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Original Article

Regenerating gene family member 4 promotes growth and migration of gastric cancer through protein kinase B pathway

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Abstract: Regenerating gene family member 4 (REG4), a secreted protein, is overexpressed in several cancers, including gastric cancer. The present study was undertaken to determine the roles of REG4 in the growth of gastric cancer in the nude mice and in the proliferation and migration in human gastric cancer cell line and its downstream signaling pathway. Gastric cancer models were elicited by intraperitoneally injecting MKN45 human gastric cancer cells and the tumor size was measured every other day. The expressions of REG4 mRNA and protein were increased in the gastric cancer tissues from gastric cancer patients. REG4 increased the gastric tumor weight and size in the nude mice, and promoted the proliferation and migration of gastric cancer cells MKN45. Adeno-associated viral (AAV)-mediated knockdown of REG4 decreased the gastric tumor weight and size in the nude mice, and suppressed the proliferation and migration of MKN45 cells. REG4 increased the expression of phosphorylated protein kinase B (Akt). Triciribine hydrate (TCN), the inhibitor of Akt, decreased the gastric tumor weight and size in the nude mice and abolished REG4-induced weight and size increase of the tumor. TCN also inhibited proliferation and migration and abolished REG4-induced proliferation and migration increase of human gastric cell line MKN45. These results indicate that REG4 promotes the growth, proliferation and migration of gastric cancer through Akt pathway.

Keywords: Regenerating gene family member 4, gastric cancer, proliferation, migration, protein kinase B

Introduction

Gastric cancer, one of the most common cancers worldwide, is usually first diagnosed in advanced stages and curative treatment is not possible [1]. It has been shown that genetic factors and environmental factors are involved in the progression of the gastric cancer [2-4]. Self-sufficiency in growth signals and metastasis are the essential alterations in cell physiology, which is involved in the unlimited proliferation and dissociation of tumor cells from the primary tumor and invasion of adjacent tissues [5]. However, the mechanisms of growth, proliferation and migration of the gastric cancer are not very clear.

Regenerating gene family (REG) consists of 17 members. REG1, REG2, REG3, REG4 as the most important members of REG family are associated with inflammation, diabetes and cancers [6]. It has been shown that REG plays an important role in tissue regeneration and in cell proliferation in epithelium origin tumors [7]. REG has been found to be up-regulated in human colorectal cancer cell lines during differentiation [8]. The expression of REG mRNA were up to 83 times more in diseased mucosa compared with the mucosa from healthy individuals [9]. REG4 levels were significantly higher in early gastric cancer patients than in controls, and in advanced gastric cancer patients than in early gastric cancer patients [10]. REG4 promotes not only growth but also *in vitro* invasiveness of pancreatic cancer cells [11]. In mice models, increased number and size of peritoneal tumors and decreased apoptosis were found in REG4 transfectants [12]. However, the precise relationship between REG4 and gastric cancer is not well understood.

Several studies suggested that REG4 activities are mediated through the epidermal growth factor receptor (EGFR)/protein kinase B (Akt) signaling pathway. It has been shown that REG4 may protect against acinar cell necrosis in

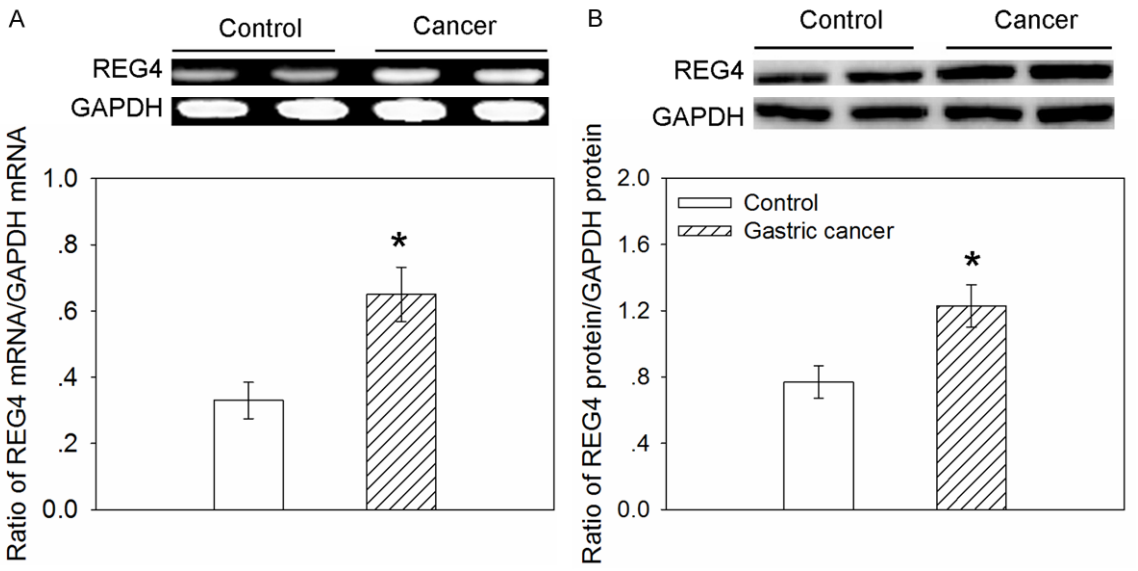

Figure 1. Expression of regenerating gene family member 4 (REG4) in the gastric cancer tissue from gastric cancer patients. A: Expression of REG4 mRNA in the gastric cancer tissue from gastric cancer patients; B: Expression of REG4 protein in the gastric cancer tissue from gastric cancer patients. Values are mean ± SE. *P<0.05 versus Control. n=6 for each group.

experimental pancreatitis by enhancing the expression of Bcl-2 and Bcl-xL via activation of the EGFR/Akt signaling pathway [13]. Using pharmacological inhibitors, it showed that PI3K/Akt is involved in REG4 invasion signals [14]. REG4 is a potent activator of the EGFR/Akt/activator protein-1 (AP-1) signaling pathway in colorectal carcinoma (CRC) [15]. However, whether Akt involved in the REG4-induced the growth, proliferation and migration of the gastric cancer is not clear. The present study was designed to determine the roles of REG4 in the growth of gastric cancer in the nude mice and in the proliferation and migration in human gastric cancer cells MKN45 and its downstream signaling pathway.

Materials and methods

Gastric cancer tissue samples

Fresh gastric cancer and corresponding normal gastric mucosa tissue samples (more than 10 cm away from the edge of the gastric cancer) were taken from gastric cancer patients, and quickly frozen in liquid nitrogen and stored at -80°C until use. No patients had received chemotherapy or radiotherapy before surgery.

Animals and gastric cancer model

Male nude mice (4-6 weeks of age and average weight of 15-20 g) were purchased from the Chinese Academy of Medical Sciences Laboratory Animal Center. The animals were housed in a temperature and humidity controlled room with a 12-hour on-off light cycle and given free access to food and water. To establish the gastric cancer mouse model, 100 μl MKN45 gastric cancer cells (5×10^7 cells/ml) in phosphate-buffered saline (PBS) were intraperitoneally injected into the nude mice [16].

Reverse transcriptase (RT)-PCR

The mRNA expressions of REG4 in human gastric cancer and normal gastric mucosa tissues were detected by RT-PCR. Especially, the primers were designed as follows: forward primer: 5'-TGCACGACCCACAGAAGAG-3', and reverse primer: 5'-GACTTGCCAGACCAGGATCT-3'; for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward primer, 5'-CCCACTCCTCCACCT TTGAC-3', and reverse primer, 5'-ATGAGGT-CCACCACCCTGTT-3'. The level of REG4 mRNA was normalized to the GAPDH mRNA level.

Western blotting

The tissues were lysed in modified radio immunoprecipitation assay (RIPA) or lysed directly in 1×sodium dodecyl sulfate (SDS) loading buffer. After process of electrophoresis and transfer, proteins on nitrocellulose membrane were

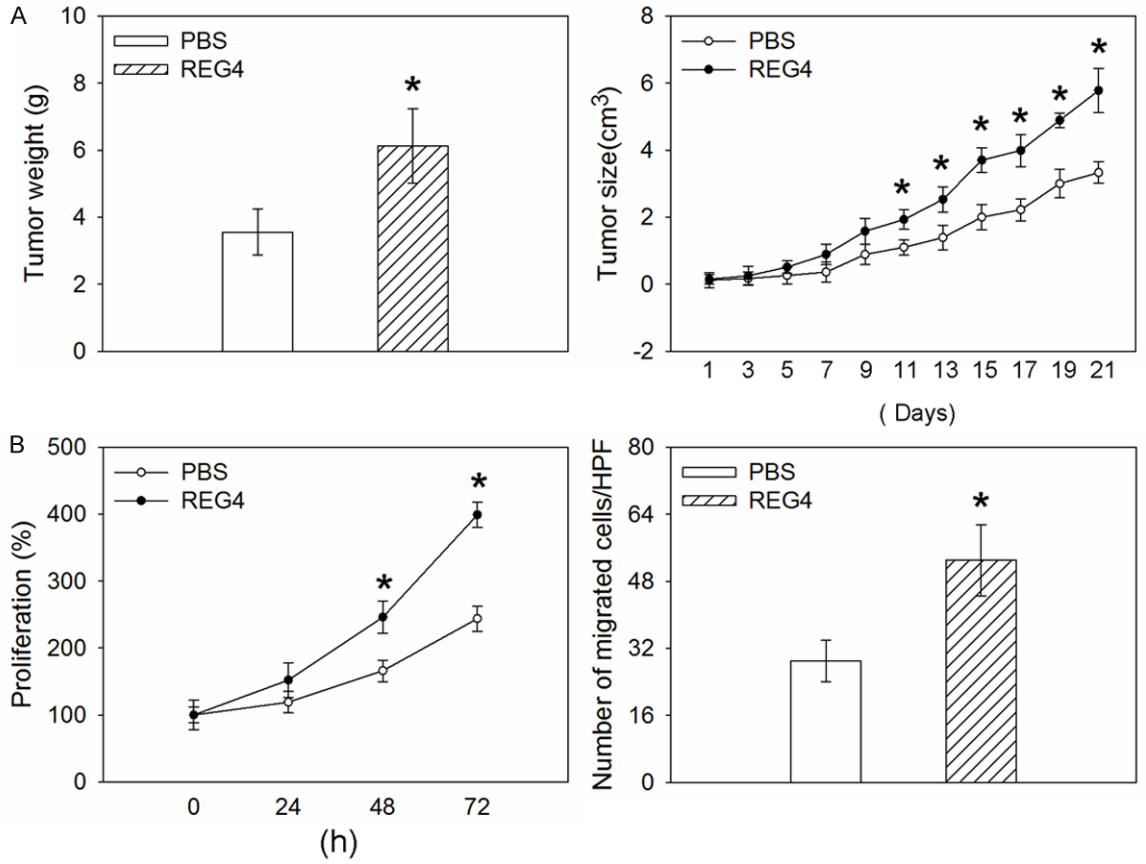

Figure 2. Effects of regenerating gene family member 4 (REG4) on the gastric cancer. A: Effects of PBS and REG4 on the tumor weight and size in the mice; B: Effects of PBS and REG4 on the proliferation and migration of MKN45 cells. The tumor weight was measured 21 days after treatment and the tumor size was measured every other day. Values are mean ± SE. *P<0.05 versus PBS. n=6 for each group.

probed with the REG4 (1:200, R&D Systems Inc., USA), Akt and phosphorylated Akt (1:500, Cell Signaling Technology, USA) and GAPDH (1:5000, Bioworld Technology Inc., USA) primary antibody followed by incubation with the secondary antibodies (1:5000; Immunology Consultants Lab, USA). The bands were visualized by enhanced chemiluminescence using ECL (Pierce Chemical) and captured on X-ray films. The total REG4 protein level was normalized to the GAPDH protein level, and phosphorylated Akt level was normalized to the total Akt protein level.

Cell line and cell culture

MKN45 human gastric cancer cell line, was purchased from the Chinese Academy of Medical Sciences Cancer Institute (Beijing, China) and cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mmol/L L-glutamine (Invitrogen) in a humid chamber at 37°C and 5% CO_2. Cells were subcultured every 3-5 days to maintain logarithmic growth until a sufficient number of cells (5×10^7 cells/ml) were obtained for transfer to the nude mice.

Cell proliferation assay

Cell proliferation was assessed by bromodeoxyuridine (BrdUrd) incorporation using a BrdUrd ELISA colorimetric assay (Roche). To determine the proliferation of MKN45 gastric cancer cells, the cells were initially plated at a density of 2×10^5 per 60 mm dish. After the cells had been incubated, they were counted using a hemocytometer and then plotted.

Cell migration assay

Cells (10^5 cells/well) were suspended in 0.5 ml of 1% FBS MEM and placed in the top chamber

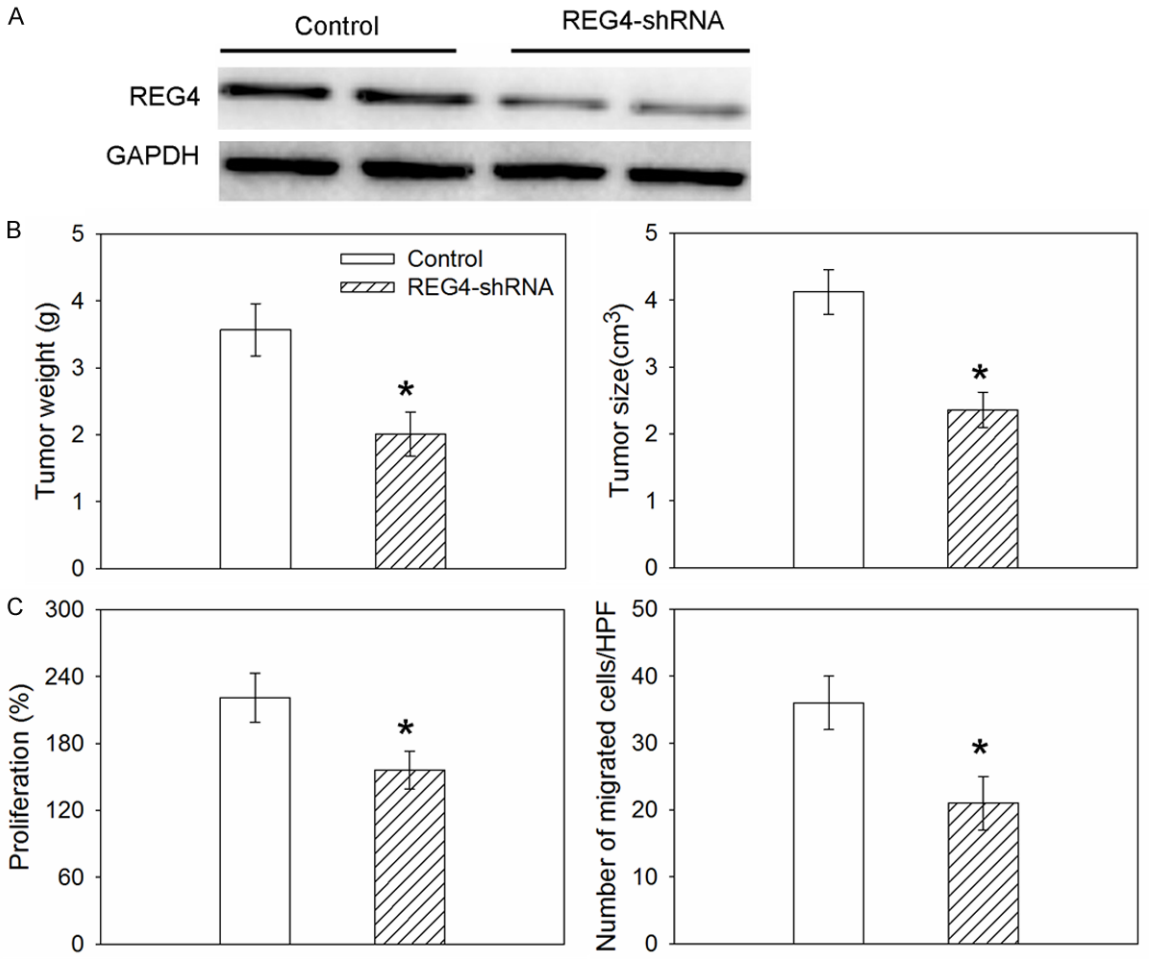

Figure 3. Effects of adeno-associated viral (AAV)-mediated knockdown of regenerating gene family member 4 (REG4) in the gastric cancer. A: The efficiency of the REG4-shRNA; B: Effects of REG4 knockdown on the tumor weight and size in the mice; C: Effects of REG4 knockdown on the proliferation and migration of MKN45 cells. The tumor weight and size were measured 21 days after treatment. Values are mean ± SE. *P<0.05 versus Control. n=6 for each group.

of the well; 0.75 ml of 10% FBS MEM was added to the bottom compartment. Following 48-h incubation, nonmigrating cells were scraped from the membrane of the top compartment, and cells that had migrated through the membrane were fixed and stained using the Protocol Diff-Quik stain set (Siemens Healthcare Diagnostics). Membranes were excised and mounted on a standard microscope slide (Matheson Scientific). The numbers of cells that migrated were determined from five random high-power fields.

Adeno-associated viral and short hairpin RNA

Adeno-associated viral (AAV) vectors encoding short hairpin RNAs targeting REG4 (AAV-shRNA-REG4) was purchased from Invitrogen (Carlsbad, CA). The following:

Constructs were selected for targeting REG4 mRNA: forward primer, 5'-gatcc CAGGAGTCCTGGGTGATAT*ACGCGTg*-3', and reverse primer, 5'-aattc*ACGCGT*ATATCACCCAGGACTCCTG-3'.

Chemicals

REG4 was purchased from R&D Systems Inc. (Minneapolis, MN, USA). Triciribine hydrate (TCN), the inhibitor of Akt, was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The chemicals were dissolved in normal PBS. The doses of REG4 and TCN in this study were 2 μmol and 25 μmol, respectively.

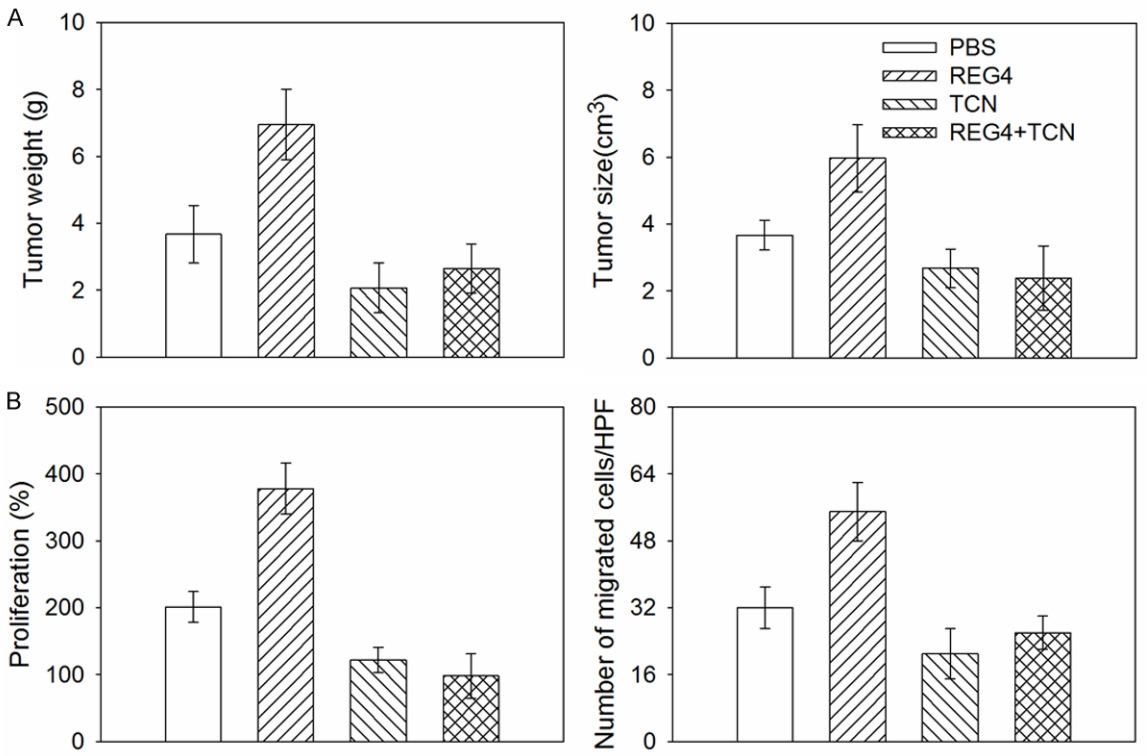

Figure 4. Effects of protein kinase B (Akt) inhibitor triciribine hydrate (TCN) on the gastric cancer. A: Effects of Akt inhibitor TCN on the tumor weight and size in the mice; B: Effects of Akt inhibitor TCN on the proliferation and migration of MKN45 cells. Values are mean ± SE. *P<0.05 versus PBS; and †P<0.05 versus REG4. n=6 for each group.

Statistical analysis

Comparisons between 2 observations were assessed by Student's paired *t*-test. One-way or two-way ANOVA was used followed by the Bonferroni test for post hoc analysis when multiple comparisons were made. All of the data were expressed as the mean ± SE. A value of $P<0.05$ was considered statistically significant.

Results

Expression of REG4 in the gastric cancer

REG4 mRNA level was increased in the gastric cancer tissues from gastric cancer patients. REG4 protein expression was also increased in the gastric cancer tissues from gastric cancer patients (**Figure 1**).

Effects of REG4 on the gastric cancer

REG4 increased the gastric tumor weight in the nude mice. The gastric tumor size was increased 11 days after treatment with REG4 compared with PBS. In human gastric cell line MKN45, REG4 promoted the proliferation and migration. The proliferation of MKN45 cell was promoted 48 h after REG4 treatment (**Figure 2**).

Effects of REG4 knockdown on the gastric cancer

To determine the efficiency and specificity of REG4-shRNA, a scrambled control vector not complementary to any known mammalian gene sequence and shRNA-REG4 were transfected into MKN45 cells. REG4-shRNA suppressed the expression of REG4 about 70% (**Figure 3A**). AAV-mediated knockdown of REG4 decreased the gastric tumor weight and size in the nude mice after 3 weeks treatment. The proliferation and migration of human gastric cell line MKN45 were suppressed after REG4-shRNA treatment (**Figure 3**).

Effects of Akt inhibitor TCN on the gastric cancer

TCN, the inhibitor of Akt, decreased the gastric tumor weight and size in the nude mice and

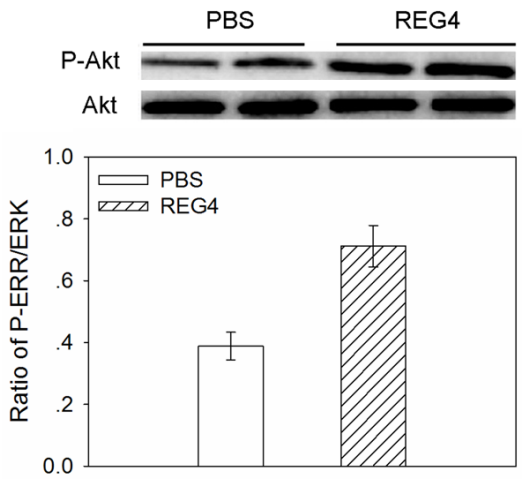

Figure 5. Effects of regenerating gene family member 4 (REG4) on protein kinase B (Akt) phosphorylation. REG4 increased the phosphorylation of Akt in the MKN45 cells 2 h after treatment. Values are mean ± SE. *P<0.05 versus PBS.

abolished REG4-induced the weight and size increase of tumor. The inhibiton of Akt by TCN also inhibited the proliferation and migration of human gastric cell line MKN45 and abolished REG4-induced the proliferation and migration in MKN45 (**Figure 4**).

Effects of REG4 on Akt phosphorylation

REG4 increased the expression of phosphorylated Akt in the MKN45 cells 2 h after treatment compared with PBS (**Figure 5**).

Discussion

REG, within the superfamily of C-type lectin, have been considered as members of a conserved protein family sharing structural and some functional properties being involved in injury, inflammation, diabetes and carcinogenesis [6, 17-20]. REG4, as the member of REG family, whose expression level was increased in several cancer tissues, including colorectal [14], pancreatic cancer [21] and gastric cancer [10]. It has been shown that upregulation of REG4 mRNA transcripts was observed in 36 of the 45 tumor specimens and was positively correlated with the invasive depth of the tumor cells and the clinical stages [22]. The mean REG4 mRNA expression levels in surgically resected gastric cancer specimens were 20 times higher than those in normal mucosa from those patients [23]. These studies suggested that REG4 may be involved in peritoneal dissemination of gastric cancers and REG4 would be a potential novel marker for peritoneal dissemination of gastric cancers. However, the precise relationship between REG4 and gastric cancer is not well understood. In the study, we demonstrated that REG4 increased the gastric tumor weight and size in the nude mice, and promoted the proliferation and migration of gastric cancer cell MKN45, and Akt pathway is involved in the effects of REG4.

Recent studies have shown that overexpression of REG4 is associated with the initiation and progression of cancer. It has been shown that REG4 protein significantly promoted the proliferation and invasiveness of pancreatic cancer cells [11]. Knockdown of REG4 in cancer cell lines inhibited anchorage-dependent and anchorage-independent (both soft-agar and spheroid assays) cell growth and induced cell cycle arrest [24]. In this study, it showed that REG4 mRNA and protein expression levels were increased in the gastric cancer tissues from gastric cancer patients which were consistent with previous studies. REG4 increased the gastric tumor weight and size in the nude mice, and promoted the proliferation and migration of gastric cancer cell line MKN45. Furthermore, AAV-mediated knockdown of REG4 decreased the gastric tumor weight and size in the nude mice and suppressed the proliferation and migration of MKN45 cells. These results demonstrated that REG4 increases the growth and promotes the migration of gastric cancer.

Some studies suggested that Akt was involved in the intracellular signaling mechanisms of REG. It has been demonstrated that REG3 stimulates beta-cell replication by activating Akt kinase [25]. REG protein mediated the anti-apoptotic effects by enhancing Akt activation [26]. REG4 is a potent activator of the EGFR/Akt signaling pathway in colorectal carcinoma [15]. Phosphorylated Akt level was increased in REG4-transfected human gastric cancer cells MKN28-R1, MKN28-R2 and TMK1-R1 [12]. In the study, we showed that REG4 increased the expression of phosphorylated Akt in the MKN45 cells. TCN, the inhibitor of Akt, decreased the gastric tumor weight and size in the nude mice and abolished REG4-induced the weight and size of increase tumor. TCN also inhibited the proliferation and migration and abolished

REG4-induced the proliferation and migration increase in human gastric cell line MKN45. These results demonstrated that REG4 increases the growth of gastric cancer in the nude mice and promotes the proliferation and migration of gastric cancer cells MKN45 through Akt pathway.

In conclusion, REG4 increased the gastric tumor weight and size in the nude mice, and promoted the proliferation and migration of gastric cancer cell MKN45. TCN, the inhibitor of Akt, decreased the gastric tumor weight and size in the nude mice and abolished REG4-induced the weight and size increase of tumor. TCN also inhibited the proliferation and migration and abolished REG4-induced the proliferation and migration increase in human gastric cell MKN45. REG4 promotes the growth, proliferation and migration of gastric cancer through Akt pathway.

Disclosure of conflict of interest

None.

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