Inhibition of RPTOR overcomes resistance to EGFR inhibition in triple-negative breast cancer cells

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Abstract. Triple-negative breast cancer (TNBC) cells frequently exhibit activated growth factor signaling and resistance to inhibitors for epidermal growth factor receptor (EGFR), despite the overexpression of EGFR protein, and this is associated with a malignant behavior and a poor prognosis. In this study, to elucidate the underlying mechanisms of resistance to EGFR inhibitor and identify inhibitors that exert a synergistic effect with EGFR inhibition, we examined the inhibitory effects of selected protein kinase inhibitors (PKIs) in combination with gefitinib on the viability of a mesenchymal stem-like (MSL) subtype TNBC cell line. MK-2206, an AKT inhibitor, and a group of mammalian target of rapamycin (mTOR) inhibitors were found to exert synergistic lethal effects in combination with gefitinib in MDA-MB-231 cells. The combination of gefitinib/MK-2206 exerted a prominent synergistic lethal effect in an MTT cell viability assay and a growth inhibitory effect in a long-term colony-forming assay in 2 MSL subtype TNBC cell lines (MDA-MB-231 and HS578T) and one basal-like (BL) subtype TNBC cell line (MDA-MB-468). Gefitinib/MK-2206 treatment synergistically decreased the mTOR signaling target substrates along with the downregulation of ribosomal protein S6 (RPS6), a marker of cell proliferation and target substrate of the AKT-mTOR signaling pathway. In addition, gefitinib markedly reduced the viability of MDA-MD-231 and HS578T cells when regulatory-associated protein of mTOR (RPTOR) was suppressed by siRNA-based knockdown (KD). These results thus suggest that RPTOR mediates, at least partially, the resistance to EGFR inhibition in TNBC cells. Therefore, targeting the mTOR complex 1 (mTORC1) pathway may be a potential strategy for the treatment of EGFR-resistant TNBC.

Introduction

Three signaling receptors are considered as important markers for the grouping of human breast cancer types and the subsequent planning of systemic therapy for breast cancer: Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (1). Triple-negative breast cancer (TNBC), which comprises 15-20% of breast cancers (2-6), is characterized by the immunohistochemical absence of both ER and PR, as well as a lack of the amplification of HER2. Due to the absence of these proteins, the treatment of TNBC with agents targeting these proteins is inadequate. Although conventional adjuvant chemotherapies employed, such as taxanes, anthracyclines, capecitabines and/or platins show a desirable response with a higher rate of pathological complete response (pCR) in initial treatment (7); drug resistance to these agents and distant metastasis commonly occur and are associated with an overall poor prognosis (8-10).

The human epidermal growth factor receptor (HER) family consists of 4 structurally related receptor tyrosine kinases: HER1 [epidermal growth factor receptor (EGFR)], HER2, HER3, and HER4 (11-13). Since EGFR is a well-established oncogenic factor and therapeutic target in certain types of human cancer, a number of humanized monoclonal antibodies and small molecule kinase inhibitors have been approved by the US Food and Drug Administration (FDA) for the treatment of several human cancers (12,13). Unlike other types of hormone therapy-sensitive breast cancer, the overexpression of EGFR is more frequently found in TNBC (14-17) and is closely associated with the aggressiveness and drug resistance of malignant cancers, including breast cancer (11,13). Up

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to 70-80% of metastatic breast cancers have been shown to overexpress EGFR but not to overexpress HER2, the basis for HER2-targeted therapy (18,19). The activation of EGFR transmits signals of cell proliferation, cell survival, drug resistance and metastasis via various signaling cascades, including RAS-mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)-AKT and SRC-signal transducer and activator of transcription (STAT) pathways (20). Although numerous EGFR target drugs, including protein kinase inhibitors (PKIs) and monoclonal antibodies, are easily available, the majority of studies on the therapeutic potential of EGFR targeted therapy have focused on lung cancer, glioblastoma, colon cancer and head and neck cancers (21 and refs therein). In addition, despite the frequent overexpression of EGFR in TNBC, the study of its potential role or therapeutic benefits in TNBC remains still limited and is still under evaluation (11,21,22).

Previously, we identified a potential therapeutic benefit of blocking EGFR and PI3K with a gefitinib/PI-103 combination treatment in basal-like (BL) subtypes of TNBC *in vitro* (23). However, a mesenchymal stem-like (MSL) subtype of TNBC cells is relatively resistant to this combination (23), probably due to the presence of alternative intracellular signaling pathways that ensure cell survival and proliferation even when EGFR is blocked (24-29). We have also reported that the additional blocking of MET coupled with EGFR inhibition effectively exerts anticancer effects in TNBC cells of the MSL subtype with a decrease in p-/total RPS6 levels (30). RPS6 is one of the downstream target of mTOR kinase signaling. In addition, the PI3K-AKT-mTOR signaling pathway, which is frequently deregulated in TNBC, is indispensable for cancer cell survival and is associated with drug resistance (31). In this study, to identify PKIs that induce synthetic lethality in combination with an EGFR inhibitor, we performed an MTT screening in MDA-MB-231 cells with PKIs, including target agents against PI3K, AKT, mTOR and SRC pathways in combination with gefitinib. As a result, we identified and further characterized an AKT inhibitor, MK-2206, as a synthetic lethal agent when used in combination with gefitinib in TNBC cells of the MSL subtype and demonstrated that the antitumor effect was associated with the downregulation of the mammalian target of rapamycin complex 1 (mTORC1)/ regulatory-associated protein of mTOR (RPTOR) signaling coupled with a downregulation in the levels of ribosomal protein S6 (RPS6).

Materials and methods

Cell culture and reagents. The MDA-MB-231, HS578T and MDA-MB-468 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Corning, Manassas, VA, USA) and 100 units/ml penicillin/streptomycin (Gibco, Grand Island, NY, USA). Cell growth was monitored by trypan blue dye (Sigma-Aldrich St. Louis, MO, USA) exclusion cell counting using the Luna Automated Cell Counter (Logos Biosystems, Gyeonggi-do, Korea). The PKIs used in these experiments were purchased from the following sources:

AZD0530, deforolimus, GDC-0941, GSK1059615, IC-87114, KU-0063794, MK-2206, Perifosine, PIK-90, PIK-75, PI-103 TG100-115, TGX221 and WYE-354 were all obtained from SelleckChem (Houston, TX, USA); (-)-Deguelin was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA); BEZ235, dasatinib, everolimus, FK506, pimecrolimus, staurosporine, temsirolimus and ZSTK474 were all obtained from LC Laboratories (Woburn, MA, USA); and LY294002, rapamycin and wortmannin were obtained from Sigma-Aldrich. Each compound was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) to create a stock solution and stored at -20°C in small aliquots.

Screening of PKIs. Synthetic lethal screening was performed as previously described (30). In brief, the MDA-MB-231 cells, plated at 1,000 cells/well in 96-well plates 24 h prior to testing, were treated with increasing concentrations of gefitinib and PKIs in duplicate in a 6x5 matrix. Cell viability was determined at 72 h after treatment by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with 4 mg/ml MTT solution as previously described (25,33). The synergism was determined by calculating the classification index (CI) as previously described (30,32) as follows: (viability of gefitinib) x (viability of PKI)/(viability of the gefitinib and PKI combination). Supra-additivity was defined as CI >1; additivity was defined as CI=1; and subadditivity was defined as CI <1 (32,33). The numbers of combination points with CI values >1.3 that were generated from individual drug concentration points were summated and further assigned as a quantitative index of synergism. The average of CIs >1.3 was used as the qualitative index of synergism.

Clonogenic cell survival assay. The cells were subcultured in 6-well plates at appropriate densities: 1,000 cells/well for the HS578T cells and 2,000 cells/well for the MDA-MB-231 and MDA-MB-468 cells. At 1 day after subculture, the cells were treated with 9 μM gefitinib, 9 μM MK-2206, or a combination of these drugs for 24 h, and then washed and supplemented with fresh normal growth media in the absence of the drugs. The cells were cultured for an additional 10-14 days following treatment with replacement of normal growth media twice per week. The colonies were stained as previously described (30,34). After washing with distilled water (DW), the colonies were imaged with a scanner. The relative amount and number of colonies were quantified as follows: The crystal violet stain for the staining of the colonies was dissolved in a solubilizing buffer [1:1 mixture (v/v) of 0.1 M sodium phosphate buffer (pH 4.5) and ethanol], and the absorbance of the dissolved crystal violet was measured by using a 680 microplate reader (Bio-Rad, Hercules, CA, USA).

Western blot analysis and antibodies. For PKI treatment, the HS578T and MDA-MB231 cells were plated at 2x10^5/60-mm dish at 1 day prior to treatment. The cells were then treated with gefitinib and MK-2206 for 24 or 2 h in the presence of normal serum-containing media. For siRNA transfection, the HS578T and MDA-MB231 cells were plated at 5x10^4 cells/60-mm dish the day prior to transfection. Following treatment with the drug or siRNA transfection, the cells were lysed with Pierce™ RIPA buffer (Thermo Fisher Scientific, Waltham,

MA, USA) containing protease phosphatase inhibitor cocktail (Thermo Fisher Scientific), and the protein concentration was determined with a BCA protein assay kit (Thermo Fisher Scientific). The following antibodies were used in this study: mTOR (#4517), phosphorylated (p-)mTOR (S2448; #2971), extracellular signal-regulated kinase (ERK)1/2 (#9102), p-ERK1/2 (T202/Y204; #4370), SRC (#2108), p-SRC (Y416; #2101), eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1; #9452), p-4E-BP1 (T37/46; #2855), RPS6 (#2217), p-RPS6 (#4856), proline-rich AKT substrate of 40 kDa (PRAS40; #2691), p-PRAS40 (S235/236; #13175), AKT (#9272), p-AKT (S473; #4060), glycogen synthase kinase (GSK)-3β (#9315), p-GSK-3β (S9); #9315), RPTOR (#2280), rapamycin-insensitive companion of mTOR (RICTOR) (#2140), poly(ADP-ribose) polymerase-1 (PARP1; #9542), cleaved PARP (Asp214; #5625), cleaved caspase-3 (Asp175; #9661) and peroxidase-conjugated secondary antibodies (anti-rabbit IgG, #7074; anti-mouse IgG, #7076) were all obtained from Cell Signaling Technology (Danvers, MA, USA); X-linked inhibitor of apoptosis protein antibody (XIAP; #610716) was from BD Sciences (San Jose, CA, USA); β-actin antibody (A330-491A) was from Bethyl Laboratories, Inc. (Montgomery, TX, USA); and β-tubulin antibody (T7816) was from Sigma-Aldrich.

Transfection of siRNA. For the siRNA-mediated knockdown of target gene expression, AccuTarget™ premade siRNA was purchased from Bioneer (Seoul, Korea) for the following target genes and control siRNA: Human RPTOR (gene ID: 57521; #1079283), human RICTOR (gene ID: 253260; #1129356) and control siRNA (SN-1003). The transfection of siRNA was carried out using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) as previously described (30). In brief, the HS578T ($1x10^5$ cells/well) or MDA-MB-231 ($5x10^4$ cells/well) cells in 6-well plates were transfected with 100 pmoles of siRNA mixed and pre-incubated with Lipofectamine 2000 in serum-free DMEM media. Following 4 h of incubation, the transfection media was supplemented with an equal volume of DMEM containing 20% FBS and 200 units/ml penicillin/streptomycin to restore the normal growth condition, and the cells were further incubated for 3 days. During the incubation, 9 μM gefitinib or DMSO (vehicle) were added 1 day after the media supplement or the last 2 h of the 3rd day of the incubation period for the cell proliferation assay and western blot analyses.

Cell counting-based cell viability assay and cell proliferation assay. The HS578T and MDA-MB231 cells were plated at $1x10^5$ cells in a 60-mm dish at 1 day prior to treatment. At the indicated time of siRNA transfection or drug treatment (0, 1, 2, 3 and 4 days), cells were detached with trypsin/EDTA (Gibco) and suspended with 0.5 ml of DMEM containing 10% FBS. Cell proliferation or number of viable cells were determined by the trypan blue exclusion method using the Luna Automated Cell Counter (Logos Biosystems) immediately after staining with trypan blue dye (Sigma-Aldrich) to avoid cell death caused by prolonged incubation (34).

Cell cycle analysis. The MDA-MB231 cells were plated at $1x10^5$ cells in a 60-mm dish at 1 day prior to treatment.

Following treatment with the drugs, both attached and floating cells were harvested and fixed with 70% ethanol at -20°C for >4 h. The nuclei were then stained with propidium iodide (Sigma-Aldrich). The chromosomal DNA content was measured using a FACSCalibur flow cytometer (BD Sciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. The data were analyzed using CellQuest Pro (BD Sciences) and the ModFit LT program (Verity Software House, Topsham, ME, USA).

Statistical analysis. Triplicate experiments were repeated 3 times. Most of the data are presented as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) with a post hoc Tukey's honest significant difference (HSD) test was used to analyze the significance of the differences between groups. Conventionally, values of P<0.05 and P<0.01 were considered to indicate statistically significant and highly statistically significant differences, respectively.

Results

Identification of a set of small molecule PKIs with synergistic effects in combination with gefitinib. In our previous studies, the combination of an EGFR inhibitor (EGFRi) and PI3K/AKT inhibitor or MET inhibitors was found to decrease the viability and proliferation of TNBC cells of the BL subtype or MSL subtype, respectively (23,30). In this study, to further identify PKIs that are effective in the presence of an EGFR inhibitor in TNBC cells of the MSL subtype, a set of known PKIs (Table I) was screened with gefitinib in the MDA-MB231 cell line, and inhibitors of the PI3K/AKT and mTOR signaling pathways were identified (Fig. 1A). For example, GSK1059615 (a PI3K/mTOR inhibitor), MK-2206 (an AKT inhibitor) and rapamycin (an mTOR inhibitor) exerted synergistic effects with gefitinib at various concentrations (Fig. 1A and B). Although MK-2206/gefitinib treatment exhibited fewer points of synergy than that of treatment with rapamycin, it exerted the highest average CI, reflecting strong synergy. The majority of the mTOR pathway inhibitors exerted a synergistic effect at 4-10 drug combination points (Fig. 1A); the overall lethal effects of rapamycin or other mTOR inhibitors in combination with gefitinib were not as potent as those of MK-2206 in terms of the average CI value (Fig. 1).

Gefitinib/MK-2206 combination decreases the viability and proliferation of TNBC cells. Among these PKIs, an allosteric AKT inhibitor, MK-2206, was further determined to exert potent synergistic anti-proliferative effects in 2 cell lines of the MSL subtype (MDA-MB-231 and HS578T cells) and 1 cell line of the BL subtype (MDA-MB-468 cells). As was expected, the MDA-MB-468 cells exhibited greater sensitivity following combination treatment with synergistic effect, as well as following treatment with gefitinib or MK-2206 alone (Fig. 2A). Potent synergistic effects were observed in the 2 cell lines of the MSL subtype. In the MDA-MB-231 cells, gefitinib and MK-2206 alone had little or no effect on cell viability; however, the combination of these inhibitors exerted marked synergistic effects (Fig. 2A). The effects of treatment with the gefitinib/MK-2206 combination were further assessed using

Table I. List of protein kinase inhibitors used in this study.

Inhibitor	Other name	Known targets [IC$_{50}$ value (nM)]	(Refs.)
AZD0530	Saracatinib	SRC (2.7), LCK (<4), YES (4), EGFR (L861Q) (4), LYN (5), EGFR (L858R) (5), FYN (10), FGR (10), BLK (11), ABL (30), EGFR (66), KIT (200)	(60)
BEZ235	NVP-BEZ235	PI3Kα (4/2), PI3Kβ (75), PI3Kδ (7), PI3Kγ (5), mTOR (20.7/2), DNAPK (5), ATM (7), ATR (21)	(61,62)
Dasatinib	BMS354825, Sprycel	BCR-ABL (0.8), SRC (0.5), LCK (0.4), YES (0.5), BLK (8), KIT (5), PDGFRβ (28), p38 (100), EGFR (180), EPHB2 (4.4), TXK (<0.3)	(63,64)
Deforolimus	Ridaforolimus AP23573 MK-8669	mTOR (0.2)	(65)
(-) Deguelin		AKT signaling, Hsp90	(66)
Everolimus	SDZ-RAD, Certican, RAD001	mTOR (0.63)	(67)
FK-506	Tacrolimus, Fujimycin, Prograf	FKBP12	(68)
GDC-0941		PI3Kα (3), PI3Kβ (33), PI3Kδ (3), PI3Kγ (75), PI3KC2β (670), mTOR (580)	(69)
GSK1059615		PK3Kα (0.4), PI3Kβ (0.6), PI3Kγ (5), PI3Kδ (2), mTOR (12)	(70)
IC-87114	D-030	PI3Kδ (130)	(71)
KU-0063794		mTOR1 (10), mTOR2 (10)	(72)
LY294002		PI3K (1400), PI3Kα (500) PI3Kδ (570), PI3Kβ (970)	(73)
MK-2206		AKT1 (5), AKT2 (12), AKT3 (65)	(74)
Perifosine	KRX-0401	AKT translocation inhibitor	(75)
PI-103		DNAPK (2), PI3Kα (8), PI3Kβ (88), PI3Kδ (48), PI3Kγ (150), mTORC1 (20), mTORC2 (83)	(71)
PIK-75		DNAPK (2), PI3Kα (5.8), PI3Kγ (76)	(71)
PIK-90		PI3KC2a (47), PI3KC2b (64), DNAPK (13), PI3Kα (11), PI3Kβ (350), PI3Kδ (58), PI3Kγ (18)	(71)
Pimecrolimus	Elidel, SDZ-ASM-981	FKBP12	(76)
Rapamycin	RAPA, Sirolimus	mTOR (0.1)	(77)
Temsirolimus	CCI779, Torisel	mTOR (1.76)	(78)
TG100-115		PI3Kγ (83), PI3Kσ (235)	(79)
TGX221		PI3Kβ (10), PI3Kδ (65)	(79)
Wortmannin	KY12420	PI3K (0.3), AKT1 (35), AKT2 (30), AKT3 (60), PKCζ (24), p38α (6), p38β (2)	(80)
WYE-354		mTOR (5)	(81)
ZSTK474		PI3Kα, PI3Kβ (17), PI3Kγ (53), PI3Kδ (6)	(82)

a long-term colony formation assay. Cells plated in 6-well plates were treated as indicated for 24 h and further cultivated in normal growth media for 14 days. All the TNBC cells tested were minimally affected by treatment with gefitinib or MK-2206 alone. However, the long-term survivals were significantly reduced by the combination treatment (Fig. 2B). The effects of the gefitinib/MK-2206 combination on cell proliferation were also assessed by viable cell counting over time. As shown in Fig. 2C, the gefitinib/MK-2206 combination markedly suppressed the proliferation of the HS578T and MDA-MB-231 cells.

The gefitinib/MK-2206 combination reduces the protein level of RPS6 in TNBC cells of the MSL subtype. To determine the intracellular signaling pathway responsible for the effects of the gefitinib/MK-2206 combination, a series of western blot analyses were carried out. First, the cells were treated with increasing concentrations of gefitinib and MK-2206 in an equimolar ratio for 24 h, and the levels of p-mTOR (S2448), p-SRC (Y416) and p-RPS6 (S235/236) were examined. Of note, both the phosphorylated and total RPS6 levels were decreased in a concentration-dependent manner both in the cells treated with MK-2206 alone and in those that were treated with the gefi-
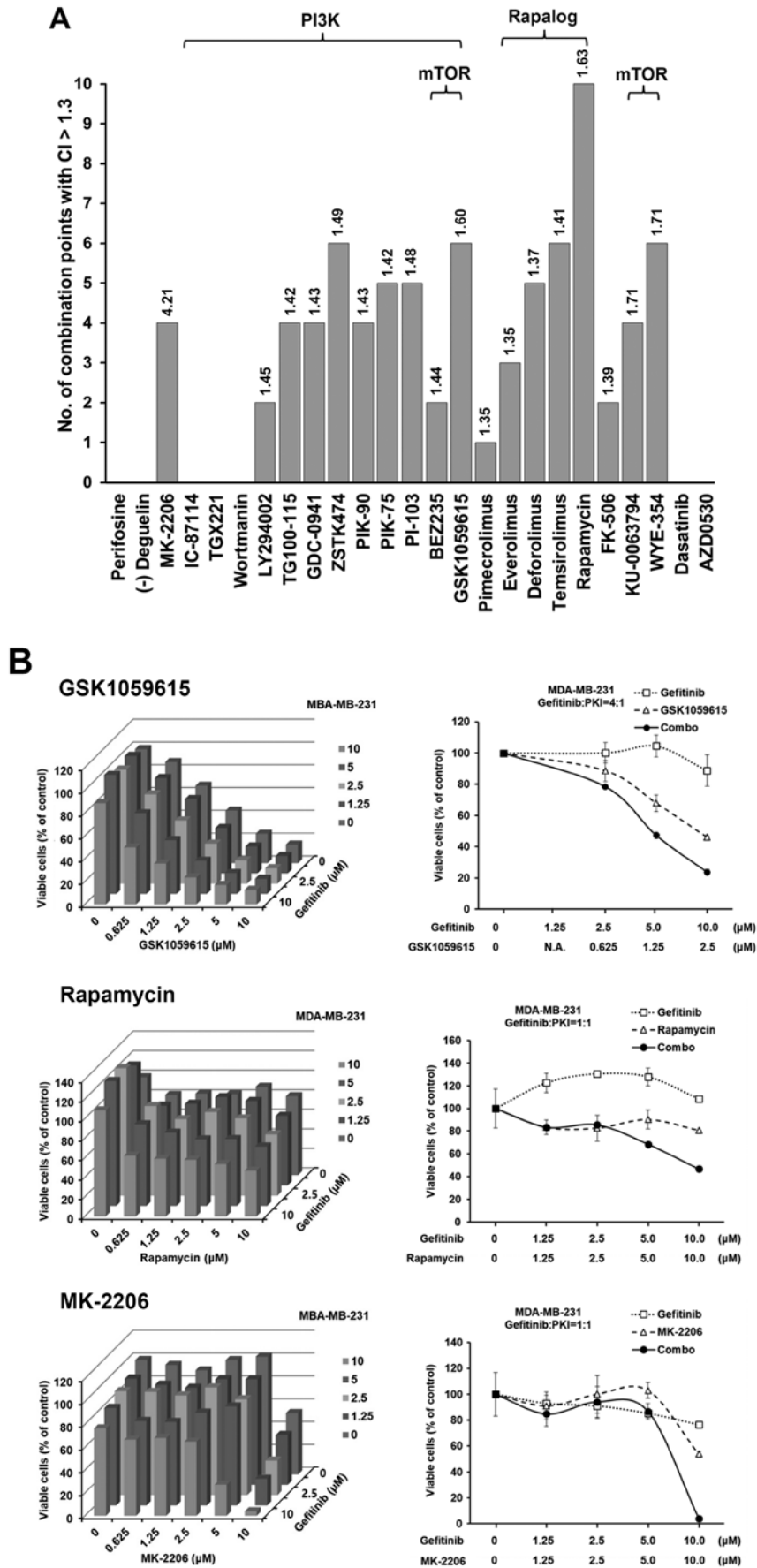
Figure 1. Identification of PKIs with synergistic effects in combination with gefitinib in MDA-MB-231 cells. (A) A set of PKIs that exerted synergistic effects with gefitinib. This synergism was indexed as described in the Materials and methods. The numbers on the tops of bar graphs are the average of the CIs of indicated drugs. (B) Representative results of MTT screening. The MDA-MB-231 cells were treated with serially increased concentrations of PKIs and gefitinib. After 72 h of treatment, cell viability was determined by MTT assay. The left panels represent the 3-dimensional cell viability graph of all of the tested concentrations of drugs. The right panels represents cell viability associated with the selected concentration ratios of the drugs.
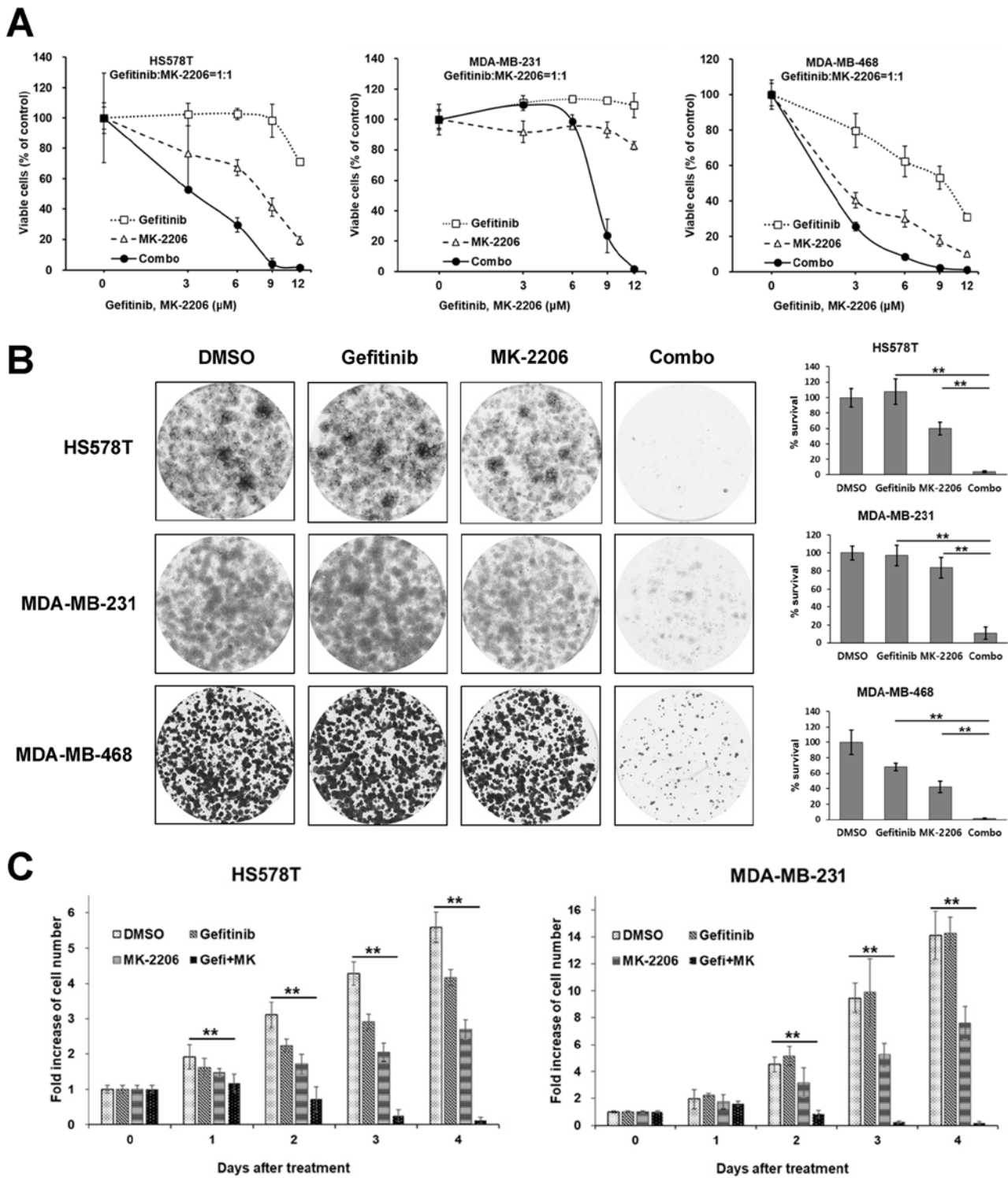
Figure 2. The gefitinib/MK-2206 combination decreases the viability and proliferation of TNBC cells. (A) Cells were treated with increasing concentrations of either gefitinib, MK-2206 or a 1:1 molar ratio combination (Combo) of these drugs for 72 h, and the ratio of viable cells was measured by MTT assay. Data are presented as the means ± SD from 3 independent experiments performed in triplicate. (B) Cells were treated with 9 μM gefitinib, 9 μM MK-2206 or a combination of these drugs for 24 h and further cultivated up to 10-14 days in normal growth media. The colonies were stained with crystal violet as described in the Materials and methods. Left panels, representative images from 3 independent experiments performed in triplicate are shown; right panels, the relative amounts of survived colonies were determined as described in the Materials and methods. **P<0.01, as obtained by the post hoc Tukey HSD test after ANOVA. (C) The HS578T and MDA-MB-231 cells were treated with 9 μM gefitinib, 9 μM MK-2206, or a combination of these drugs for the indicated number of days, and the number of viable cells was determined by counting viable cells with trypan blue dye staining as described in the Materials and methods. Integrated data are presented as the means ± SD from 3 independent experiments performed in triplicate. **P<0.01, as obtained by the post hoc Tukey HSD test after ANOVA.

tinib/MK-2206 combination (Fig. 3A). However, the decrease in the phosphorylated and total RPS6 levels was more prominent with the combination treatment. Previously, we found

a similar synergistic decrease in the RPS6 levels following combination treatment with gefitinib and SU11274, a MET inhibitor, in the same TNBC cell line (30). As the overexpres-
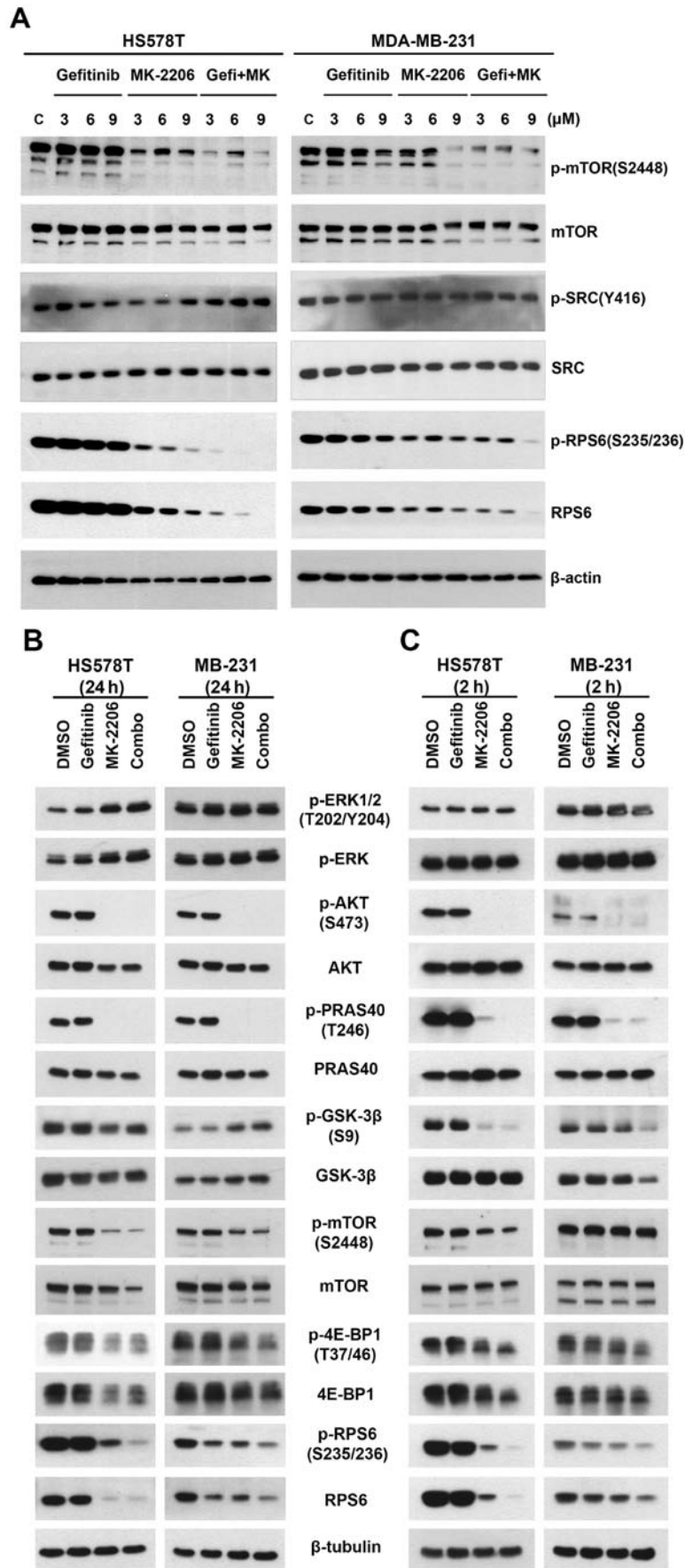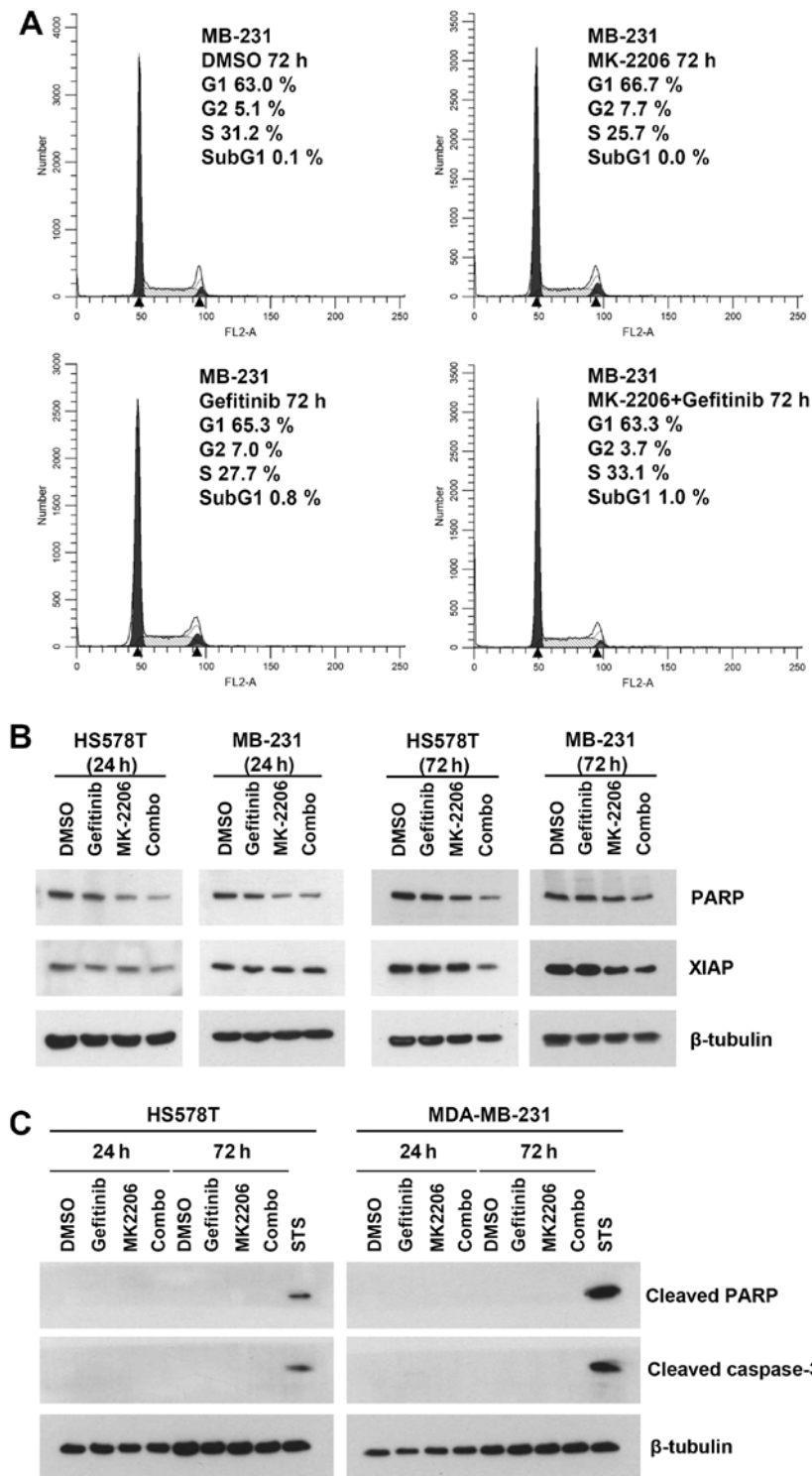
Figure 3. The gefitinib/MK-2206 combination decreases the level of RPS6 protein in TNBC cell lines of the MSL subtype. (A) The HS578T and MDA-MB-231 cells were treated with increasing concentratios of the compounds (0, 3, 6 and 9 μM) as indicated for 24 h, and western blot analysis was performed with the indicated antibodies. β-actin was used as a loading control. (B and C) The HS578T and MDA-MB-231 cells were treated with 9 μM of the drugs as indicated for 24 h (B) or 2 h (C), and western blot analysis was performed with the indicated antibodies. β-tubulin was used as a loading control. Combo, combined treatment with gefitinib and MK-2206.

Figure 4. The Gefitinib/MK-2206 combination decreases XIAP expression in TNBC cells of the MSL subtype. (A) The MDA-MB-231 cells were treated with 9 μM gefitinib, 9 μM MK-2206, or a combination of these drugs 72 h, fixed with ice-cold 70% ethanol, and stained with PI to analyze the DNA content. The PI-stained cells were subjected to flow cytometric analysis with a FACSCalibur flow cytometer with CellQuest Pro software for initial fluorescence-activated cell sorting (FACS) data mining and further analyzed with ModFit 4.1 software. (B) The HS578T and MDA-MB-231 cells were treated with 9 μM of the drugs as indicated for 24 or 72 h, and the cell lysates were subjected to western blot analysis with the indicated antibodies. β-tubulin was used as a loading control. (C) The HS578T and MDA-MB-231 cells were treated with 9 μM of gefitinib, MK-2206 or combination of these (Combo) for 24 or 72 h; staurosporine (STS) was used at a concentration of 25 nM for 24 h as a positive control. The cell lysates were subjected to western blot analysis with the indicated antibodies. β-tubulin was used as a loading control.

sion of EGFR in breast cancer is often accompanied by the co-overexpression of SRC, providing synergistic tumorigenic effects between EGFR and SRC (35), TNBC cells of the MSL type exhibit greater sensitivity to dasatinib, an SRC and ABL kinase inhibitor (5,36). Therefore, in this study, we also examined the changes in SRC expression following treatment with gefitinib/MK-2206. The level of p-SRC (Y416) (37) was not affected under these conditions (Fig. 3A). However, the level

of p-mTOR (S2448) (38) was more markedly decreased by the gefitinib/MK-2206 combination than with treatment with either agent alone (Fig. 3A).

The effect of the gefitinib/MK-2206 combination was further analyzed with a fixed concentration of both PKIs. Unlike p-ERK1/2 (T202/Y204), which exhibited no apparent change in the MDA-MB-231 cells and even increased in the HS578T cells, the expression of p-AKT (S473) and p-PRAS40 (T246) (39), a downstream substrate of AKT, disappeared in the cells treated with MK-2206 and the gefitinib/MK-2206 combination (Fig. 3B). In addition, the level of p-mTOR was decreased by the combination treatment. The phosphorylation of 4E-BP1, which is known to be mediated by mTORC1 (39,40), at the threonine 37 and 46 residues, was apparently diminished by gefitinib/MK-2206 combination treatment.

To identify the immediate intracellular signaling changes that occur prior to the onset of autonomous feedback regulation, we further assessed the immediate-early phosphorylation status of the AKT and mTOR signaling molecules. As shown in Fig. 3C, the level of p-ERK1/2 (T202/Y204) was not affected, even in the HS578T and MDA-MB-231 cells treated with the gefitinib/MK-2206 combination, suggesting that ERK1/2 may be uncoupled from EGFR in these cells. By contrast, p-AKT (S473) expression disappeared both in the cells treated with MK-2206 alone and in those treated with the gefitinib/MK-2206 combination (Fig. 3C). Accordingly, the phosphorylation of AKT substrates such as PRAS40 (T246) and GSK-3β (S9) was reduced in MK-2206-treated cells and further decreased in gefitinib/MK-2206 combination-treated cells within 2 h. However, the phosphorylation status of mTOR (S2448) differed between the HS578T and MDA-MB-231 cells. The level of p-mTOR was synergistically decreased in the HS578T cells, whereas the change in p-mTOR expression in the MDA-MB-231 cells was not as evident. Under these conditions, the level of p-4E-BP1 (T37) was synergistically decreased by the gefitinib/MK-2206 combination treatment. Of note, the levels of phosphorylated and total RPS6 were decreased as early as 2 h after treatment in both cell lines, and the gefitinib/MK-2206 combination further decreased the levels of phosphorylated/total RPS6 in both the HS578T and MDA-MB231 cells. A similar early decrease in p-RPS6 and RPS6 expression within 2 h was also previously observed with MET inhibitor/gefitinib combination treatment in the same TNBC cell lines (30).

The gefitinib/MK-2206 combination decreases XIAP expression without affecting the levels of apoptotic markers. As the gefitinib/MK-2206 combination effectively inhibited cell proliferation and clonogenicity, we analyzed cell cycle progression following treatment with the drug. The MDA-MB-231 cells were treated as indicated for 72 h and were then subjected to flow cytometric analysis. As shown in Fig. 4A, no apparent cell cycle arrest or accumulation of apoptotic cells was observed in the MDA-MB-231 cells. We also used western blot analysis to examine PARP cleavage and XIAP expression following treatment of the HS578T and MDA-MB-231 cells with the drugs for 24 or 72 h. As shown in Fig. 4B, the expression of full-length PARP was decreased by treatment with the drugs in both cell lines. However, the cleaved product of PARP was not observed. The level of XIAP was decreased in

the cells treated with the gefitinib/MK-2206 combination for 72 h. Additional western blot analyses were performed using antibodies for the cleaved forms of caspase-3 and PARP, and staurosporine-treated samples were simulatnously tested as positive controls. Staurosporine induced the cleavage of both caspase-3 and PARP in the TNBC cells tested. On the contrary, no evident cleavage of capase-3 and PARP was observed in the TNBC cells treated with gefitinib, MK-2206 or their combination (Fig. 4C).

Gefitinib with RPTOR knockdown decreases the level of p-/total RPS6 and the viability of TNBC cells of the MSL subtype. In addition to MK-2206, several mTOR pathway inhibitors and a subset of PI3K/AKT pathway inhibitors consistently exhibited synergism with gefitinib in the initial PKI screening (Fig. 1). Based on these findings, we speculated that the resistance to EGFR inhibition and the effects of the gefitinib/MK-2206 combination in TNBC cells of the MSL subtype are to be attributed to the mTOR pathway. mTOR is a protein kinase in two distinct protein complexes, mTORC1 and mTORC2. Therefore, to further elucidate which mTOR pathway contributes to the resistance to EGFR inhibition, the mTORC1 and mTORC2 pathways were selectively blocked by the siRNA-based knockdown (KD) of RPTOR or RICTOR, respectively, in both the MDA-MB231 and HS578T cell lines. RPTOR KD decreased the level of phosphorylated/total RPS6, and the effect was potentiated by the addition of gefitinib in both cell lines (Fig. 5A). However, RICTOR KD with gefitinib treatment did not affect the level of RPS6 protein. As RPTOR KD potentiated the de-phosphorylation of RPS6, we examined the effects of the gefitinib/RPTOR KD combination on cell proliferation. As shown in Fig. 5B, the percentage cell viability was significantly decreased to approximately 50% inhibited by the combination of RPTOR KD and gefitinib treatment. To determine the synergistic inhibitory effects on cell proliferation, the CI values (33) of each group after RPTOR KD and/or gefitinib treatment were calculated. The CI values from 5 independent cell proliferation experiments indicated that this combination was supra-additive, with mean CI values of 1.38±0.31 and 1.34±0.25 in the HS578T and MDA-MB-231 cells, respectively.

Discussion

Due to the lack of appropriate therapies and a poor prognosis of patients with TNBC, the identification of a 'druggable' target is an unmet requirement for the development of an effective TNBC therapy. As numerous types of cancer, including breast cancer have limited driver oncogene activation or tumor suppressor gene mutations (41), proper control of the key surrogate/redundant pathway crosstalk involved in EGFR resistance can provide appropriate therapeutic strategies against the particular type of breast cancer with EGFR activation/overexpression (22-24,26-28). Among the TNBC subgroups classified according to Lehmann *et al*, the BL and MSL subtype exhibits activated EGFR signaling (5). In addition to the activation of the epithelial-mesenchymal transition, the MSL-type TNBC exhibits relatively greater resistance to EGFR inhibitors than the BL subtype, regardless of the overexpression status of EGFR (23,30).
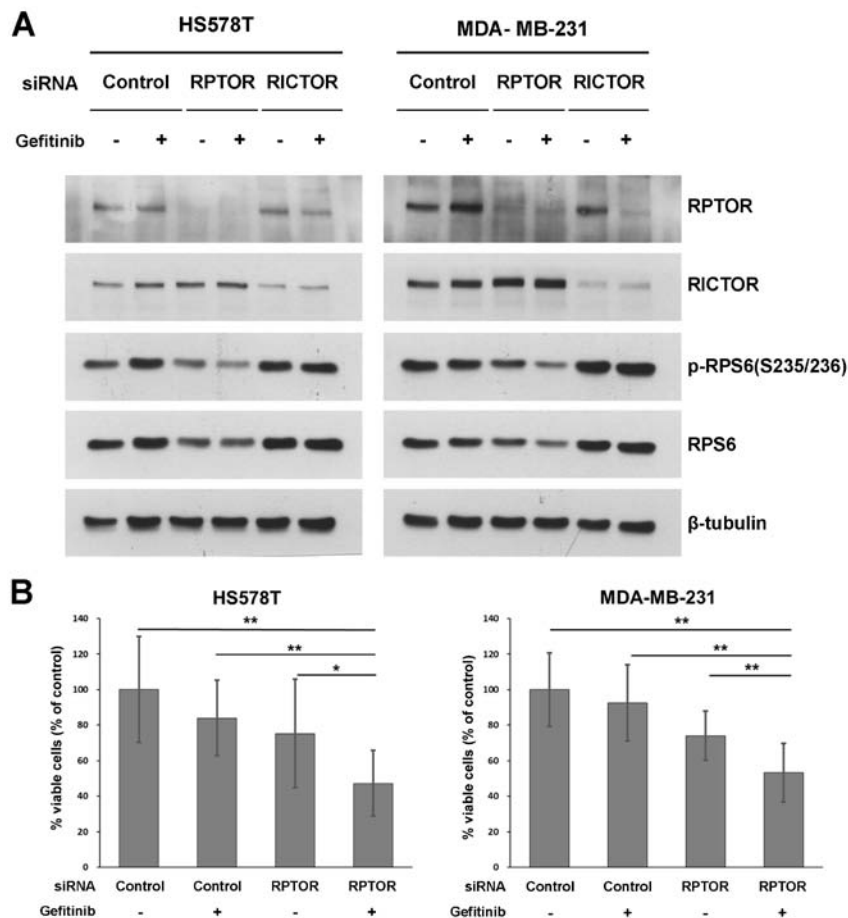
Figure 5. Gefitinib treatment with RPTOR KD decreases the level of phosphorylated/total RPS6 and the viability of TNBC cells of the MSL subtype. The HS578T and MDA-MB-231 cells (plated at $1x10^5$ cells per 60-mm dish at 1 day prior to treatment) were transfected with the indicated siRNA. (A) After 72 h of siRNA transfection, the cells were treated with gefitinib (9 μM) or DMSO for 2 h, and then western blot analysis was performed with the indicated antibodies. β-tubulin was used as a loading control. (B) After the addition of 2X normal serum-containing media with either gefitinib (9 μM) or DMSO, the cells were further incubated for 72 h, and the cell proliferation rate was determined by viable cell counting using an automated cell counter. The viability was plotted as percentage viability compared with control samples (control siRNA-transfected and DMSO-treated). These results were obtained from 3 independent experiments with triplicates. Data are shown as the means ± SD. *$P<0.05$ and **$P<0.01$, as obtained by a post hoc Tukey HSD test after ANOVA.

In this study, we screened a series of known PKIs in the presence of gefitinib in typical EGFR inhibitor-resistant TNBC cells of the MSL subtype (MDA-MB-231 cells) and identified that MK-2206, an AKT inhibitor, exerts a potent lethal effect with gefitinib combination treatment. In addition, a group of inhibitors against mTOR kinase, including rapamycin, temsirolimus, WYE-354 and GSK1059615 also synergistically inhibited cell viability with gefitinib at multiple points of combination than those of MK2206. However, to investigate resistance to gefitinib in TNBC cells of the MSL-subtype, the investigation of the effects of the MK-2206/gefitinib combination, which exhibited potent lethal synergistic effects (average CI value, 4.21; Fig. 1A), would be more feasible to dissect the downstream signaling cascade to unveil the mechanism of resistance to EGFR inhibitors. MK-2206 treatment alone, which effectively blocked AKT activation and phosphorylation of its downstream substrates, had minimal effect in TNBC cells, indicating that EGFR signaling is indispensable for TNBC cell viability. In addition, gefitinib alone had little effect on the TNBC cells of the MSL subtype tested, whereas the gefitinib/MK-2206 combination was synthetic lethal. The synergistic effects of the gefitinib/MK-2206 combination were also demonstrated by a long-term clonogenic assay following

a 24-h treatment with this combination. These results suggest that the AKT-mTOR pathway may contribute to resistance to EGFR inhibitors in MSL TNBC cells.

The decreased phosphorylation of both mTOR and 4E-BP1 (Thr 37/46) suggested that the nodal signaling pathways of the MK-2206/gefitinib combination converge on mTOR signaling. As a downstream signal transducer of the PI3K/AKT pathway, mTORC1, composed of mTOR, RPTOR, PRAS40, mammalian lethal with SEC13 protein 8 (mLST8) and DEP-domain-containing mTOR-interacting protein (DEPTOR), mediates protein synthesis for cell proliferation and cell cycle progression by promoting mRNA translocation as a serine-threonine kinase (42,43). A closely related, but structurally heterologous complex, mTORC2 [composed of mTOR, RICTOR, GβL, mammalian stress-activated protein kinase interacting protein-1 (mSIN1), Protor 1/2 and DEPTOR] mediates AKT activation and cytoskeletal actin remodeling (43,44). mTOR pathway activation is more frequent in TNBC than non-TNBC and is associated with a poor prognosis in early-stage TNBC (45,46). In addition, the activated PI3K mutation and loss of PTEN function, along with the recent identification of mTOR activation in TNBC, rationalizes the use of mTOR-targeted therapy (31,47). The safety and

efficacy of an oral mTOR inhibitor, everolimus, was previously evaluated in phase II clinical studies of patients with metastatic or recurrent breast cancer (48). In a recent report of phase II clinical trials, the use of everolimus in combination with paclitaxel and cisplatin neoadjuvant therapy in TNBC patients revealed more adverse effects without a clinically significant improvement (49). Other independent phase II clinical trials of everolimus combined with conventional chemotherapeutics, such as paclitaxel with anthracyclines also revealed no significant benefit in TNBC patients (50). To date, several phase I/II clinical trials targeting EGFR and mTOR pathways in an advanced setting of breast cancers, including TNBC have been carried out or are still undergoing: Erlotinib and everolimus treatment in metastatic breast cancer (NCT00574366, completed); lapatinib and everolimus treatment in TNBC (NCT01272141, terminated); letrozole, lapatinib and everolimus treatment in advanced endocrine resistant breast cancer (NCT01499160, terminated); temsirolimus, cisplatin and erlotinib treatment in TNBC (NCT00998036, completed); lapatinib and erlotinib treatment in HER2-positive metastatic breast cancer (NCT01283789, active); and everolimus, lapatinib and capecitabine treatment in HER2-positive breast cancer with CNS metastasis (NCT01783756, active) (31,51). While the effectiveness of the combination of EGFR inhibitors and rapalogs with the appearance of apoptotic cells has been reported in some TNBC cell lines, the mechanisms responsible for these synergistic effects have not been reported (52). The phosphorylation of eukaryotic translation initiation factor 4B (eIF4B) in TNBC is a possible fragile point in EGFRi/rapalog synergy (53). As a component of mTORC1, RPTOR is a stoichiometric adaptor molecule for mTOR kinase (54). In the present study, the suppression of mTORC1 via siRNA-mediated RPTOR KD with gefitinib decreased the level of both phosphorylated and total RPS6 protein and inhibited cell viability. These results suggest that mTORC1 provides, at least partially, resistance to EGFR inhibitors in TNBC cells of the MSL subtype, and targeting mTORC1 is a potential alternative therapeutic strategy to overcome resistance to EGFR inhibitors in a subset of TNBCs. Previously, we identified RPS6 dephosphorylation/degradation as a readout pathway of the gefitinib/MET inhibitor combination treatment, and RPS6 KD eventually reduced TNBC cell proliferation (30). A high level of phosphorylated RPS6 is closely associated with active mTOR and is a poor prognostic marker of various cancers including non-small cell lung cancer (NSCLC), hepatocellular carcinoma, sarcoma and pancreatic neuroendocrine tumors (55-58). Importantly, the inhibition of mTOR reduces the level of p-RPS6 in renal carcinoma cells (59).

In this study, we demonstrated the necessity for mTORC1-targeted therapy in EGFRi-resistant TNBC cells and that the dephosphorylation/degradation of RPS6 is closely associated with the mTORC1 signaling pathway along with the survival of a subgroup of TNBC cells. Although we demonstrated an association between resistance to gefitinib and mTORC1 activation, other possible crosstalk mechanisms need to be explored to overcome resistance to EGFR inhibitors and for the successful intervention and control of TNBC. In addition, whether the regulation of RPS6 protein is under the control of a cancer-specific growth signaling pathway remains unclear. Further investigations are warranted in order to dissect the RPS6 de-phosphorylation and/or the degradation pathway and to identify the cancer-specific altered homeostasis of the RPS6 pathway to further develop therapeutic targets in malignant cancers, including TNBCs.

Acknowledgements

Competing interests

The authors declare that they have no competing interests.

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