FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 58, No. 2, 2020
pp. 96–107

ORIGINAL PAPER

VIA MEDICA

Overexpression of Rictor protein and Rictor-*H. pylori* interaction has impact on tumor progression and prognosis in patients with gastric cancer

Fang Wang[1], Xiaoqi Lou[1], Yanfeng Zou[2], Dingtao Hu[1], Jiatao Liu[3], Jie Ning[1], Yang Jiao[1], Zuoyang Zhang[4], Feng Yang[4], Lulu Fan[1], Hanqing Yu[1], Wei Wei[5], Hua Wang[1, 6], Guoping Sun[1*]

[1]Department of Oncology, the First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui, China

[2]Department of Epidemiology and Biostatistics, School of Public Health, Anhui Medical University, Hefei 230032, Anhui, China

[3]Department of Pharmacy, the First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui, China

[4]Department of Pathology, the First Affiliated Hospital of Anhui Medical University, Hefei 230022, Anhui, China

[5]Institute of Clinical Pharmacology, Anhui Medical University, Hefei 230032, Anhui, China

[6]Institute for Liver Diseases of Anhui Medical University, Hefei 230032, Anhui, China

Abstract

Introduction. Growing evidence indicates that Rictor (Rapamycin-insensitive companion of mTOR) is overexpressed across several malignancies and associated with poor survival. However, only limited data indicate that Rictor plays a role in gastric cancer (GC). We sought to explore the prognostic value of Rictor in GC and present interaction analysis between Rictor expression and *H. pylori* status regarding their effects over the prognosis of GC patient.

Materials and methods. 250 GC tissues and 124 lymph node metastases were collected for the detection of Rictor by immunohistochemistry. Cox regression model was used to assess the association between Rictor expression and patient prognosis. Functional experiments were examined in transfected cells using Rictor siRNA. Additive and multiplicative interactions of Rictor and *H. pylori* were evaluated.

Results. In this study, the positive rate of Rictor was 51.6% (129/150) in GC tissues. Multivariate analyses showed that Rictor was independent unfavorable predictor for OS (HR = 1.554, 95% CI = 1.076–2.244, P = 0.019) and DFS (HR = 1.556, 95% CI = 1.081–2.240, P = 0.017). Patients with upregulated Rictor in the primary tumor and lymph node metastases had the worst prognosis. We observed significant additive and multiplicative interactions between Rictor expression and *H. pylori* status for OS and DFS ($P < 0.05$). Our in vitro experiment showed that knockdown of Rictor could suppress cell proliferation, induce apoptosis and inhibit tumor migration and invasion.

Conclusion. Our results demonstrate that Rictor, acting as an oncogene, might be a potential prognostic biomarker and therapeutic target in GC. We suggest that Rictor expression and *H. pylori* status may be a prognostic marker in gastric cancer. *(Folia Histochemica et Cytobiologica 2020, Vol. 58, No. 2, 96–107)*

Key words: gastric cancer; Rictor; *H. pylori*; prognosis; progression; IHC; siRNA

***Correspondence address:** Dr. Guoping Sun,
Department of Oncology,
the First Affiliated Hospital of Anhui Medical University,
218 Jixi Road, Hefei 230022, Anhui, China
phone: +86055162923615, fax +86055162923615
e-mail: sungp@ahmu.edu.cn

Introduction

Cancer remains one of the most important global threats in public health. Recent statistical reports document that gastric cancer (GC) ranks as the fifth most common cancer and the third leading cause of cancer death [1]. Despite progress in the clinical management of GC, treatment of the disease remains challenging owing to the complexity of the disease and difficulty in early diagnosis. For early staged GC, the only curative treatment option is surgical resection [2]. Unfortunately, for patients with advanced-stage due to the poor prognosis, it remains a major clinical concern. The effects of conventional therapies, including chemotherapy and radiation, on survival benefits are limited. Targeted therapeutic strategies, such as angiogenesis and epidermal growth factor receptor (EGFR) inhibitors have exhibited significant antitumor activity [3]. The process of tumorigenesis involves diverse gene alterations, including epigenetic changes, oncogene activation, inactivation of tumor suppressor genes, and abnormal expression of cancer-related genes. Therefore, there is an urgent need for the discovery of novel genes that govern the progression of GC and novel therapeutic targets.

The mammalian target of rapamycin (mTOR) is known as a conserved serine-threonine protein kinase. It could regulate cell growth, metabolism as well as survival [4], and functions through two complexes: mTORC1 and mTORC2 [5]. Rapamycin insensitive companion of mTOR (Rictor) is an essential subunit of mTORC2 complex [6]. Rictor plays a vital role in the feedback loops and cross-talk in the PI3K/AKT/ /mTOR signal transduction pathway. Since Akt activation is essential for many events of the metastatic pathway in cancer, Rictor could serve as a novel therapeutic target. Targeted inhibition of mTORC2 activity through Rictor gene silencing could promote cisplatin-induced apoptosis and prevent cell migration in osteosarcoma and breast cancer [7–8]. Rictor upregulation was associated with invasive behavior and poor prognosis in several malignancies [9–11]. Currently, only a limited number of reports exist regarding the roles of Rictor in GC [10].

There is evidence that *H. pylori* infection is identified as a beneficial prognostic indicator for GC, indicating different growth patterns between *H. pylori* (+) and *H. pylori* (–) GC [12–13]. Meanwhile, the infection of *H. pylori* is associated with mTOR signaling [14–15]. Relatively little is known about whether there is an interaction between Rictor expression and *H. pylori* status in the prognosis of GC.

The objective of the current study was to explore the functional significance of Rictor in primary carcinomas and lymph node metastases, as well as the prognostic value in GC. Here, we also presented interaction analysis between Rictor expression and *H. pylori* status regarding their effects over GC patient prognosis. In addition, we characterized the role of Rictor in GC proliferation, migration, invasion and apoptosis.

Materials and methods

Patients and tissue specimens. This study was conducted in the First Affiliated Hospital of Anhui Medical University, and 250 pathologically diagnosed GC patients who underwent surgery from November 2007 to November 2009 were recruited. The current and our previous studies [12] were conducted based on the same study population, and it should be noted that there can be overlap between the patients of the two studies. We excluded patients with Siewert type I cardia adenocarcinoma and patients who received neoadjuvant treatment before surgery in the study.

In order to collect prognosis data, patients who underwent surgical resection were followed up according to a standard scheme [16]. At the first 2 years after the surgery, patients were followed up every 3 months. At the following 2 years, patients were followed up every 6 months, then yearly afterward. The follow-up program consisted of clinical examination, hematologic analyses, measurement of tumor markers, abdominal ultrasonography and chest radiography (once half a year), and endoscopy of the upper digestive tract (once a year). Computed tomography (CT) or positron emission tomography (PET)/CT was conducted for the surveillance of patients to detect recurrence after surgery. The follow-up plan lasted from the surgery until the date of death or the last date of follow-up via hospital visit or telephone. The follow-up deadline was August 15, 2018. Overall survival (OS) and disease-free survival (DFS) of patients were calculated. Overall survival (OS) was recorded from pathological diagnosis/recruitment to the date of death or the date of last follow-up. Disease-free survival (DFS) was recorded from the date of pathological diagnosis/ /recruitment to the date of disease recurrence or the date of last follow-up. The Ethics Committee of Anhui Medical University approved the study, and all of the participants enrolled in the study gave the written informed consent.

Immunohistochemistry. The H&E stained sections of each tumor were reexamined by a pathologist (ZZY) for histological analyses. Formalin-fixed paraffin-embedded (FFPE) sections of 250 GC tissues and 124 lymph node metastases were obtained for the detection of Rictor by immunohistochemical (IHC) method. Firstly, all the tissue sections were dewaxed with xylene, followed by antigen retrieving in a microwave for 20 min. In order to block endogenous peroxidase activity, we have immersed each slide in 3% hydrogen peroxide for 10 min. Then, these

slides were incubated with the primary mouse monoclonal anti-Rictor antibody (1:700 diluted sc-271081, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Thereafter, at room temperature (RT), these slides were incubated with horseradish peroxidase (HRP) labeled goat anti-mouse/anti-rabbit immunoglobulin (PV-6000, ZSGB Biotechnology, Beijing, China) for 20 min. Finally, the slides were stained with 3,3'-diaminobenzidine (DAB). The Rictor protein level was determined using semi-quantitative IHC detection system [17]. Assessment of IHC staining was performed by two pathologists (YF and ZZY) independently. We chose 10 high power fields (HPF) for each the tissue section and calculated the average number of positive cells. We assessed the percentage of positively stained cells (none = 0, less than 10% = 1, 10% to 50% = 2, greater than 50% = 3) as well as the staining intensity (none = 0, light yellow = 1, pale brown = 2, dark brown = 3). Finally, an immunoreactivity score was calculated using the sum of the percentage score and intensity score, ranging from 0 to 6. The immunoreactivity score of 0–2 was considered negative for Rictor immunostaining, otherwise positive [10].

IHC assay was performed on 250 pairs of GC and non-cancerous (located at least 5 cm from the tumor) tissues for the detection of *H. pylori*. The methods have been described in our previous studies [12]. We considered the patients as *H. pylori* positive if either the tumor or non-tumoral tissues was categorized as positive. Otherwise, if both the tumor and non-tumoral tissues were categorized as negative, we considered the patients as *H. pylori* negative.

Cell culture and transfection. BGC-823 and SGC-7901 human GC cell lines were purchased from the Cell Resource Centre, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China. The cells were grown in the RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) to which 10% fetal bovine serum, penicillin (100 μ/ml) and streptomycin (100 mg/ml) were added. All cells were cultured at 37° and with a humidified atmosphere of 5% CO_2. We used RNA interference (siRNA) (final concentration of 100 nM, GenePharma, Shanghai, China) for Rictor knock-down. According to the manufacturer's protocol, we have plated BGC-823 and SGC-7901 cells in 6-well or 96-well plates at a density of 2×10^5 or $5\sim 6 \times 10^3$ cells per well for transfection, respectively. When cells grew to 40% ~ 60% confluence, they were transfected with 100 nM Rictor siRNA or the corresponding negative control (100 nM, GenePharma) using Lipofectamine 3000 transfection agent (Invitrogen). For example, in 6-well plates, 10 μl of Rictor siRNA was absorbed by RNA free enzyme gun head (Axygen, Tewksbury, MA, USA) and dissolved in 125 μl of Opti-MEM reduced serum culture medium (No:31985-062, GIBCO BRL, Grand Island, NY, USA) for transfection, and gently mixed. 7.5 μl Lipofectamine 3000 transfection agent was absorbed by RNA free enzyme gun head and dissolved in 125 μl of Opti-MEM reduced serum culture medium for transfection, and gently mixed. The reagent was blown and left at RT for 5 min. Then, the transfection mixture was added into the six-well plate. The transfected cells were incubated under 5% CO_2 at 37° for subsequent experiments.

Western blotting. Western blot assays were conducted to screen the specific Rictor siRNA sequence. RIPA buffer and protease inhibitors were used to extract lysates from cultured cells 48 h post-transfection. We have loaded the same amount of cell lysate for electrophoresis. Protein samples were separated using 6% SDS-PAGE. Then, we transferred the samples onto PVDF membranes (Millipore, Bedford, MA, USA) on ice overnight for about 14 h with constant pressure of 70 V, following by blocking with 5% non-fat milk for 2 h at RT. We incubated the membranes with mouse monoclonal anti-Rictor antibody (1:1000; ab56578, Abcam, Cambridge, MA, USA) overnight at 4°C. The membranes were then incubated with HRP-conjugated secondary antibody for 2 h at 37°C. Visualization was performed by using ECL substrate. We used β-actin as the loading control.

Cell viability assay. CCK-8 assay (Bestbio, Beijing, China) was used to measure effects of Rictor siRNA on cell viability. Cell suspensions (100 μl, 3×10^3 cells/well) were seeded into 96-well plates and transfected. After transfection, 10 μl of the CCK-8 reagent was added into each well at 24 h, 48 h and 72 h and the plates were incubated for another 1 h at 37°C. Then we used a microplate reader to record the absorbance at 450 nm. We performed at least three independent experiments. For each time point, each experimental group included six replicate wells.

Transwell cell migration/invasion assays. Transwell assay was used to determine the abilities of cell migration and invasion. We used 24-well plates with poly-carbonate transwell filters (Corning Incorporated, Corning, NY, USA) for the assay. For cell invasion, the membrane of the chamber was pre-coated with 60 μl extracellular Matrigel (Cat. no. 356234, BD Biosciences, San Jose, CA, USA). The extracellular Matrigel was freshly 1:7 diluted with serum-free medium to form a matrix barrier. At 37°C, it was incubated for 3 h before invasion assay. 48 h after the transfection, SGC-7901 cells and BGC-823 cells were harvested, and resuspended in serum-free medium to a concentration of 1×10^5 cells per well. These cells were loaded into each upper chamber with a volume of 100 μl and 750 μl of RPMI-1640 medium containing 10% FBS was added to the lower chamber. They were incubated for 24 h/48 h (SGC-7901 cell/BGC-823 cell) to measure the effects of Rictor siRNA on cell migration potential and incubated for 72 h to assess the effects of Rictor siRNA on cell invasion potential. Then, a cotton swab was used to scrape off the upper surface of the well. We fixed

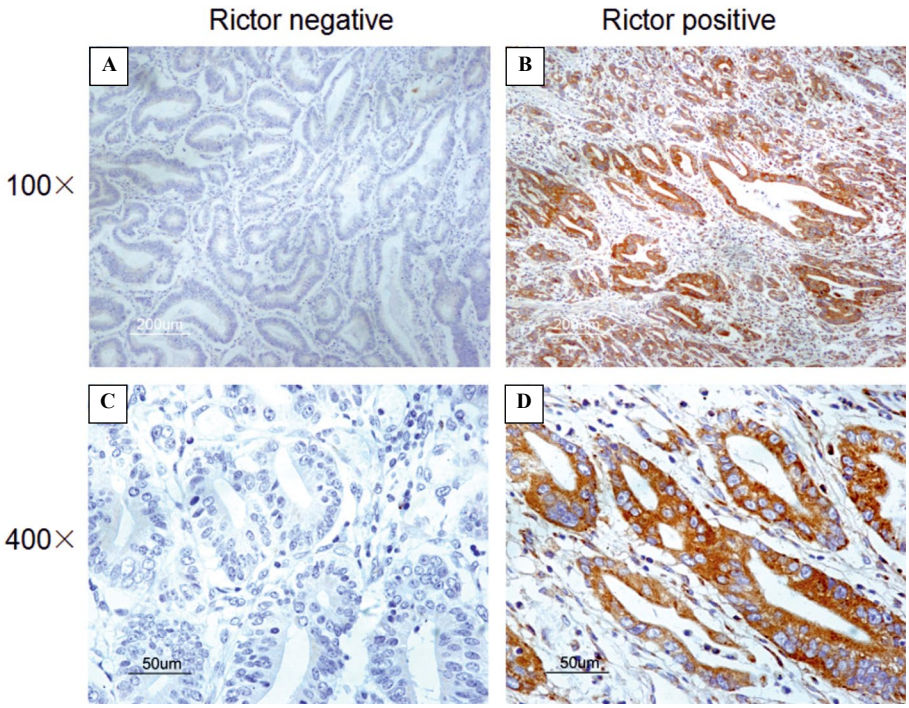


Figure 1. Immunoreactivity of Rictor in gastric cancer tissues. **A.** Negative expression of Rictor in gastric tumor specimens. **B.** Positive expression of Rictor in gastric tumor specimens. **C.** Negative expression of Rictor in gastric tumor specimens. **D.** Positive expression of Rictor in gastric tumor specimens. Magnifications: A and B — 100× C and D — 400×.

cells in 90% ethanol, stained with 0.1% crystal violet and then counted these cells on the lower surface of the well. We randomly selected five low-magnification areas (×100) to calculate the cell numbers.

Apoptosis assays. Annexin-V fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (Bestbio) was used for apoptosis assays by flow cytometry. Cells were collected 48 h after transfection and centrifuged at 300 *g* for 5 min. Then, cells were washed twice in phosphate-buffered saline (PBS) and centrifuged in the same condition. Subsequently, these cells were re-suspended in 400 μl 1 × Binding Buffer. Afterwards, in the dark, these cells were stained with 5 μl Annexin-V FITC and 10 μl PI at 4°C for 15 min. The cells were then subjected to flow cytometry (Cytomics FC 500, Beckman Coulter, Brea, CA, USA) analysis, and quantified by using FlowJo (Version 7.6.2, http://www.flowjo.com/index.php).

Statistical analysis. Assays were independently performed at least three times and one representative experiment data were displayed. The statistical analyses of data were carried out by using SPSS version 13.0 software (SPSS Inc., Chicago, IL, USA). Correlations between Rictor expression level and clinicopathological characteristics of patients were identified by using Chi-square test. For continuous variables, differences among groups were identified by using *t*-test or ANOVA. OS and DFS were assessed by using Kaplan-Meier method. Log-rank test was used to compare the survival distributions between different groups. Univariate and multivariate Cox proportional hazard regression models were used to assess the prognostic value of Rictor in GC. The additive interactions were estimated by using direct counting and Chi-square test. We performed the tests using a 2 × 2 factorial design in order to calculate the attributable proportion (AP) and relative excess risk (RERI) [18–19]. The multiplicative interactions were estimated by using Cox's proportional hazards regression models. *P* value less than 0.05 indicated significantly difference.

Results

Rictor expression in gastric cancer

The expression of Rictor protein in GC tissues was detected by using IHC method. We found that 129 of 250 (51.6%) cancer tissues presented Rictor immunoreactivity (Rictor-IR) which was observed only in the cytoplasm of tumor cells (Fig. 1). Correlations between Rictor status and clinicopathological characteristics of the patients are shown in Table 1. The expression of Rictor was correlated with lymph node metastasis (χ^2 = 9.251, P = 0.002) and TNM stage (χ^2 = 10.840, P = 0.013). The rate of Rictor-IR was 38.2% for stage I, 48.0% for stage II, 62.1% for stage III, 75.0% for stage IV, respectively. The positive

Table 1. Relationships between Rictor expression and clinicopathological factors of patients

Factors	Cases	Rictor positive/n (%)	χ^2-value	P-value
Gender				
Male	191	96 (50.3)	0.580	0.446
Female	59	33 (55.9)		
Age (years)				
≤ 61	125	66 (52.8)	0.144	0.704
> 61	125	63 (50.4)		
WHO Grading				
Well differentiated	7	2 (28.6)	5.038	0.081
Moderately differentiated	78	34 (43.6)		
Poorly differentiated	165	93 (56.4)		
Lauren classification				
Intestinal	161	81(50.3)	0.422	0.810
Diffuse	73	40 (54.8)		
Mixed	16	8 (50.0)		
Location				
Proximal (cardia or fundus)	57	25 (43.9)	1.771	0.183
Distal (corpus, antrum or pylorus)	193	104 (53.9)		
Depth of invasion				
T1	40	15 (37.5)	4.888	0.180
T2	14	6 (42.9)		
T3	170	95 (55.9)		
T4	26	13 (50.0)		
Lymph node				
Negative	126	53 (42.1)	9.251	**0.002**
Positive	124	76 (61.3)		
TNM stage				
I	68	26 (38.2)	10.840	**0.013**
II	75	36 (48.0)		
III	103	64 (62.1)		
IV	4	3 (75.0)		
***H. pylori* status**				
Negative	98	58 (59.2)	3.712	0.054
Positive	152	71 (46.7)		

Abbreviations: WHO — World Health Organization; *H. pylori* — *Helicobacter pylori*.

staining rate of Rictor in GC patients with more advanced stage was significantly higher than in those with low grade stage. The positive staining rate of Rictor was 76 of 124 (61.3%) in lymph node metastasis group, compared to 53 of 126 (42.1%) in non-lymph node metastasis group. We also observed a relatively higher positive rate of Rictor in tissues with poor differentiation ($\chi^2 = 5.038$, P = 0.081) and in tissues without *H. pylori* infection than in tissues with *H. pylori* infection ($\chi^2 = 3.712$, P = 0.054); however, these differences did not reach statistical significance. We did not find significant correlations between Rictor expression and gender, age, Lauren classification, tumor location as well as depth of invasion in GC patients (all P > 0.05). Our results indicated that high Rictor expression was correlated with more

aggressive behavior including advanced stage and lymphatic metastasis.

Prognostic value of Rictor in gastric cancer

We explored the role of Rictor expression in prognosis of GC. The log-rank test was used to assess the association between Rictor expression and the prognosis of patients after surgery. The median OS in patients with positive Rictor status was 78.3 months (95% confidence interval (CI) = 28.8–127.8), while the mean OS was 94.7 months (95% CI = 87.2–102) in patients with negative Rictor status (P = 0.008). The median DFS in patients with positive Rictor status was 65.2 months (95% CI = 28.5–102.0), while the mean DFS was 90.5 months (95% CI = 82.3–98.7) in patients with negative Rictor status (P = 0.006). The OS and DFS curves are shown in Figure 2.

Then, we determined the relationship of Rictor status with DFS and OS in GC patients with surgical resection by using Cox proportional hazard model (Table 2). Univariate analyses indicated that Rictor status was associated with the OS of GC patients, showing that patients with Rictor-IR had relatively shorter survival time (HR = 1.617, 95% CI = 1.131–2.311, P = 0.008). Univariate analyses also showed that patients with Rictor (+) had relatively shorter DFS (HR = 1.631, 95% CI = 1.147–2.319, P = 0.006). When adjusting for gender, age, TNM stage, WHO grading, Lauren classification, invasion depth, tumor location and lymph node metastases, multivariate analyses showed that positive Rictor status was independent predictor for OS (HR = 1.554, 95% CI = 1.076–2.244, P = 0.019) and DFS (HR = 1.556, 95% CI = 1.081–2.240, P = 0.017). Our results revealed that Rictor IHC status may be associated with the prognosis of GC.

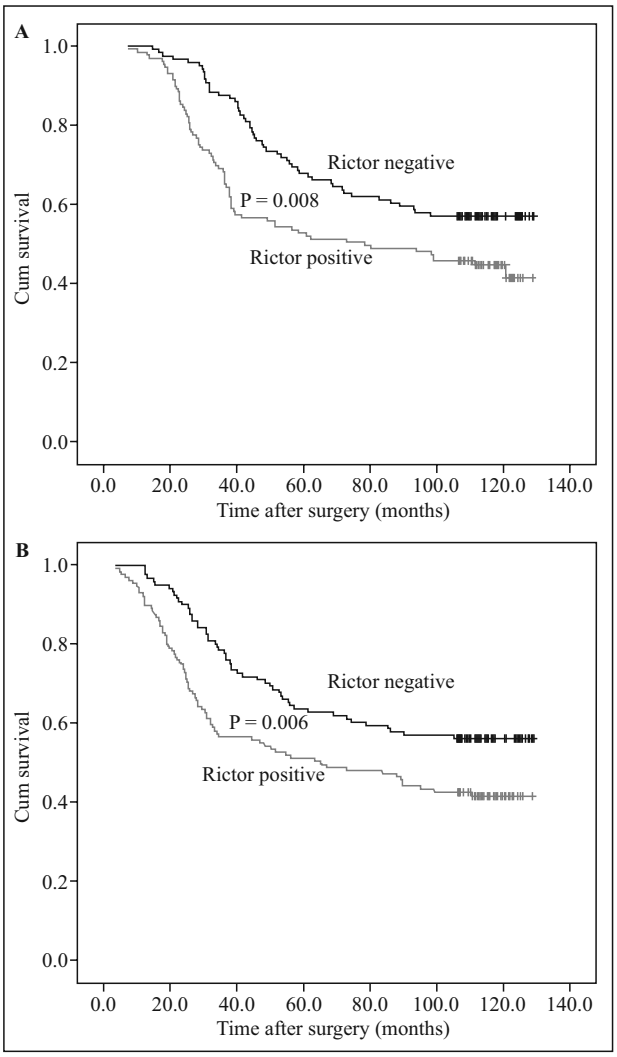


Figure 2. Overall survival (**A**) and Disease-free survival (**B**) in the entire group of 250 gastric cancer patients with respect to Rictor expression assessed by immunohistochemistry.

Rictor expression in primary carcinomas and corresponding lymph node metastases related to clinical outcome

Furthermore, we performed IHC analysis of Rictor on 124 lymph node metastases specimens. In 47 cases, Rictor-IR was found both in primary carcinomas and lymph node metastases whereas in 32 cases we did not detect Rictor-IR in these locations. In 45 cases, the expression of Rictor was found either in primary carcinomas or lymph node metastases. The Rictor-IRs in primary carcinomas and lymph node metastases were consistent ($\kappa = 0.369$, $P < 0.001$).

Then, patients were divided into three groups depending on the status of Rictor-IR in primary carcinomas and lymph node metastases. Univariate analysis revealed obvious survival differences among the three groups, but not significantly different (P = 0.090) (Fig. 3). The median OS of patients with Rictor-IR in both primary carcinomas and lymph node metastases was 33.6 months (95% CI = 22.7–44.4), 68.3 months (95% CI = 30.0–107.0) in patients with lack of Rictor-IR in both primary carcinomas and lymph node metastases (P = 0.036). The median OS of patients with Rictor-IR was 48.9 months (95% CI = 25.1–72.7) in either primary carcinomas or lymph node metastases. Rictor expression both in the primary carcinomas and lymph node metastases was correlated with the worst prognosis of GC patients (Fig. 3).

Additive and multiplicative interaction analysis of Rictor expression and H. pylori infection involved in the prognosis of patients with gastric cancer

H. pylori infection was considered as an independent protective factor for GC patients in our previous studies

Table 2. Univariate and multivariate Cox regression analysis of Rictor expression and survival of patients

Survival	Univariate analysis		Multivariate analysis[a]	
	HR (95% CI)	P-value	HR (95% CI)	P-value
OS				
Rictor negative	1.0 [Reference]	–	1.0 [Reference]	–
Rictor positive	1.617 (1.131–2.311)	**0.008**	1.554 (1.076–2.244)	**0.019**
DFS				
Rictor negative	1.0 [Reference]	–	1.0 [Reference]	–
Rictor positive	1.631 (1.147–2.319)	**0.006**	1.556 (1.081–2.240)	**0.017**

[a]Adjusted for gender, age, TNM stage, WHO grading, Lauren classification, tumor location, depth of invasion and lymph node metastasis. Abbreviations: HR — hazard ratio; CI — confidence interval; OS — overall survival; DFS — disease-free survival.

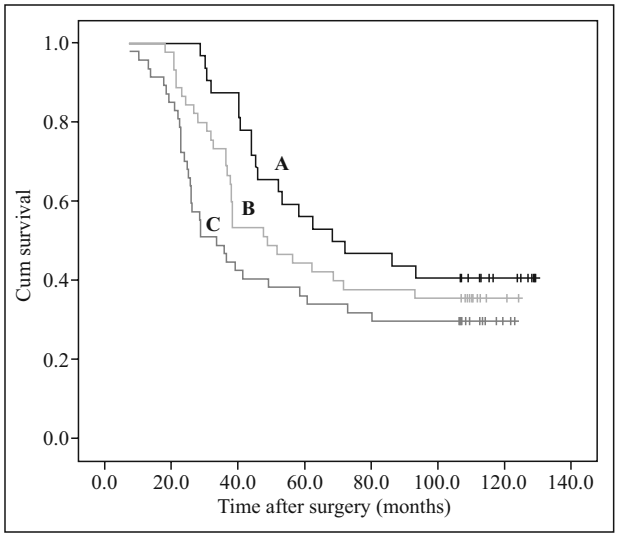


Figure 3. Overall survival of 124 gastric cancer patients with lymph node metastases according to different Rictor status in primary tumor and lymph node metastases (P = 0.090). **A.** Rictor was negative in both primary tumor and lymph node metastases. **B.** Rictor was positive in either primary tumor or lymph node metastases. **C.** Rictor was positive in both primary tumor and lymph node metastases. P = 0.276 (A *vs.* B), P = 0.036 (A *vs.* C), P = 0.209 (B *vs.* C).

[12, 20]. The update survival data resulted in similar findings. In order to explore the interaction between Rictor expression and *H. pylori* infection involved in patient prognosis, additive and multiplicative models were used for analysis. The results of interaction analysis for OS and DFS are shown in Table 3. A significant additive interaction was observed between Rictor expression and *H. pylori* status for OS (HR = 2.586, 95% CI = 1.613–4.146, P < 0.0001) and DFS (HR = 2.662, 95% CI = 1.680–4.218, P < 0.0001). The AP due to interaction was 0.343 for OS and 0.393 for DFS. The RERI due to interaction was 0.886 for OS and 1.047 for DFS. When adjusting for other prognostic factors including gender, age, TNM stage, WHO grading, Lauren classification, invasion depth, tumor location and lymph node metastasis, an additive interaction was still observed between Rictor expression and *H. pylori* status for OS (HR = 2.506, 95% CI = 1.532–4.098, P = 0.0003) and DFS (HR = 2.550, 95% CI = 1.573–4.134, P = 0.0001). The AP due to interaction was 0.413 for OS and 0.433 for DFS. The RERI due to interaction was 1.035 for OS and 1.104 for DFS. We have also observed significant multiplicative interaction between Rictor expression and *H. pylori* status for OS (HR = 2.165, 95% CI = 1.475–3.179, P < 0.0001) and DFS (HR = 2.276, 95% CI = 1.576–3.306, P < 0.0001) by Cox's proportional hazards regression. When adjusting for other prognostic factors including gender, age, TNM stage, WHO grading, Lauren classification, tumor location, invasion depth as well as lymph node metastasis, the results showed a consistent tendency toward a multiplicative interaction between Rictor expression and *H. pylori* status for OS (HR = 2.197, 95% CI = 1.472–3.277, P < 0.001) and DFS (HR = 2.255, 95% CI = 1.525–3.333, P < 0.0001).

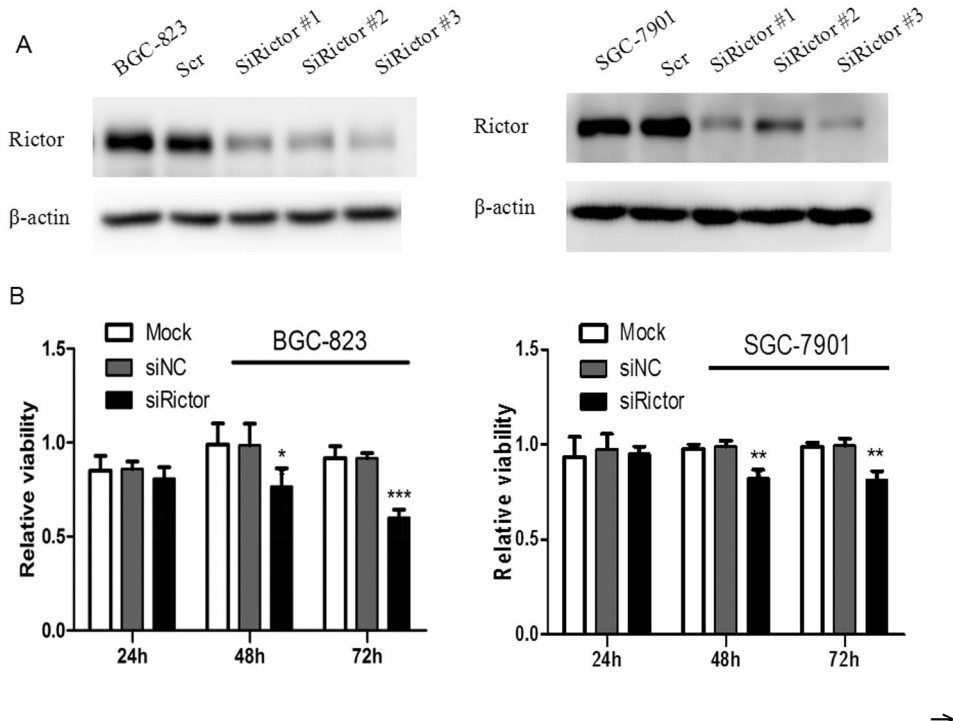
Effects of Rictor on gastric cancer cell proliferation, migration, invasion and apoptosis

To evaluate the implication of Rictor in GC cell proliferation, migration, invasion and apoptosis, specific siRNAs against Rictor were generated for Rictor silencing. As shown in Figure 4A, Rictor expression was inhibited in the cell lines BGC-823 and SGC-7901 by using RNA interference technology. A panel of three Rictor siRNA sequences was transiently transfected into GC cells, screening for the sequence providing the greatest Rictor knockdown. Western blot analysis revealed a dramatically reduction of Rictor protein expression in GC cells 48 h post transfection with siRictor, especially for sequence siRic.3, which was used for all further studies.

Table 3. Proportional hazard models depicting the additive and multiplied interaction between Rictor expression and *H. pylori* status involved in gastric cancer patients' prognosis

Model with interaction term	HR (95%CI)	P-value	HR[a] (95%CI)	P[a]-value
Overall survival (OS)				
Additive				
H. pylori+/Rictor–	1.0 [Reference]	–	1.0 [Reference]	–
H. pylori–/Rictor–	1.355 (0.775–2.369)	0.287	1.230 (0.694–2.181)	0.479
H. pylori+/Rictor+	1.345 (0.830–2.180)	0.229	1.241 (0.760–2.027)	0.389
***H. pylori*–/Rictor+**	2.586 (1.613–4.146)	**< 0.0001**	2.506 (1.532–4.098)	**0.0003**
Multiplied				
*H. pylori**Rictor	2.165 (1.475–3.179)	**< 0.0001**	2.197 (1.472–3.277)	**< 0.001**
Disease-free survival (DFS)				
Additive				
H. pylori+/Rictor–	1.0 [Reference]	–	1.0 [Reference]	–
H. pylori–/Rictor–	1.322 (0.759–2.305)	0.325	1.235 (0.700–2.181)	0.466
H. pylori+/Rictor+	1.294 (0.801–2.088)	0.292	1.210 (0.743–1.972)	0.443
***H. pylori*–/Rictor+**	2.662 (1.680–4.218)	**< 0.0001**	2.550 (1.573–4.134)	**0.0001**
Multiplied				
***H. pylori**Rictor**	2.276 (1.576–3.306)	**< 0.0001**	2.255 (1.525–3.333)	**< 0.0001**

[a]Adjusted for other prognostic factors: sex, age, TNM stage, WHO grading, Lauren classification, tumor location, depth of invasion and lymph node metastasis. Abbreviations: HR — hazard ratio; CI — confidence interval; OS — overall survival; DFS — disease-free survival; H.pylori — Helicobacter pylori.



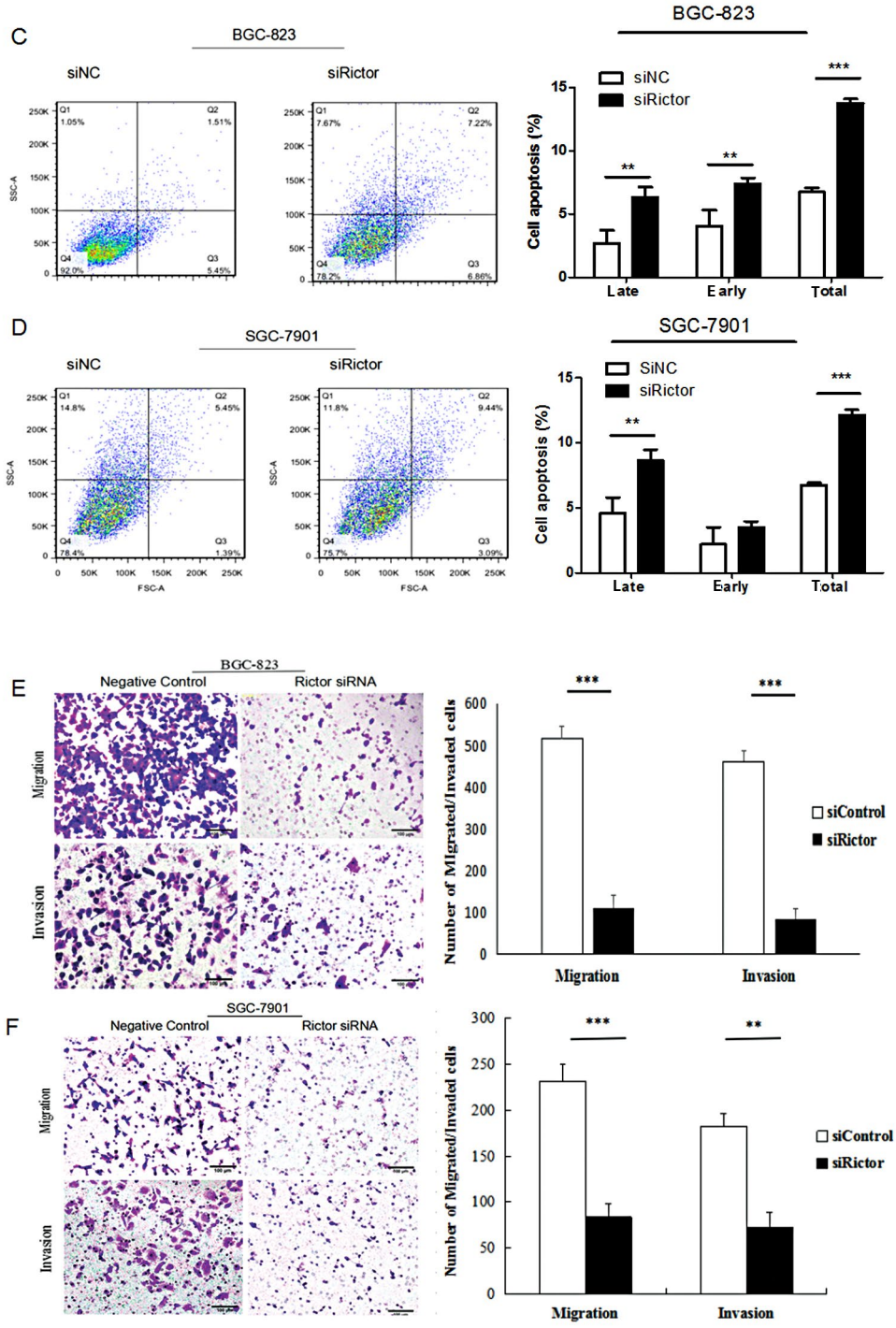


Figure 4. Functional effects of Rictor siRNA on gastric cancer cell proliferation, apoptosis, migration and invasion. **A.** Knockdown of Rictor by siRNA was confirmed by Western blot in BGC-823 and SGC-7901 cells. β-actin served as an internal control. **B.** Suppression of Rictor significantly inhibited gastric cancer cell proliferation. **C–D.** Suppression of Rictor significantly induced cell apoptosis. **E–F.** Suppression of Rictor significantly inhibited the migration and invasion of BGC-823 and SGC-7901 cells. Figure is representative of 3 experiments with similar results. Data represent the mean ± SEM. Bars represent mean and whiskers SD. *P < 0.05, **P < 0.01, ***P < 0.001.

We next examined the biological effects of siRNA--mediated Rictor knockdown on GC cells. CCK-8 assay was used to investigate the functions of Rictor regarding GC cell proliferation (Fig. 4B). Compared with the control group, the proliferation rate in BGC-823 cells transfected with siRictor was significantly

decreased at 48 h and 72 h of incubation ($P < 0.05$). Similarly, the proliferation rate in SGC-7901 cells transfected with siRictor also showed a significant decrease at 48 h and 72 h of incubation ($P < 0.05$). All of these data showed that knockdown of Rictor could impair the growth of GC cells.

Annexin V staining and cytometry analysis were used to assess the effects of Rictor on GC cell apoptosis. Knocking down Rictor expression significantly promoted cell apoptosis in BGC-823 and SGC-8901 cells (Fig. 4C, D). These results indicated that Rictor may act as an oncogene involved in the promotion of GC cell proliferation and inhibition of GC cell apoptosis.

Transwell assays were used to explore the influence of Rictor on GC cell migration and invasion. Migration and invasion assays indicated that pore transfer capacity of GC cells was significantly decreased in cells transfected with siRictor compared to control group (Fig. 4E, F). These results revealed that Rictor played a promotion role in the migration and invasion of GC cells.

Discussion

In this study, Rictor was identified to be often upregulated in GC tissues. We found that overexpression of Rictor was associated with tumor stage and lymph node metastasis. A relatively higher positive rate of Rictor was observed in tissues with poor differentiation and negative *H. pylori* status. Through long period of follow-up, univariate and multivariate analyses revealed that the patients with positive Rictor status had relatively shorter survival time. Patients with Rictor upregulated both in the primary carcinomas and lymph node metastases had the worst prognosis. The additive and multiplicative interactions between Rictor expression and *H. pylori* status involved in patient prognosis were identified for the first time. Our in vitro experiment showed that knockdown of Rictor could suppress GC cell proliferation, induce apoptosis and inhibit tumor migration and invasion. Collectively, our findings demonstrated that Rictor, acting as an oncogene, may be a potential prognostic biomarker and therapeutic target in GC.

In previous studies, elevated Rictor expression was associated with some clinicopathological factors such as age, invasion depth, tumor size, pathological stage, lymph node metastasis and tumor thrombus in some malignancies [9–11]. Consistent with these findings, we also found the association between Rictor expression and TNM stage as well as lymph node metastasis. Rictor was significantly upregulated in advanced-stage GC tissues and lymph node metastases. In the current work, we explored the survival time of different groups of patients depending on the status of Rictor on primary carcinomas and lymph node metastases. Rictor immunoreactivity in primary carcinomas as well as in the corresponding metastatic lymph nodes correlated with the worst clinical outcome. These results suggest that Rictor may be involved in the process of cancer metastases, indicating the pattern of more aggressive behavior which can be harbored on metastases.

To our knowledge, only a few studies [10, 11] focused on the relationship between Rictor expression and GC prognosis. In the study of Bian *et al.*, Rictor expression was not an independent prognostic indicator when adjusting other factors [10]. In our study, we had the chance to explore such an association based on a cohort of GC patients. Several years ago, the GC cohort was established at the study department, and we collected and preserved the gastric tumor as well as normal tissues of GC patients who underwent surgery [12]. Based on the same study population, a long follow-up was possible and the prognosis data of the patients were prospectively recorded. Our data has shown Rictor as an independent unfavorable prognostic indicator for OS and DFS both in univariate and multivariate analysis.

This is the first study to show a synergistic relationship between Rictor overexpression and negative *H. pylori* status in predicting patient prognosis. The current study demonstrated significant additive and multiplicative interactions between Rictor expression and *H. pylori* status. This was suggestive of a much poorer survival for patients whose GC tissues were Rictor positive (+) and *H. pylori* negative (–). In our previous study, *H. pylori* (+) and *H. pylori* (–) GC tissues exhibited different biological behavior and prognosis, indicating different genetic alterations. It can be speculated that Rictor overexpression as potential oncogenic driver for *H. pylori* negative GC. Future studies will be needed to investigate whether patients with GC can be stratified based on Rictor amplification and *H. pylori* infection status to derive clinical benefits from target therapies. The molecular mechanisms need to be further elucidated.

Rictor is a key component of mTORC2, functions in actin cytoskeleton, cell proliferation and survival. Existing evidence has demonstrated the oncogenic roles of mTORC2/Rictor in regulating cancer cell migration, invasion and metastasis in breast, prostate, colorectal cancers and gliomas [21–25]. Cui *et al*. has identified that over-activation of mTORC2 in glioma could promote proliferation and migration of neoplastic cells [23]. Tumor growth of pancreatic cancer can be impaired by the inhibition of mTORC2 component RICTOR [26]. Cheng *et al*. found that patients with

a new subset of lung cancer with Rictor amplification could benefit from mTOR1/2 inhibitors [27]. Similar results were identified across solid tumors by Kim *et al*., which led to further pre-clinical and clinical investigation with AZD2014 in Rictor-amplified GC [28]. Other authors showed that inhibition of mTORC2/ /Rictor in colon cancer reduced cell proliferation *in vitro* and the formation of tumor xenografts *in vivo* [21]. These studies suggested the importance of Rictor in various cancers. In the study, we confirmed the effects of Rictor on GC cells. Consistent with the role of Rictor as a potential cancer promoter, the results showed that the invasion and migration abilities of GC cells were decreased whereas apoptosis of tumor cells was increased when Rictor was knocked down. The proliferation ability of GC cells was also impaired by inhibition of Rictor. It has been viewed as a promising approach for targeting Rictor in GC therapy, since Rictor may play important role in GC development.

Taken together, Rictor may be an unfavorable prognostic factor for GC patients. To our knowledge, this report is the first to demonstrate significant additive and multiplicative interactions between Rictor expression and *H. pylori* status in predicting patient prognosis. In addition, the present study provided the evidence that Rictor functions as an oncogene that facilitates GC cell proliferation and promotes cell migration and invasion. The results suggest therapeutic potential of targeting Rictor in gastric cancer.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (grant number: 81602115 to F. Wang, grant number: 81572430, 81872047 to G. Sun and grant number: 81522009 to H. Wang), the Foundation of Supporting Program for the Excellent Young Faculties in Universities of Anhui Province in China (gxyq2019012).

Conflict of interest

The authors declare no conflict of interest.

References

1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018; 68(6): 394–424, doi: 10.3322/caac.21492, indexed in Pubmed: 30207593.
2. Toiyama Y, Tanaka K, Kitajima T, et al. Serum angiopoietin-like protein 2 as a potential biomarker for diagnosis, early recurrence and prognosis in gastric cancer patients. Carcinogenesis. 2015; 36(12): 1474–1483, doi: 10.1093/carcin/bgv139, indexed in Pubmed: 26420253.
3. Yuan DD, Zhu ZX, Zhang X, et al. Targeted therapy for gastric cancer: Current status and future directions (Review). Oncol Rep. 2016; 35(3): 1245–1254, doi: 10.3892/or.2015.4528, indexed in Pubmed: 26718131.
4. Betz C, Hall MN. Where is mTOR and what is it doing there? J Cell Biol. 2013; 203(4): 563–574, doi: 10.1083/jcb.201306041, indexed in Pubmed: 24385483.
5. Guertin DA, Sabatini DM. Defining the role of mTOR in cancer. Cancer Cell. 2007; 12(1): 9–22, doi: 10.1016/j.ccr.2007.05.008, indexed in Pubmed: 17613433.
6. Sarbassov DD, Ali SM, Kim DH, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol. 2004; 14(14): 1296–1302, doi: 10.1016/j.cub.2004.06.054, indexed in Pubmed: 15268862.
7. Li H, Lin J, Wang X, et al. Targeting of mTORC2 prevents cell migration and promotes apoptosis in breast cancer. Breast Cancer Res Treat. 2012; 134(3): 1057–1066, doi: 10.1007/s10549-012-2036-2, indexed in Pubmed: 22476852.
8. Wang X, Lai P, Zhang Z, et al. Targeted inhibition of mTORC2 prevents osteosarcoma cell migration and promotes apoptosis. Oncol Rep. 2014; 32(1): 382–388, doi: 10.3892/or.2014.3182, indexed in Pubmed: 24840134.
9. Jiang WJ, Feng RX, Liu JT, et al. RICTOR expression in esophageal squamous cell carcinoma and its clinical significance. Med Oncol. 2017; 34(3): 32, doi: 10.1007/s12032-017-0894-5, indexed in Pubmed: 28132115.
10. Bian Y, Wang Z, Xu J, et al. Elevated Rictor expression is associated with tumor progression and poor prognosis in patients with gastric cancer. Biochem Biophys Res Commun. 2015; 464(2): 534–540, doi: 10.1016/j.bbrc.2015.07.001, indexed in Pubmed: 26159923.
11. Wen SY, Li CH, Zhang YL, et al. Rictor is an independent prognostic factor for endometrial carcinoma. Int J Clin Exp Pathol. 2014; 7(5): 2068–2078, indexed in Pubmed: 24966915.
12. Wang F, Sun GP, Zou YF, et al. Helicobacter pylori infection predicts favorable outcome in patients with gastric cancer. Curr Oncol. 2013; 20(5): e388–e395, doi: 10.3747/co.20.1417, indexed in Pubmed: 24155636.
13. Meimarakis G, Winter H, Assmann I, et al. Helicobacter pylori as a prognostic indicator after curative resection of gastric carcinoma: a prospective study. Lancet Oncol. 2006; 7(3): 211–222, doi: 10.1016/s1470-2045(06)70586-1.
14. Li Na, Tang B, Jia YP, et al. CagA Protein Negatively Regulates Autophagy and Promotes Inflammatory Response via c-Met-PI3K/Akt-mTOR Signaling Pathway. Front Cell Infect Microbiol. 2017; 7: 417, doi: 10.3389/fcimb.2017.00417, indexed in Pubmed: 28983474.
15. Hu G, Guo L, Ye G. Helicobacter pylori infection impairs gastric epithelial barrier function via microRNA-100-mediated mTOR signaling inhibition. Mol Med Rep. 2018; 18(1): 587–594, doi: 10.3892/mmr.2018.8971, indexed in Pubmed: 29749451.
16. Marrelli D, Pinto E, Stefano ADe, et al. Clinical utility of CEA, CA 19-9, and CA 72-4 in the follow-up of patients with resectable gastric cancer. Am J Surg. 2001; 181(1): 16–19, doi: 10.1016/s0002-9610(00)00549-3, indexed in Pubmed: 11248169.
17. Gulhati P, Cai Q, Li J, et al. Targeted inhibition of mammalian target of rapamycin signaling inhibits tumorigenesis of colorectal cancer. Clin Cancer Res. 2009; 15(23): 7207–7216, doi: 10.1158/1078-0432.CCR-09-1249, indexed in Pubmed: 19934294.
18. Andersson T, Alfredsson L, Källberg H, et al. Calculating measures of biological interaction. Eur J Epidemiol. 2005;

20(7): 575–579, doi: 10.1007/s10654-005-7835-x, indexed in Pubmed: 16119429.
19. Hughes T, Adler A, Kelly JA, et al. BIOLUPUS Network. Evidence for gene-gene epistatic interactions among susceptibility loci for systemic lupus erythematosus. Arthritis Rheum. 2012; 64(2): 485–492, doi: 10.1002/art.33354, indexed in Pubmed: 21952918.
20. Wang F, Liu J, Zou Y, et al. MicroRNA-143-3p, up-regulated in H. pylori-positive gastric cancer, suppresses tumor growth, migration and invasion by directly targeting AKT2. Oncotarget. 2017; 8(17): 28711–28724, doi: 10.18632/oncotarget.15646, indexed in Pubmed: 28404925.
21. Roulin D, Cerantola Y, Dormond-Meuwly A, et al. Targeting mTORC2 inhibits colon cancer cell proliferation in vitro and tumor formation in vivo. Mol Cancer. 2010; 9: 57, doi: 10.1186/1476-4598-9-57, indexed in Pubmed: 20226010.
22. Gulhati P, Bowen KA, Liu J, et al. mTORC1 and mTORC2 regulate EMT, motility, and metastasis of colorectal cancer via RhoA and Rac1 signaling pathways. Cancer Res. 2011; 71(9): 3246–3256, doi: 10.1158/0008-5472.CAN-10-4058, indexed in Pubmed: 21430067.
23. Cui Y, Zhao J, Yi L, et al. microRNA-153 Targets mTORC2 Component Rictor to Inhibit Glioma Cells. PLoS One. 2016; 11(6): e0156915, doi: 10.1371/journal.pone.0156915, indexed in Pubmed: 27295037.
24. Guan B, Wu K, Zeng J, et al. Tumor-suppressive microRNA-218 inhibits tumor angiogenesis via targeting the mTOR component RICTOR in prostate cancer. Oncotarget. 2017; 8(5): 8162–8172, doi: 10.18632/oncotarget.14131, indexed in Pubmed: 28030804.
25. Werfel TA, Wang S, Jackson MA, et al. Selective mTORC2 Inhibitor Therapeutically Blocks Breast Cancer Cell Growth and Survival. Cancer Res. 2018; 78(7): 1845–1858, doi: 10.1158/0008-5472.CAN-17-2388, indexed in Pubmed: 29358172.
26. Schmidt KM, Hellerbrand C, Ruemmele P, et al. Inhibition of mTORC2 component RICTOR impairs tumor growth in pancreatic cancer models. Oncotarget. 2017; 8(15): 24491–24505, doi: 10.18632/oncotarget.15524, indexed in Pubmed: 28445935.
27. Cheng H, Zou Y, Ross JS, et al. RICTOR Amplification Defines a Novel Subset of Patients with Lung Cancer Who May Benefit from Treatment with mTORC1/2 Inhibitors. Cancer Discov. 2015; 5(12): 1262–1270, doi: 10.1158/2159-8290.CD-14-0971, indexed in Pubmed: 26370156.
28. Kim ST, Kim SY, Klempner SJ, et al. Rapamycin-insensitive companion of mTOR (RICTOR) amplification defines a subset of advanced gastric cancer and is sensitive to AZD2014-mediated mTORC1/2 inhibition. Ann Oncol. 2017; 28(3): 547–554, doi: 10.1093/annonc/mdw669, indexed in Pubmed: 28028034.

Submitted: 8 January, 2020
Accepted after reviews: 16 June, 2020
Available as AoP: 25 June, 2020