Okamoto et al. 1

Revised version: MCT-11-0638

Overcoming erlotinib resistance in EGFR mutation–positive non–small cell lung cancer cells by targeting survivin

Kunio Okamoto,1 Isamu Okamoto,1 Erina Hatashita,1 Kiyoko Kuwata,1 Haruka Yamaguchi,1 Aya Kita,2 Kentaro Yamanaka,2 Mayumi Ono,3 and Kazuhiko Nakagawa1

1Department of Medical Oncology, Kinki University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan. 2Drug Discovery Research, Astellas Pharma Inc., 21 Miyukigaoka, Tsukuba-shi, Ibaraki 305-8585, Japan. 3Department of Pharmaceutical Oncology, Graduate School of Pharmaceutical Sciences, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan.

Running title: Overcoming EGFR-TKI resistance in EGFR-mutant NSCLC

Key words: PTEN, survivin, EGFR mutation, apoptosis, non–small cell lung cancer

Address correspondence and reprint requests to: Isamu Okamoto, Department of Medical Oncology, Kinki University Faculty of Medicine, 377-2 Ohno-higashi, Osaka-Sayama, Osaka 589-8511, Japan.
Tel.: +81-72-366-0221. Fax: +81-72-360-5000. E-mail: chi-okamoto@dotd.med.kindai.ac.jp

The authors declare no potential conflict of interest.

Word count (excluding references): 5089 Number of figures: 6
Abstract

Loss of PTEN (phosphatase and tensin homolog) was recently shown to contribute to resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in EGFR mutation–positive non–small cell lung cancer (NSCLC) through activation of the protein kinase AKT. We previously showed that down-regulation of the expression of the antiapoptotic protein survivin by EGFR-TKIs contributes to EGFR-TKI–induced apoptosis in EGFR mutation–positive NSCLC cells. We have now investigated the role of survivin expression in EGFR-TKI resistance induced by PTEN loss. The EGFR-TKI erlotinib did not affect survivin expression or induce apoptosis in EGFR mutation–positive NSCLC cells with PTEN loss. Down-regulation of survivin either by transfection with a specific siRNA or by exposure to the small-molecule survivin suppressor YM155 reversed erlotinib resistance in such cells in vitro. Furthermore, combination therapy with YM155 and erlotinib inhibited the growth of tumors formed by EGFR mutation–positive, PTEN-deficient NSCLC cells in nude mice to a greater extent than did treatment with either drug alone. These results thus indicate that persistent activation of signaling by the AKT-survivin pathway induced by PTEN loss underlies a mechanism of resistance to erlotinib-induced apoptosis in EGFR mutation–positive NSCLC. They further suggest that the targeting of survivin has the potential to overcome EGFR-TKI resistance in EGFR mutation–positive NSCLC.
Introduction

Approximately 70% of individuals with non–small cell lung cancer (NSCLC) who harbor somatic mutations in exons of the epidermal growth factor receptor (EGFR) gene that encode the tyrosine kinase domain of the receptor experience substantial tumor regression when treated with the EGFR tyrosine kinase inhibitors (TKIs) gefitinib or erlotinib (1). However, most patients, even those who show a marked response to initial treatment, develop acquired resistance to EGFR-TKIs after varying periods of time (2). To date, several major mechanisms of such acquired resistance, including secondary mutation of EGFR, amplification of MET, and overexpression of hepatocyte growth factor, have been identified, and the development of pharmaceutical agents that target these mechanisms is under way (3-7). In addition, some patients are intrinsically resistant to EGFR-TKIs, even though their tumors harbor activating mutations of EGFR (8). Further characterization of the mechanisms of EGFR-TKI resistance is thus important in order to provide a basis for the development of effective therapies for patients who develop such resistance.

The deletion or inactivation of the phosphatase and tensin homolog (PTEN) gene occurs in a variety of tumor types, including melanoma as well as lung, bladder, renal, breast, endometrial, and thyroid cancer, and there are no related proteins that can compensate for the loss of PTEN function (9). The loss of PTEN results in misregulation of AKT-dependent signaling, which plays a key role in the progression of malignant cancer (10). Recent studies have shown that PTEN loss contributes to EGFR-TKI resistance in EGFR mutation–positive lung cancer through activation of the protein kinase AKT (11, 12). We recently found that EGFR-TKIs down-regulate survivin expression through inhibition of the phosphoinositide 3-kinase (PI3K)–AKT signaling pathway, and that such down-regulation of survivin contributed to EGFR-TKI–induced apoptosis in EGFR mutation–positive NSCLC cells (13). Survivin is a member of the inhibitor of apoptosis (IAP) family of proteins and has been shown to inhibit caspases and to prevent caspase-mediated cell death (14). Persistent survivin
expression might therefore be expected to result in resistance to EGFR-TKIs in EGFR mutation–positive NSCLC cells with PTEN loss.

We now show that erlotinib does not affect survivin expression or induce apoptosis in EGFR mutation–positive NSCLC cells with PTEN loss. We further examined whether persistent survivin expression was associated with the sensitivity of cells to EGFR-TKIs and whether modulation of survivin expression might overcome resistance to these drugs in EGFR mutation–positive NSCLC cells with PTEN loss.

Materials and Methods

Cell culture and reagents

The human NSCLC cell lines HCC827, and H1650 were obtained from American Type Culture Collection (Manassas, VA). The NSCLC line PC9 was obtained as described previously (15). The NSCLC lines PC9/GEF1-1 and PC9/GEF2-1 were generated and characterized as described previously (12). We screened all cell lines for the presence of EGFR mutations by direct DNA sequencing of exons 18 to 21 as described previously (15) for this study. All cells were passaged for ≤3 months before the renewal from frozen, early-passage stocks obtained from the indicated sources. Cells were regularly screened for mycoplasma with the use of a MycoAlert Mycoplasma Detection Kit (Lonza, Tokyo, Japan). All cells were cultured under a humidified atmosphere of 5% CO2 at 37°C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% FBS. Erlotinib (Supplemental Fig. 1A) was obtained from Kemprotec (Middlesbrough, UK), and YM155 (Supplemental Fig. 1B) was obtained from Astellas Pharma.

Immunoblot analysis

Cells were washed twice with ice-cold PBS and then lysed in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, and leupeptin (1 µg/ml). The protein
concentration of the cell lysates was determined with the use of the Bradford reagent (Bio-Rad, Hercules, CA), and equal amounts of protein were subjected to SDS-PAGE on a 7.5% gel. The separated proteins were transferred to a nitrocellulose membrane, which was then exposed to 5% nonfat dried milk in PBS for 1 h at room temperature before incubation overnight at 4°C with primary antibodies. Rabbit polyclonal antibodies to human phosphorylated EGFR (pY1068), to phosphorylated or total forms of AKT, to phosphorylated or total forms of extracellular signal–regulated kinase (ERK), to XIAP, to IAP-1, to BCL-xl, to BCL-2, to MCL-1, to PARP, to BIM, and to PTEN were obtained from Cell Signaling Technology (Danvers, MA); those to survivin were from Santa Cruz Biotechnology (Santa Cruz, CA); and those to β-actin were from Sigma. Mouse monoclonal antibodies to EGFR were obtained from Invitrogen (Carlsbad, CA). All antibodies were used at a 1:1000 dilution, with the exception of those to β-actin (1:200). The nitrocellulose membrane was then washed with PBS containing 0.05% Tween 20 before incubation for 1 h at room temperature with horseradish peroxidase–conjugated goat antibodies to rabbit (Sigma) or mouse (Santa Cruz Biotechnology) immunoglobulin G. Immune complexes were finally detected with chemiluminescence reagents (Perkin-Elmer Life Science, Waltham, MA).

**Gene silencing**

Cells were plated at 50 to 60% confluence in six-well plates or 25-cm² flasks and then incubated for 24 h before transient transfection for 24 h with siRNAs mixed with the Lipofectamine reagent (Invitrogen). Small interfering RNAs specific for PTEN (5′-UGAACCUGAUCUAUAUAGATT-3′) or survivin (5′-GAAGCAGUUUGAAGAAUUA-3′) mRNAs as well as a corresponding scrambled (control) siRNA were obtained from Nippon EGT (Toyama, Japan).

**Annexin V binding assay**

The binding of annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining Kit (Roche, Basel, Switzerland). Cells were harvested by exposure to trypsin-EDTA, washed
with PBS, and centrifuged at 200 × g for 5 min. The cell pellets were resuspended in 100 μl of Annexin-V-FLUOS labeling solution, incubated for 10 to 15 min at 15° to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ).

**Cell growth inhibition assay**

Cells were transferred to 96-well flat-bottomed plates and cultured for 24 h before exposure for 72 h to various concentrations of erlotinib. Tetra Color One (5 mM tetrazolium monosodium salt and 0.2 mM 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku Kogyo, Tokyo, Japan) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems, Franklin, MA). Absorbance values were expressed as a percentage of that for untreated cells.

**Growth inhibition assay in vivo**

All animal studies were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kinki University. The ethical procedures followed conformed to the guidelines of the United Kingdom Coordinating Committee on Cancer Prevention Research. Tumors cells (5 × 10^6) were injected subcutaneously into the axilla of 5- to 6-week-old female athymic nude mice (BALB/c nu/nu; CLEA Japan, Tokyo). Treatment was initiated when tumors in each group of six mice achieved an average volume of 300 to 600 mm³. Treatment groups consisted of vehicle control, erlotinib alone (10 mg/kg), YM155 alone (5 mg/kg), and erlotinib plus YM155. Erlotinib was administered by oral gavage daily for 31 days, with control animals receiving a 0.5% (w/v) aqueous solution of hydroxypropylmethylcellulose as vehicle. Continuous infusion of YM155 has been found to induce tumor regression and intratumoral survivin suppression in established human hormone-refractory prostate cancer, non-Hodgkin lymphoma, melanoma, and NSCLC.
xenografts (16-20). YM155 was thus administered over 7 consecutive days (days 1 to 7) with the use of an implanted micro-osmotic pump (Alzet model 1003D; Durect Cupertino, CA). Tumor volume was determined from caliper measurements of tumor length \((L)\) and width \((W)\) according to the formula \(LW^2/2\). Both tumor size and body weight were measured twice per week.

**Statistical analysis**

Quantitative data are presented as means ± SE from three independent experiments or for six animals per group. The significance of differences in the percentage of annexin V–positive cells or in tumor size was evaluated with the unpaired two-tailed Student’s \(t\) test. A \(P\) value of <0.05 was considered statistically significant.

**Results**

**Differential apoptotic responses of \(EGFR\) mutation–positive NSCLC cell lines to erlotinib**

We first examined the effects of the EGFR-TKI erlotinib on apoptosis in \(EGFR\) mutation–positive NSCLC cell lines (HCC827, PC9, and H1650) by staining with annexin V. HCC827, PC9, and H1650 cells harbor an \(EGFR\) allele with an activating mutation, whereas H1650 cells also show homozygous deletion of \(PTEN\). Erlotinib induced a marked increase in the proportion of apoptotic cells among HCC827 and PC9 cells, whereas it was without effect in H1650 cells, despite these cells harboring an activating mutation in \(EGFR\) (Fig. 1A).

Immunoblot analysis of the cleaved form of PARP confirmed that erlotinib did not induce apoptosis in H1650 cells (Fig. 1B). We and others previously showed that PI3K-AKT-survivin and MEK-ERK-BIM signaling pathways play important roles in EGFR-TKI–induced apoptosis (13, 21-23). We therefore next compared the effects of erlotinib on the expression level or phosphorylation status of EGFR, AKT, ERK, PTEN, survivin, and BIM in the three NSCLC cell lines by immunoblot analysis. Erlotinib induced the dephosphorylation of EGFR as well as that of AKT and ERK in the erlotinib-sensitive cell lines HCC827 and PC9 (Fig. 1C).
addition, erlotinib induced down-regulation of survivin expression and up-regulation of BIM expression in HCC827 and PC9 cells, consistent with our previous observations (13). However, whereas erlotinib induced the dephosphorylation of EGFR and ERK as well as the up-regulation of BIM expression in H1650 cells, it had no effect of the levels of AKT phosphorylation or survivin expression (Fig. 1C). The expression of other IAP or BCL family members, including XIAP, IAP-1, BCL-2, BCL-xI, and MCL-1, was not substantially affected by erlotinib in any of the cell lines examined. Given that PTEN inhibits AKT activation by catalyzing the dephosphorylation of phosphatidylinositol 3,4,5-trisphosphate, the product of class I PI3K action, these results suggested that the resistance of some EGFR mutation–positive cells to erlotinib-induced apoptosis may be associated with persistent activation of the AKT-survivin pathway.

The effect of erlotinib on survivin expression correlates with that on apoptosis

We have previously established EGFR-TKI (gefitinib)–resistant sublines (PC9/GEF1-1 and PC9/GEF2-1) of PC9 cells that harbor the activating EGFR mutation of the parental cells but which have also lost PTEN expression, with the loss of PTEN contributing to their EGFR-TKI resistance (12). Both staining with annexin V and immunoblot analysis of PARP revealed that erlotinib did not induce a substantial level of apoptosis in PC9/GEF1-1 or PC9/GEF2-1 cells (Fig. 2A, B), consistent with our previous observations (12). To investigate whether loss of PTEN expression affects the modulation of AKT-survivin signaling by erlotinib, we examined the effects of erlotinib on the expression level or phosphorylation status of EGFR, AKT, and survivin in these cell lines by immunoblot analysis. Similar to our observations with H1650 cells, erlotinib did not affect the levels of AKT phosphorylation or survivin expression in PC9/GEF1-1 and PC9/GEF2-1 cells, whereas it still induced the dephosphorylation of EGFR and ERK as well as the up-regulation of BIM expression (Fig. 2C). These findings thus suggested that persistent expression of survivin in the presence of erlotinib contributes to the resistance to this drug associated with PTEN loss in EGFR mutation–positive NSCLC cells.
Knockdown of PTEN induces persistent survivin expression and reduces erlotinib sensitivity in EGFR mutation–positive cells

To investigate the contribution of PTEN to survivin expression and erlotinib resistance, we examined the effects of siRNA-mediated depletion of PTEN in PC9 and HCC827 cells. Transfection of these cells with PTEN siRNA attenuated the dephosphorylation of AKT and down-regulation of survivin expression, without affecting the dephosphorylation of ERK and up-regulation of BIM, induced by erlotinib (Fig. 3A). Staining with annexin V also revealed that depletion of PTEN resulted in inhibition of erlotinib-induced apoptosis (Fig. 3B). Moreover, knockdown of PTEN reduced the sensitivity of HCC827 and PC9 cells to the inhibitory effect of erlotinib on cell growth (Fig. 3C). These results suggested that PTEN loss contributes to persistent survivin expression in the presence of erlotinib and to erlotinib resistance in EGFR mutation–positive cells.

Knockdown of survivin reverses erlotinib resistance induced by loss of PTEN in EGFR mutation–positive cells

To investigate further the contribution of persistent survivin expression to erlotinib resistance associated with PTEN loss, we examined the effect of siRNA-mediated depletion of survivin on erlotinib-induced apoptosis in parental PC9 cells and the EGFR-TKI–resistant sublines PC9/GEF1-1 and PC9/GEF2-1. Whereas erlotinib induced down-regulation of survivin expression only in the parental PC9 cells, transfection with survivin siRNA resulted in specific depletion of survivin in both the parental and EGFR-TKI–resistant NSCLC cells (Fig. 4A). The erlotinib-induced up-regulation of BIM expression was apparent in all three cell lines in the absence or presence of survivin siRNA (Fig. 4A). The survivin siRNA had no significant effect on erlotinib-induced apoptosis in PC9 cells, as revealed by staining with annexin V (Fig. 4B). In contrast, depletion of survivin by transfection with the survivin siRNA sensitized PC9/GEF1-1 and PC9/GEF2-1 cells to the proapoptotic effect of erlotinib (Fig. 4B). A second siRNA targeted to a different region of survivin mRNA yielded similar results (data not shown).
These observations thus suggested that persistent expression of survivin contributes to erlotinib resistance in EGFR mutation–positive cells with loss of PTEN.

**YM155 reverses erlotinib resistance induced by loss of PTEN in EGFR mutation–positive cells in vitro**

We next examined the effect of the combination of erlotinib and YM155 on apoptosis in NSCLC cells with EGFR-TKI resistance due to loss of PTEN. YM155 is a small-molecule agent that specifically inhibits survivin expression in various types of cancer cell lines in vitro and in vivo (16-20). Treatment of PC9/GEF1-1 or PC9/GEF2-1 cells, or of parental PC9 cells, with YM155 resulted in down-regulation of survivin expression (Fig. 5A). Erlotinib induced up-regulation of BIM in all three cell lines in the absence or presence of YM155 (Fig. 5A). Whereas YM155 had no significant effect on erlotinib-induced apoptosis in PC9 cells, it sensitized PC9/GEF1-1 and PC9/GEF2-1 cells to the proapoptotic effect of erlotinib (Fig. 5B). In addition, whereas YM155 did not affect the sensitivity of PC9 cells to the antiproliferative effect of erlotinib, it increased that of PC9/GEF1-1 and PC9/GEF2-1 cells to this action of erlotinib (Fig. 5C). These results thus suggested that down-regulation of survivin expression by YM155 promoted the proapoptotic and antiproliferative effects of erlotinib in EGFR mutation–positive cells with loss of PTEN.

**YM155 reverses erlotinib resistance induced by loss of PTEN in EGFR mutation–positive cells in vivo**

We also examined the effects of siRNA-mediated depletion of survivin and of YM155 on erlotinib-induced apoptosis in H1650 cells. Transfection with the survivin siRNA resulted in specific depletion of survivin and sensitized H1650 cells to the proapoptotic effect of erlotinib (Fig. 6A). YM155 also down-regulated survivin expression and sensitized H1650 cells to the induction of apoptosis by erlotinib (Fig. 6B). In addition, YM155 increased the sensitivity of H1650 cells to the inhibitory effect of erlotinib on cell growth in vitro (Fig. 6C). Finally, to determine whether the enhancement of the proapoptotic effect of erlotinib by YM155 in
EGFR-TKI–resistant cells observed in vitro might also be apparent in vivo, we injected H1650 cells into nude mice to elicit the formation of solid tumors. After tumor formation, the mice were treated with erlotinib, YM155, or both drugs. Combined treatment with erlotinib and YM155 inhibited tumor growth to a greater extent than did treatment with either drug alone (Fig. 6D, E). These data suggested that YM155 enhances the response of EGFR mutation–positive tumor cells with loss of PTEN to erlotinib both in vitro and in vivo.

Discussion

Tyrosine kinases have come to be recognized as key regulators of cancer cell proliferation and apoptosis as well as of tumor angiogenesis, and they are therefore considered potential targets for anticancer therapies (24). Several strategies for targeting these kinases have been pursued, the most successful of which has been the development of small-molecule TKIs (25-27). However, increasing evidence has demonstrated the development of acquired resistance to these drugs, and extensive preclinical studies are ongoing in order to provide insight into the molecular mechanisms underlying such resistance (28). We and others have recently shown that loss of PTEN contributes to EGFR-TKI resistance in EGFR mutation–positive lung cancer through the activation of AKT (11, 12). Loss of PTEN was also found to reduce responsiveness to EGFR-TKIs in patients with recurrent glioblastoma expressing EGFR variant type III, a constitutively active mutant form of EGFR (29). In addition, restoration of PTEN expression was shown to increase the susceptibility to EGFR-TKI–induced apoptosis in EGFR mutation–positive NSCLC cells with PTEN loss (11). These observations thus support the notion that PTEN loss is associated with resistance to EGFR-TKIs in EGFR-driven tumors.

The precise molecular mechanism by which PTEN loss induces resistance to EGFR-TKIs has remained unclear, however. We have now shown that erlotinib did not induce apoptosis or down-regulation of survivin expression in H1650 cells, which harbor an EGFR allele with an activating mutation and also no longer manifest PTEN expression. Similar results
were obtained with EGFR-TKI–resistant PC9/GEF sublines of PC9 cells that have also lost PTEN expression and manifest persistent phosphorylation of AKT. We further found that an siRNA specific for PTEN mRNA inhibited the dephosphorylation of AKT and down-regulation of survivin induced by erlotinib in EGFR mutation–positive NSCLC cells. These results are consistent with our previous observation that inhibition of the EGFR-PI3K-AKT pathway contributed to down-regulation of survivin expression by EGFR-TKIs in EGFR mutation–positive NSCLC cells (13). Whereas the mechanism of survivin down-regulation by EGFR-TKIs remains unclear, previous studies have suggested that the PI3K-AKT pathway regulates survivin expression through modulation of transcriptional factors (30, 31). We confirmed that erlotinib down-regulated the amount of survivin mRNA in EGFR mutation–positive NSCLC cells without PTEN loss (data not shown), suggesting that survivin down-regulation by erlotinib is mediated, at least in part, at the transcriptional level. These results suggest that the PI3K-AKT pathway and its downstream transcriptional factors are targeted by EGFR-TKIs in the regulation of survivin expression.

In the present study, we also demonstrated that down-regulation of survivin expression either by transfection with survivin siRNA or by exposure to YM155 reversed in vitro or in vivo the resistance of EGFR mutation–positive NSCLC cells to erlotinib induced by PTEN loss. On the other hand, similar to EGFR mutation–positive cells without PTEN loss, the combination of erlotinib and YM155 did not manifest a synergistic antiproliferative effect in NSCLC cells harboring wild-type EGFR alleles (data not shown), suggesting that sensitization to EGFR-TKIs by YM155 is specific for EGFR-TKI–resistant cells with both an EGFR mutation and PTEN loss. Our observations thus show that PTEN loss activates AKT signaling, and that this pathway contributes to the persistence of survivin expression in the presence of erlotinib and to EGFR-TKI resistance in EGFR mutation–positive NSCLC cells.

In addition to the PI3K-AKT-survivin pathway, we and others have previously shown that the induction of BIM expression through inhibition of the MEK-ERK pathway is important
for EGFR-TKI–induced apoptosis (21-23, 32). Although erlotinib induced up-regulation of the proapoptotic protein BIM in EGFR mutation–positive cells regardless of PTEN status, the proapoptotic effect of erlotinib was not observed in cells with PTEN loss. These results suggest that the PI3K-AKT-survivin signaling pathway might be more important than the MEK-ERK-BIM pathway in the regulation of survival in EGFR mutation–positive cells with loss of PTEN. However, we found that the addition of YM155 to erlotinib resulted in both the down-regulation of survivin and the up-regulation of BIM, leading to a greater increase in the number of apoptotic cells compared with that observed with either agent alone, in EGFR mutation–positive cells with PTEN loss. These results suggest that, although the relative contributions of the PI3K-AKT-survivin and MEK-ERK-BIM signaling pathways to cell survival may vary among cells, concomitant down-regulation of survivin and up-regulation of BIM are necessary for the induction of a substantial level of apoptosis in EGFR mutation–positive cells. Nuclear factor–κB and FAS receptor signaling were also recently shown to regulate EGFR-TKI sensitivity in EGFR mutation–positive NSCLC cells (33), suggesting that multiple mechanisms underlie EGFR-TKI resistance.

We have found that the persistent activation of AKT-survivin signaling by PTEN loss represents a mechanism of primary or acquired resistance to erlotinib in EGFR mutation–positive cells. Our results show that erlotinib down-regulates survivin expression in EGFR-TKI–sensitive NSCLC cells but not in EGFR-TKI-resistant NSCLC cells with PTEN loss. Although we previously demonstrated that the expression of PTEN was reduced in tumor specimens from NSCLC patients showing acquired resistance to EGFR-TKIs (12), analysis of serial tumor samples obtained before and during treatment with these drugs will be required to determine whether survivin expression is affected by such treatment. Moreover, we found that YM155 significantly enhanced the antitumor effect of erlotinib in EGFR mutation–positive NSCLC cells with PTEN loss both in vitro and in vivo. YM155, a small-molecule inhibitor of the expression of the antiapoptotic protein survivin, is currently in clinical development as the
first survivin suppressant (18, 19, 34-36). This drug was found to exhibit a favorable safety-tolerability profile and moderate single-agent activity in a recent phase II trial with patients with advanced, refractory NSCLC (37). Our results now suggest that further studies of combination therapy with YM155 and erlotinib are warranted in NSCLC patients with EGFR-TKI resistance induced by PTEN loss.

**Grant Support**
This study was supported by KAKENHI (grants-in-aid for scientific research) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.
References


platinum-based drugs. Br J Cancer 2010;103:36-42.


**Figure Legends**

**Figure 1.** Differential apoptotic responses of *EGFR* mutation–positive NSCLC cell lines to erlotinib. *A*, HCC827, PC9, or H1650 cells were incubated in complete medium with or without erlotinib (100 nM) for 48 h, after which the proportion of apoptotic cells was determined by staining with fluorescein isothiocyanate–conjugated annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from three independent experiments. *B*, Cells were incubated in complete medium with or without erlotinib (100 nM) for 48 h, lysed, and subjected to immunoblot analysis with antibodies to PARP or to β-actin (loading control). Bands corresponding to the intact and cleaved (cl) forms of PARP are indicated. *C*, Cells were incubated in complete medium containing the indicated concentrations of erlotinib for 24 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of EGFR, AKT, or ERK or to the indicated proteins. Data in *B* and *C* are representative of three independent experiments.

**Figure 2.** Effect of erlotinib on survivin expression correlates with sensitivity to erlotinib in *EGFR* mutation–positive NSCLC cells. *A*, PC9, PC9/GEF1-1, or PC9/GEF2-1 cells were incubated in complete medium with or without erlotinib (100 nM) for 48 h, after which the proportion of apoptotic cells was determined by staining with fluorescein isothiocyanate–conjugated annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from three independent experiments. *B*, Cells were incubated in complete medium with or without erlotinib (100 nM) for 48 h, lysed, and subjected to immunoblot analysis with antibodies to PARP or to β-actin. *C*, Cells were incubated in complete medium containing the indicated concentrations of erlotinib for 24 h, after which cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins. Data in *B* and *C* are representative of three independent experiments.
**Figure 3.** Knockdown of PTEN induces persistent survivin expression and reduces erlotinib sensitivity in EGFR mutation–positive cells. A, HCC827 or PC9 cells were transfected with PTEN or scrambled (control) siRNAs for 24 h and then incubated in complete medium with or without erlotinib (100 nM) for 24 h. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to the indicated proteins. Data are representative of three independent experiments. B, Cells transfected as in A were incubated with or without erlotinib (100 nM) for 48 h, after which the proportion of apoptotic cells was determined by staining with fluorescein isothiocyanate–conjugated annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from three independent experiments. *P < 0.05 for the indicated comparisons. C, Cells transfected as in A were cultured in complete medium containing the indicated concentrations of erlotinib for 72 h, after which cell viability was assessed. The number of viable cells is expressed as a percentage of the corresponding value for cells not exposed to erlotinib. Data are means from three independent experiments.

**Figure 4.** Knockdown of survivin expression reverses erlotinib resistance induced by loss of PTEN in EGFR mutation–positive NSCLC cells. A, PC9, PC9/GEF1-1, or PC9/GEF2-1 cells were transfected with survivin or scrambled (control) siRNAs for 24 h and then incubated in complete medium with or without erlotinib (100 nM) for 24 h. Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to survivin, to BIM, or to β-actin. Data are representative of three independent experiments. B, Cells transfected as in A were incubated with or without erlotinib (100 nM) for 48 h, after which the proportion of apoptotic cells was determined by staining with fluorescein isothiocyanate–conjugated annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from three independent experiments. *P < 0.05 for the indicated comparisons.

**Figure 5.** YM155 reverses erlotinib resistance induced by loss of PTEN in EGFR
mutation–positive NSCLC cells in vitro. A, PC9, PC9/GEF1-1, or PC9/GEF2-1 cells were incubated in complete medium with or without erlotinib (100 nM), YM155 (10 nM), or both drugs for 24 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to the indicated proteins. Data are representative of three independent experiments. B, Cells were incubated in complete medium with or without erlotinib (100 nM), YM155 (10 nM), or both drugs for 48 h, after which the proportion of apoptotic cells was determined by staining with fluorescein isothiocyanate–conjugated annexin V and propidium iodide followed by flow cytometry. Data are means ± SE from three independent experiments. *P < 0.05 for the indicated comparisons. C, Cells were cultured in complete medium containing the indicated concentrations of erlotinib with or without 10 nM YM155 for 72 h, after which cell viability was assessed. The number of viable cells is expressed as a percentage of the corresponding value for cells not exposed to erlotinib. Data are means from three independent experiments.

**Figure 6.** YM155 reverses erlotinib resistance induced by loss of PTEN in *EGFR* mutation–positive cells in vivo. A, H1650 cells were transfected with survivin or scrambled (control) siRNAs for 24 h and then incubated in complete medium with or without erlotinib (2 µM) for 24 h (left) or 48 h (right). Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to survivin, to BIM, or to β-actin (left), or the proportion of apoptotic cells was determined by staining with fluorescein isothiocyanate–conjugated annexin V and propidium iodide followed by flow cytometry (right). B, Cells were incubated in complete medium with or without erlotinib (2 µM), YM155 (20 nM), or both drugs for 24 h (left) or 48 h (right). Cell lysates were then prepared and subjected to immunoblot analysis with antibodies to the indicated proteins (left), or the proportion of apoptotic cells was determined by staining with fluorescein isothiocyanate–conjugated annexin V and propidium iodide followed by flow cytometry (right). Data in A and B are representative of (left) or means ± SE from (right) three
independent experiments. *$P < 0.05$ for the indicated comparisons. $C$, Cells were cultured in complete medium containing the indicated concentrations of erlotinib with or without 10 nM YM155 for 72 h, after which cell viability was assessed. The number of viable cells is expressed as a percentage of the corresponding value for cells not exposed to erlotinib. Data are means from three independent experiments. $D$, Nude mice with tumor xenografts established by subcutaneous injection of H1650 cells were treated with vehicle (control), erlotinib (10 mg/kg), YM155 (5 mg/kg), or the combination of erlotinib and YM155 as described in Materials and Methods. Tumor volume was measured at the indicated times after the onset of treatment. Data are means ± SE for six mice per group. *$P < 0.05$ for the combination of erlotinib plus YM155 versus control or either erlotinib or YM155 alone. $E$, Representative mice treated as in $D$ showing tumors at the end of the 31-day treatment period.
Figure 1

A

![Bar graph showing Annexin V-positive cells (%) for different conditions.

B

![Western blots for different proteins under various conditions.

C

<table>
<thead>
<tr>
<th>EGFR mutation positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC827</td>
</tr>
<tr>
<td>Erlotinib (µM):</td>
</tr>
<tr>
<td>pEGFR</td>
</tr>
<tr>
<td>EGFR</td>
</tr>
<tr>
<td>PTEN</td>
</tr>
<tr>
<td>pAKT</td>
</tr>
<tr>
<td>AKT</td>
</tr>
<tr>
<td>Survivin</td>
</tr>
<tr>
<td>XIAP</td>
</tr>
<tr>
<td>IAP-1</td>
</tr>
<tr>
<td>pERK</td>
</tr>
<tr>
<td>ERK</td>
</tr>
<tr>
<td>BIM</td>
</tr>
<tr>
<td>BCL-xI</td>
</tr>
<tr>
<td>BCL-2</td>
</tr>
<tr>
<td>MCL-1</td>
</tr>
<tr>
<td>Actin</td>
</tr>
</tbody>
</table>
Figure 2

A

B

C

Table of Protein Expression

<table>
<thead>
<tr>
<th>Protein</th>
<th>Parent</th>
<th>GEF1-1</th>
<th>GEF2-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erlotinib (µM)</td>
<td>0</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>pEGFR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTEN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAKT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AKT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Survivin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XIAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IAP-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pERK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BIM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCL-xl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCL-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCL-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cl-PARP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4
Molecular Cancer Therapeutics

Overcoming erlotinib resistance in \(\text{EGFR}^+\) non-small cell lung cancer cells by targeting survivin

Kunio Okamoto, Isamu Okamoto, Erina Hatashita, et al.

\textit{Mol Cancer Ther} Published OnlineFirst November 10, 2011.

Updated version: Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-11-0638

Supplementary Material: Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2011/11/04/1535-7163.MCT-11-0638.DC1

Author Manuscript: Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts: Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions: To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions: To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.