Knockdown of Rap1b Enhances Apoptosis and Autophagy in Gastric Cancer Cells via the PI3K/Akt/mTOR Pathway

Yazhou Li,*† Yang Liu,‡ Feiyu Shi,† Liang Cheng,† and Junjun She†

*Department of Interventional Radiology, Hi-Tech People Hospital, BaoJi, China
†Department of General Surgery, First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China
‡Department of Orthopaedics, Honghui Hospital, Xi'an Jiaotong University, Xi'an, China

Gastric cancer (GC) is the fourth most common malignancy and the second leading cause of cancer mortality around the world. However, the regulatory mechanisms of GC tumorigenesis and cancer cell motility are completely unknown. We investigated the role of a RAS-related protein (Rap1b) in the progression of GC. Our results showed that the expression of Rap1b is aberrantly upregulated in GC tissue samples and human GC cell lines, and the high expression of Rap1b indicated a positive correlation with poor prognosis in patients with GC. Inhibition of endogenous Rap1b dramatically reduced the cell cycle progression but strongly enhanced the apoptosis capacity of human GC cell lines MKN-28 and SGC-7901 cells compared with the control group. Western blotting assay showed that Rap1b inhibition resulted in a significant increase in the ratio of LC3-II to LC3-I, and the levels of p62 protein were decreased in both MKN-28 and SGC-7901 cells. Furthermore, PI3K/Akt/mTOR activation was found to be maintained in a low level in the normal gastric mucosal epithelial cells, while it was significantly upregulated in GC cells, which could be decreased by Rap1b inhibition. The PI3K inhibitor LY294002 was enhanced but activator insulin-like growth factor 1 (IGF-1) blocked the Rap1b silencing-induced enhancement of apoptosis and autophagy in MKN-28 and SGC-7901 cells. In conclusion, we demonstrate that Rap1b expression is aberrantly increased in GC, resulting in the inhibition of autophagy and apoptosis of GC cells by the PI3K/Akt/mTOR pathway. This might provide a new understanding and represent a novel therapeutic target for human GC.

Key words: Gastric cancer (GC); Rap1b; Autophagy; Apoptosis; PI3K/Akt/mTOR pathway

INTRODUCTION

Gastric cancer is the fourth most common malignancy and the second leading cause of cancer mortality around the world. Gastric cancer has a high migratory potential and a poor prognosis, with a 5-year survival rate of 20% (1). According to the difference in clinicopathological entities, gastric cancer can be classified with multiple histological subtypes, including adenocarcinomas (approximately 90%), non-Hodgkin's lymphomas and leiomyosarcomas (10%), and adenosquamous, undifferentiated carcinomas, squamous, choriocarcinomas, rhabdomyosarcomas, carcinoid tumors, hemangiopericytomas, and others (rare) (2). Although there are distinct clinical and genetic features, most subtypes of gastric cancer are malignant tumors of the stomach (3). Despite significant improvements in surgery and radiotherapy/chemotherapeutic treatments, the prognosis of patients with gastric cancer is very poor with a high mortality. Therefore, more knowledge about the pathology of

gastric cancer is needed to develop the therapeutic strategy for human gastric cancer.

Rap1b is an important member of the small GTPase Ras family. As a molecular switch, Ras facilitates the alternation between inactive guanosine diphosphate (GDP) and active guanosine 5′-triphosphate (GTP), and plays a crucial role in the regulation of cell growth and differentiation. It is reported that Rap1 can modulate the activation of the ERK/MAP kinase pathway via B-Raf to regulate the cell–matrix adhesion and cell–cell junctions by activating integrins via RAPL and RIAM (4). The available data indicate that Rap1b expression is aberrantly altered in various cancer-related diseases and involved in the pathogenesis of cancers, including human colorectal carcinoma, esophageal squamous cell carcinoma, squamous carcinomas, and ovarian cancer (5–7). In terms of gastric cancer, Rap1b was recently identified to be highly expressed and is significantly associated with malignant behavior and poor prognosis in gastric

cancer (8). However, the specific role and potential mechanism of Rap1b in human gastric cancer tumorigenesis and cancer cells motility are not clear.

Autophagy and apoptosis, two different types of programmed cell death, occur in multicellular organisms and have important roles in maintaining metabolic homeostasis. By "self-eating" or "self-killing," autophagy and apoptosis caused by stress can degrade the damaged protein and organelles to make damaged cells survive or program death. The dysfunction of autophagy and apoptosis has severe pathophysiological consequences and is significantly associated with tumor angiogenesis (9–11). In the present study, we aimed to determine the expression of Rap1b in human gastric cancer tissues and cells. Furthermore, we addressed whether and how Rap1b affects the autophagy and apoptosis of gastric cancer cells in vitro. We also investigated the interaction between Rap1b and the PI3K/Akt/mTOR pathway to further elucidate the potential role of Rap1b in gastric cancer.

MATERIALS AND METHODS

Human Gastric Cancer Samples and Cell Cultures

Frozen normal gastric tissues (12 cases) and gastric cancer tissue samples (44 cases) were obtained from Hi-Tech People Hospital. The protocol was approved by the Ethics Committee of the Hi-Tech People Hospital. All patients gave informed consent.

The human gastric cancer cell lines MKN-45 and MKN-28 and gastric adenocarcinoma cell lines SGC-7901 and AGS were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in DMEM containing 10% fetal bovine serum (FBS; Invitrogen) at 37°C in 5% CO_2 atmosphere. Human normal gastric mucosal epithelial cell line GES1 was obtained from ATCC and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco/Invitrogen) at 37°C.

Quantitative RT-PCR

Total RNA was isolated from tissues or cells using TRIzol. The expression of Rap1b was measured using the CellAmp Direct RNA Prep kit for qPCR and Protein Analysis Kit (Takara, Dalian, China). Ct values of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as the internal control to normalize the relative expression of target genes. Relative expression levels were presented using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting

Total protein was isolated from tissues or cell lysates using lysis buffer. Equal amounts of protein were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Invitrogen) and then were transferred to polyvinylidene difluoride (PVDF) membranes. Primary

antibodies [rabbit anti-Rap1b, mTOR p-mTOR (S2481), 1:1,000 dilution (Abcam); mouse anti-LC3-II, LC3-I, 1:2,000 dilution (VIVA Bioscience); rabbit anti-p62, 1:1,000 dilution (Santa Cruz Biotechnology); rabbit anti-phospho-Akt, anti-total Akt, 1:1,000 dilution (Abcam); mouse anti-p85, 1:1,000 dilution (Abcam); mouse anti-β-actin, 1:2,500 dilution (ABclonal)] were reacted with the blots overnight at 4°C. Then secondary antibodies were incubated with the blots for 1 h, followed by ECL (Amersham Pharmacia, Piscataway, NJ, USA).

Cell Transfection

The Rap1b siRNA and nontargeting negative control siRNA were purchased from Santa Cruz Biotechnology. Cells were cultured to approximately 80% confluence and then transfected with specific siRNA or negative control siRNA by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell Cycle Analysis

Transfected cells were cultured for 48 h, harvested with ice-cold phosphate-buffered saline, and then resuspended in binding buffer containing 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, 10 mM HEPES (pH 7.4), and 5% bovine serum albumin with 5×10^6 cells in a volume of 250 μl. Cells were then incubated with 0.6 μg/ml propidium iodide (PI) for 15 min in the dark. The FACSCalibur system (Becton Dickinson) was applied to analyze the cell cycle.

Cell Apoptosis Assay

Transfected cells were cultured for 48 h and were maintained in the serum-free DMEM for another 12 h. Cells were harvested with ice-cold phosphate-buffered saline and then resuspended in binding buffer with 5×10^6 cells in a volume of 250 μl. Cells were then incubated with 0.6 μg/ml PI and 0.5 μg/ml annexin V-fluorescein isothiocyanate (FITC) for 15 min in the dark. The FACSCalibur system (Becton Dickinson) was applied to analyze the apoptosis of cells.

Statistical Analysis

Data are expressed as mean ± SEM. The differences between groups were measured by GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). All experiments were performed at least three times. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Rap1b Is Overexpressed in Human Gastric Cancer Tissue and Cell Lines

To identify whether Rap1b has a regulatory role in the progression of human gastric cancer, we first detected the expression profile of Rap1b in gastric cancer tissues from

patients by reverse transcription polymerase chain reaction (RT-PCR) analysis. As shown in Figure 1A, Rap1b was expressed at low levels in normal gastric tissues, whereas it was aberrantly upregulated in the gastric cancer tissues. Additionally, the upregulation of Rap1b expression showed a positive correlation with poor prognosis in patients with gastric cancer, as seen in Figure 1B.

We further investigated the expression of Rap1b in human gastric cancer cell lines MKN-45 and MKN-28 and gastric adenocarcinoma cell lines SGC-7901 and AGS. Compared to the normal gastric mucosal epithelial cell line GES1, the levels of Rap1b were markedly increased in all gastric cancer cell lines (Fig. 1C). These results suggest that the upregulation of Rap1b expression might be a general character in gastric cancer.

Knockdown of Rap1b Enhances the Apoptosis of Gastric Cancer Cells In Vitro

We then employed Rap1b siRNA knockdown to further evaluate the effects of high Rap1b levels on the tumorigenesis of human gastric cancer. Human gastric cancer cell lines MKN-28 and SGC-7901 were chosen to be the objects of our study in vitro according to the difference of Rap1b expression in all four gastric cancer cell lines. Compared to the nontargeting negative control siRNA and control groups, the expression of Rap1b was significantly decreased with a loss of approximately 60% in MKN-28 and SGC-7901 cells transfected with specific siRNA, as measured by RT-PCR analysis (Fig. 2A). Further, the suppression of endogenous Rap1b dramatically reduced the cell cycle progression in MKN-28 and SGC-7901 cells in comparison with the control groups (Fig. 2B). On the contrary, the apoptosis capacity of

MKN-28 and SGC-7901 cells was strongly enhanced by Rap1b silencing, as shown in Figure 2C. These data indicate that high levels of endogenous Rap1b might play a positive role in the development of gastric cancer by promoting gastric cancer cell cycle progression and inhibiting cancer cells apoptosis.

Knockdown of Rap1b Enhances the Autophagy of Gastric Cancer Cells

Autophagy is a cellular stress adaptation response. We next investigated whether the autophagy of gastric cancer cells is affected by endogenous Rap1b. The ratio of LC3-II to LC3-I is a reliable indicator of autophagy. As seen in Figure 3, Rap1b inhibition resulted in a significant increase in the ratio of LC3-II to LC3-I in both MKN-28 and SGC-7901 cells compared to the control. We also measured the levels of p62 protein, a negative indicator of autophagy, in MKN-28 and SGC-7901 cells. A decrease of p62 levels was found in cultured gastric cancer cells transfected with Rap1b siRNA in comparison with the control. Taken together, these data suggest that high Rap1b levels might be a potential suppressor for cell autophagy in gastric cancer.

PI3K/Akt/mTOR Pathway Is Involved in the Regulation of Rap1b to Gastric Cancer Cells Motility

The PI3K/Akt/mTOR signaling pathway is known to be important in tumor metastasis and angiogenesis during cancer progression (12). To evaluate the possible mechanism by which Rap1b affects the apoptosis and autophagy capacity in gastric cancer, we analyzed the expression profile of key molecules in the PI3K/Akt/mTOR signaling pathway by Western blot. As seen in
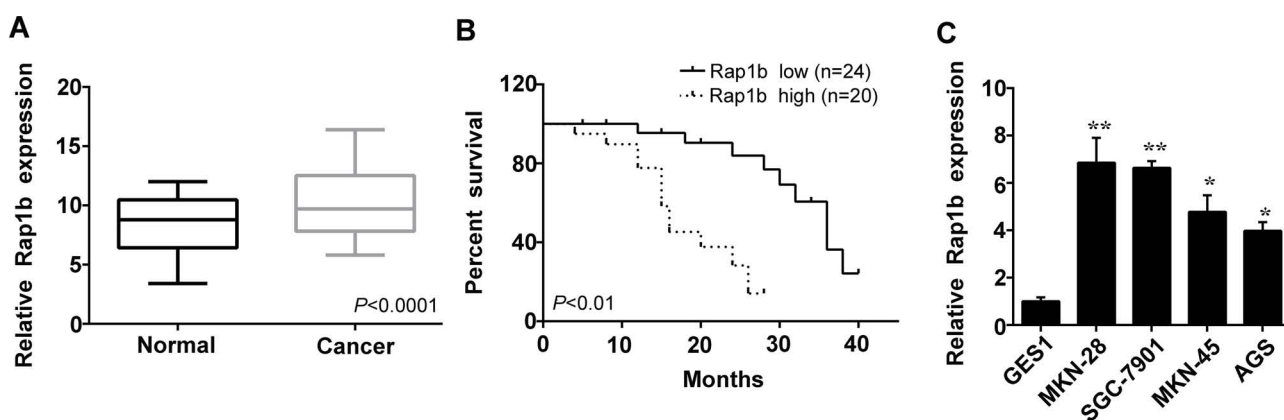

Figure 1. Expression of Rap1b in human gastric cancer tissues and cells. (A) Reverse transcription polymerase chain reaction (RT-PCR) was used to measure the expression levels of Rap1b in normal gastric tissues (Normal) and gastric cancer tissues. (B) The correlation between Rap1b levels and the survival rate in patients with gastric cancer was analyzed using the Kaplan–Meier test. Rap1b high indicates high Rap1b mRNA levels (more than threefold higher than normal tissue), and Rap1b low represents low Rap1b mRNA levels (less than threefold). (C) The levels of Rap1b in the human gastric cancer cell lines MKN-45, MKN-28, SGC-7901, and AGS as well as in normal gastric mucosal epithelial cell line GES1 were examined using RT-PCR. $*p < 0.05$ and $**p < 0.01$ versus GES1.
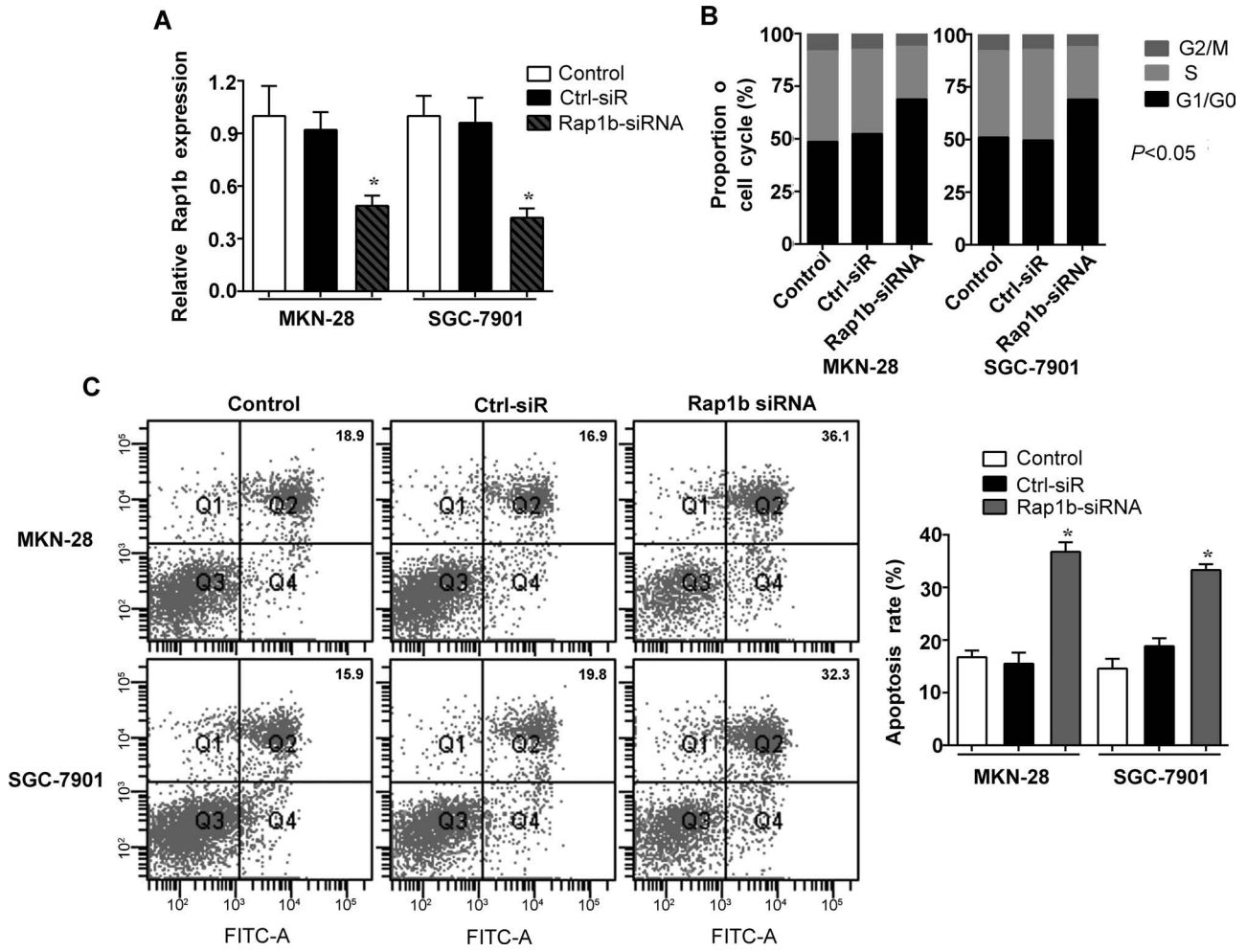
Figure 2. Knockdown of Rap1b enhances the apoptosis of gastric cancer cells. MKN-28 and SGC-7901 cells were cultured (Control), followed by transfection with Rap1b siRNA or nontargeting control siRNA (Ctrl-siR). (A) The expression of Rap1b was measured after 48 h by RT-PCR to confirm the efficiency of Rap1b silencing. (B) The cell cycle and (C) apoptosis of cells were tested by flow cytometric analysis. *$p < 0.05$ versus Control.
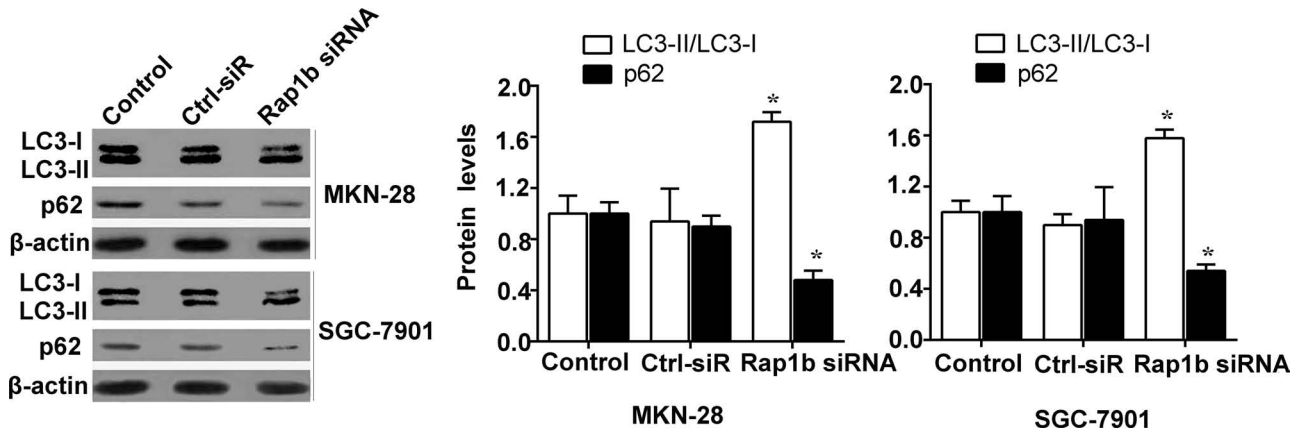

Figure 3. Knockdown of Rap1b enhances the autophagy of gastric cancer cells. MKN-28 and SGC-7901 cells were cultured (Control), followed by transfection with Rap1b siRNA or nontargeting control siRNA (Ctrl-siR). The expression of LC3-I, LC3-II, and p62 protein was detected by Western blotting. *$p < 0.05$ versus Control.

Figure 4A, the p85 subunit of PI3K was expressed in a low level in the normal gastric mucosal epithelial cell line GES1, while it was significantly upregulated in the gastric cancer cell lines MKN-28 and SGC-7901. In addition, Rap1b inhibition resulted in a significant decrease in the expression of p85 in MKN-28 and SGC-7901 cells. A similar change in the pattern of phosphorylated Akt levels was also observed in MKN-28 and SGC-7901 cells, although there is no significant change in the levels of total Akt. Consistently, the activation of mTOR was also enhanced in gastric cancer cells but reduced by Rap1b silencing.

Further, we applied the PI3K inhibitor LY294002 and activator insulin-like growth factor 1 (IGF-1) to verify the role of PI3K/Akt/mTOR signaling. The Rap1b silencing-induced enhancement of apoptosis and autophagy in MKN-28 and SGC-7901 cells was enhanced by LY294002 but was blocked by IGF-1 treatment (Fig. 4B and C). These data suggest that the PI3K/Akt/mTOR pathway could be activated by Rap1b overexpression in gastric cancer cells and be involved in the regulation of Rap1b to gastric cancer cells motility.

DISCUSSION

As a member of the small GTPase Ras family, Rap1b has been demonstrated to be aberrantly expressed and involved in the development of several cancers (13). In colorectal carcinoma, Rap1b was found to be directly targeted by miR-100 and miR-139 and overexpressed in cancer tissues, contributing to the proliferation and invasion of human colorectal cancer cells (6,14). Highly expressed Rap1b is also associated with the enhanced
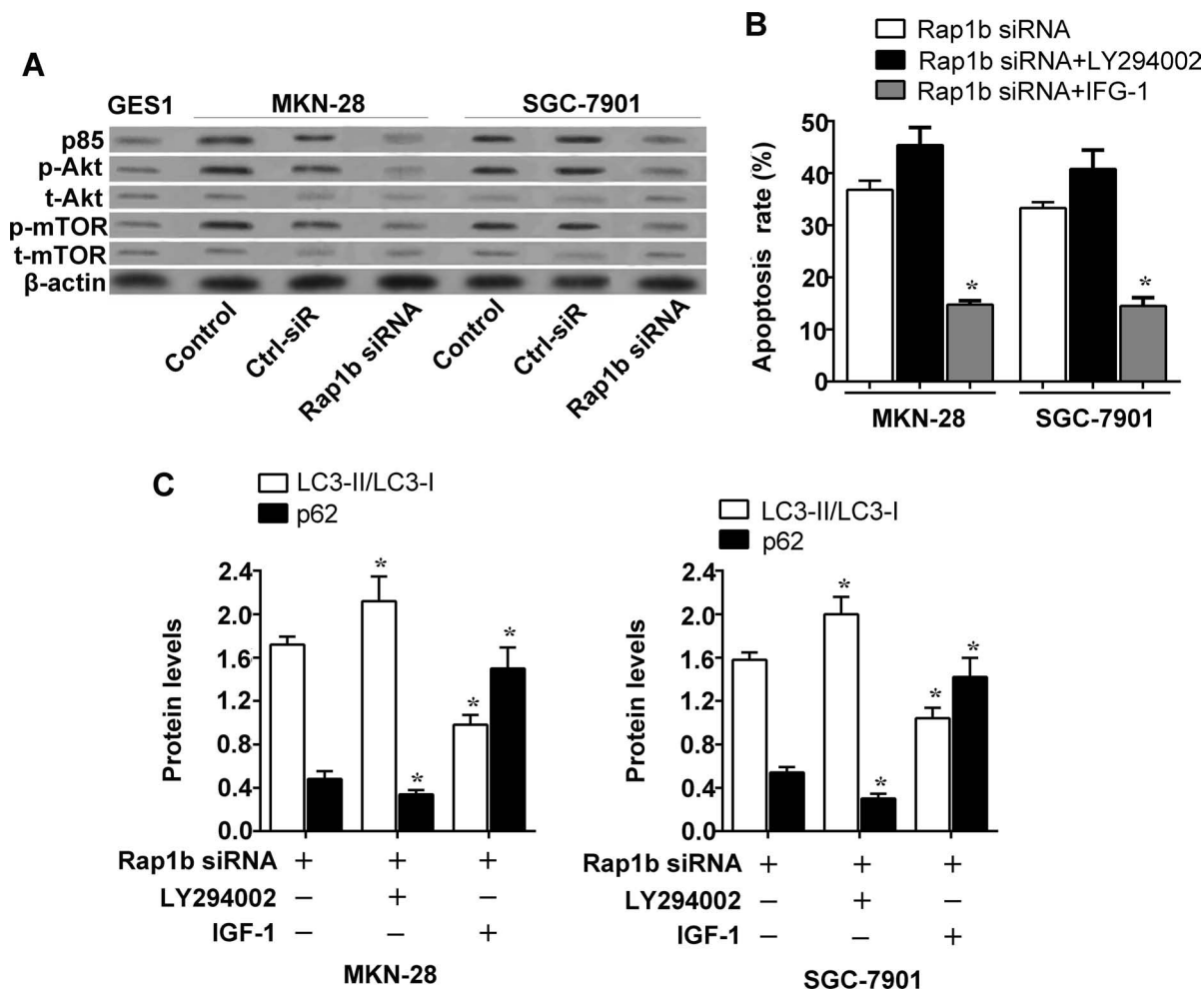

Figure 4. Effect of Rap1b on the PI3K/Akt/mTOR pathway in gastric cancer cells. (A) The levels of p85, total Akt, phosphorylated Akt, total mTOR, and phosphorylated mTOR were tested by Western blotting in MKN-28 and SGC-7901 cells as well as GES1. (B and C) MKN-28 and SGC-7901 cells were transfected with Rap1b siRNA, followed by treatment with 200 nM LY294002 (AbMole, Shanghai, China) or 10 ng/ml IGF-1 (R&D Systems China, Shanghai, China) for 12 h. Apoptosis assay was performed on cells, and cell extracts were prepared for Western blotting using antibody specific for LC3-I, LC3-II, and p62. *$p < 0.05$ versus Rap1b siRNA.

proliferation and invasion as well as repressed apoptosis in esophageal squamous cell carcinoma, which is inhibited by miR-518b (5). Lin et al. reported that glucocorticoid-induced miR-708 could result in the reduction of invasion and abdominal metastasis in ovarian cancer cells by directly targeting Rap1b (7). In this study, we showed that Rap1b is aberrantly upregulated in the tumor tissues of gastric cancer patients as well as in four human gastric cancer cell lines. Notably, high levels of Rap1b presented a positive relationship with the poor prognosis of gastric cancer patients. These observations are in line with a previous study demonstrating that highly expressed Rap1b contributed to the gastric cancer malignant behavior and poor prognosis (8).

Apoptosis is an important process of programmed cell death. Disruption of cell apoptosis is one of the basic pathologies involved in the carcinogenesis of human carcinomas, including human gastric cancer (15,16). In a previous study, the effect of Rap1b on cell apoptosis has been reported. Sun et al. found that Rap1b moderates the glucose-induced apoptosis in renal tubular cells by ameliorating the dysfunction of mitochondria (17). In the present article, we found that the reduced apoptotic capacity of gastric cancer cells was significantly increased by Rap1b repression. Additionally, the cell cycle progression of cancer cells was inhibited by Rap1b silencing, indicating the effect of Rap1b on cell apoptosis in the progression of gastric cancer.

Another approach we took to identify the role of Rap1b was to investigate the effect of Rap1b on autophagy in gastric cancer cells. Autophagy is another type of programmed cell death and is significantly associated with metabolic homeostasis (18). In the initial phase, cell responds to the stimulus signals and forms the autophagosome, in which the lysosomal membrane is fused to deliver those damaged contents, including impaired proteins and organelles, for further degradation (15,18). During the process of autophagy, the ratio of LC3-II to LC3-I is a reliable indicator of autophagy. In the autophagosome, LC3-I is fused with phosphatidylamine to form LC3-phosphatidylamine (LC3-II), which is essential for autophagosome activity. The ubiquitin binding protein p62 is a negative indicator of autophagy, and it is accumulated when autophagy is disrupted (19,20). Increasing evidence demonstrates that the disruption of autophagy contributes to the development of tumor angiogenesis (21,22). On the contrary, induced autophagy by the specific ingredient celecoxib or celastrol could inhibit cancer cell growth by induction of autophagy (12,16). Herein, we hypothesized that Rap1b might also regulate autophagy in gastric cancer cells and found that highly expressed Rap1b is associated with the inhibition of autophagy. When Rap1b expression is suppressed, the autophagy capacity is upregulated in gastric cancer cells.

Previous studies have reported that apoptosis and autophagy signaling can be activated by various pathways. The PI3K/Akt/mTOR pathway is a key signaling pathway with pleiotropic functions and has been demonstrated to be involved in many malignant tumors (12,23,24). In tumorigenic cells, PI3K could be activated by hormones, growth factors, and other stimuli. The activated PI3K produces a messenger PIP3, which makes inactive Akt undergo a conformational shift (25,26). Then the Ser124/Thr450 and Thr308/Ser473 sites are successively phosphorylated, resulting in the activation of Akt signal. Activated Akt further triggers the phosphorylation activation of mTOR to regulate cell growth, apoptosis, and autophagy in the development of tumors, including gastric cancer (27–29). We hypothesized that PI3K/Akt/mTOR signaling might be implicated in the regulation of Rap1b in gastric cancer cell apoptosis and autophagy. Results showed that the PI3K/Akt/mTOR signaling is significantly activated in parallel with the Rap1b overexpression in gastric cancer cells. In addition, the PI3K/Akt/mTOR pathway is involved in the regulation of Rap1b to the apoptosis and autophagy capacity of gastric cancer cells.

In conclusion, we have demonstrated that Rap1b expression is aberrantly increased in gastric cancer, resulting in the inhibition of autophagy and apoptosis of gastric cancer cells by the PI3K/Akt/mTOR pathway. This might provide new understanding and represent a novel therapeutic target for human gastric cancer.

REFERENCES

1. Martel, C. D.; Forman, D.; Plummer, M. Gastric cancer: Epidemiology and risk factors. Gastroenterol. Clin. North Am. 42(2):219–240; 2013.
2. Uemura, N.; Okamoto, S.; Yamamoto, S.; Matsumura, N.; Yamaguchi, S.; Yamakido, M.; Taniyama, K.; Sasaki, N. Schlemper, R. J. Helicobacter pylori infection and the development of gastric cancer. N. Engl. J. Med. 346(1):65–67; 2002.
3. Caldas, C.; Carneiro, F.; Lynch, H. T.; Yokota, J.; Wiesner, G. L.; Powell, S. M.; Lewis, F. R.; Huntsman, D. G.; Pharoah, P. D.; Jankowski, J. A. Familial gastric cancer: Overview and guidelines for management. J. Med. Genet. 36(12):873–880; 2015.
4. Yu, L.; Jingliang, Y.; Pradip, D.; Hua-Chen, C.; Akira, Y.; Christopherson, K. W.; Paranavitana, N. C.; Xiaodong, P.; Chaekyun, K.; Veerendra, M. Rap1a null mice have altered myeloid cell functions suggesting distinct roles for the closely related Rap1a and 1b proteins. J. Immunol. 179(12): 8322–8331; 2007.
5. Zhang, M.; Zhou, S.; Zhang, L.; Zhang, J.; Cai, H.; Zhu, J.; Huang, C.; Wang, J. miR-518b is down-regulated, and involved in cell proliferation and invasion by targeting Rap1b in esophageal squamous cell carcinoma. FEBS Lett. 586(19):3508–3521; 2012.

6. Peng, H.; Luo, J.; Hao, H.; Hu, J.; Xie, S. K.; Ren, D.; Rao, B. MicroRNA-100 regulates SW620 colorectal cancer cell proliferation and invasion by targeting RAP1B. Oncol. Rep. 31(5):2055–2062; 2014.

7. Lin, K. T.; Yeh, Y. M.; Chuang, C. M.; Yang, S. Y.; Chang, J. W.; Sun, S. P.; Wang, Y. S.; Chao, K. C.; Wang, L. H. Glucocorticoids mediate induction of microRNA-708 to suppress ovarian cancer metastasis through targeting Rap1B. Nat. Commun. 6:5917; 2015.

8. Yang, Y.; Li, M.; Yan, Y.; Zhang, J.; Sun, K.; Qu, J. K.; Wang, J. S.; Duan, X. Y. Expression of RAP1B is associated with poor prognosis and promotes an aggressive phenotype in gastric cancer. Oncol. Rep. 34(5):2385–2394; 2015.

9. Xie, B.; Zhou, J.; Shu, G.; Liu, D. C.; Zhou, J.; Chen, J.; Yuan, L. Restoration of klotho gene expression induces apoptosis and autophagy in gastric cancer cells: Tumor suppressive role of klotho in gastric cancer. Cancer Cell Int. 13(1):18; 2013.

10. Balcer-Kubiczek, E. K.; Garofalo, M. C. Molecular targets in gastric cancer and apoptosis. Netherlands: Springer; 2009.

11. Hanahan, D.; Weinberg, R. A. Hallmarks of cancer: The next generation. Cell 144(5):646–674; 2011.

12. Liu, M.; Li, C. M.; Chen, Z. F.; Ji, R.; Guo, Q. H.; Li, Q.; Zhang, H. L.; Zhou, Y. N. Celecoxib regulates apoptosis and autophagy via the PI3K/Akt signaling pathway in SGC-7901 gastric cancer cells. Int. J. Mol. Med. 33(6):1451–1458; 2014.

13. Mitra, R. S.; Zhang, Z.; Henson, B. S.; Kurnit, D. M.; Carey, T. E.; D'Silva, N. J. Rap1A and rap1B ras-family proteins are prominently expressed in the nucleus of squamous carcinomas: Nuclear translocation of GTP-bound active form. Oncogene 22(40):6243–6256; 2003.

14. Guo, H.; Hu, X.; Ge, S.; Qian, G.; Zhang, J. Regulation of RAP1B by miR-139 suppresses human colorectal carcinoma cell proliferation. Int. J. Biochem. Cell Biol. 44(9):1465–1472; 2012.

15. Li, B.; Chen, R.; Chen, L.; Qiu, P.; Ai, X.; Huang, E.; Huang, W.; Chen, C.; Liu, C.; Lin, Z.; Xie, W. B.; Wang, H. Effects of DDIT4 in methamphetamine-induced autophagy and apoptosis in dopaminergic neurons. Mol. Neurobiol.; 2016.

16. Lee, H. W.; Jang, K. S. B.; Choi, H. J.; Jo, A.; Cheong, J. H.; Chun, K. H. Celastrol inhibits gastric cancer growth by induction of apoptosis and autophagy. BMB Rep. 47(12):697–702; 2014.

17. Sun, L.; Xie, P.; Wada, J.; Kashihara, N.; Liu, F. y.; Zhao, Y.; Kumar, D.; Chugh, S. S.; Danesh, F. R.; Kanwar, Y. S. Rap1b GTPase ameliorates glucose-induced mitochondrial dysfunction. J. Am. Soc. Nephrol. 19(12):2293–2301; 2008.

18. Kyu Yeoun, W.; Young, K. G.; Sung-Jig, L.; Ji-Youn, S.; Youn Wha, K.; Yong-Koo, P.; Juhie, L.; Hee Seung, C. Autophagy is related to the hedgehog signaling pathway in human gastric adenocarcinoma: Prognostic significance of Beclin-1 and Gli2 expression in human gastric adenocarcinoma. Pathol. Res. Pract. 211(4):308–315; 2015.

19. Hyun Soo, P.; Gi-Young, K.; Taek-Jeong, N.; Nam, D. K.; Yung, H. C. Antiproliferative activity of fucoidan was associated with the induction of apoptosis and autophagy in AGS human gastric cancer cells. J. Food Sci. 76(3):T77–T83; 2011.

20. Levine, B.; Kroemer, G. Autophagy in the pathogenesis of disease. Cell 132(1):27–42; 2008.

21. Vigen, R. A.; Kodama, Y.; Viset, T.; Fossmark, R.; Waldum, H.; Kidd, M.; Wang, T. C.; Modlin, I.; Chen, D.; Zhao, C. M. Mo1560 impaired autophagy in gastric carcinoids and adenocarcinoma of both rodent models and patients. Gastroenterology 142(5):S-628; 2012.

22. Azhar, R.; Bo, Y.; Lili, Z.; Muhammad, K.; Hong, Y.; Tonghui, M. Cytotoxic effect of evodiamine in SGC-7901 human gastric adenocarcinoma cells via simultaneous induction of apoptosis and autophagy. Oncol. Rep. 27(5):1481–1487; 2012.

23. Kumar, D.; Shankar, S.; Srivastava, R. K. Rottlerin induces autophagy and apoptosis in prostate cancer stem cells via PI3K/Akt/mTOR signaling pathway. Cancer Lett. 343(2):179–189; 2014.

24. Li, Z.; Handong, W.; Jianguo, X.; Jianhong, Z.; Ke, D. Inhibition of cathepsin S induces autophagy and apoptosis in human glioblastoma cell lines through ROS-mediated PI3K/AKT/mTOR/p70S6K and JNK signaling pathways. Toxicol. Lett. 228(3):248–259; 2014.

25. Franke, T. F.; Hornik, C. P.; Segev LShostak, G. A.; Sugimoto, C. PI3K/Akt and apoptosis: Size matters. Oncogene 22(56):8983–8998; 2003.

26. Wang, D.; Chen, J.; Chen, H.; Duan, Z.; Xu, Q.; Wei, M.; Wang, L.; Zhong, M. Leptin regulates proliferation and apoptosis of colorectal carcinoma through PI3K/Akt/mTOR signalling pathway. J. Biosci. 37(1):91–101; 2012.

27. Dunlop, E. A.; Tee, A. R. mTOR and autophagy: A dynamic relationship governed by nutrients and energy. Semin. Cell Dev. Biol. 36(5):121–129; 2014.

28. Tapia, O.; Riquelme, I.; Leal, P.; Sandoval, A.; Aedo, S.; Weber, H.; Letelier, P.; Bellolio, E.; Villaseca, M.; Garcia, P. The PI3K/AKT/mTOR pathway is activated in gastric cancer with potential prognostic and predictive significance. Virchows Arch. 465(1):25–33; 2014.

29. Ying, J.; Xu, Q.; Liu, B.; Zhang, G.; Chen, L.; Pan, H. The expression of the PI3K/AKT/mTOR pathway in gastric cancer and its role in gastric cancer prognosis. OncoTargets Ther. 8:2427–2433; 2015.