Am J Transl Res 2017;9(9):4317-4330
www.ajtr.org /ISSN:1943-8141/AJTR0059711

Original Article

Targeting mTORC2 component rictor inhibits cell proliferation and promotes apoptosis in gastric cancer

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Received May 30, 2017; Accepted August 25, 2017; Epub September 15, 2017; Published September 30, 2017

Abstract: The mammalian target of rapamycin (mTOR) kinase acts downstream of phosphoinositide 3-kinase/Akt and plays an important role in tumor growth and progression of gastric cancer. It is well characterized that mTOR complex1 (mTORC1) controls cell metabolism and proliferation, whereas the contribution of mTOR complex2 (mTORC2) and its key component, Rictor, remains poorly understood. Therefore, we investigated clinical significance of Rictor expression by immunohistochemical analysis of 391 tissue samples from gastric cancer patients. In addition, the roles of Rictor in cell proliferation, apoptosis, migration and invasion in vitro were evaluated by RNA interference. The results showed that over expression of Rictor was associated with increased tumor size, depth of tumor invasion, lymph node metastasis and advanced TNM stage, together with poorer overall and relapse-free survival. Stable sh-RNA mediated down-regulation of Rictor significantly inhibited SGC7901 and MGC803 gastric cancer cells proliferation, migration and invasion. Furthermore, Rictor knockdown attenuated cell cycle progression and enhanced apoptosis, synergistic with treatment of mTORC1 inhibitor rapamycin owing to abrogating the feedback activation of Akt. Our findings identify Rictor as an important mediator of tumor progression and metastasis, providing the rationale for targeting both mTORC1 and mTORC2 as part of therapeutic strategy for gastric cancer.

Keywords: Rictor, Gastric cancer, mTOR, RNA interference, rapamycin

Introduction

Gastric carcinoma is the fourth most common malignancy worldwide and serves the third leading cause of cancer deaths [1]. Although a steady decline of incidence was noted in western countries, gastric cancer still remains the second common mortality in China [2]. Tumor stage and potential for radical resection are two most important factors for long-term survival. However, the majority of patients are diagnosed with either advanced stage or inoperable metastasis. Conventional treatment including chemotherapy and radiotherapy is far from satisfactory, resulting in an overall 5-year survival rate less than 30%. The poor outcomes suggest a need for novel targeted agents that may confer a better survival benefit in patients with advanced or recurrent disease [3]. Molecular therapies targeting human epidermal growth factor receptor-2 (HER2) and vascular endothelial growth factor receptor (VEGFR) are now becoming the foci for gastric cancer treatment and have been used in clinic [4, 5]. Moreover, the mammalian target of rapamycin (mTOR) is under investigation and may present as another potential therapeutic target.

mTOR is a Serine/Threonine protein kinase which belongs to the phosphoinositide 3-kinase (PI3K) related kinase family and plays a pivotal role in cell metabolism, growth and survival [6]. Based on its sensitivity to rapamycin, mTOR exits in two structurally and functionally distinct multiprotein complexes known as mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2). Regulatory associated protein of mTOR (Raptor) and rapamycin insensitive companion of mTOR (Rictor) are two core proteins for mTORC1 and mTORC2, respectively. mTORC1, the sensitive target of rapamycin, responds to a spectrum of stimuli such as growth factors, energy status and inflammation, mediating phosphorylation of downstream p70S6 ribosomal kinase (S6K)

and eukaryotic translation initiation factor 4E binding protein-1 (4E-BP1). When activated, mTORC1 stimulates cap-dependent protein translation and promotes anabolic progress [7]. mTORC2, which is insensitive to rapamycin treatment, contributes to cell growth and survival by phosphorylation of its main substrate Akt and related kinases, in addition to regulating cell cycle dependent actin cytoskeleton and cell migration [8, 9]. Dysregulation of mTOR signaling pathway has been noted in a variety of human cancers [10]. Moreover, mTOR was found to be activated in gastric cancer, indicating a clear rationale for the use of mTOR inhibitor as targeted therapy [11, 12]. However, targeting mTOR with rapamycin analogue everolimus has been less successful than expected in the recent clinical trial [13]. This is of particular interest since mTOR inhibitor dominantly targets mTORC1/S6K pathway while does not involve mTORC2/Akt inhibition. Furthermore, inhibition of mTORC1 by rapamycin relieves a negative feedback loop from S6K to insulin-like growth factor-1 receptor (IGF-1R), which results in feedback activation of Akt [14, 15]. This paradoxical procedure indicates a problem as activated Akt promotes cell survival and enables resistance to anti-tumor effect of mTOR inhibitor [16].

Rictor, the key component of mTORC2, is an upstream kinase of several AGC protein family members including Akt, SGK, and PKC. Activation of Rictor/mTORC2 modifies actin organization and promotes cell proliferation by phosphorylation of these main substrates. mTORC2 has been implicated as the major hydrophobic kinase to phosphorylate the Ser473 residue of Akt, thus placing mTOR both upstream and downstream of Akt [8]. Since Akt signaling is hyperactivated in gastric cancer, targeting Rictor might be a promising therapeutic strategy. Recent studies showed that Rictor had critical oncogenic roles in regulating cell proliferation and metastasis in a variety of cancer cell types such as melanoma [17], pheochromocytoma [18], colorectal cancer [19], breast cancer [20] and renal cell carcinoma [21]. Specifically, prostatic oncogenesis induced by PTEN loss is dependent on Rictor dosage [22]. Moreover, overexpression of Rictor promotes ligand-induced activation of IGF-1R and cell proliferation, indicating Rictor participates in feedback activation of Akt induced by rapamycin [23]. Growing evidence has revealed that Rictor is implicated in pathogenesis and progression of various malignancies, however, its prognostic role and biological functions in gastric cancer remain obscure. In the present study, the expression of Rictor in gastric cancer tissues was detected by immunohistochemistry, and its correlation with clinicopathological features and survival was observed. Subsequently, we demonstrated that inhibition of Rictor had pronounced effect on gastric cancer cell proliferation and invasion in vitro, which was enhanced in combination with mTORC1 inhibitor rapamycin. These data suggested that Rictor may serve as a novel prognostic marker and therapeutic target in gastric cancer.

Materials and methods

Gastric cancer patients and specimens

A total of 391 gastric cancer patients diagnosed and treated by surgery at Renji Hospital from 2009 to 2011 were enrolled in this study. Among them, 357 cases without distant metastasis accepted gastrectomy with standard D2 lymph node dissection. The other 34 metastatic patients with primary tumor complications such as obstruction or bleeding underwent palliative stomach resection. Pathological TNM stage was determined according to the 7th edition of Union for International Cancer Control (UICC) guidelines. Follow-up data were available for all participants. Overall survival (OS) time was determined from the date of surgery to the follow-up deadline or date of death. Relapse-free survival (RFS) time was defined as from the date of surgery to the last observation or the date of clinical recurrence diagnosed by imaging or surgical exploration. Ethical approval was obtained from the hospital's ethics committee, and informed consents were obtained from all patients.

Antibodies, reagents and cell lines

Primary antibodies used were rabbit monoclonal anti-Rictor (Bethyl Laboratory), anti-pAktSer473 and anti-Akt1 (Epitomics), rabbit polyclonal anti-pS6Ser235/236 (Cell Signaling Technology), anti-GAPDH (Affinity BioReagents) antibody. Goat anti-rabbit IgG polyclonal antibody was from Abcam Company. Rapamycin was purchased from Cell Signaling Technology. Human gastric cancer cell lines SGC7901 and MGC803 were obtained from Shanghai Institute of Cell Biology. Viral packaging HEK293T cell

was from American Type Culture Collection and stored in State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute. The RNA interference lentiviral vector system was purchased from GenePharma Co., Ltd (Shanghai, China). The system includes LV2 shuttle vector, pPACK-GAG, pPACK-REV and pPACK-VSV-G packaging vectors.

Immunohistochemistry

Immunohistochemical staining was performed using a two-step protocol. After microwave antigen retrieval, tissue slides were incubated with Rictor primary antibody (1:400) at 4°C overnight. Following 60 min incubation with secondary antibody at room temperature, sections were developed in DAB solution under microscope observation and counterstained with hematoxylin. The results of immunoreactivity were evaluated by two independent investigators without prior knowledge of patients' clinical details. In the light of a semiquantitative grading system, scoring was determined based on proportion of stained cells (no staining = 0; <1/3 staining = 1; 1/3 to 2/3 staining = 2; >2/3 staining = 3) and staining intensity (none = 0; week = 1; medium = 2; strong = 3). According to sum of the two variables, the final staining score was defined as negative (0~2) and positive (3~6).

Cell culture, plasmid trasfections and lentiviral transductions

Human gastric cancer cell lines SGC7901 and MGC803 were cultured in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. HEK293T cells were grown in DMEM with 10% (v/v) FBS and antibiotics as above at 37°C in a humidified incubator under 5% CO_2 condition. For generation of stable knockdown cells, four short hairpin RNAs (shRNA) against human Rictor gene were constructed in pGLV2-U6-Puro vector: sh-1 (RICTOR-homo-1435, 5'-GCAGCCTTGAACTGTTTAA-3'), sh-2 (RICTOR-homo-2203,5'-GCCTGCAGACTCTATGCAACA-3'), sh-3 (RICTOR-homo-333, 5'-GCTACGAGCGCTTCGATATCT-3'), and sh-4 (RICTOR-homo-4625, 5'-GCAACCAACTGAGTGCAATAT-3'). A plasmid carrying nontargeting sequence was used to create negative control (sh-NC) cells. For lentivirus packaging, vectors were cotransfected with three accessory packaging plasmids into HEK293T cells using lipofectamine 2000 (Invitrogen, US). After 48 h of incubation, the virus-containing supernatant was collected, filtered and overlaid onto SGC7901 and MGC803 cells in the presence of 5 μg/ml polybrene for 48 h. The infected cells were then selected by puromycin (6 μg/ml), and after one week, the puromycin concentration was reduced to 2 μg/ml and maintained in culture medium.

Cell lysate and western blotting

Cells with 90% confluence were harvested, washed and lysed with lysis buffer on ice. Protein concentration of the resulting lysates was determined by BCA assays (Bio-Rad Laboratories, US). Equal amounts of samples were separated by SDS-PAGE and transferred to PVDF membrane (Millipore, US). Membranes were blocked with 5% (w/v) bovine serum albumin in Tris-buffered saline and incubated overnight at 4°C with primary antibodies against Rictor (1:4000), pAktSer473 (1:1000), Akt-1 (1:1000), pS6Ser235/236 (1:4000) or GAPDH (1:15000). Subsequently, membranes were washed and incubated with secondary antibodies at room temperature for 1 h. The bound antibodies were detected by Odyssey infrared Imaging System (LI-COR Bioscience, US).

Transwell migration, invasion assays and wound-healing assay

Cells were trypsinized and resuspended in serum-free medium at a concentration of 1×10^5 cells/ml. Approximate 2×10^4 cells were seeded in the upper chamber (Millipore, US) seated on 24-well plate, and 600 μl complete medium with 10% FBS was added to each well of the plate as the lower chamber. After 24 h of incubation, non-filtered cells were removed using a cotton swab. Migratory cells on the under side of membrane were fixed and stained with 0.1% crystal violet, counted by an inverted microscope in five different fields at 200× magnification. For invasion assay, similar procedures were performed except that the basement membrane of upper chamber was coated with 100 μl Matrigel (BD, US). In addition, 5×10^4 cells were seeded in the chambers and the invading cells were counted after 48 h of incubation. For wound-healing assay, cells were seeded in 12-well plates and grown over 95% confluence. After making a straight scratch by using a 10 μl pipette tip, cells were incubated in medium containing 1% FBS. Images were captured 0 h, 24 h and 48 h after scratching at the

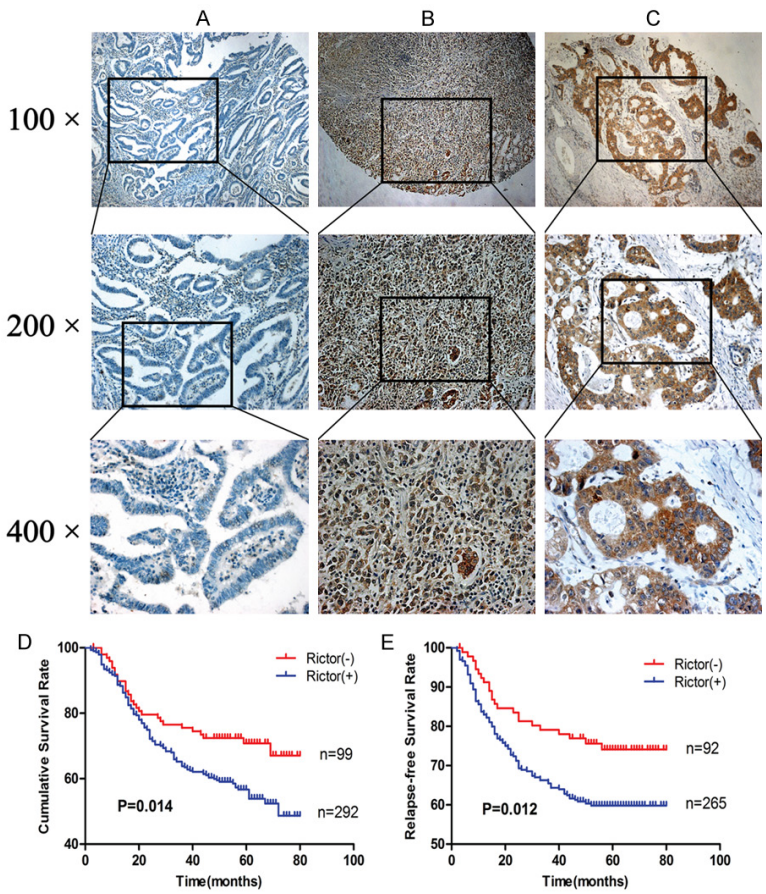


Figure 1. Expression of Rictor by immunohistochemistry in gastric cancer tissues. Representative immunohistochemical staining from 391 samples are shown. Ninety-nine cases were negative for Rictor (A), and 292 cases were positive staining exhibited in poorly differentiated (B) or well differentiated adenocarcinoma (C). Kaplan-Meier analysis with log-rank test showed that Rictor positive expression predicted poor overall survival in all 391 patients (D) and poor relapse-free survival in 357 patients who underwent radical resection (E).

same location of the well, and the relative wound distances were measured by using Image-Pro Plus 6.0 software.

Cell viability assay

Cells were seeded onto 96-well plate at a density of 2×10^3 per well with 100 μl complete medium and cultured for 0 h to 96 h. Then 10 μl of Cell Counting Kit-8 (CCK-8, Dojindo, Japan) solution was added to each well and incubated for 2 h. Cell viability was detected by the absorbance reading at 450 nm using a microplate reader. The experiment was performed in quintuplicate and repeated twice.

Cell cycle analysis

A total of 2×10^5 cells were seeded in 6-well plates and incubated overnight followed by another 12 h of serum starvation to synchronize cell cycles. Afterwards, the media was replaced by complete medium and cultured for 48 h. Cells were harvested, washed and fixed in 70% ethanol overnight at 4°C. Fixed cells were then washed and stained with propidium iodide (PI, 10 μg/ml) and RNase A (100 μg/ml) at room temperature for 30 min. Cells were subjected to flow cytometry using a Becton Dickinson FACScan. The percentages of cells in different cell cycle phases were analyzed with ModFit software.

Apoptosis assay and detection of Caspase3/7 activity

Cells were cultured in 6-well plates until about 50% confluence. Then the media was replaced by serum-free medium with or without rapamycin to induce apoptosis. After 48 h, cells were harvested, washed with PBS and resuspended. About 1×10^5 cells in 100 μl binding buffer were double stained with 5 μl PI and 5 μl FITC-Annexin V using the FITC-Annexin V Apoptosis Kit (BD, US). After 15 min incubation at room temperature protected from light, another 400 μl binding buffer was added into samples. Cell apoptotic status was measured via flow cytometry and analyzed by CellQuest software. Caspase 3/7 activity was detected using a Caspase-Glo 3/7 assay kit (Promega, US) according to the manufacturer's instruction. Briefly, after 48 h of cell apoptosis induction by serum-free medium, 100 μl Caspase-Glo reagent was added into 100 μl cell suspension in 96-well plate and incubated at room temperature for 2 h. The luminescence was read on a SpectraMax microplate reader (Molecular Devices, US).

Statistical analysis

All statistical analyses were performed using SPSS software (version 17.0). The association of Rictor expression with various clinicopatho-

logical parameters was analyzed using the chi-square test. Cumulative survival was estimated by the Kaplan-Meier method and analyzed by the log-rank test to compare survival distributions. All experiments in vitro were conducted at least in triplicate and repeated twice. Quantitative data were described as mean ± SD. One-way analysis of variance (ANOVA) was used to compare the differences between multiple groups. A *P* value less than 0.05 was considered statistically significant.

Results

Expression of Rictor is correlated with clinicopathological features and prognosis in patients with gastric cancer

In gastric cancer tissues, positive immunostaining for Rictor was predominantly observed in cytoplasm of tumor cells, granularly or diffuse distribution. The expression of Rictor was classified as negative in 99 (25.3%) cases (**Figure 1A**) and positive in 292 (74.7%) cases (**Figure 1B**, **1C**). Statistical analyses indicated that Rictor expression was significantly higher in patients with increased age, larger tumor size, deeper invasion, presence of lymph node metastasis and advanced TNM stage. The results also showed a trend towards correlation between Rictor expression and tumor thrombus, albeit the trend did not reach statistical significance. There was no significant association with gender, tumor location, distant metastasis, grade of differentiation or perineural invasion (**Table 1**). Kaplan-Meier survival analysis with log-rank test revealed a correlation between Rictor positive expression and poorer survive. For all 391 patients, the 5-year cumulative survival rate of Rictor negative group and positive group was 70.8% and 56.7%, respectively (**Figure 1D**). In addtion, among 357 patients who underwent radical resection, the 5-year relapse-free survival rate was 74.1% and 59.8%, respectively (**Figure 1E**). Taken together, these results indicated the association of Rictor with tumor progression and prognosis in gastric cancer.

Knockdown of Rictor inhibits proliferation of gastric cancer cells, and the effect is enhanced by rapamycin

We hypothesized that Rictor played an important role in gastric cancer progression by regulating cell growth and proliferation. To validate this hypothesis, we chose two shRNA vectors (sh-1 and sh-3) from four interference sequences that markedly suppressed the expression of Rictor in SGC7901 and MGC803 cells, and the RNAi-mediated knockdown was verified by western blotting (**Figure 2A**). CCK-8 assays revealed that down regulation of Rictor significantly inhibited cell proliferation of both gastric cancer cells after 48 h of incubation (**Figure 2B**). Next, we looked at rapamycin, an inhibitor of mTORC1, to determine its effect on SGC7901 cells. As shown in **Figure 2C**, rapamycin treatment resulted in increase of pAktSer473 and decrease of pS6 levels in a dose-independent manner. Moreover, the addition of 10 nM rapamycin further reduced cell viability of both cells (**Figure 2D**). These findings suggest that knockdown of Rictor can inhibit proliferation of gastric cancer cells, and the effect may be enhanced when in combination with mTORC1 inhibitor.

Knockdown of Rictor attenuates migration and invasion of gastric cancer cells

Migration and invasion are critical steps in initial progression of cancer that facilitate metastasis. We used transwell assay and wound healing test to determine the role of Rictor in gastric cancer cell migration and invasion. After 24 h of incubation, down regulation of Rictor significantly suppressed cell migratory potentials compared with control by traswell migration assay (**Figure 3A**). Similarly, knockdown of Rictor inhibited SGC7901 and MGC803 cells wound healing by using a scratch assay in low serum medium, the sh-1 and sh-3 cells failed to fill the gap 48 h after scratch (**Figure 3B**). Furthermore, invasion of cells was assessed by using a modified chamber coated with Matrigel to resemble the extracellular environment. The results also indicated knockdown of Rictor attenuated invasion of both cells after 48 h of incubation, albeit less strongly than that in migration assay (**Figure 3C**). Taken together, our findings suggest that Rictor is required for gastric cancer cells migration and invasion, regardless of chemotaxin.

Knockdown of Rictor mediates cell cycle arrest and potentiates rapamycin induced apoptosis in gastric cancer cells

To get insight into the mechanism by which Rictor knockdown inhibits cell proliferation, we assessed the effect of Rictor on cell cycle progression by flow cytometry. As shown in **Figure 4A**, the percentage of cells in S phase

Table 1. Correlation of Rictor expression with clinicopathological characteristics in gastric cancer patients

Factors	Cases	Number of patients (%)		X^2 test	*P* value
		Rictor (-)	Rictor (+)		
Gender					
Male	258	63 (24.4)	195 (75.6)	0.326	0.568
Female	133	36 (27.1)	97 (72.9)		
Age (yr)					
≤60	176	55 (31.3)	121 (68.8)	5.953	**0.015**
>60	215	44 (20.5)	171 (79.5)		
Location					
Upper	25	6 (24.0)	19 (76.0)	0.227	0.973
Central	154	39 (25.3)	115 (74.4)		
Lower	206	52 (25.2)	154 (74.8)		
Total	6	2 (33.3)	4 (66.7)		
Size					
< 5 cm	200	60 (30.0)	140 (70.0)	4.743	**0.029**
≥ 5 cm	191	39 (20.4)	152 (79.6)		
Depth of invasion					
T1+T2	129	41 (31.8)	88 (68.2)	4.242	**0.039**
T3+T4	262	58 (22.1)	204 (77.9)		
Lymph node metastasis					
Negative	161	55 (34.2)	106 (65.8)	11.316	**0.001**
Positive	230	44 (19.1)	186 (80.9)		
Distant metastasis					
M0	357	92 (25.8)	265 (74.2)	0.441	0.507
M1	34	7 (20.6)	27 (79.4)		
TNM stage					
I	98	35 (35.7)	63 (64.3)	9.161	**0.002**
II	97	27 (27.8)	70 (72.2)		
III	162	30 (18.5)	132 (81.5)		
IV	34	7 (20.6)	27 (79.4)		
WHO grading					
Grade 1	71	20 (28.2)	51 (71.8)	2.168	0.141
Grade 2	120	19 (15.8)	101 (84.2)		
Grade 3	200	60 (30.0)	140 (70.0)		
Tumor thrombus					
Negative	337	91 (27.0)	246 (73.0)	3.656	0.056
Positive	54	8 (14.8)	46 (85.2)		
Perineural invasion					
Negative	365	95 (26.0)	270 (74.0)	1.454	0.228
Positive	26	4 (15.4)	22 (84.6)		

Data are analyzed by chi-square test, and bold values are statistically significant.

decreased and the percentage of cells in G0/G1 phase increased markedly in sh-1 and sh-3 cells compared with control group. Moreover, 10 nM of rapamycin was added into sh-NC and sh-3 cells, and we found that rapamycin treatment also resulted in G0/G1 arrest, although the degree was weaker than Rictor knockdown. However, no synergistic effect of rapamycin and Rictor knockdown was observed in SGC7901 cells (**Figure 4B**). Furthermore, we investigated the effect of Rictor down regulation on cellular apoptosis induced by serum starvation. As measured by Annexin V/PI staining and flow cytometry, the apoptotic rate was significantly higher in Rictor shRNA-transfected cells than in control cells (**Figure 4C**). Similarly, in presence of 10 nM rapamycin, rate of apoptosis elevated remarkably in both Rictor knockdown and negative control groups, while the effect was synergistic in condition of mTORC1 inhibition combined with Rictor knockdown (**Figure 4D**). To further characterize this cell specific apoptotic effect of targeting Rictor, we analyzed the levels of caspase 3 and caspase 7, two key molecules in the canonical apoptosis pathway. Consistent with apoptosis measured by flow cytometry, the relative caspase 3/7 activities of sh-1 and sh-3 cells were significantly elevated compared with sh-NC cells after 48 h of serum starvation. Moreover, the addition of 10 nM rapamycin resulted in an obvious increase in caspase 3/7 activity, with the greatest levels achieved in cells that were both Rictor down-regulated and exposed to rapamycin (**Figure 5A**). All these findings demonstrated that Rictor knockdown can induce apoptosis of gastric cancer cells, either alone or combined with mTORC1 inhibitor rapamycin.

Combination of Rictor knockdown and rapamycin treatment blocks the feedback activation of Akt in gastric cancer cells

In view of the fact that blocking mTORC1 leads to activation of Akt by up-regulating upstream receptor tyrosine kinase signaling such as IGF-

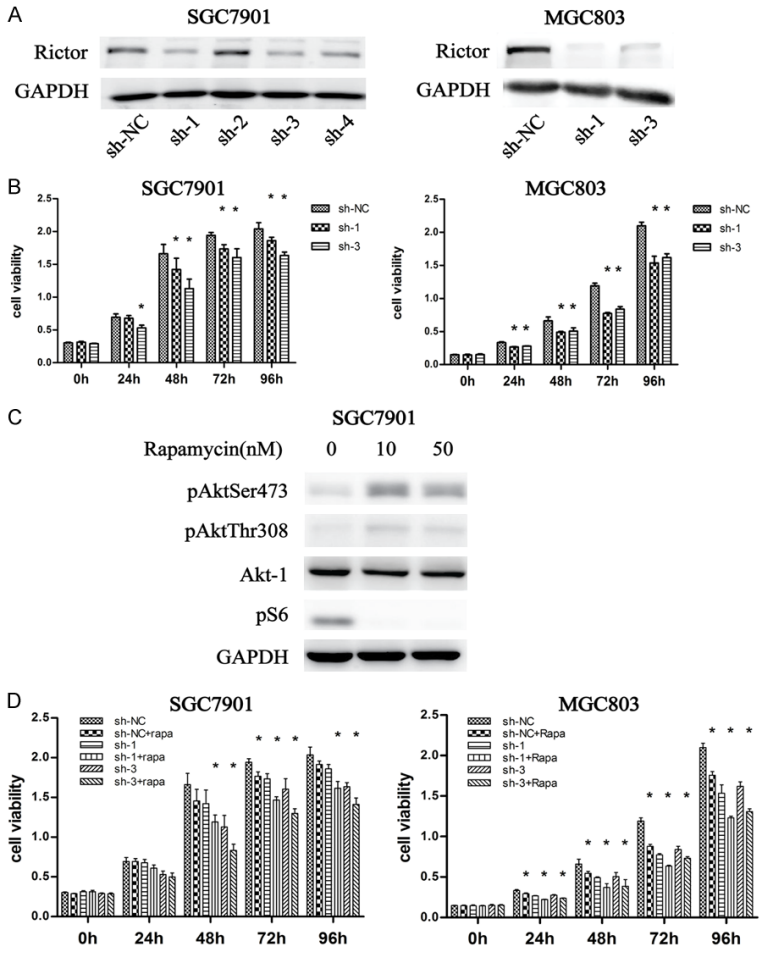


Figure 2. shRNA mediated Rictor knockdown inhibits gastric cancer cell proliferation. A. Western blotting analysis of Rictor expression in cells transfected with shRNA plasmids or mock vector, GAPDH served as a loading control. B. Cell proliferation was impaired in sh-Rictor cells assessed by CCK-8 assay, values were mean ± SD of quintuplicate wells (*, *P<0.05* versus sh-NC). C. Rapamycin treatment resulted in increase of pAktSer473 and decrease of pS6 levels in SGC7901 cells. D. Rapamycin further reduced cell viability in Rictor knockdown cells (*, *P<0.05* versus sh-Rictor alone).

1R, it is hypothesized that the mTORC2 component Rictor might mediate this effect. We then investigated the mechanism of synergy between inhibition of mTORC2 by targeting Rictor and inhibition of mTORC1 by rapamycin. As expected, pS6, a downstream target of mTORC1, was significantly inhibited after rapamycin treatment. However, pAktSer473 was activated by rapamycin, suggesting the feedback loop activation by mTORC1 inhibition (**Figure 2C**). Interestingly, Rictor knockdown blunted the phosphorylation of AktSer473 induced by rapamycin, while led to an enhanced phosphorylation of S6 (**Figure 5B**). These results indicate that Rictor indeed plays an important role in mTOR-regulated feedback activation of Akt, and simultaneous inhibition of the two mTOR complexes could be effective for preventing mTOR inhibitor related escape mechanisms in gastric cancer cells (**Figure 5C**).

Discussion

mTOR integrates oncogenic PI3K/Akt signaling and critical downstream pathways that controls cell metabolism, growth and survival, and also mediates tumorigenesis [6, 10]. It has been reported that p-mTOR was over-expressed in human gastric cancer and presented as prognostic marker, suggesting investigational mTOR inhibitor may provide a novel therapeutic approach [11]. However, mTOR exerts additional functions when combined with Rictor to form mTORC2, while the expression and role of Rictor remains unidentified in gastric cancer. In the present study, we demonstrated that Rictor was widely expressed in gastric cancer, and this over-expression was significantly correlated with tumor size, depth of invasion, lymph node metastasis and TNM stage, indicating that Rictor involved in tumor growth and metastasis. In addition, Kaplan-Meier analysis showed that Rictor positive expression predicted poorer overall and relapse free survival. Previous literature manifested that Rictor over-expression was associated with tumor progression and poor prognosis in hepatocelluar carcinoma [24], endometrial carcinoma [25], and pituitary adenoma [26]. However, several studies argued about the role of Rictor in colorectal cancer [19, 27] and breast cancer [20, 28], making the prognostic value controversial. Our results are consistent with the majority of the former findings, speculating that Rictor positive expression is implicated in progression and metastasis of gastric cancer, which might serve as a novel biomarker and therapeutic target.

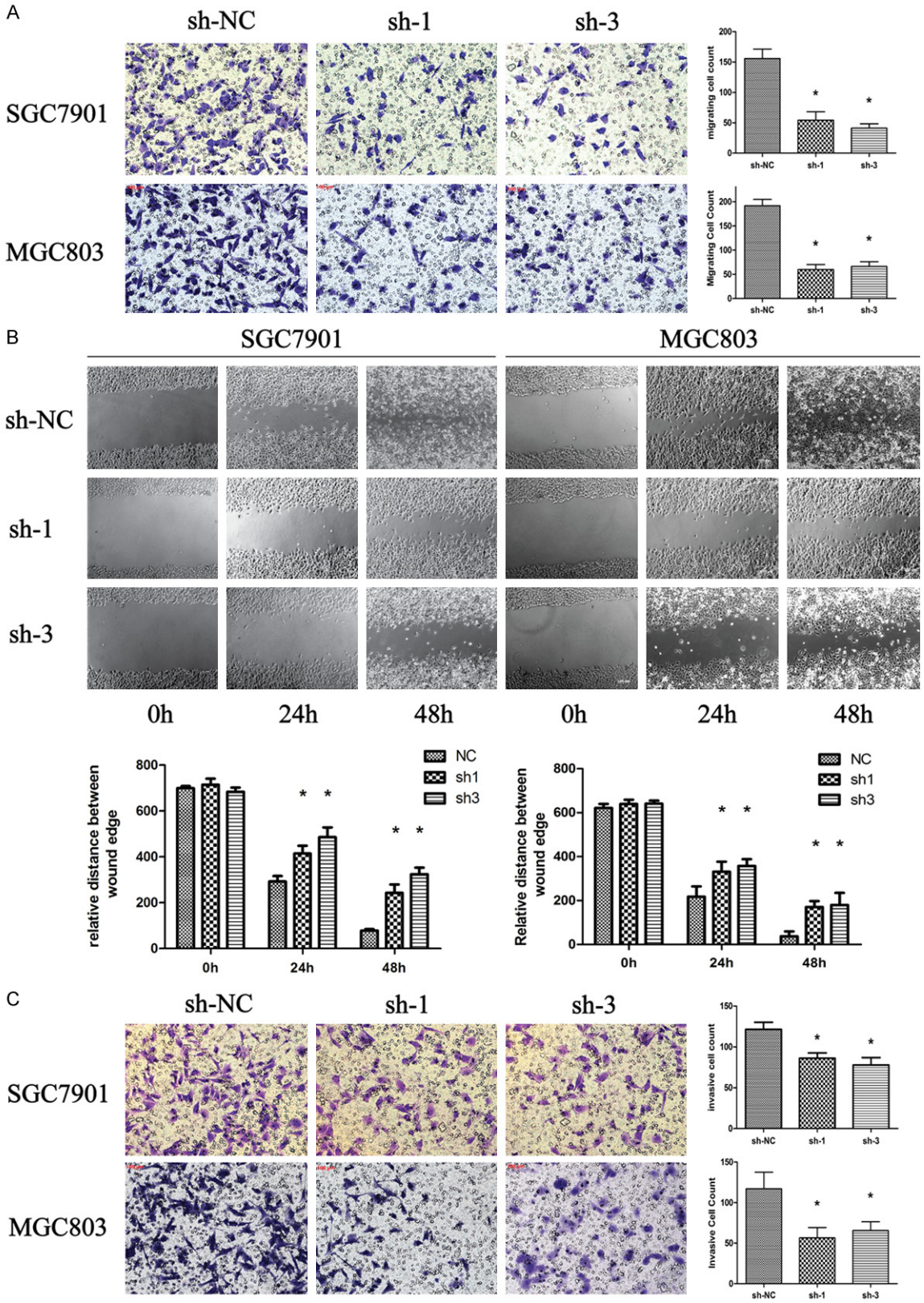
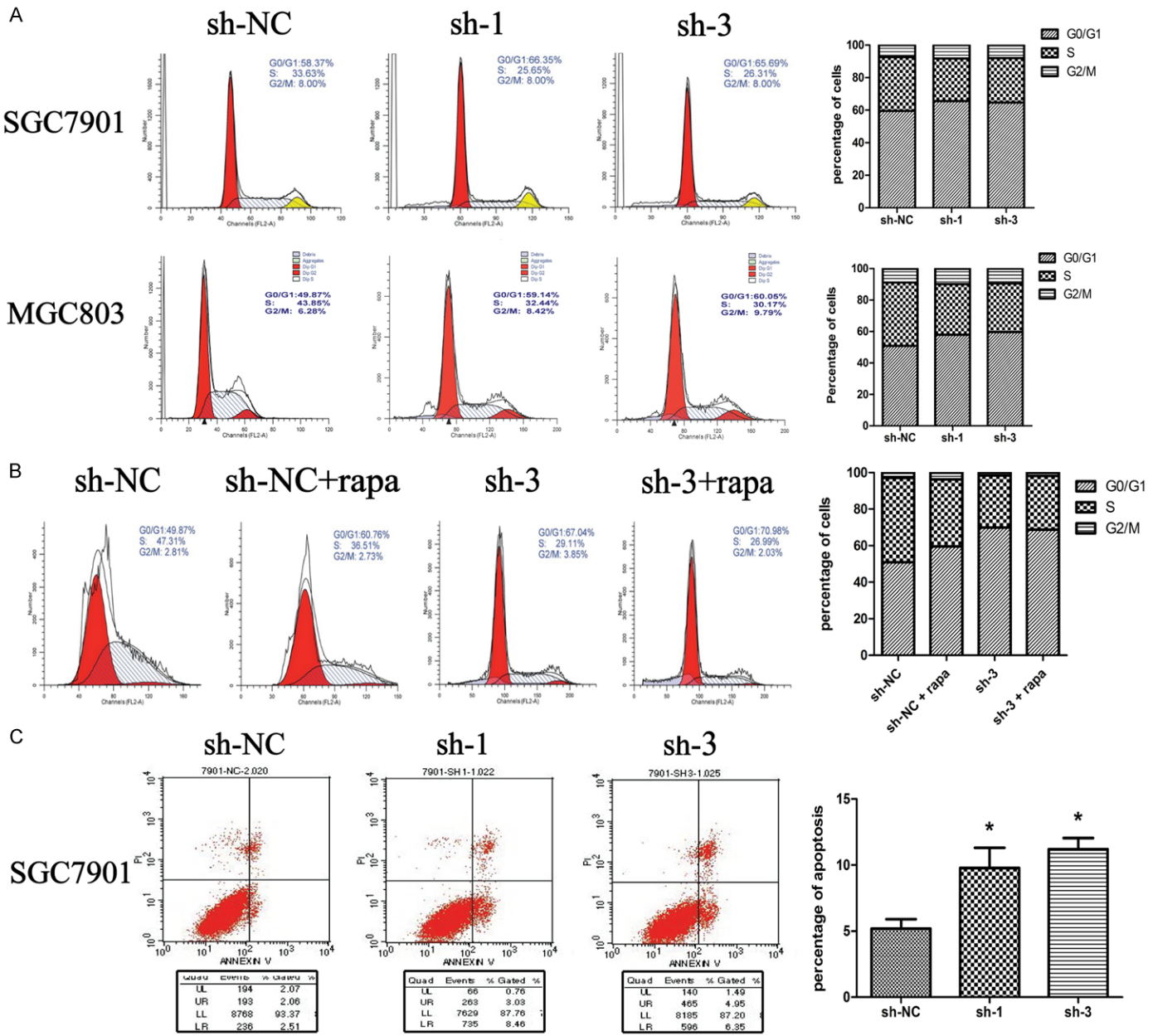


Figure 3. Down-regulation of Rictor attenuates migration and invasion of gastric cancer cells. A. Representative images of transwell migration assay carried out after 24 h of incubation (×200), values were mean ± SD of five independent fields per well. B. Wound healing assay carried out 0 h, 24 h and 48 h after scratch, the gap distance on cell monolayer was measured (×100). C. Transwell invasion assay conducted after 48 h of incubation (×200) (*, *P<0.05* versus sh-NC).


A
sh-NC
sh-1
sh-3
SGC7901
G0/G1:58.37%
S: 33.63%
G2/M: 8.00%
G0/G1:66.35%
S: 25.65%
G2/M: 8.00%
G0/G1:65.69%
S: 26.31%
G2/M: 8.00%
MGC803
G0/G1:49.87%
S: 43.85%
G2/M: 6.28%
G0/G1:59.14%
S: 32.44%
G2/M: 8.42%
G0/G1:60.05%
S: 30.17%
G2/M: 9.79%
percentage of cells
Percentage of cells
G0/G1
S
G2/M
B
sh-NC
sh-NC+rapa
sh-3
sh-3+rapa
G0/G1:49.87%
S: 47.31%
G2/M: 2.81%
G0/G1:60.76%
S: 36.51%
G2/M: 2.73%
G0/G1:67.04%
S: 29.11%
G2/M: 3.85%
G0/G1:70.98%
S: 26.99%
G2/M: 2.03%
sh-NC + rapa
sh-3 + rapa
C
sh-NC
sh-1
sh-3
SGC7901
7901-NC-2.020
7901-SH1-1.022
7901-SH3-1.025
ANNEXIN V
Quad Events % Gated %
UL 194 2.07
UR 193 2.06
LL 8768 93.37
LR 236 2.51
UL 66 0.76
UR 263 3.03
LL 7629 87.76
LR 735 8.46
UL 140 1.49
UR 465 4.95
LL 8185 87.20
LR 596 6.35
percentage of apoptosis

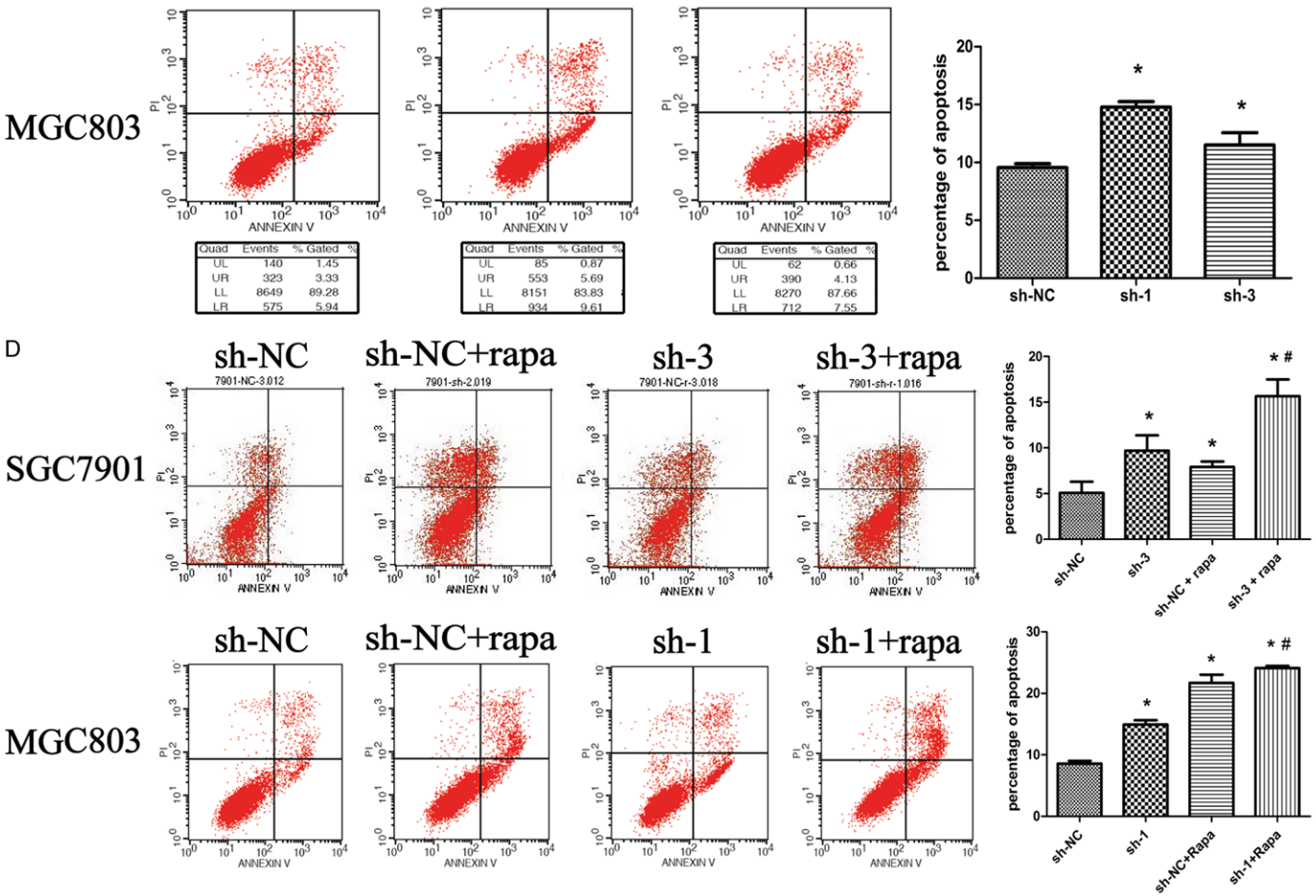


Figure 4. Rictor knockdown promotes cell cycle arrest and enhances rapamycin induced apoptosis in gastric cancer cells. A. Cell cycle progression analysis using flow cytometry showed G0/G1 phase arrest in sh-Rictor cells, the average percentage of distributions was from triplicate wells. B. Analysis of SGC7901 cell cycle progression in sh-NC and sh-3 cells treated with or without rapamycin. C. Knockdown of Rictor induced cell apoptosis assessed by Annexin V/PI staining and flow cytometry, values presented as mean ± SD were determined by triplicate wells (*, *P<0.05* versus sh-NC). D. Compared to the control, sh-Rictor induced cell apoptosis, which was further enhanced by treatment of rapamycin (*, *P<0.05* versus sh-NC; #, *P<0.05* versus sh-Rictor).

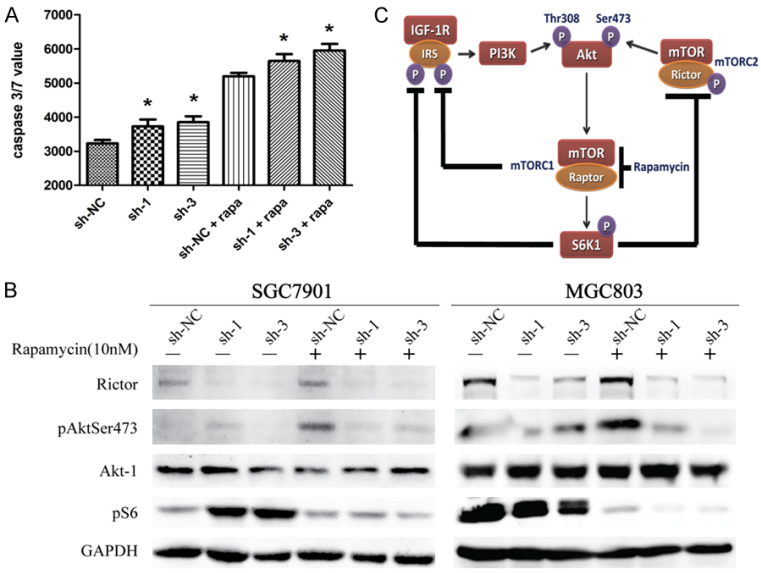


Figure 5. Rictor knockdown blocks feedback activation of Akt induced by rapamycin. A. Caspase 3/7 activity assay in SGC7901 cells treated with or without rapamycin, values were mean ± SD from triplicates (*, *P<0.05* versus sh-NC). B. Western blotting analyses of specific proteins of mTORC1 and mTORC2 pathways in cell lysates treated with or without rapamycin, GAPDH served as loading control. C. Models for the synergistic effects of rapamycin treatment combined with Rictor or mTORC2 inhibition.

Growing evidence has shown that PI3K/Akt/mTOR signaling pathway plays a critical role in pathogenesis of gastric cancer. The present study of Rictor expression by immunohistochemistry in human cancer tissues suggested the hypothesis that targeting Rictor/mTORC2 may attenuate tumor growth. As a result, we found that stable knockdown of Rictor inhibited proliferation of gastric cancer cells in vitro. To gain insight into the mechanism, we analyzed cell cycle profiles and revealed enrichment of G0/G1 phase in Rictor depleted cells compared to controls. In addition, Rictor knockdown promoted apoptosis detected by flow cytometry and caspase 3/7 activity, which coincided with inhibition of Akt phosphorylation, suggesting that tumor growth could be efficiently blunted by Rictor down-regulation. The effects of Rictor on cell proliferation, apoptosis and cell cycle arrest have been observed in melanoma [29], lung cancer [30], and malignant pheochromocytoma [18]. Moreover, over-expression of Rictor contributes to glioma formation through Akt-cyclinD1 and PKCα signaling pathway [31, 32]. Targeting Rictor/mTORC2 as an anticancer therapy is attractive since 68% of gastric cancer patients show elevated Akt levels, and mTORC2 is a critical kinase to phosphorylate Ser473 residue to make full activation of Akt. Furthermore, mTORC2 is dispensable in normal epithelium or cultured mouse embryonic fibroblasts, while it is required under conditions of elevated PI3K activity which occurs in cancer [22]. This indicates that inhibition of Rictor/mTORC2 might be more deleterious to cancer cells than to normal cells, leading to less toxicity by selective mTORC2 inhibitor.

Our results also support the hypothesis that Rictor plays a critical role in gastric cancer metastasis. Pathological investigation indicated that Rictor expression was significantly higher in lymph node positive patients than negative ones, together with a trend of correlation with tumor thrombus. Cancer metastasis is a complex and multi-step progress, including degradation and detachment from extracellular matrix, migration from primary site, and finally invasion into adjacent or distant organs. Previous studies have suggested potential role of Rictor in cancer invasion and metastasis. Signaling pathways via RhoA and Rac1 are known as downstream substrates by which Rictor may regulates epithelial-mesenchymal transition and motility of colorectal cancer [19]. Meanwhile, Rictor knockdown reduces cellular chemotactic capacity and ablates pulmonary metastasis via interacting with PKCζ in breast cancer [20]. On the contrary, up-regulation of Rictor gene transcription by TNFα or IL-6 contributes to renal cell carcinoma invasion in vitro and metastasis in vivo [21]. Recent literature stated that Rictor may act as scaffold protein to form complexes with integrin-linked kinase [33], or may directly regulate cell migration and adhesion excluding the role of mTORC2 [34, 35]. In the present study, we found that knockdown of Rictor attenuated gastric cancer cell migration and invasion associated with mTORC2 specific Akt phosphorylation, which was similar to its role in bladder cancer and glioma [36, 37]. Thus, by activating Akt pathway, Rictor promotes inva-

sion and enhances metastatic ability of gastric cancer cells.

During the last decades, aberrant activation of mTOR has been observed in cancers, and the first generation mTOR inhibitor everolimus has achieved clinical efficacy in renal cell cancer and neuroendocrine tumors [38, 39]. Everolimus also showed inhibitory effect on gastric cancer cell growth in vitro and in vivo. However, the result of phase III clinical trial with everolimus monotherapy in gastric cancer patients was less successful than expected [13]. It is increasingly recognized that rapamycin analogues are partial mTOR inhibitors predominantly targeting S6K1 while does not block mTORC2 and 4E-BP1 sufficiently [12]. In addition, the presence of several negative feedback loops results in activation of PI3K/Akt signaling due to incomplete mTOR inhibition. The effect involves IGF-1R/IRS-1 system and mTORC2, since S6K1 can prevent an inhibitory phosphorylation of IRS-1 [14, 15] and Rictor [40, 41]. In turn, inhibition of mTORC1 impairs the activity of S6K1 on IRS-1 and Rictor, leading to full activation of Akt owing to phosphorylation on both residues at Thr308 and Ser473, respectively (**Figure 5C**). Thus it is rational to speculate targeting both mTOR complexes might be more efficient than targeting individual. Previous study reported that Her-2 expression was up-regulated in gastric cancer cells treated with rapamycin, which was abrogated by using siRNA-Rictor [42]. We also found that knockdown of Rictor achieved similar tumor suppression as rapamycin treatment. Moreover, Rictor down-regulation made further efforts to inhibite cell proliferation, enhance cell cycle arrest and induce apoptosis when co-treated with rapamycin, via blocking the feedback phosphorylation of AktSer473. These results indicated that inhibition of Rictor/mTORC2 may prevent undesired oncogenic effect of mTORC1 inhibitor. Recently, new generation of ATP-competitive and selective mTOR inhibitors targeting both mTORC1 and mTORC2 were under development [43]. These agents showed improvement of antitumor efficacy superior to rapamycin, or could overcome everolimus resistance [18, 44]. Our findings provide the rationale for further investigation toward mTOR kinase inhibitor targeting both mTOR complexes or specifically targeting mTORC2, as an effective therapeutic candidate against gastric cancer in the future [45].

In summary, the current study provide substantial new evidence that Rictor is involved in gastric cancer cell proliferation and metastasis, indicating that Rictor may serve as a novel prognostic marker and therapeutic target for gastric cancer.

Acknowledgements

This study was supported by National Natural Science Foundation of China (No. 81272743). We would like to thank Dr. Ying-Yan Yu and Jun Ji from Shanghai Institute of Digestive Surgery for assistance in immunohistochemistry. We are grateful to Dr. Yan-Yun Gu and Hong-Li Zhang from Shanghai Institute of Endocrine and Metabolic Diseases for their help with in vitro studies.

Disclosure of conflict of interest

None.

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