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Original Article

RSK4 activation confers paclitaxel resistance in gastric cancer

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Abstract: Paclitaxel (PTX) has shown encouraging activity in the treatment of advanced gastric cancer. However, the fact that more than half of GC patients respond poorly to PTX-based chemotherapies demonstrates the urgent need for biomarkers of PTX sensitivity and strategy of overcoming drug resistance in GC patients. We determined the sensitivity of eight GC cells to Paclitaxel and discovered a difference in sensitivities. In mechanism, Paclitaxel-resistant cells shows stronger RSK4 phosphorylation than Paclitaxel-resistant cells. RSK4 inhibition using RNA knock down or RSK4 inhibitor in combination with Paclitaxel led to dramatic apoptosis and cell death in Paclitaxel-resistant cell. This combination caused marked in vivo tumor regressions in Paclitaxel-resistant cell xenograft. Furthermore, activation of RSK4 is mediated by activation of AKT and ERK, Inhibition of AKT or ERK can inactivate RSK4 and overcome Paclitaxel resistance. These data show that combined Paclitaxel and RSK4 inhibition as a potential therapeutic approach for Paclitaxel resistant gastric cancer.

Keywords: Gastric cancer, paclitaxel, RSK4, AKT

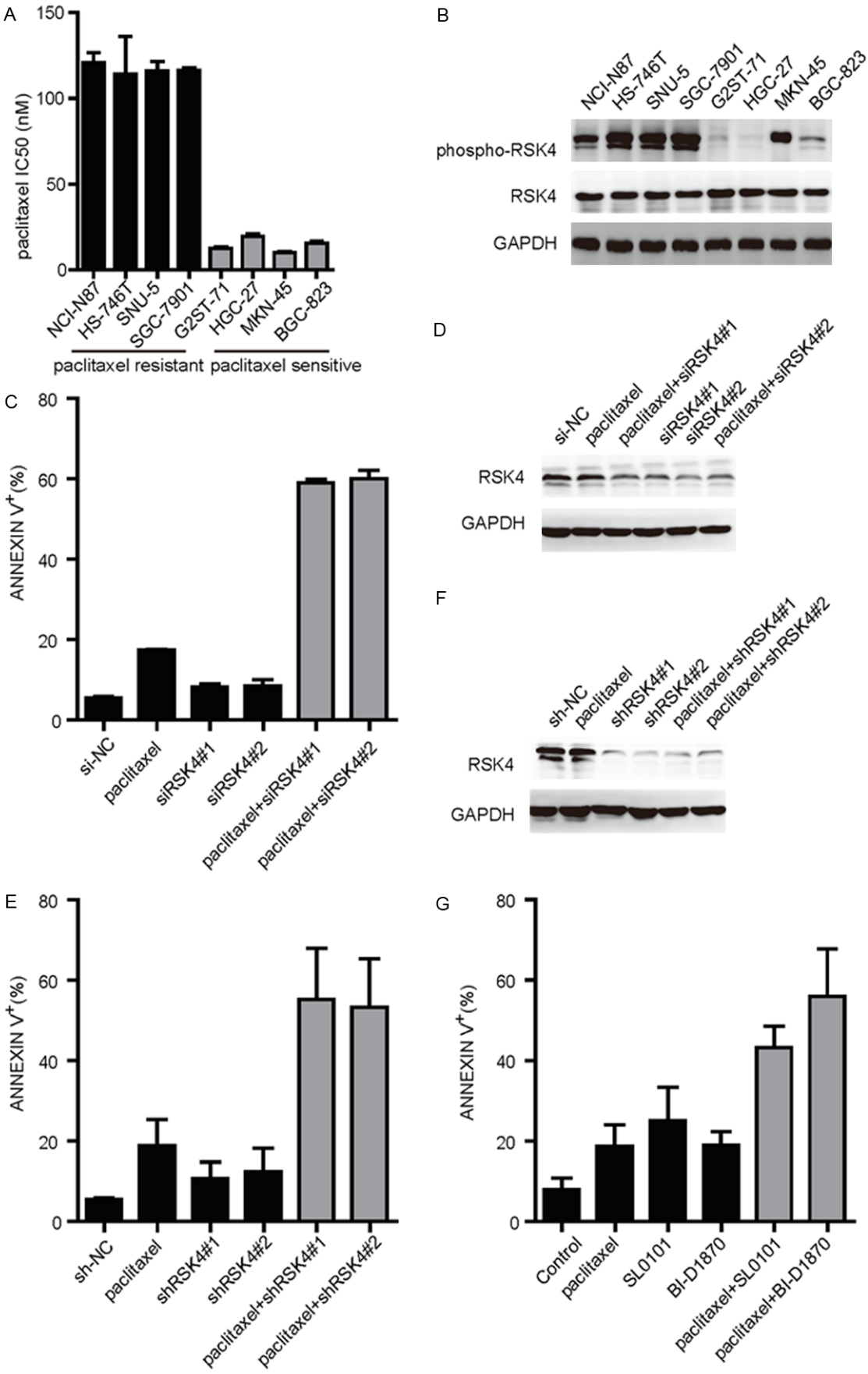
Introduction

Gastric cancer (GC) remains one of the major causes of cancer-related deaths, currently ranked at third, despite its decreasing incidence [1-3]. About one million new cases of GC were estimated to have occurred in 2012, making it currently the fifth most common malignancy in the world, behind cancers of the lung, breast, colorectum and prostate. More than 70% of diagnosed GC cases are registered in developing countries [4, 5].

The disease presents as localized disease in only one-third of patients and as locally advanced or metastatic disease in the remaining two-thirds of patients. Surgery remains the primary mainstay in the treatment of GC. Despite the curative-intent resections, at approximately 70% of cases [6]. In such patients, 5-year survival rates do not exceed 25% and prognosis remains poor [7].

Most GC patients, including those with early-stage disease, relapse at some point during the course of their disease. Among available treatment modalities, only systemic chemotherapy has demonstrated a superior survival rate in this group of patients [8]. Most patients do not respond or relapse within a short time from the end of first-line therapy. Approximately 20%-30% of patients receive further treatment with second-line chemotherapy [9]. Various cytotoxic agents (5-fluorouracil, cisplatin, mitomycin C, methotrexate, docetaxel, paclitaxel [6], nab-paclitaxel, pemetrexed, irinotecan and oxaliplatin) have been previously studied extensively either as monotherapy or in combination in the second-line setting. The median overall survival of patients receiving second-line therapy ranges from 3.5 to 10.7 months, with an objective response rate of 4.8-52.3% [11]. To characterize this heterogeneity, it is necessary to develop prognostic and/or predictive markers to identify which GC patients will benefit from chemotherapy, thus leading to an improved prognosis.

The 90 kDa ribosomal S6 kinase (RSK) family of proteins is a group of highly conserved Ser/Thr kinases that regulate diverse cellular processes, such as cell growth, cell motility, cell surviv-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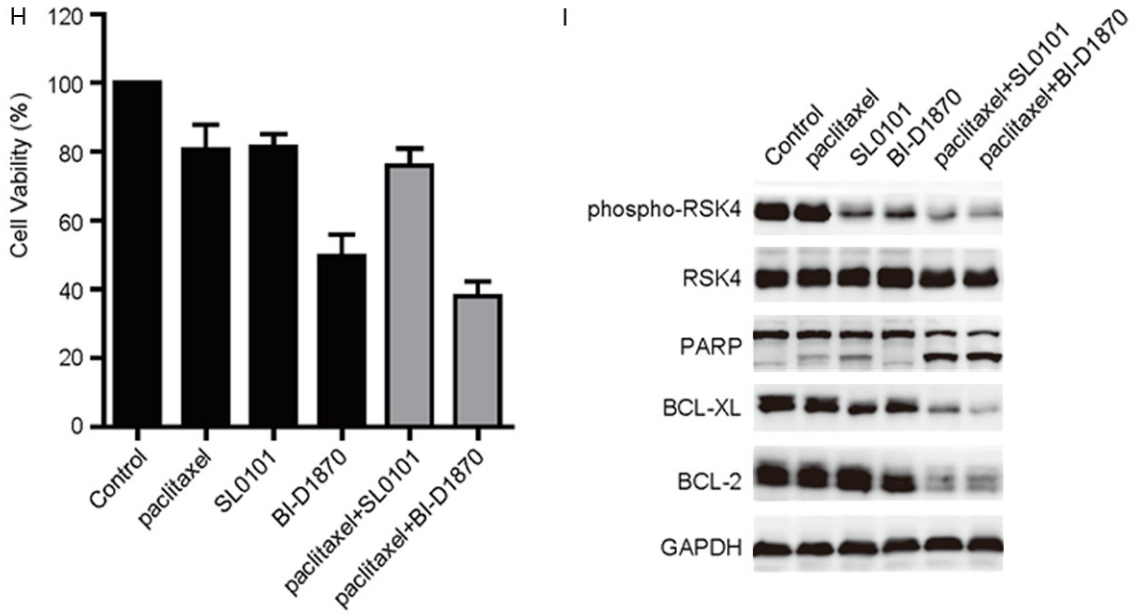

Figure 1. Activation of RSK4 is associated with PTX resistance in GC cells. A. *In vitro* IC_{50} of PTX in eight cancer cell lines. GC cell lines were treated with indicated concentrations of PTX for 72 h and IC_{50} was measured using CCK8 assay. B. Western blot analysis of activation of RSK4 in PTX-resistant GC cells. C. Apoptosis induction of PTX-resistant NCI-N87 GC cells treated with combined RSK4 siRNA and PTX treatment. D. Western blot confirmation of RSK4 levels upon RSK4 siRNA knockdown. E. Apoptosis induction of PTX resistant NCI-N87 GC cells treated with combined RSK4 shRNA and PTX treatment. F. Western blot confirmation of RSK4 levels upon RSK4 shRNA knockdown. G. Apoptosis induction of NCI-N87 cells treated with combined PTX and RSK4 inhibitor SL0103 or BI-D1870. H. Inhibition of cell viability in NCI-N87 cells treated with combined PTX and RSK4 inhibitor SL0103 or BI-D1870. I. Western blot detection of cleaved PARP, BCL2 and BCL-XL levels in NCI-N87 cells treated with combined PTX and RSK4 inhibitor SL0103 or BI-D1870.

al and cell proliferation [12, 13]. RSKs are downstream effectors of the Ras extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling cascade [13]. Significant advances in the field of RSK and ERK/MAPK signaling have revealed novel RSK substrates and new RSK regulatory mechanisms [13]. RSK1-3 are important mediators of growth factor stimulation of cellular proliferation, survival, and differentiation and are activated via coordinated phosphorylation by ERK and 3-phosphoinositide-dependent protein kinase-1 (PDK1). RSK4 is a predominantly cytosolic protein with very low expression that contains several characteristics of the RSK family kinases, including the presence of two functional kinase domains and a C-terminal docking site for ERK [14]. Previous studies showed that the RSK4 constitutively active serine/threonine-protein kinase exhibits growth-factor-independent kinase activity and may participate in p53/TP53-dependent cell growth arrest signaling and play an inhibitory role during embryogenesis [15].

Previous reports also showed that RSK4 I is involved resistance to kinase inhibitor drugs [16]. Overexpression of RSK4 promotes cellular proliferation by PI3K pathway blockade via inhibiting apoptosis and regulating cellular translation through phosphorylation of ribosomal proteins S6 and eIF4B. By combining PI3K inhibitors with MEK or RSK inhibitors, we reversed the resistance phenotype of breast cancer cell lines and PDX models with activated RSK, and we proposed that this combination may be clinically effective in patients with RSK-activated breast cancers [17]. RSK4 overexpression also causes Sunitinib resistance in kidney carcinoma and melanoma cell lines. However, reduction of RSK4 by siRNA was sufficient to sensitize kidney and melanoma cell lines against Sunitinib [16].

Here, we report that RSK4 activation conferred chemoresistance and predicted an unfavorable postoperative prognosis of patients who have stage I through III GC, especially for those who received chemotherapy. These novel findings

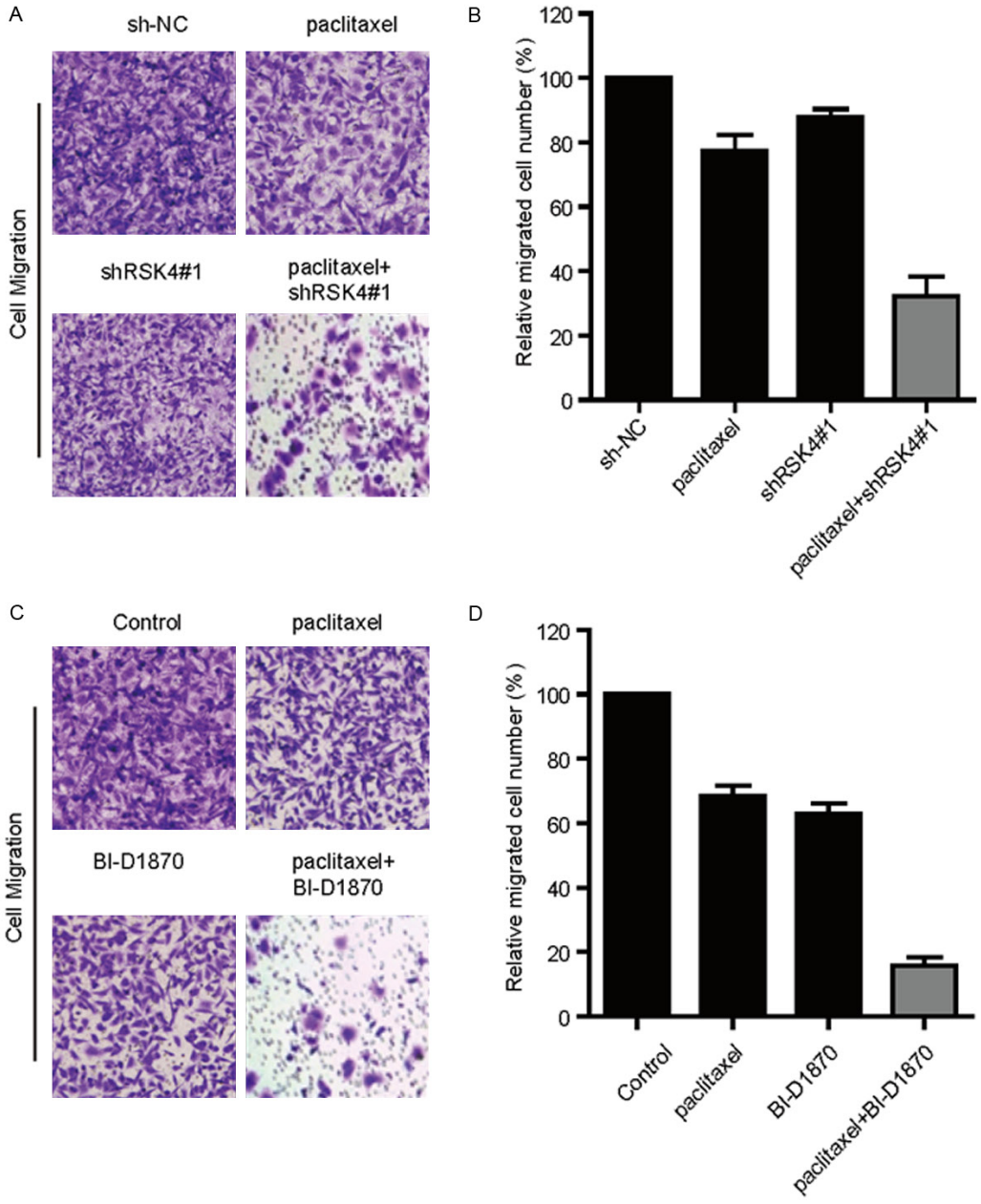
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Figure 2. Combined inhibition of RSK4 with PTX treatment inhibits cell metastasis in PTX-resistant GC cells. A and B. RSK4 knockdown using shRNA combined with PTX inhibited cell metastasis in PTX-resistant GC cells using transwell assays. C and D. RSK4 inhibitor SL0103 combined with PTX inhibited cell metastasis in PTX-resistant GC cells using transwell assays. E. Western blot detection of vimentin, α-SMA, E-cadherin and N-cadherin levels in NCI-N87 cells treated with RSK4 shRNA combined with 100 nM PTX for 48 h. F. Western blot detection of vimentin, α-SMA, E-cadherin and N-cadherin levels in NCI-N87 cells treated with RSK4 inhibitor 5 μM BI-D1870 combined with 100 nM PTX.

may shed light on the role of RSK4 activation in chemoresistance and provide a predictive and prognostic factor for GC prognosis [18].

Results

Activation of RSK4 in paclitaxel resistant gastric cancer cells

To probe the mechanism of the PTX primary resistance in gastric cancer, we first tested the *in vitro* efficacy of PTX in eight GC cell lines. Only four cell lines (NCI-N87, HS746T, SNU5 and SGC-7901) responded to PTX, while for the other four cell lines (G2ST-71, HGC-27, MKN-45 and BGC-823) showed limited cell growth inhibition (**Figure 1A**). Next, we examined RSK phosphorylation and activation in all eight GC cell lines by western blot (**Figure 1B**). We found a strong correlation of RSK4 kinase activation with response to PTX in the eight cell lines; the four PTX resistant cell lines showed stronger RSK4 phosphorylation and activation than the four PTX sensitive cell lines. These data imply that constitutive activation of RSK4 could predict PTX resistance in GC.

Combined inhibition of RSK4 with PTX induces cell death in paclitaxel resistant GC cells

Previous studies showed that RSK4 could mediate resistance to PI3K pathway inhibitors in breast cancer. We hypothesized that constitutive activation of RSK4 could mediate PTX primary insensitivity and primary drug resistance.

To test this hypnosis, we first knocked down RSK4 expression by small interfering RNA (siRNA) as well as short hairpin RNA (shRNA) in the NCI-N87 cell line, which is the most PTX resistant GC cell line, followed by treatment with PTX for 48 h. We then detected cell apoptosis using Annexin V staining. Neither RSK4 siRNA nor PTX treatment alone was able to significantly induce cell apoptosis, whereas the combination of RSK4 siRNA and PTX treatment dramatically increased the percentage of apoptotic cells (**Figure 1C**, **1D**). RSK4 shRNA also showed the same result (**Figure 1E**, **1F**). These findings suggest that RSK4 activation is commonly engaged in gastric tumors and contributes significantly to the overall response to PTX inhibition. We also used the RSK4 kinase inhibitors SL0101 and BI-D1780 and observed significant reversion of PTX resistance (**Figure 1H**). Western blot showed PARP cleavage and decrease of anti-apoptotic proteins BCL2 and BCL-XI, further demonstrating the important role of RSK4 activation in mediating PTX resistance.

Combined inhibition of RSK4 with PTX treatment induces cell metastasis in PTX-resistant GC cells

We next performed transwell experiments to test the possibility that RSK4 inhibition combined with paclitaxel could synergistically inhibit GC cell metastasis. Interestingly, cell metastasis was not significantly inhibited by individual treatments but increased significantly after combined RSK4 shRNA and PTX (**Figure 2A** and **2B**). Similar results were obtained with the combination of the RSK4 inhibitor BI-D1870 and PTX treatment (**Figure 2C** and **2D**). Immunoblotting revealed downregulation of vimentin and α-SMA, as well as upregulation of E-cadherin and N-cadherin in cells treated with RSK4 shRNA and PTX (**Figure 2E**). Similar results were obtained with the RSK4 inhibitor BI-D1870 and PTX treatment (**Figure 2F**). These data indicate a pivotal involvement of vimentin and α-SMA, as well as dysregulation of E-cadherin and N-cadherin in cell metastasis inhibition by the combination of the RSK4 inhibitor BI-D1870 and PTX.

AKT and ERK activation is associated with RSK4 activation in PTX-resistant GC cells

We next examined the mechanism that mediates constitutive activation of RSK4 in PTX-resistant cell lines. As RSK4 has been reported to be activated by PI3K-AKT signaling as well as MEK-ERK signaling [9], we examined whether one of these two pathways mediated RSK4 activation and PTX resistance. We performed western blot analysis of AKT activation and ERK activation in the eight GC cell lines. Surprisingly,

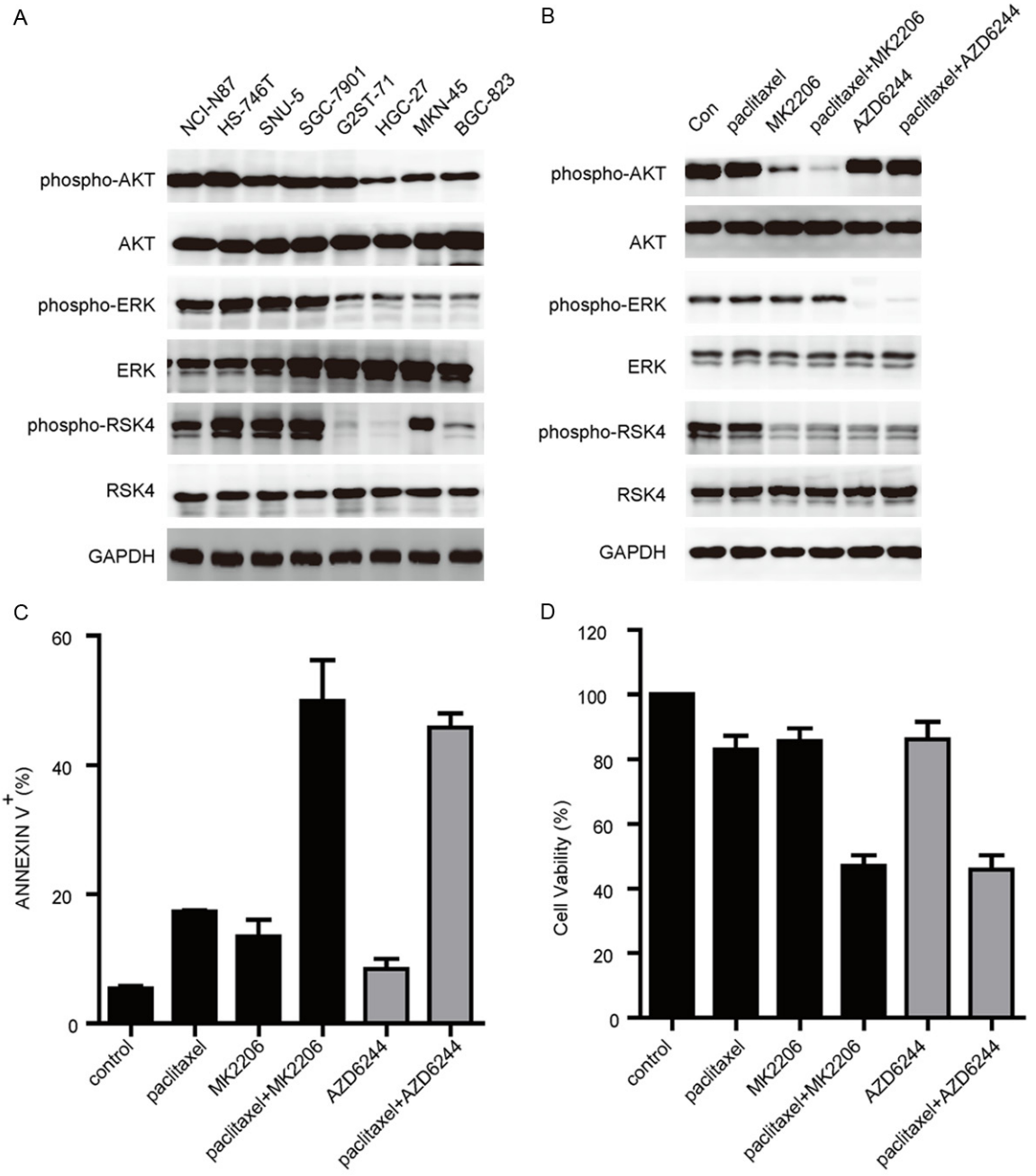

Figure 3. AKT and ERK activation is associated with RSK4 activation in PTX-resistant GC cells. A. Constitutive activation of AKT and ERK in PTX-resistant GC cells compared with PTX-sensitive GC cells. B. Inhibition of RSK4 phosphorylation in cells treated with AKT inhibitor MK2206 or ERK inhibitor AZD6244 with PTX. C. Apoptosis induction of NCI-N87 cells treated with 100 nM PTX with 1 μM MK2206 or 1 μM AZD6244 for 72 h. D. Cell viability inhibition of NCI-N87 cells treated with 100 nM PTX with 1 μM MK2206 or 1 μM AZD6244 for 72 h.

the four PTX resistant cell lines showed stronger AKT and ERK phosphorylation than the four PTX sensitive cell lines (**Figure 3A** and **3B**), which significantly in association with the activation status of RSK4. These results implied activated AKT and ERK may play a key role in RSK4 activation in the PTX resistant cells.

To examine this possibility in more details, we used AKT inhibitor MK2206 and ERK inhibitor AZD6244, separately and combined with PTX. Western blot result showed that MK2206 as well as AZD6244 could downregulate RSK4 activation. We also used MK2206 or AZD6244 combined with PTX and observed a significant

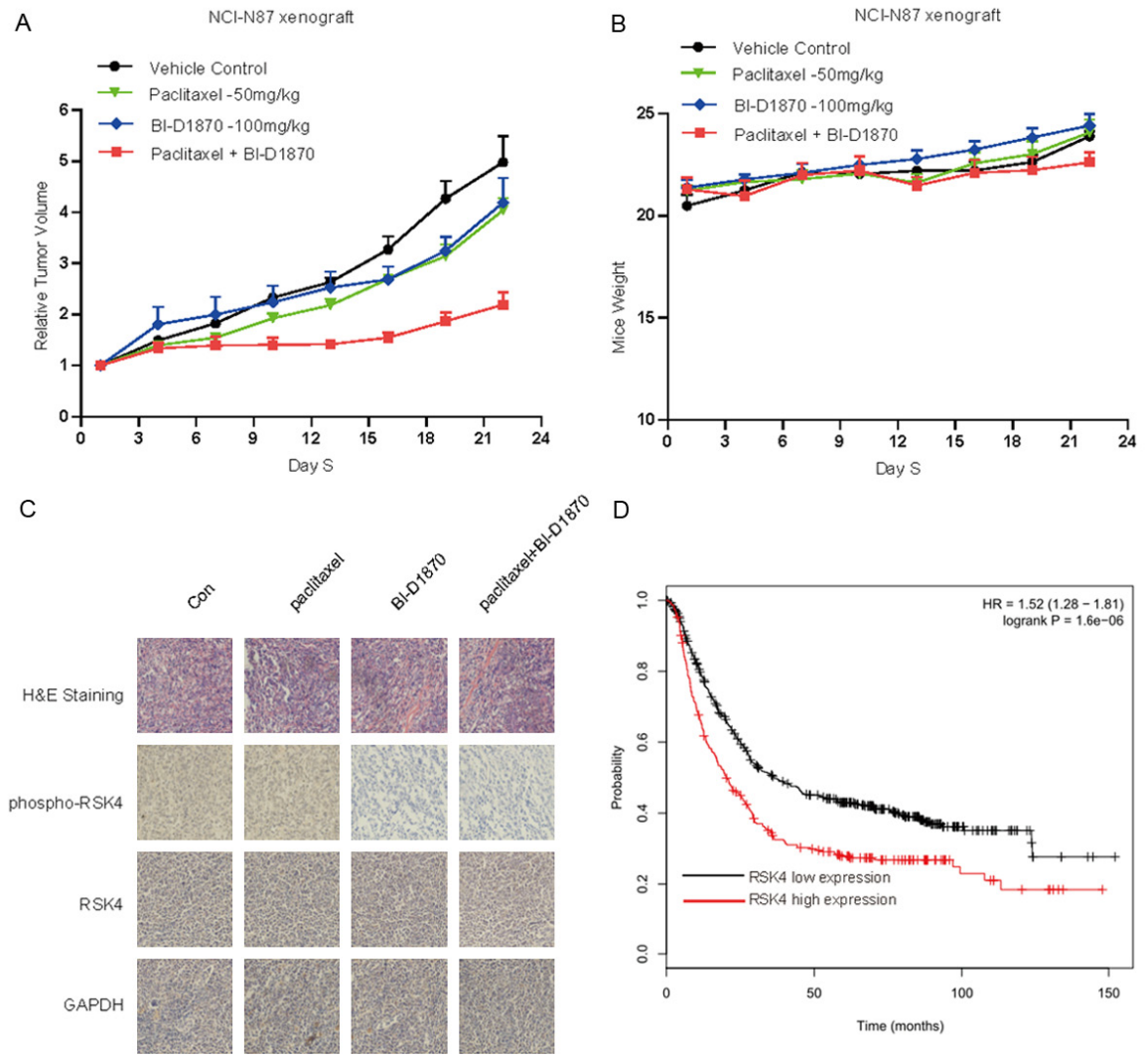

Figure 4. RSK4 inhibitor combined with PTX enhances regression of gastric tumors. A. Relative tumor growth of NCI-N87 GC in mice treated with 100 mg/kg/day PTX and 100 mg/kg/day RSK4 inhibitor BI-D1870, either alone or in combination for 22 days. B. Body weight changes of mice bearing NCI-N87 GC treated with 100 mg/kg/day PTX and 100 mg/kg/day RSK4 inhibitor BI-D1870, either alone or in combination for 22 days. C. Representative immunostaining images of phospho-RSK levels in tumor samples after indicated administration of drugs for 22 days. D. Kaplan-Meier survival analysis of association between RSK4 expression and prognosis in GC patient.

reversion of PTX resistance by detecting apoptosis using ANNEXIN V staining (**Figure 3C**) and cell viability by CCK8 detection (**Figure 3D**). These results support the conclusion that constitutive activation of AKT and ERK is the mechanism that mediates RSK4 activation and further mediates PTX resistance.

RSK4 inhibitor combined with PTX enhances regression of gastric tumors

The finding that RSK4 activation plays a key role in PTX resistance in GC raises the possibility that RSK4 activation could reduce the efficacy of PTX inhibitors *in vivo*. We used one GC xenograft model (NCI-N87 cells) to examine whether inhibiting RSK4 combined with PTX could affect the growth of tumors. Tumors were treated with BI-D1780 and/or PTX. Single inhibition of BI-D1780 and PTX had moderate impact on tumor growth. However, tumor growth was significantly reduced when PTX was combined with BI-D1780 (**Figure 4A**).

We then assessed the efficiency of SL0101 and PTX inhibition on signaling pathway. Immunostaining of NCI-N87 xenograft tumors showed strong staining of RSK4 phosphoryla-

tion; administration of BI-D1780 significantly inhibited RSK4 activation, indicating that BI-D1780 potently blocked its targets (**Figure 4C**). Collectively, our data demonstrate that inhibition of the RSK4 combined with PTX synergistically reduced tumor growth. This strongly suggests that the combination of PTX and RSK4 inhibitors may be more effective in the treatment of GC than either inhibitor alone.

Because there are no data available to assess the association between RSK4 activation and GC, we assessed the correlation of RSK4 expression and GC survival analysis in the clinic using Kaplan Meier-plotter database. Kaplan-Meier survival plots showed that higher RSK4 expression has poorer prognosis than lower RSK4 expression in GC patients, which further indicates the importance of RSK4 in GC progression.

Discussions

GC is the fourth most common malignancy and the second most common cause of cancer-related death worldwide [19], and currently no standard first-line treatment regimen for gastric cancer is in place. For decades, chemotherapy such as paclitaxel, fluoropyrimidine, platinum-based dual or triplet therapies have been the most widely used regimens for metastatic gastric cancer (MGC), which have shown efficacy and manageable toxicity, but only about portion patients respond to it. Identification of factors that predict efficacy, find out mechanism for drug resistance and apply rational drug combination in order to improve clinical outcome is necessary.

RSK4, which belongs to the RSK superfamily, is a multi-functional protein that exerts different functions in different tissue types or under different conditions [14]. Currently, the specific function of RSK4 in cancer remains unclear, and the association between RSK4 and chemoresistance, such as PTX resistanceis unexploited. Our data showed that RSK4 activation is associated with PTX drug resistance. Either RSK4 inhibitor, RSK4 siRNA or RSK4 shRNA could increase drug sensitivity of GC cells to PTX. Our *in vivo* results also support the synergetic anti-cancer effect of the drug combination. Our results suggest that combining RSK4 inhibitors with PTX inhibitors may be effective in the treatment of PTX-resistant GC, and suggests potential application to other types of cancers.

AKT, a serine/threonine kinase, is a key molecule in protecting cells from apoptosis, and the AKT-mediated survival signaling pathway is an attractive target for cancer chemotherapy [20-22]. The activation of AKT inactivates the expression of caspase 9 and regulates the expression of the apoptosis-inducing FAS ligand [23, 24]. It also phosphorylates I κB, promoting I κB degradation, thereby increasing the activity of the well-known cell survival factor, NF-κB. The expression of AKT is altered in various human tumors, and this aberrant expression may contribute to chemoresistance [25-27]. AKT-mediated chemoresistance is likely to result from overall anti-apoptotic activity of AKT and activation of the PI3K signaling cascade, which leads to multidrug resistance. Evidence also showed that RSK4 involved kinase inhibitor drug resistance. Overexpression RSK4 supports cellular proliferation under PI3K pathway blockade by inhibiting apoptosis and regulating cellular translation through phosphorylation of ribosomal proteins S6 and eIF4B. Combining inhibitors of PI3K with inhibitors of MEK or RSK, we can reverse the resistance phenotype exhibited by breast cancer cell lines and PDX models with activated RSK and propose that this therapeutic combination may be clinically effective in patients with RSK-activated breast cancers [17]. Our result support the conclusion that constitutively activation of AKT and ERK is the mechanism that mediated RSK4 activation and further mediated PTX resistance.

Next we examined whether inhibiting RSK4 combined with PTX could affect the growth of tumors. Results demonstrated that BI-D1780 treatment and/or PTX single inhibition of BI-D1780 and PTX had moderate impact on tumor growth. However, tumor growth was significantly reduced when PTX was combined with BI-D1780. Collectively, In vivo result demonstrate the synergistically anti-cancer effect combined RSK4 inhibition with PTX in PTX resistant gastric cancer. These finding could guild the clinical usage of rational drug combination in gastric cancer, especially in those PTX resistant gastric cancer patients.

Experimental procedures

Cell lines

Eight GC cell lines NCI-N87, HS746T, SNU5, SGC-7901, G2ST-71, HGC-27, MKN-45 and BGC-823, were obtained from the American

Type Culture Collection (Rockville, MD, USA). Cell lines were routinely monitored by short tandem repeat analysis. Cell lines were cultured in RPMI 1640 supplemented with 10% FBS (Gibco), 50 U/ml penicillin, and 50 U/ml streptomycin at 37°C in a 5% CO_2 incubator.

Antibodies and reagents

Antibodies against AKT, p-AKT (Thr473), ERK, p-ERK (Thr202/Tyr204), PARP, BCL2 and BCL-XL were obtained from Cell Signaling Technology. Antibodies against RSK4 and p-RSK4 (S232) were purchased from Santa Cruz Biotechnology. Anti-GAPDH antibody was obtained from Abcam. Paclitaxel, BI-D1870, SL0101-MK2206 and AZD6244 were purchased from Selleck (Texas, USA).

Western blot

Tumors and cells were harvested in RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 1% sodium deoxycholate, with protease and phosphatase inhibitors). Protein concentrations were determined using BCA assays (Thermoscientific). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Immobilon-P; Millipore). Membranes were blocked for 1 h at room temperature with 3% milk in 1 x TBST buffer, followed by incubation overnight with primary antibodies and then incubation with secondary HRP-conjugated anti-mouse or anti-rabbit antibodies at 1:5,000 for 1 h at room temperature. The antigen-antibody reaction was visualised with an enhanced chemiluminescence assay (Thermoscientific). The results shown are representative of at least three independent experiments.

siRNA transfection

Cells were plated at 3×10^5 cells/ml in OPTI-MEM serum-free medium and transfected with a specific siRNA duplex using Lipofectamine RNAiMAX Reagent Agent (Life Technologies) according to the manufacturer's instructions. siRNAs were purchased from Sigma-Aldrich.

Cell viability assay

Cells were seeded into a 96-well plate overnight and then treated with indicated drugs. CCK8 assay (Life Technologies) was carried out after incubation for 72 h. Untreated cells served as the indicator of 100% cell viability. The absorbance (optical density, OD) was read at a wavelength of 515 nm on an ELISA plate reader. Cell viability rate was calculated as follows: (OD treated/OD control) ×100%.

Cell apoptosis assay

Cells were treated for 48 h with the indicated agents and detached using trypsin-EDTA, resuspended in growth medium and counted. Cells (1×10^6) were washed with cold PBS, resuspended in 100 μl of binding buffer, and propidium iodide and FITC-labelled antibody against Annexin V were added according to the manufacturer's protocol (Vazyme Biotech). At least 1×10^4 cells per sample were analysed with a FACS can flow cytometer (Becton Dickinson).

Animal model

Animal studies were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. NCI-N87 cells were suspended in 100 ml PBS and injected subcutaneously. Tumor-bearing mice were randomized into four groups based on tumor volume prior to the initiation of treatment, which started when average tumor volume was at least 100 mm^3. SL0101 (100 mg/kg) or/and PTX (500 mg/kg) were given orally daily on 6 consecutive days followed by 1 day without the drug for 15 days. Tumor size was measured using calipers every other day using the formula (length × $width^2$)/2. Mice were sacrificed and tumor tissues were collected 4 h after the last dosing.

Statistical analysis

Statistical significance was analyzed using two tail student's t test, and $P < 0.05$ was considered significant.

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Disclosure of conflict of interest

None.

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