EGFR mutations. We speculate that lung SqCC would respond more rapidly to EGFR-TKI treatment than would ADC, through an activated bypass pathway in a feedback loop. Our results show different biological behaviors between lung squamous carcinoma and adenocarcinoma. Thus, the effect of histological types on molecular targeted treatment should be considered before therapy with EGFR-TKI.

Materials and Methods

This study was approved by the Institutional Ethics Committee of Peking University Cancer Hospital. Written informed consent was obtained from all patients. All animal studies reported here were approved by the Animal Care Committee of the National Institute of Biological Sciences (Beijing). *SI Materials and Methods* contains summaries of patient information, cell line details, and reagent information. Details of the methods, including gene aberrance detection, IHC, ICC, cell viability tests, Western blot analysis, soft agar foci formation, RNA sequencing analysis, protein chip analysis, and the s.c. in vivo experiments can be found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. This work was supported by Beijing Nova Program Grants xx2014B051 and xx2012070 from the Beijing Municipal Commission of Science and Technology, National Basic Science 973 Grants 2010CB835400 and 2012CB837400 from the Chinese Ministry of Science and Technology; National Natural Sciences Foundation Key Program 81330062; Education Ministry Innovative Research Team Program IRT13003; Fund for Peking University–Tsinghua University Joint Center for Life Sciences Clinical Investigator; Special Research Foundation of State Key Laboratory of Medical Genomics; National High Technology Research and Development Program 863 (SS2015AA020403); Beijing Technology Project Z141100000214013; Center for Molecular and Translational Medicine; Collaborative Innovation Center for Cancer Medicine; Special Research Foundation for the Doctoral Program of Higher Education; and Beijing Excellent Talents Project 2013D003034000019.

1. Brambilla E, Travis WD, Colby TV, Corrin B, Shimosato Y (2001) The new World Health Organization classification of lung tumours. *Eur Respir J* 18(6):1059–1068.
2. Travis WD, Brambilla E, Riely GJ (2013) New pathologic classification of lung cancer: Relevance for clinical practice and clinical trials. *J Clin Oncol* 31(8):992–1001.
3. Sequist LV, et al. (2011) Genotypic and histological evolution of lung cancers acquiring resistance to EGFR inhibitors. *Sci Transl Med* 3(75):75ra26.
4. Mok TS, et al. (2009) Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. *N Engl J Med* 361(10):947–957.

5. Maemondo M, et al.; North-East Japan Study Group (2010) Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N Engl J Med* 362(25):2380–2388.
6. Mitsudomi T, et al.; West Japan Oncology Group (2010) Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): An open label, randomised phase 3 trial. *Lancet Oncol* 11(2):121–128.
7. Rosell R, et al.; Spanish Lung Cancer Group in collaboration with Groupe Français de Pneumo-Cancérologie and Associazione Italiana Oncologia Toracica (2012) Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): A multicentre, open-label, randomised phase 3 trial. *Lancet Oncol* 13(3):239–246.
8. Zhou C, et al. (2011) Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): A multicentre, open-label, randomised, phase 3 study. *Lancet Oncol* 12(8):735–742.
9. Kwak EL, et al. (2010) Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. *N Engl J Med* 363(18):1693–1703.
10. Shaw AT, et al. (2009) Clinical features and outcome of patients with non-small-cell lung cancer who harbor EML4-ALK. *J Clin Oncol* 27(26):4247–4253.
11. Shukuya T, et al. (2011) Efficacy of gefitinib for non-adenocarcinoma non-small-cell lung cancer patients harboring epidermal growth factor receptor mutations: A pooled analysis of published reports. *Cancer Sci* 102(5):1032–1037.
12. Network TCGAR; Cancer Genome Atlas Research Network (2012) Comprehensive genomic characterization of squamous cell lung cancers. *Nature* 489(7417):519–525.
13. Kim Y, et al. (2014) Integrative and comparative genomic analysis of lung squamous cell carcinomas in East Asian patients. *J Clin Oncol* 32(2):121–128.
14. Hata A, et al. (2013) How sensitive are epidermal growth factor receptor-tyrosine kinase inhibitors for squamous cell carcinoma of the lung harboring EGFR gene-sensitive mutations? *J Thorac Oncol* 8(1):89–95.
15. Rekhtman N, et al. (2012) Clarifying the spectrum of driver oncogene mutations in biomarker-verified squamous carcinoma of lung: Lack of EGFR/KRAS and presence of PIK3CA/AKT1 mutations. *Clin Cancer Res* 18(4):1167–1176.
16. Roggli VL, et al. (1985) Lung cancer heterogeneity: A blinded and randomized study of 100 consecutive cases. *Hum Pathol* 16(6):569–579.
17. Zheng C, Sun YH, Ye XL, Chen HQ, Ji HB (2011) Establishment and characterization of primary lung cancer cell lines from Chinese population. *Acta Pharmacol Sin* 32(3): 385–392.
18. Bean J, et al. (2007) MET amplification occurs with or without T790M mutations in EGFR mutant lung tumors with acquired resistance to gefitinib or erlotinib. *Proc Natl Acad Sci USA* 104(52):20932–20937.
19. Zoncu R, Efeyan A, Sabatini DM (2011) mTOR: From growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 12(1):21–35.
20. Langenfeld E, Hong CC, Lanke G, Langenfeld J (2013) Bone morphogenetic protein type I receptor antagonists decrease growth and induce cell death of lung cancer cell lines. *PLoS One* 8(4):e61256.
21. Attisano L, Wrana JL (2002) Signal transduction by the TGF-beta superfamily. *Science* 296(5573):1646–1647.
22. Kretzschmar M, Liu F, Hata A, Doody J, Massagué J (1997) The TGF-beta family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. *Genes Dev* 11(8):984–995.
23. Cancer Genome Atlas Research Network (2014) Comprehensive molecular profiling of lung adenocarcinoma. *Nature* 511(7511):543–550.
24. Choi YL, et al. (2013) EGFR mutation testing in patients with advanced non-small cell lung cancer: A comprehensive evaluation of real-world practice in an East Asian tertiary hospital. *PLoS One* 8(2):e56011.
25. Marchetti A, et al. (2005) EGFR mutations in non-small-cell lung cancer: Analysis of a large series of cases and development of a rapid and sensitive method for diagnostic screening with potential implications on pharmacologic treatment. *J Clin Oncol* 23(4): 857–865.
26. Bai H, et al. (2009) Epidermal growth factor receptor mutations in plasma DNA samples predict tumor response in Chinese patients with stages IIIB to IV non-small-cell lung cancer. *J Clin Oncol* 27(16):2653–2659.
27. Tanaka T, et al. (2010) Frequency of and variables associated with the EGFR mutation and its subtypes. *Int J Cancer* 126(3):651–655.
28. Pao W, Girard N (2011) New driver mutations in non-small-cell lung cancer. *Lancet Oncol* 12(2):175–180.
29. Tseng JS, et al. (2012) Retrospective study of erlotinib in patients with advanced squamous lung cancer. *Lung Cancer* 77(1):128–133.
30. Miyamae Y, et al. (2011) Significance of epidermal growth factor receptor gene mutations in squamous cell lung carcinoma. *Oncol Rep* 25(4):921–928.
31. Serizawa M, Takahashi T, Yamamoto N, Koh Y (2013) Combined treatment with erlotinib and a transforming growth factor-beta type I receptor inhibitor effectively suppresses the enhanced motility of erlotinib-resistant non-small-cell lung cancer cells. *J Thorac Oncol* 8(3):259–269.
32. Fei ZH, Yao CY, Yang XL, Huang XE, Ma SL (2013) Serum BMP-2 up-regulation as an indicator of poor survival in advanced non-small cell lung cancer patients. *Asian Pac J Cancer Prev* 14(9):5293–5299.