Clinical significance of Akt2 in advanced pancreatic cancer treated with erlotinib

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Abstract. Akt2 is an isoform of Akt, and an association between Akt2 and resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) has been suggested in pancreatic cancer (PC) *in vitro*. In this study, we investigated the association between Akt2 expression as evaluated using immunohistochemistry and the outcome of patients with advanced PC who had received treatment with erlotinib (an EGFR-TKI). Although the difference was not significant, patients with high levels of Akt2 expression tended to have a poorer response and a shorter progression-free survival period after treatment with erlotinib plus gemcitabine than those with low expression levels (P=0.16 and 0.19, respectively). *In vitro*, an *Akt2*-amplified PC cell line and *Akt2*-overexpressed cell lines exhibited resistance to anti-EGFR therapies, including erlotinib, but combined treatment with BYL719 (a PI3K inhibitor) cancelled this resistance. Our findings suggest that Akt2 might be associated with the resistance to anti-EGFR therapies, especially the use of erlotinib against PC, and that this resistance can be overcome by combined treatment with a PI3K inhibitor. Akt2 expression could become a predictive biomarker for erlotinib resistance in PC.

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Abbreviations: CRC, colorectal cancer; CT, computed tomography; EGFP, enhanced green fluorescent protein; EGFR, epidermal growth factor receptor; EUS-FNA, endoscopic ultrasonography-guided fine needle aspiration; FBS, fetal bovine serum; HNSCC, head and neck squamous cell carcinoma; LAd, lung adenocarcinoma; LSq, lung squamous cell carcinoma; NSCLC, non-small cell lung cancer; OS, overall survival; PC, pancreatic cancer; PFS, progression-free survival; TCGA, the Cancer Genome Atlas; TKI, tyrosine kinase inhibitor; SD, standard deviations

Key words: pancreatic cancer, Akt2, anti-EGFR therapy, erlotinib, PI3K inhibitor

Introduction

Pancreatic cancer (PC) remains a deadly disease. Gemcitabine has been considered as the standard therapy for patients with unresectable or metastatic disease for over a decade (1,2). Recently, overall survival (OS) has been significantly prolonged using combination therapies, such as gemcitabine plus erlotinib, a combination of oxaliplatin, irinotecan, fluorouracil and leucovorin (FOLIFRINOX), a combination of nab-paclitaxel and gemcitabine, or a combination of nanoliposomal irinotecan, fluorouracil and leucovorin (NAPOLI-1) (3-6). However, despite such recent progress, the OS rate of PC patients is still <5% (1,2). Erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), was the first drug approved for the treatment of PC after showing a survival benefit when combined with gemcitabine over traditional gemcitabine alone (3). Although PC has been well characterized at the genetic level, the molecular mechanisms linking genetic changes to the aggressive nature of this disease remain unclear (7). Multiple genetic alterations, such as K-ras mutation or the loss of *p53* and *SMAD4*, are thought to influence the progression of PC (8). Nevertheless, to date, the inhibition of EGFR by erlotinib is the only targeted approach to demonstrate a survival benefit.

The PI3K/Akt/mTOR pathway, which is located downstream of the EGFR pathway and regulates cell survival and apoptosis, is frequently upregulated or altered in many cancers, and components of the PI3K/Akt/mTOR pathway can also be targeted in the treatment of many cancers (9). Among them, the inhibition of mTOR with rapalogs has initially shown a clinical efficacy in some solid tumors (10). More recently, many agents in clinical development have been designed to inhibit other components of this pathway, including Akt, PI3K and PTEN.

Akt, the major downstream component of the PI3K/Akt/mTOR pathway, is a serine/threonine kinase that plays a critical role in regulating diverse cellular function including cell gowth, proliferation, survival, glucose metabolism, genome stability, transcription and protein synthesis, and neurovascularization (9). The Akt family has three isoforms: Akt1, Akt2 and Akt3. These isoforms are structually homologous but exhibit distinct features. Akt1 and Akt2 are ubiquitously expressed, whereas Akt3 is found predominantly in the brain, heart and kidneys (11). The Akt isoforms are known to carry specific genetic alterations in different tumor types. *Akt1* amplification has been detected in gastric adenocarcinoma, and the selective activation of *Akt3* in combination with a loss of PTEN has been found in sporadic melanoma (12). In contrast, amplification or high levels of expression of *Akt2* are frequently found in human pancreatic, lung, colorectal, ovarian, and breast cancers (12-19), and high *Akt2* expression levels are positively correlated with the aggressiveness of cancer or poor survival rates in colorectal, ovarian, and breast cancers (16-19). In PC, the activation of the PI3K/Akt/mTOR pathway is a biological indicator of aggressiveness (20), and a recent report has shown that EGFR-TKI resistance in PC is associated with the upregulation of the PI3K/Akt/mTOR pathway *in vitro* (21). Thus, high Akt2 expression levels have been hypothesized to induce resistance to the EGFR-TKI, erlotinib, in patients with PC. In this study, we investigated the association between the Akt2 expression level and the outcome of patients with advanced PC who had received erlotinib treatment as well as the contribution of Akt2 to resistance to anti-EGFR therapies *in vitro*.

Materials and methods

Patients and clinical specimens. Twenty-six patients with advanced PC that received Tarceva® (erlotinib) treatment in combination with gemcitabine at Kindai University Hospital between 2010 and 2014 were included. Among them, 22 patients who had been diagnosed based on the results of endoscopic ultrasonography-guided fine needle aspiration (EUS-FNA) participated in this study. Progression-free survival (PFS) was defined as the time from the initiation of erlotinib treatment until the first observation of disease progression or death from any cause, while OS was defined as the time from the initiation of erlotinib treatment until death from any cause. The stage of disease was classified according to the clinical TNM staging system. Tumor response was evaluated using computed tomography (CT) according to the Response Evaluation Criteria in Solid Tumors. This study was performed retrospectively and was approved by the ethics committee of the Kindai University Faculty of Medicine.

Immunohistochemistry. The immunohistochemical method used in this study has been previously described (22). Briefly, 4-μm tissue sections from formalin-fixed, paraffin-embedded blocks were sectioned and placed onto charged slides. The slides were then deparaffinized and hydrated; endogenous peroxidase activity was blocked using 3% H_2O_2 in methanol and normal goat serum. The slides were incubated in a rabbit polyclonal antibody specific for Akt2 (1:200; Proteintech Chicago, IL, USA) overnight at 4˚C. Immunohistochemical staining was performed using the rabbit Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA), and the slides were developed in diaminobenzidine (DAB kit, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocols, then counterstained with hematoxylin. The staining assessment was performed using ImageJ software (http://imagej.nih.gov/ij/).

Cell culture and reagents. The HCC827, PC-9, Ma-1, and H358 cell lines [human non-small cell lung cancer (NSCLC) cell lines] and the BxPC-3 and PANC-1 cell lines (human PC cell lines) were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS; Sigma-Aldrich). The CCK81 cell line [a human colorectal cancer (CRC) cell line] and the gpIRES-293 cell line were maintained in DMEM medium (Nissui Pharmaceutical, Tokyo, Japan) with 10% FBS. All the cells were maintained in a 5% CO_2-humidified atmosphere at 37˚C. Erlotinib and BYL719 were purchased from Selleck Chemicals (Houston, TX, USA).

Copy number assay. The *Akt2* copy number was determined using a commercially available and predesigned TaqMan Copy Number assay (Applied Biosystems, Foster City, CA, USA), as described previously (23). Genomic DNA was extracted from each of the cell lines using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The primer ID used for *Akt2* was Hs04028824_cn. The *TERT* locus was used for the internal reference copy number. Human genomic DNA (Takara, Shiga, Japan) was used as a normal control. A PCR analysis was performed using the ABI PRISM 7900HT Sequence Detection system (Applied Biosystems), and the results were analyzed using CopyCaller software version 2.0 (Applied Biosystems). The experiment was performed in triplicate.

Real-time reverse-transcription PCR (RT-PCR). A total of 1 μg of RNA was isolated from the cells using Isogen reagent (Nippon Gene, Tokyo, Japan) and then converted to cDNA using the Gene Amp RNA-PCR kit (Applied Biosystems). Real-time PCR was performed using SYBR Premix Ex Taq and Thermal Cycler Dice (Takara) under the following conditions: 95˚C for 5 min, followed by 50 cycles of 95˚C for 10 sec and 60˚C for 30 sec, as described previously (23). *GAPDH* was used to normalize the expression levels in the subsequent quantitative analyses. The experiment was performed in triplicate. The primers used for this study were as follows: *Akt2* F, CCGCCTGTGCTTTGTGATGG; R, TTTCCAGCTTGATG TCGCGG. *GAPDH* F, GCACCGTCAAGGCTGAGAAC; and R, ATGGTGGTGAAGACGCCAGT.

In vitro growth inhibition assay. The growth-inhibitory effects of the drugs were examined using a 3-(4, 5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT; Sigma-Aldrich), as described previously (23). The experiment was performed in triplicate.

Plasmid construction, viral production, and stable transfectants. The methods used in this section have been previously described (24). Complementary DNA (cDNA) encoding human full length *Akt2* was prepared by PCR using Prime

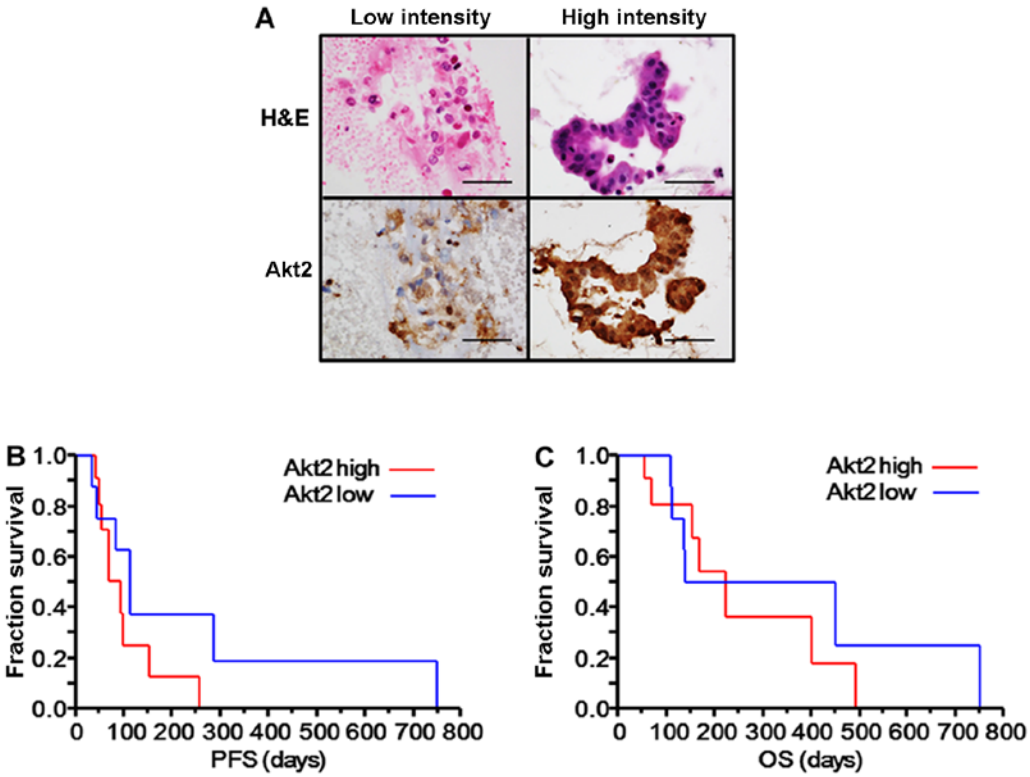


Figure 1. Akt2 expression and outcome of patients with advanced pancreatic cancer (PC) treated with erlotinib. (A) Hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining for Akt2 in patients with advanced PC. Deparaffinized sections from endoscopic biopsied tumors were analyzed using H&E and IHC staining. The staining intensity was determined by the relative mean grey value (mean grey value/maximum grey value) using ImageJ software, and the patients were divided into two groups: a low intensity group (relative mean grey value <70%) and a high intensity group (relative mean grey value ≥70%). Scale bar, 50 μm. (B) Kaplan-Meier curve of progression-free survival (PFS). Patients with a high Akt2 intensity tended to have a shorter PFS than those with a low intensity (median PFS; 92 vs. 113 days, P=0.19). (C) Kaplan-Meier curve of overall survival (OS). Patients with a high Akt2 intensity tended to have a shorter OS than those with a low intensity (median OS; 224 vs. 295 days, P=0.59).

STAR HS DNA polymerase (Takara) and the following primers: forward, 5'-GGGAATTCGCCGCCATGAATGAGG TGTCTGTCATCAAAG-3'; reverse, 5'-CCCTCGAGGCCCA GTCACTCGCGGATGCTGGC-3'. The full length *Akt2* was subcloned into a pCR-Blunt II-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) as *Eco*RI-*Xho*I fragments. *Akt2* in the TOPO cloning vector was cut out and transferred to a pQCLIN retroviral vector (Clontech Laboratories, Inc., Palo Alto, CA, USA) together with the enhanced green fluorescent protein (EGFP) following the internal ribosome entry site sequence (IRES) to monitor the expression of the inserts indirectly. The nucleotide sequence of the construct was verified by DNA sequence analysis. A pVSV-G vector (Clontech) for the constitution of the envelope and the pQCLIN-IG constructs were cotransfected into gpIRES-293 cells using FuGENE6 transfection reagent (Roche Diagnostics, Basel, Switzerland). After 48 h of transfection, the culture medium was collected and viral particles were concentrated by centrifugation at 15,000 x g for 3 h at 4˚C. The viral pellet was suspended in fresh DMEM medium. The titer of the viral vector was calculated using the EGFP-positive cells that were infected by the serial dilution of virus-containing media, and the multiplicity of infection was determined. The vectors and the stable viral transfectant BxPC-3, Ma-1, and CCK81 cell lines were designated as pQCLIN-EGFP, pQLCIN-Akt2, BxPC-3/EGFP, BxPC-3/Akt2, Ma-1/EGFP, Ma-1/Akt2, CCK81/EGFP, and CCK81/Akt2, respectively.

Western blot analysis. The western blot analysis was performed as described previously (24). Subconfluent cells were washed with cold phosphate-buffered saline (PBS) and lysed using lysis A buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.0), 5 mM EDTA, 50 mM sodium chloride, 50 mM pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, and a protease inhibitor mix, Complete™ (Roche Diagnostics). Whole-cell lysates were separated using SDS-PAGE and were blotted onto a polyvinylidene fluoride membrane. The membrane was blocked for 1 h with 5% skim milk in a TBS buffer (pH 8.0) with 0.1% Tween-20. The membrane was then washed 3 times with TBS and incubated overnight with the primary antibody at 4˚C. After washing 3 times with TBS, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membrane was then washed, followed by visualization using an ECL detection system and LAS-4000 (GE Healthcare, Buckinghamshire, UK). Rabbit antibodies specific for EGFR, phospho-EGFR, Akt, phospho-Akt, caspase-3, cleaved caspase-3, and β-actin were obtained from Cell Signaling (Beverly, MA, USA). To evaluate the influence of the drugs on phosphorylation and an apoptosis-related molecule, the cells were stimulated for 1-3 and 24 h, respectively.

Database analysis. To analyze the prevalence of *Akt2* amplification and high expression levels, the cBioPortal for Cancer

Genomics database (http://www.cbioportal.org/public-portal/) was searched (25,26). Within the database, The Cancer Genome Atlas (TCGA) datasets (http://cancergenome.nih.gov/) of several cancers are analyzed.

Statistical analysis. Continuous variables were analyzed using Student's t-test, and the results were expressed as the average and standard deviations (SD). The univariate relationship between each independent variable was examined using the Fisher's exact test. The statistical analyses were two-tailed and were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) and JMP Pro 11 (SAS Institute, Cary, NC, USA). $P<0.05$ was considered statistically significant.

Results

Akt2 expression and patient characteristics. A total of 22 patients with advanced PC and a good performance status (0-1) who had been diagnosed based on the results of EUS-FNA were enrolled in this study. The patients with PC ranged in age from 17 to 70 years, with a median age of 61 years, and the male: female ratio was 12:10. To evaluate the relationship between Akt2 expression and prognosis, an immunohistochemical analysis was performed using biopsy specimens from these patients (Fig. 1A). Three of the 22 specimens could not be evaluated properly using immunohistochemistry for Akt2 because of the poor conditions of the samples and were excluded from additional analyses. The staining intensity was determined as the relative mean grey value (mean grey value/maximum grey value) using ImageJ software, and the patients were divided into two groups: a low intensity group (relative mean grey value <70%, n=8) and a high intensity group (relative mean grey value ≥70%, n=11). No significant differences in the patient characteristics were observed between the two groups, but patients with a high Akt2 intensity tended to have a poorer response to erlotinib plus gemcitabine (0/11 vs. 2/8, P=0.16) (Table I). Though the difference was not significant, patients with a high Akt2 intensity also tended to have a shorter PFS (median PFS: 92 vs. 113 days, P=0.19) (Fig. 1B) and OS after the initiation of erlotinib plus gemcitabine (median OS: 224 days vs. 295 days, P=0.59) (Fig. 1C). These results suggest that Akt2 might be associated with erlotinib resistance in advanced PC.

Akt2 copy number, Akt2 gene expression, and Akt2 protein expression in diverse cancer cell lines. To investigate the relationship with Akt2 expression and the response to anti-EGFR therapies, we evaluated the Akt2 gene copy numbers, Akt2 gene expression, and Akt2 protein expression in several cancer cell lines for which anti-EGFR therapies are commonly used. Seven diverse cancer cell lines (four NSCLC cell lines, one CRC cell line, and two PC lines) were used because the PANC-1 cell line has been reported to exhibit *Akt2* amplification and the other cell lines are sensitive to anti-EGFR therapies. The Akt2 gene copy number, Akt2 gene expression, and Akt2 protein expression for these cell lines were estimated using a copy number assay, real-time RT-PCR, and western blotting, respectively. The PANC-1 cell line had high copy numbers of the Akt2 gene (40 copies) and the Akt2 gene expression level was also markedly elevated, whereas the other cell lines had neither a high *Akt2* copy number nor a high expression level (Fig. 2A and B). Western blot analysis also revealed that Akt2 protein was highly expressed in the PANC-1 cell line (Fig. 2C).

Table I. Patient characteristics and associations with Akt2 expression.

Patients characteristics	Akt2 intensity		P-value[b]
	Low (n=8)	High (n=11)	
Age, years			
<60	4	5	1.0
≥60	4	6	
Gender			
Male	4	7	0.66
Female	4	4	
T stage			
T1-3	5	5	0.65
T4	3	6	
N stage			
N0	3	7	0.37
N1-3	5	4	
M stage			
M0	3	2	0.60
M1	5	9	
Treatment line of erlotinib plus gemcitabine			
First line	4	5	1.0
Second line or later	4	6	
Response to erlotinib plus gemcitabine			
PR	2	0	0.16
SD or PD	6	11	
Median PFS (days)[a]	113	92	0.19
Median OS (days)[a]	295	224	0.59

PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression-free survival; OS, overall survival. [a]PFS and OS were defined as the time from the initiation of erlotinib treatment until disease progression or death from any cause and the time from the initiation of erlotinib treatment until death from any cause, respectively. [b]PFS and OS were analyzed using the log-rank test, and the others were analyzed using the Fisher's exact test.

Combined effect of erlotinib and a PI3K inhibitor in an Akt2-amplified and highly expressed PANC-1 cell line. To investigate the influence of *Akt2* on the resistance to anti-EGFR therapies, the effect of erlotinib against the PANC-1 cell line (an *Akt2*-amplified and highly expressed cell line) was tested using an MTT assay. As is shown in Fig. 3A, the PANC-1 cell

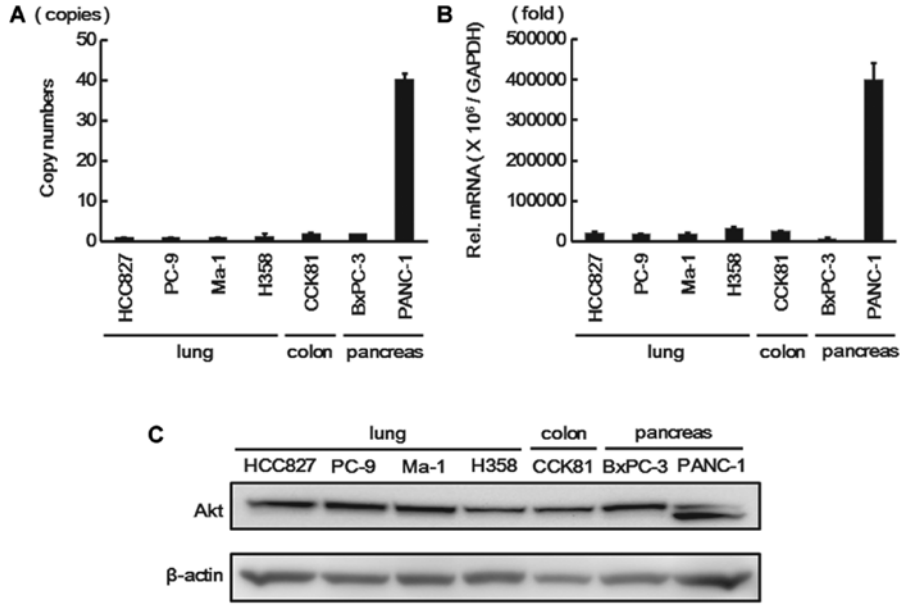


Figure 2. *Akt2* copy number, *Akt2* expression, and Akt2 protein expression in diverse cancer cell lines. (A) *Akt2* copy number. The copy number for *Akt2* was determined using a TaqMan Copy Number assay. A copy number gain for *Akt2* was observed only in the PANC-1 cell line (40 copies). Columns, mean of independent triplicate experiments; error bars, SD. (B) *Akt2* expression. The *Akt2* expression level was estimated using real-time RT-PCR. *GAPDH* was used to normalize the expression levels. The *Akt2* expression level in the PANC-1 cell line was particularly high compared with those in the other cell lines. Columns, mean of independent triplicate experiments; error bars, SD. (C) Akt2 protein expression. Western blotting was performed to confirm Akt expression. Akt2 protein was highly expressed in the PANC-1 cell line, compared with the other cell lines. β-actin was used as an internal control.

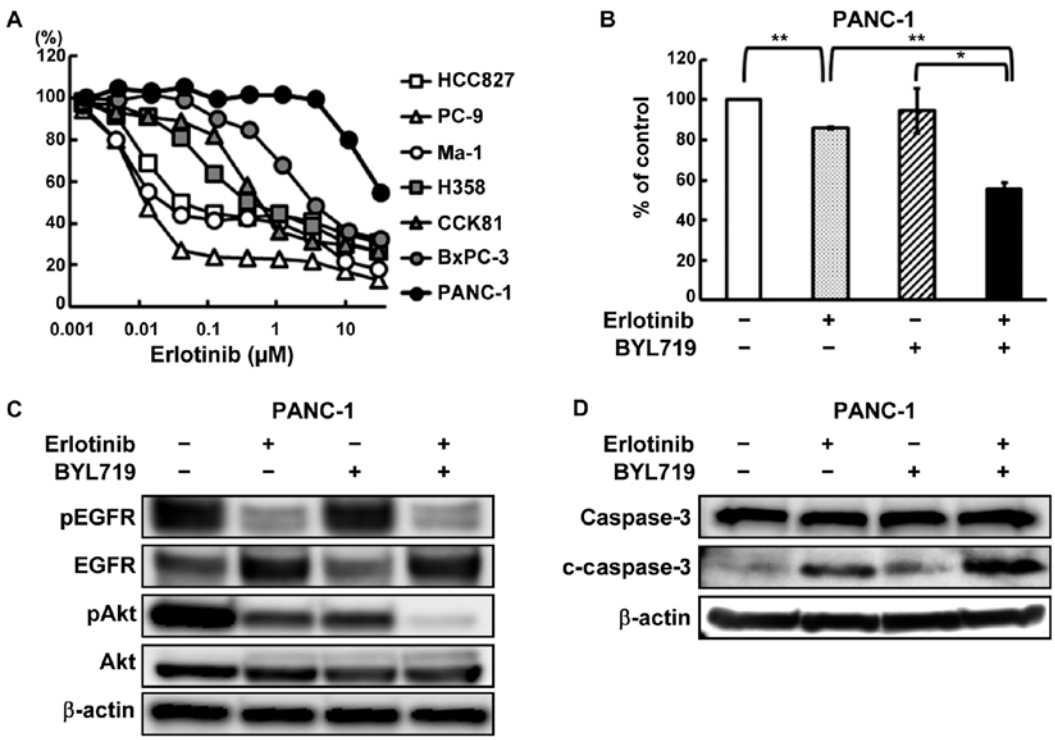


Figure 3. Combined effect of erlotinib and BYL719 in an *Akt2*-amplified and highly expressed PANC-1 cell line. (A) Growth inhibitory effects of erlotinib in diverse cancer cell lines. The cells were exposed to each concentration of erlotinib for 72 h, and the growth inhibitory effects were evaluated using an MTT assay. The HCC827, PC-9, and Ma-1 cell lines (non-small cell lung cancer cell lines harboring *EGFR* mutaions) were hypersensitive to erlotinib. The PANC-1 cell line was particularly resistant to erlotinib. Lines, mean of independent triplicate experiments. (B) Growth inhibitory effects of erlotinib and/or BYL719 in the PANC-1 cell line. The cells were treated with 10 μM of erlotinib and/or 2 μM of BYL719 for 72 h, and the growth inhibitory effects were evaluated using an MTT assay. The combined treatment of erlotinib and BYL719 inhibited the cellular growth more intensively, compared with erlotinib monotherapy. Columns, mean of independent triplicate experiments; error bars, SD; *P<0.05; **P<0.01. (C) Phosphorylation of EGFR and Akt in the PANC-1 cell line. The cells were treated with 10 μM of erlotinib and/or 2 μM of BYL719, and the samples were collected 1 h after drug stimulation. The phophorylation of Akt persisted in the erlotinib monotherapy, while the combined treatment with BYL719 decreased the phosphorylation of Akt. β-actin was used as an internal control. pEGFR, phospho-EGFR; pAkt, phopho-Akt. (D) Expression of an apoptosis-related molecule in the PANC-1 cell line. Twenty-four hours after the cells were exposed to the drugs (erlotinib, 10 μM; BYL719, 2 μM), the samples were collected. Although erlotinib or BYL719 monotherapy did not increase the expression of cleaved caspase-3, the combined treatment increased the expression. β-actin was used as an internal control. c-caspase-3, cleaved caspase-3.

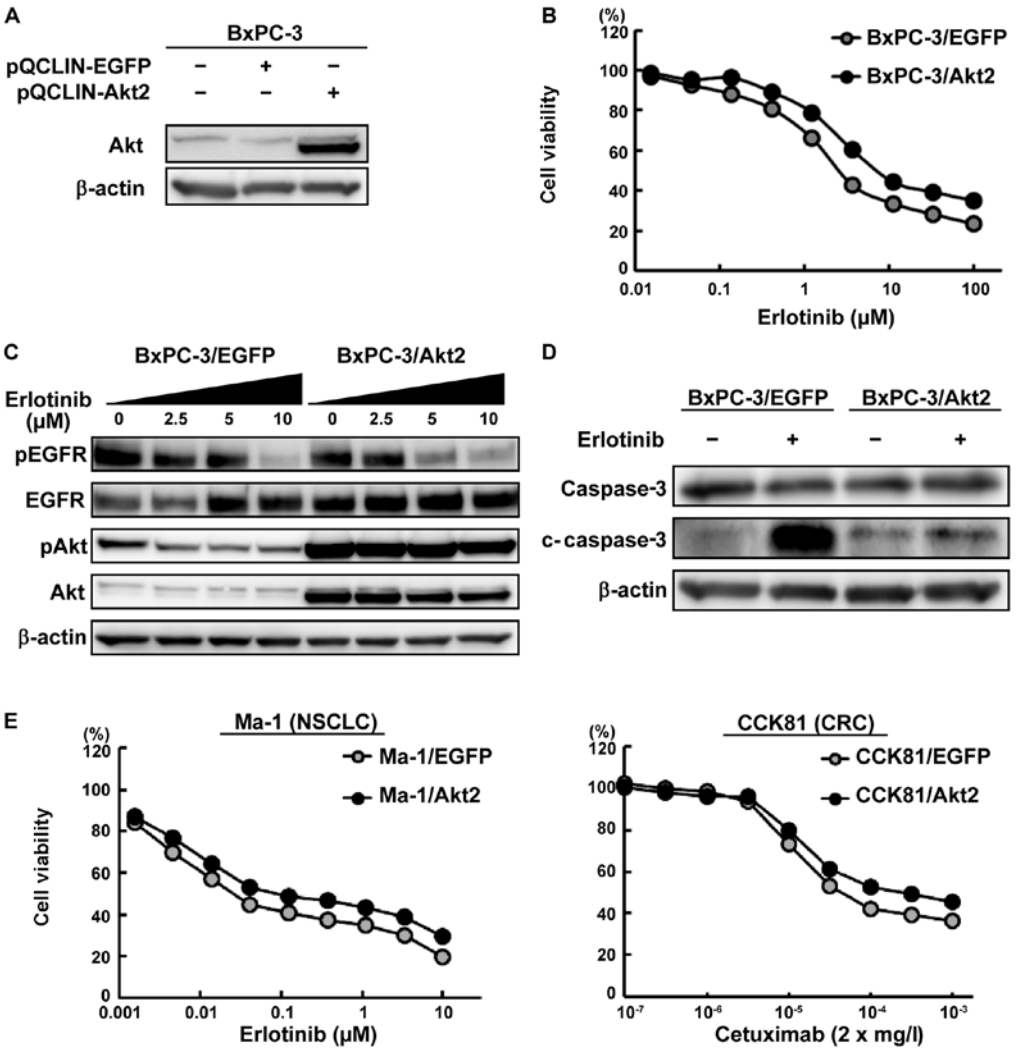
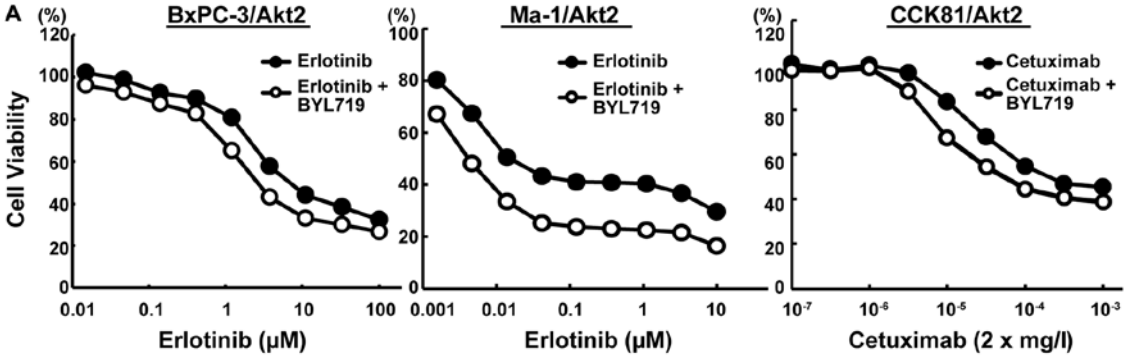


Figure 4. Influence of *Akt2*-overexpression on the efficacy of anti-EGFR therapies against PC, colorectal cancer (CRC) or non-small cell lung cancer (NSCLC) cell lines. (A) Akt2 protein expression in the BxPC-3 cell lines. To investigate the influence of Akt2, an *Akt2*-overexpressed cell line was retrovirally created. An *EGFP*-overexpressed cell line was used as a control. The BxPC-3/Akt2 cell line exhibited the overexpression of Akt2 protein. β-actin was used as an internal control. (B) Growth inhibitory effects of erlotinib in the BxPC-3 cell lines. The cells were exposed to each concentration of erlotinib for 72 h, and the growth inhibitory effects were evaluated using an MTT assay. The sensitivity to erlotinib was weakened in the BxPC-3/Akt2 cell line, compared with the control. Lines, mean of independent triplicate experiments. (C) Phosphorylation of EGFR and Akt in the Bx-PC3 cell lines. Three hours after the cells were treated with the indicated concentration of drugs, the samples were collected. Erlotinib inhibited the phosphorylation of EGFR and Akt in a dose-dependent manner in the BxPC-3/EGFP cell line. In contrast, the phosphorylation of Akt strongly persisted in the BxPC-3/Akt2 cell line regardless of the inhibition of the phosphorylation of EGFR. β-actin was used as an internal control. pEGFR, phospho-EGFR; pAkt, phopho-Akt. (D) Expression of an apoptosis-related molecule in the BxPC-3 cell lines. Twenty-four hours after the cells were treated with the drug (erlotinib, 2.5 μM), the samples were collected. Erlotinib increased the expression of cleaved caspase-3 to a greater extent in the BxPC-3/EGFP cell line but did not increase the expression in the BxPC-3/Akt2 cell line. β-actin was used as an internal control. c-caspase-3, cleaved caspase-3. (E) Growth inhibitory effects in NSCLC and CRC cell lines. The Ma-1/Akt2 cell line (human NSCLC cell line) and CCK81/Akt2 cell line (human CRC cell line) were resistant to erlotinib and cetuximab, respectively. The cells were exposed to each concentration of erlotinib/cetuximab for 72 h, and the growth inhibitory effects were evaluated using an MTT assay. Lines, mean of independent triplicate experiments.

line was particularly resistant to erlotinib, compared with the other cell lines that did not exhibit either amplification or a high *Akt2* expression level. Next, the combined effect of erlotinib and BYL719, a PI3K inhibitor, was examined in the PANC-1 cell line. The combined treatment of erlotinib and BYL719 inhibited the cellular growth more intensively than erlotinib monotherapy (Fig. 3B). This combined effect in the PANC-1 cell line has already been reported (21), but we also confirmed the efficacy of combination therapy in this study. Whereas the phosphorylation of Akt persisted after erlotinib monotherapy, the combined treatment decreased the phosphorylation of Akt (Fig. 3C). In contrast to erlotinib or BYL719 monotherapy, the combined treatment of erlotinib and BYL719 increased the expression level of an apoptosis-related molecule, cleaved caspase-3 (Fig. 3D).

Akt2 overexpression led to resistance to anti-EGFR therapies. To evaluate the contribution of Akt2 to the EGFR-TKI response, a stably *Akt2*-overexpressed PC cell line was created. The BxPC-3 cell line was mainly used because this PC cell line is intermediately sensitive to erlotinib with no *Akt2* amplification or high expression (Figs. 2 and 3A). The stably *EGFP*-expressed cell line was used as a control. *Akt2*-overexpression was confirmed using a western blot analysis (Fig. 4A). Then, we



B

		BxPC-3/Akt2 (Erlotinib)	Ma-1/Akt2 (Erlotinib)	CCK81/Akt2 (Cetuximab)
IC_{50} of Erlotinib / Cetuximab	Monotherapy	6.6 μM	0.018 μM	0.79 mg/L
	With BYL719	2.5 μM	0.0042 μM	0.076 mg/L
P-value		P < 0.01	P < 0.02	P < 0.01

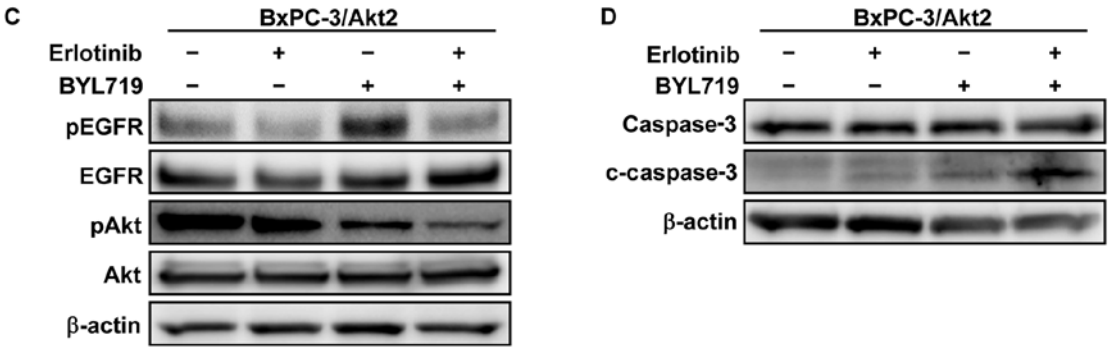


Figure 5. Combined effect of anti-EGFR therapy and BYL719 in the Akt2-overexpressed cell lines. (A) Growth inhibitory effects of the combined treatment of erlotinib/cetuximab and BYL719 in the *Akt2*-overexpressed cell lines. Cells were exposed to each concentration of erlotinib/cetuximab with or without BYL719 (BxPC-3, 4 μM; CCK81 and Ma-1, 2 μM) for 72 h, and the growth inhibitory effects were evaluated using an MTT assay. The combined treatment with erlotinib/cetuximab and BYL719 strongly inhibited the cellular growth compared with erlotinib/cetuximab monotherapy. Lines, mean of independent triplicate experiments. (B) IC_{50} values of erlotinib/cetuximab with or without BYL719 in the *Akt2*-overexpressed cell lines. The IC_{50} values of erlotinib/cetuximab were significantly lower in the combination therapy than in the monotherapy. (C) Phosphorylation of EGFR and Akt in the BxPC-3/Akt2 cell line. Three hours aftter the cells were exposed to the drugs (erlotinib, 5 μM; BYL719, 4 μM), the samples were collected. The phosphorylation of Akt was inhibited to a greater degree by the combined treatment than the erlotinib monotherapy. β-actin was used as an internal control. pEGFR, phospho-EGFR; pAkt, phopho-Akt. (D) Expression of an apoptosis-related molecule in the BxPC-3/Akt2 cell line. Twenty-four hours after the cells were exposed to the drugs (erlotinib, 5 μM; BYL719, 4 μM), the samples were collected. In contrast to erlotinib or BYL719 monotherapy, the combined treatment increased the expression of cleaved caspase-3 to a greater extent. β-actin was used as an internal control. c-caspase-3, cleaved caspase-3.

performed a growth inhibition assay using this cell line and found that the BxPC-3/Akt2 cell line was resistant to erlotinib (Fig. 4B). In contrast, Akt2-overexpression was not associated with gemcitabine resistance. In western blot analyses, erlotinib inhibited the phosphorylation of EGFR and Akt in a dose-dependent manner in the control cells. In contrast, the phosphorylation of Akt strongly persisted in the BxPC-3/Akt2 cell line, although erlotinib inhibited the phosphorylation of EGFR (Fig. 4C). The erlotinib-induced elevation in the expression level of cleaved caspase-3 in the BxPC-3/Akt2 cell line was lower than that in the control cells (Fig. 4D). Furthermore, similar experiments were also performed in other cell lines (Ma-1 and CCK81), demonstrating the resistance to anti-EGFR therapies in *Akt2*-overexpressed cell lines (Fig. 4E). These results indicate that Akt2 might be related to resistance to anti-EGFR therapies.

Combined effect of anti-EGFR therapies and a PI3K inhibitor in the Akt2-overexpressed cell lines. To investigate the effect of a PI3K inhibitor on the drug resistance induced by *Akt2* overexpression, we performed growth inhibition assays using erlotinib/cetuximab and BYL719 in the *Akt2*-overexpressed lines. The combined treatment with erlotinib/cetuximab and BYL719 considerably inhibited the cellular growth, compared with the monotherapy (Fig. 5A). The IC_{50} values of erlotinib/cetuximab were significantly lower in the combination therapy than in the monotherapy (Fig. 5B). In the BxPC-3/Akt2 cell line, the phosphorylation of Akt was also markedly inhibited by the combined treatment with BYL719 compared with erlotinib alone (Fig. 5C). Compared with monotherapy, the combined treatment with BYL719 increased the expression of cleaved caspase-3 to a greater extent (Fig. 5D). These results indicate that Akt2 can be associated with resistance to

Table II. High RNA expression and gene amplification of *Akt2* in TCGA database.

	Amplification		RNA expression	
Cancer type	No. of samples	No. of samples with amplification (%)	No. of samples	No. of samples with a high expression level (%)
PC	145	11 (8)	178	25 (14)
LAd	230	3 (1.3)	230	21 (9.1)
LSq	178	8 (4.5)	178	24 (13.5)
CRC	212	3 (1.4)	244	17 (7)
HNSCC	279	4 (1.4)	279	27 (9.7)

PC, pancreatic cancer; LAd, lung adenocarcinoma; LSq, lung squamous cell carcinoma; CRC, colorectal adenocarcinoma; HNSCC, head and neck squamous cell carcinoma. Gene amplification and RNA expression were evaluated by genome sequencing and RNA sequencing, respectively. High RNA expressed samples were defined as those with a Z-score >2.0.

anti-EGFR therapies via the PI3K-Akt pathway and that such resistance can be overcome by a PI3K inhibitor.

Akt2 amplification and high Akt2 expression levels in several cancers from TCGA datasets. Next, we analyzed the TCGA database to investigate the frequencies of *Akt2* amplification and high expression levels in several cancers. Data for PC, lung adenocarcinoma, lung squamous cell carcinoma, CRC, and head-and-neck squamous cell carcinoma (HNSCC) were analyzed since EGFR-TKIs or anti-EGFR monoclonal antibodies are clinically used for the treatment of these cancers. A high *Akt2* expression level was defined as a Z-score >2.0 using RNA sequencing. Samples with a high *Akt2* expression level were found at a relative high frequency of 7-14%. In contrast, the frequency of *Akt2* amplification was not so high, except for PC (8%), and *Akt2* amplification was correlated with the *Akt2* expression level. Among all the cancers that were examined, the frequencies of both *Akt2* amplification and a high expression level were the highest for PC (Table II).

Discussion

In this study, we revealed the possible association between high Akt2 expression levels and erlotinib resistance in clinical specimens of advanced PC. In addition, *in vitro* experiments also demonstrated this association and the efficacy of combined treatment with a PI3K inhibitor for overcoming resistance. Although a previous *in vitro* study demonstrated an association between *Akt2* and erlotinib resistance (21), to the best of our knowledge, this is the first study to show this association in clinical specimens of advanced PC.

EGFR is a cell membrane growth factor receptor characterized by tyrosine kinase activity that plays a crucial role in the control of key cellular transduction pathways (27). At present, targeting EGFR is one of the most effective anticancer strategies available, and anti-EGFR therapies are now widely used for the treatment of PC, NSCLC, CRC, and HNSCC (27). For advanced PC, erlotinib is the first drug for which a superiority of combination therapy with gemcitabine in terms of OS and PFS, has been documented in a large randomized trial, but the achievable improvement has remained limited (3). This unsatisfactory outcome has encouraged a number of studies which have attempted to identify molecular markers capable of predicting the efficacy of erlotinib in patients with PC (1,27). *EGFR* mutation or amplification has been a potential predictor of the response to EGFR-TKI treatment or patient prognosis in several cancers (27), and EGFR-TKIs are known to be effective against NSCLC harboring *EGFR* mutations (28). Several studies, however, have demonstrated that neither *EGFR* amplification nor *EGFR* mutation is a predictive biomarker for the response to erlotinib in PC (29,30). *KRAS* mutations, which act downstream of the EGFR pathway, have been characterized as a predictive biomarker for resistance to anti-EGFR antibodies in CRC (31). In PC, however, *K-ras* mutations, which occur in 90% of PC, have not been recognized as a predictive biomarker for resistance to erlotinib in combination with gemcitabine (29). These previous studies highlight the need to explore alternative explanations for responses to erlotinib and to identify markers that can predict the efficacy of erlotinib in patients with PC. Recently, alterations of the PI3K-Akt pathway, which is active downstream of the EGFR pathway, have been implicated as potential mechanisms of resistance to anti-EGFR therapies in CRC and lung adenocarcinoma (32-35). Amplification and high expression levels of Akt2 are also reportedly associated with resistance to erlotinib in PC *in vitro* (21). The present study showed a tendency toward a poorer response and a shorter PFS and OS for erlotinib plus gemcitabine in patients with advanced PC harboring high Akt2 expression. Futhermore, *in vitro* experiments showed that Akt2 is associated with the resistance to anti-EGFR therapies, and a TCGA dataset showed that the frequencies of both *Akt2* amplification and high expression levels are relatively high in PC. These findings suggest that Akt2 expression could be a predictive biomarker for resistance to erlotinib in PC. In addition, in our *in vitro* experiments, the combined treatment of erlotinib and a PI3K inhibitor was able to overcome this resistance, indicating that this combined treatment might be a promising starategy for the treatment of patients who are resistant to anti-EGFR therapies because of high Akt2 expression levels.

A previous report showed that a high Akt2 expression detected using immunohistochemistry was observed in 40%

of cases, consistent with the results of the present study, but a correlation with the survival of patients with PC was not seen (14). In the previous study, all the patients had received curative surgical resections and did not receive erlotinib treatment (14). In contrast, we focused on patients with advanced PC treated with erlotinib plus gemcitabine and showed a tendency toward a poorer outcome among patients with high Akt2 expression levels. The PI3K/Akt/mTOR pathway regulates cell survival and apoptosis (9), and *Akt2* expression is reportedly associated with prognosis and aggressiveness in other cancers (16-19). In addition, Akt2, which is active downstream of the EGFR pathway, seems to be associated with erlotinib resistance. Therefore, the tendency toward a poorer outcome among patients with advanced PC with high Akt2 expression levels who had been treated with erlotinib plus gemcitabine, as observed in the present study, seems reasonable.

The present study had some limitations. First, the study was relatively small and was performed retrospectively, and we could not show a significant correlation between Akt2 expression and the response to erlotinib. Only a few patients with advanced PC are eligible for surgical resection (1), and cytological examinations such as EUS or CT-guided FNA, which is an invasive method, are not rountinely performed when cancer is almost certainly diagnosed using dynamic CT imaging (36). Consequently, predictive biomarkers in advanced PC are difficult to investigate and studies like ours are very rare. At our institute, however, EUS-FNA is commonly conducted (37). Therefore, the present study might be valuable from the aspect of its use of rare biopsy specimens despite the small number of available samples. In addition, the present study, showing Akt2 expression as a biomarker candiate, encourages the use of aggresive biopsies in patients with advanced PC. Recently, evaluation of gene amplification has become feasible using a liquid biopsy, which detects cell-free circulating tumor DNA in the blood (38,39). Liquid biopsies are less invasive than EUS-FNA and might be useful for the detection of biomarkers such as gene amplification.

As a second limitation, we could not evaluate Akt2 gene amplification and expression because of the poor sample conditions. Due to the small amount of biopsied tissue obtained using EUS-FNA, we could not extract a sufficient amount of DNA/RNA for such analyses. Instead, we analyzed the TCGA database to investigate the frequencies of *Akt2* amplification and high expression levels. The frequencies of *Akt2* amplification and high expression in PC are not as high as those of other gene alterations, such as those of the *K-ras* mutation. Recently, however, molucular targeted therapy for low-frequency alterations has been developed. For example, the frequencies of *ALK* rearrangements and uncommon *EGFR* mutations in NSCLC are as low as 5 and 10%, respectively (40,41). Patients with *ALK* rearrangements are well known to respond dramatically to crizotinib (40), and in a recent post hoc analysis and our *in vitro* studies, afatinib was reported to be effective for patients harboring uncommon *EGFR* mutations, including exon 18 mutations, the *S768I* mutation, and the *L861Q* mutation (42-44). Thus, the low frequencies of gene alterations shoud not be overlooked, and effective biomarkers for the detection and treatment of PC are eagerly anticipated, but not yet defined. Therefore, Akt2 expression in PC might be valuable in treatment with erlotinib. To eliminate these limitations and to confirm our findings, further analyses including multi-center studies are desirable.

In conclusion, we found an association between high Akt2 expression levels and resisitance to erlotinib in both clinical specimens of advanced PC and several cell lines. A high Akt2 expression level might be a predictive biomarker for resistance to anti-EGFR therapies, especially erlotinib, in patients with PC. Our present study was, however, very small and was performed retrospectively. Therefore, further large-scale studies are needed to confirm these findings.

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