

miR-488 acts as a tumor suppressor gene in gastric cancer

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Abstract MicroRNAs (miRNAs) are small, non-coding RNAs that modulate development, cell proliferation, and apoptosis. The deregulated expression of microRNAs is found in carcinogenesis including gastric cancer (GC). In this study, we showed that the expression levels of miR-488 were downregulated in GC tissues compared to in non-tumor tissues. In addition, the expression of miR-488 was also lower in GC cell lines in contrast with the gastric epithelial cell line (GES). In addition, the expression level of miR-488 was negatively correlated with the TNM stage in GC patients, and lower miR-488 expression was found in tumors with advanced TNM stage. The ectopic expression of miR-488 suppressed the GC cell proliferation, cell cycle, colony information, and migration. PAX6 was identified as a direct target gene of miR-488 in HGC-27. Moreover, we found that the expression level of PAX6 was upregulated in the GC tissues compared with the non-tumor tissues. The PAX6 expression level was correlated with the cancer TNM stage, and higher PAX6 expression was found in tumors with advanced TNM stage. Furthermore, there was an inverse correlation between PAX6 and miR-488 expression levels in GC tissues. Therefore, these studies demonstrated that miR-488 might act as a tumor suppressor miRNA in the development of GC.

Keywords Gastric cancer · microRNA · miR-488 · PAX6

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Introduction

Gastric cancer (GC) remains one of the most common malignancies and ranks as the second most common cause of cancer-related death worldwide [1–5]. Although the clinical outcome of GC has gradually improved due to the advancement in surgery resection and chemotherapy, the overall prognosis of advanced GC remains poor, with a 5-year survival rate of only 20–30 % [6–9]. Early diagnosis of GC is difficult since few symptoms are manifested during the early stage [10–13]. Therefore, it is essential to have a more improved and detailed understanding of the mechanisms underlying GC to identify novel molecular markers to improve GC therapy.

MicroRNAs (miRNAs) are a class of 20–25 nucleotides long non-coding RNAs that inhibit the translation and stability of messenger RNAs (mRNAs) via binding to the 3'-untranslated region (3'-UTR) [14–16]. MiRNAs play important roles in the various processes of cancer progression, including development, cell apoptosis, proliferation, metastasis, invasion, inflammation, and epithelial to mesenchymal transition (EMT) [15, 17–20]. Increasing evidences have suggested that miRNAs are aberrantly expressed in several human cancers such as breast cancer, hepatocellular carcinoma, lung cancer, prostate cancer, bladder cancer, Ewing's sarcoma, and renal cell carcinoma [21–27]. miRNAs can function as tumor suppressors or oncogenes in the development and progression of malignancies [25, 28, 29].

Previous studies showed that miR-488 played important roles in several diseases [33–37]. For example, Sikand et al. found that overexpression of miR-488 suppressed the prostate cancer cell proliferation and promoted prostate cancer cell apoptosis through regulating androgen receptor (AR) expression [38]. Song et al. demonstrated that miR-488 played a positive role for chondrocyte cartilage/differentiation development by repressing MMP-13 expression by targeting ZIP-8

expression [39]. However, the role of miR-488 is still unknown in the GC development. In our study, we demonstrated that miR-488 was downregulated in GC, and miR-488 played a tumor suppressor role through suppressing GC cell proliferation and migration via targeting PAX6.

Materials and methods

Samples and cell lines cultured and cell transfection

Primary GC tissues and adjacent normal tissues were collected from GC patients who underwent surgery in our hospital. None of these patients received chemotherapy or radiotherapy before the operation. All patients were signed with informed consent and approved by the Ethics Review Board of the First Affiliated Hospital of Xi'an Jiaotong University. The human GC cell lines HGC-27, SGC-7901, MGC-803, and MKN-45 were obtained from the Cell Resource Center of the Chinese Academy of Medical Sciences (Beijing, China) and were kept in RPMI1640 (HyClone, Rockville, MD, USA). miRNA mimic and scramble were purchased from GenePharma Company (Shanghai, China). Cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's information.

Real-time quantitative polymerase chain reaction

Total RNA was isolated from tissues or cultured cells with Trizol reagent (Invitrogen, Carlsbad, CA). RT-qPCR was performed to measure the expression of miR-488 and PAX6 on the iQ5 Real-Time PCR Detection System (Bio-Rad, CA). The sequences of the specific primers were shown: PAX6 sense forward, 5' TTCAGCACCAGTGTCTACCA 3'; reverse, 5'GCTGTAGGTGTTTGTGAGGG 3'; GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-3'; reverse, 5'-TGGTGAAGACGCCAGTGGA-3'.

Cell growth, colony information, cell cycle, and migration assay

Cell growth was detected by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. MTT solution (0.5 mg/mL) was put to the cells and then the cells were continued to be cultured for 4 h. Dimethyl sulfoxide (DMSO) was increased and the optical density was measured at 450 nm. For cell migration, wound healing analysis was performed. A wound was performed using a pipette tip, and the cells were cultured for additional 48 h. The closure rate of the wound was measured. For cell cycle analysis, cells were incubated with DNA Prep Stain and DNA Prep LPR for 30 min. Cell cycle was measured using flow cytometry. For

cell colony information, cells were cultured at 12-well plates for 2 weeks. The colony number was calculated and counted.

Western blot analysis

The protein was isolated from tissues or cells by using RIPA lysis. The protein was separated by 10 % SDS-PAGE and transferred to the membrane. Then, the membrane was blocked with milk and incubated with primary antibody at 4 °C overnight. The primary antibody was showed as the following: PAX6 and GAPDH (Sigma). Signals were measured with chemoluminescent (Pierce, USA).

Dual luciferase reporter assay

Cells were cultured in a 96-well plate and transfected with miR-488 mimic or scramble and plasmid. After transfection for 48 h, the luciferase activity was measured by the dual luciferase analysis (Promega) and the Renilla luciferase gene was used as control.

Histology

The tissue was fixed with buffered formalin overnight, embedded in paraffin, and then marked with hematoxylin and eosin (H&E).

Statistical analysis

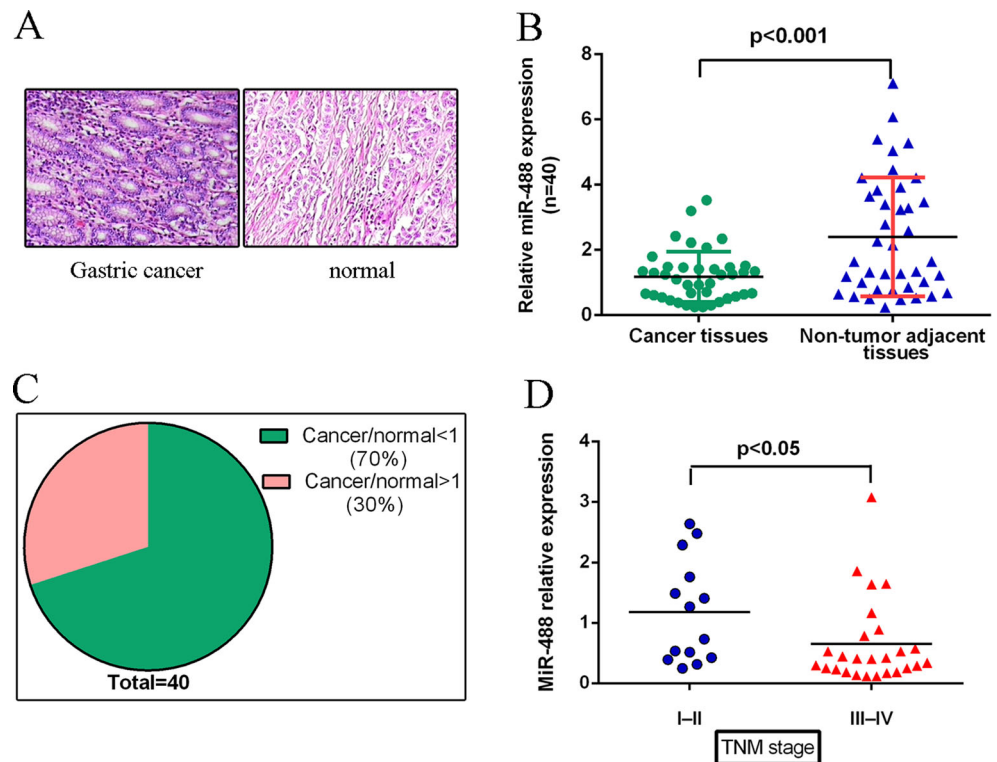
Data was shown as means \pm SD. ANOVA was performed for comparison for more than two groups and Student's *t* test was performed to compare the differences of the two groups. *P* value less than 0.05 was considered significant.

Result

miR-488 was downregulated in GC tissues

The representative GC tissues were diagnosed using hematoxylin and eosin (H&E) staining (Fig. 1a). We found lower expression levels of miR-488 in GC tumor tissues than in non-tumor tissues by using qRT-PCR (Fig. 1b). miR-488 expression was downregulated in 28 patients (28/40, 70 %) compared with the adjacent tissues (Fig. 1c). Moreover, the miR-488 expression level was negatively correlated with the cancer TNM stage, and the lower miR-488 expression was found in tumors with advanced TNM stage (Fig. 1d).

Fig. 1 miR-488 was downregulated in GC tissues. **a** One patient who was diagnosed as gastric cancer in hematoxylin and eosin (H&E) staining. **b** The expression of miR-488 was detected using qRT-PCR. **c** miR-488 expression was downregulated in 28 patients (28/40, 70 %) in contrast with the adjacent tissues. **d** The miR-488 expression level was negatively correlated with the cancer TNM stage, and lower miR-488 expression was found in tumors with advanced TNM stage



Restored expression of miR-488 suppressed GC cell proliferation and cell cycle

miR-488 was downregulated in these GC cell lines (SGC-7901, MGC-803, HGC-27, and MKN-45) compared with the GES (Fig. 2a). In addition, miR-488 was upregulated after transfected with miR-488 mimic (Fig. 2b). The ectopic expression of miR-488 suppressed the HGC-27 cell proliferation (Fig. 2c). Moreover, overexpression of miR-488 increased the proportion of HGC-27 cells in G0/G1-phase and suppressed the proportion of HGC-27 cells in S-phase (Fig. 2d). Overexpression of miR-488 inhibited the cyclin D1 expression in the HGC-27 cells (Fig. 2e).

miR-488 overexpression suppressed GC cell colony information and migration

Overexpression of miR-488 suppressed HGC-27 cell information (Fig. 3a). Moreover, the ectopic expression of miR-488 inhibited the HGC-27 cell migration (Fig. 3b).

miR-488 targets the 3'-UTR of PAX6

Overexpression of miR-488 suppressed the PAX6 mRNA expression in HGC-27 cell (Fig. 4a). Moreover, we also demonstrated that the ectopic expression of miR-488 inhibited the protein expression of PAX6 (Fig. 4b and c). We used the TargetScan and found that there was a putative binding site

for miR-488 in the 3'-UTR of PAX6 at 4721–4727 bp (Fig. 4d). To verify the predictions, the luciferase reporter assay was done in the HGC-27 cell. The relative luciferase activity was decreased about 60 % in the HGC-27 cell containing the PAX6 wild-type 3'UTR. However, this effect was abolished in HGC-27 cell containing the PAX6 mutant-type 3' UTR (Fig. 4e).

Upregulation of PAX6 was inversely associated with miR-488 expression in GC

We found that higher expression levels of PAX6 in GC tumor tissues than in non-tumor tissues by (Fig. 5a) PAX6 expression was upregulated in 32 patients (32/40, 80 %) compared with the adjacent tissues (Fig. 5b). The PAX6 expression level was correlated with the cancer TNM stage, and higher PAX6 expression was found in tumors with advanced TNM stage (Fig. 5c). The expression of PAX6 was upregulated in these GC cell lines (SGC-7901, MGC-803, HGC-27 and MKN-45) compared with the GES (Fig. 5d). Moreover, there was an inverse correlation between PAX6 and miR-488 expression levels in GC tissues (Fig. 5e)

Discussion

In this study, we showed that the expression levels of miR-488 were downregulated in GC tumor tissues

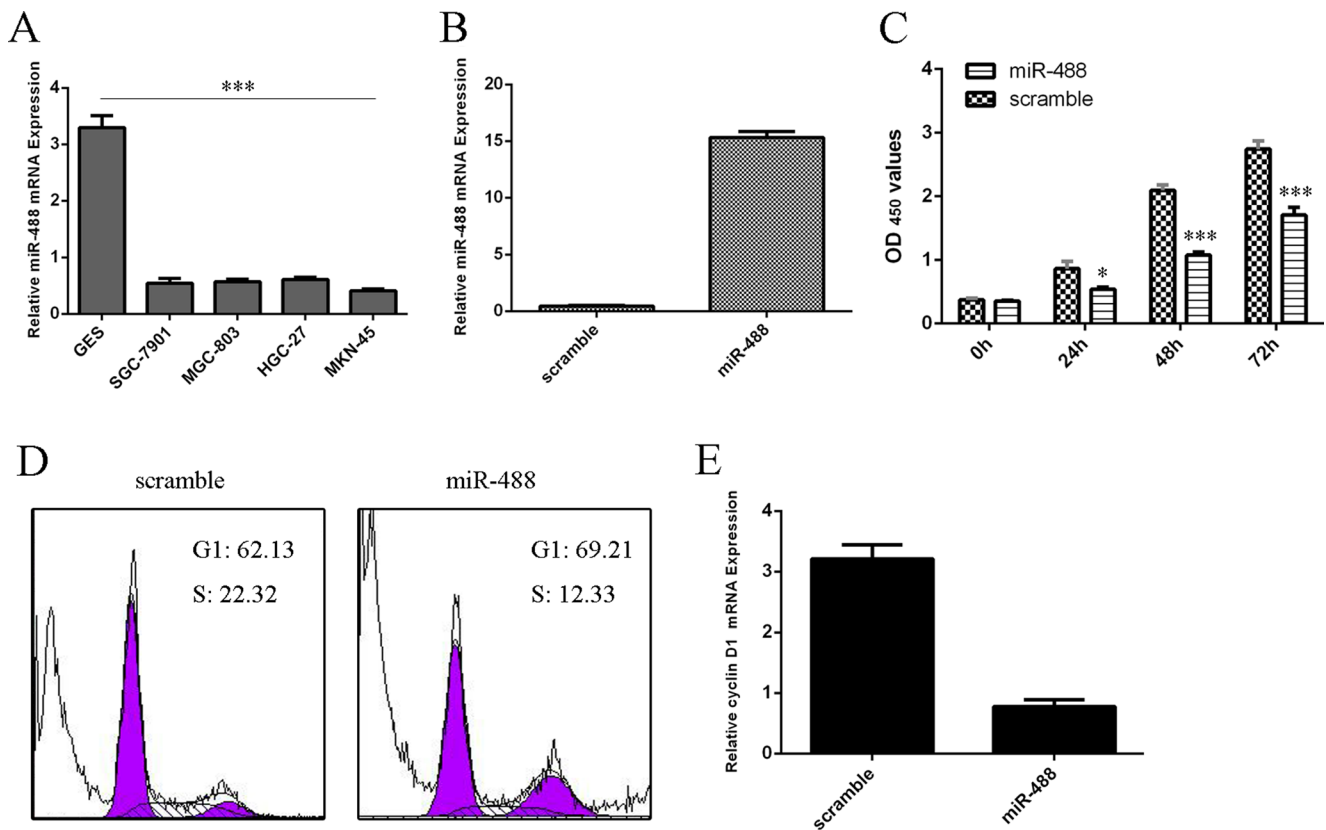


Fig. 2 Restoration of miR-488 expression suppressed GC cell proliferation and cell cycle. **a** The expression of miR-488 was measured by using qRT-PCR. **b** The expression of miR-488 was detected by using qRT-PCR in the HGC-27 cell. **c** Restoration of miR-488 expression

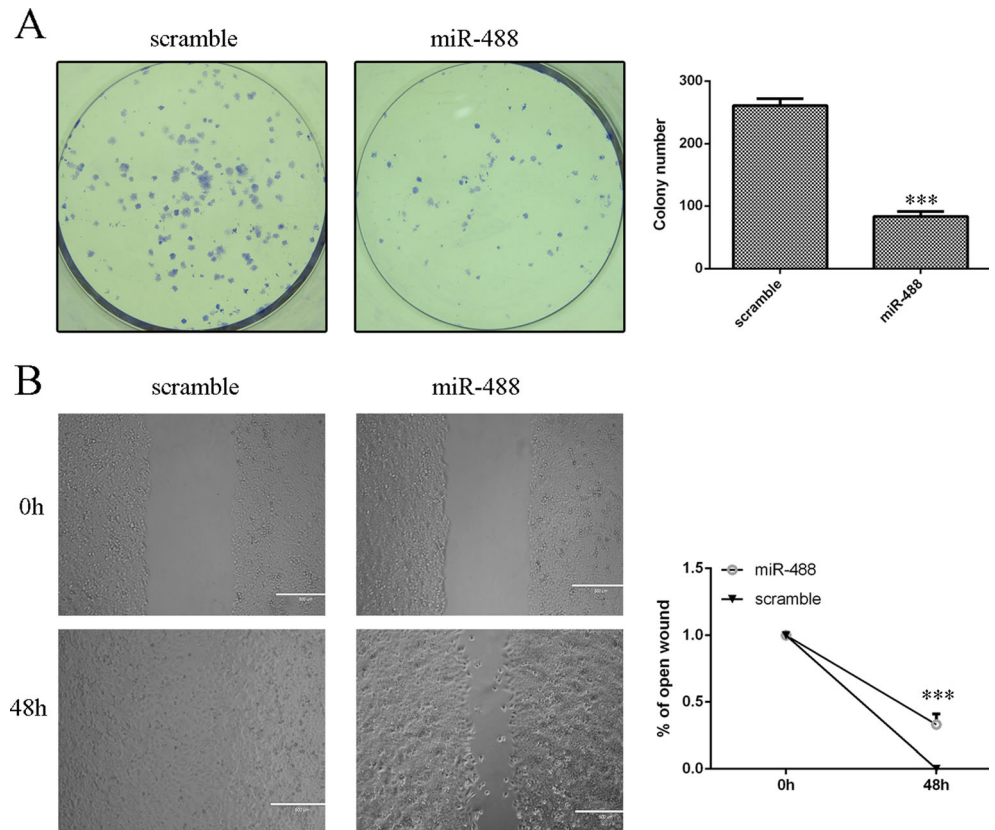
suppressed GC cell proliferation. **d** Overexpression of miR-488 inhibited GC cell cycle. **e** The mRNA expression of cyclin D1 was measured by using qRT-PCR. * $p < 0.05$ and *** $p < 0.001$

compared with in non-tumor tissues and the expression of miR-488 was also lower in GC cell lines (SGC-7901, MGC-803, HGC-27 and MKN-45) compared with the GES. In addition, the miR-488 expression level was negatively correlated with the cancer TNM stage, and lower miR-488 expression was found in tumors with advanced TNM stage. The ectopic expression of miR-488 suppressed GC cell proliferation, cell cycle, colony information, and migration. PAX6 was identified as a direct target gene of miR-488 in HGC-27. Moreover, we found that PAX6 was upregulated in the GC tumor tissues compared to the non-tumor tissues. The PAX6 expression level was correlated with the cancer TNM stage, and higher PAX6 expression was found in tumors with advanced TNM stage. Furthermore, there was an inverse correlation between PAX6 and miR-488 expression levels in GC tissues. Therefore, this data demonstrated that miR-488 might act as a tumor suppressor miRNA in the development of GC.

Emerging evidences have showed that miRNAs play crucial roles in the development of tumors, and the deregulated expressions of miRNAs are found in a number of cancers including GC [21, 30–32]. Previous

studies also demonstrated that miR-488 played critical roles in several diseases [33–37]. For example, Sikand et al. found that overexpression of miR-488 suppressed the prostate cancer cell proliferation and promoted prostate cancer cell apoptosis through regulating the androgen receptor (AR) expression [38]. Song et al. demonstrated that miR-488 played a positive role for chondrocyte cartilage/differentiation development through repressing MMP-13 expression by targeting ZIP-8 expression [39]. However, the role of miR-488 is still unknown in the GC development. In our study, we firstly measured the expression level of miR-488 in the GC tissues. We found that miR-488 was downregulated in GC tumor tissues compared with in non-tumor tissues. Moreover, miR-488 expression level was negatively correlated with the cancer TNM stage, and lower miR-488 expression was found in tumors with advanced TNM stage. Furthermore, we also found that miR-488 expression was lower in GC cell lines (SGC-7901, MGC-803, HGC-27, and MKN-45) compared with the GES. In addition, we demonstrated that the ectopic expression of miR-488 suppressed the GC cell proliferation, cell cycle, colony information, and migration.

Fig. 3 miR-488 overexpression suppressed GC cell colony information and migration. **a** Overexpression of miR-488 suppressed the HGC-27 cell information. **b** Restoration of miR-488 expression suppressed the HGC-27 cell migration. ****p* < 0.001



In this study, we demonstrated that PAX6 was a direct target gene of miR-488 in GC. PAX6 belongs to the family member of the PAX gene and can encode a transcription factor

with crucial roles in various diseases [40–42]. PAX6 is a crucial transcription factor in pancreas, eyes, and central nervous system development [43–45]. Recent studies demonstrated

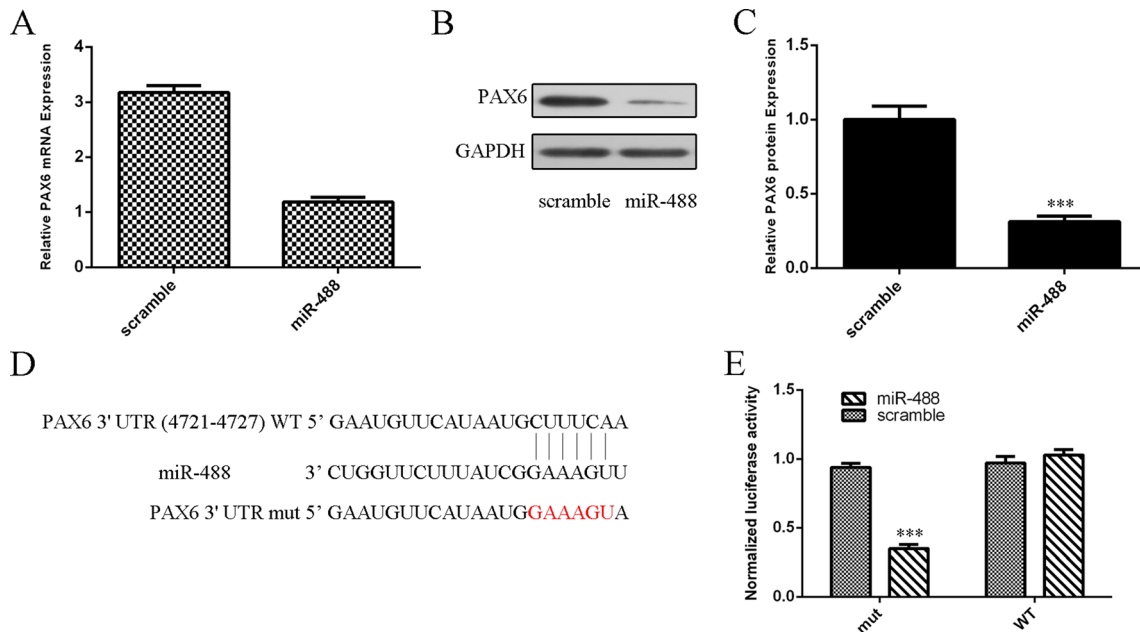
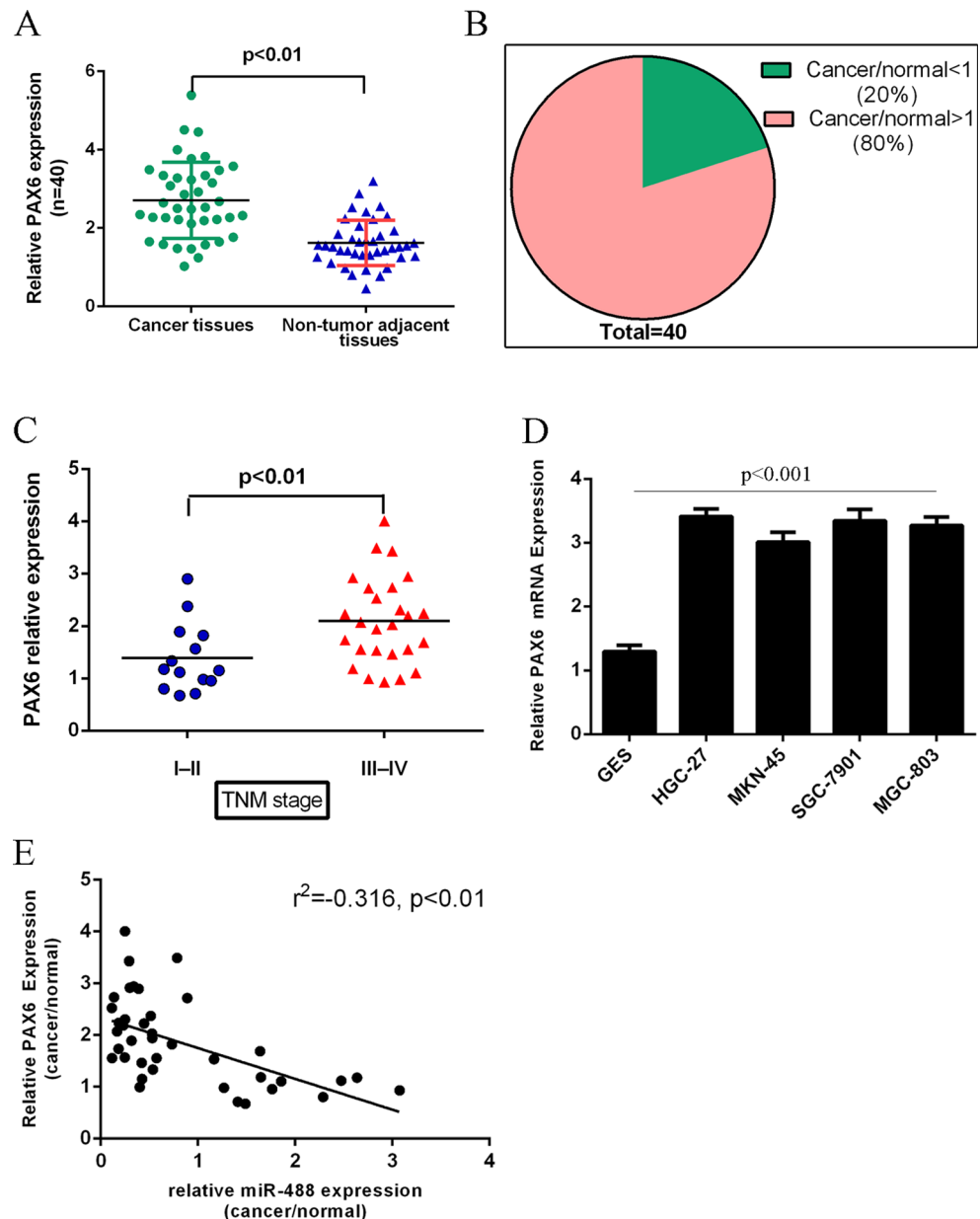


Fig. 4 miR-488 targets the 3'-UTR of PAX6. **a** Overexpression of miR-488 suppressed the mRNA expression of PAX6. **b** The protein expression of PAX6 was measured by using western blot. **c** The relative expression of PAX6 was shown. **d** The potential miR-488 binding site at the 3'-UTR

of PAX6 mRNA was computationally predicted by TargetScan. **e** Luciferase activity assay was performed to measure the interaction between PAX6 3'-UTR and miR-488. ****p* < 0.001

Fig. 5 Upregulation of PAX6 was inversely associated with miR-488 expression in GC. **a** The expression of PAX6 was detected using qRT-PCR. **b** PAX6 expression was upregulated in 32 patients (32/40, 80 %) in contrast with the adjacent tissues. **c** The PAX6 expression level was correlated with the cancer TNM stage, and higher PAX6 expression was found in tumors with advanced TNM stage. **d** The expression of PAX6 in the cell lines was measured by using qRT-PCR. **e** There was an inverse correlation between PAX6 and miR-488 expression in the GC tissues



that PAX6 acted as an oncogene in some tumors [46, 47]. For example, the expression of PAX6 was increased in pancreatic tumors, retinoblastoma, and intestinal tumors [44, 46, 47]. The expression of PAX6 was also upregulated in breast and brain cancer cell [48, 49]. Zhao et al. found that the PAX6 expression level was increased in the lung cancer tissues in comparison with normal tissues [50]. Overexpression of PAX6 increased the cell cycle progression through activating MAPK pathway in lung cancer. In line with this previous data, we demonstrated that PAX6 was upregulated in the GC tumor tissues compared to the non-tumor tissues. The expression level of PAX6 was correlated with the cancer TNM stage, and higher PAX6 expression was found in tumors with

advanced TNM stage. Furthermore, there was an inverse correlation between PAX6 and miR-488 expression levels in the GC tissues.

In this study, we demonstrated that miR-488 was downregulated in GC, and miR-488 played a tumor suppressor role through suppressing GC cell proliferation and migration via targeting PAX6. Our data suggest that the ectopic expression of miR-488 might be a therapeutic strategy for GC treatment.

Compliance with ethical standards

Conflicts of interest None

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