

RESEARCH PAPER



## Knockdown of RREB1 inhibits cell proliferation via enhanced p16 expression in gastric cancer

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### ABSTRACT

Gastric cancer (GC) is the most common gastrointestinal malignancy worldwide. However, the molecular mechanisms of the progression of GC are not fully understood. Ras-responsive element binding protein 1 (RREB1) is an oncogene in many types of cancer that is involved in various biological processes, such as DNA damage repair, cell growth and proliferation, cell differentiation, fat development, and fasting glucose balance. In this study, we demonstrate the role of RREB1 in gastric cancer. First, by immunohistochemistry staining (IHC) and bioinformatics analysis, we demonstrated the expression of RREB1 in gastric cancer and paired normal gastric tissues. Then, we established RREB1 overexpression and knockdown cell lines via lentiviral transfection and detected cell proliferation by using MTT, colony-forming, cell cycle and apoptosis assays *in vitro*. We demonstrated the effect of RREB1 on cell proliferation *in vivo* by using a subcutaneous xenograft tumor model in nude mice. Finally, by using Western blotting and IHC, we demonstrated the possible mechanism by which RREB1 affects cell proliferation. The IHC and bioinformatics analyses demonstrated that RREB1 was highly expressed in gastric cancer and showed that RREB1-expressing patients had a larger tumor size and more lymphovascular invasion than RREB1-negative patients. Knockdown of RREB1 inhibited cell proliferation *in vivo* and *in vitro*. Knockdown of RREB1 enhanced p16 expression *in vivo* and *in vitro*, and p16 expression was negatively related to RREB1 in gastric cancer tissue. RREB1 was highly expressed in gastric cancer, and knockdown of RREB1 inhibited cell proliferation via enhanced p16 expression.

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### KEYWORDS

Gastric cancer; RREB1; cell proliferation; p16



## 1. Introduction

Gastric cancer (GC) is one of the most common malignant cancers worldwide, especially in East Asia [1]. Among all cancers, gastric cancer ranks fifth in terms of incidence and third in terms of mortality. In China, most patients are diagnosed with GC in advanced stages, which leads to a poor prognosis and causes a serious threat to human health [2].

Self-sufficiency in growth signals is one of the most important characteristic for cancer, in which the Ras related pathway plays a vital role [3]. When Ras related pathway is activated in cancer, the malignant cell will acquire unlimited energy sporting for growth. Thus, inhibit Ras related pathway is very useful for treatment of cancer. However, there is only a small number of drug

target Ras, and the effect of these drug is limited. Therefore, to discover more Ras related target is urgently for develop new drugs that inhibit Ras [4].

RREB1 is a zinc finger transcription factor that binds specifically to the distal Ras-responsive element (RRE) and may be involved in Ras/Raf-mediated cell differentiation [5]. RREB1 has been identified as a potential human oncogene, and it is overexpressed in thyroid cancer, where it results in increased transactivation of the calcitonin gene in response to Ras signal transduction [6]. RREB1 suppressed the expression of the p16INK promoter, and the development of pristane-induced plasma cell tumors in BALB/c mice was attributable to a polymorphism in the RREB1 binding site [7]. KRas and RREB1 are experimentally

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determined targets of miR-143 and miR-145, which demonstrates the existence of a feedback circuit that potentiates KRas-mediated tumorigenesis via suppression of the antitumorigenic miR-143/145 locus [8]. RREB1 was proven to induce upregulation of AGAP2-AS1 to regulate cell proliferation and migration in pancreatic cancer partly by suppressing ANKRD1 and ANGPTL4 by recruiting EZH2 [9]. Jie Su and his colleagues demonstrated that RREB1 provides a molecular link between the Ras and TGF- $\beta$  pathways for coordinated induction of developmental and fibrogenic epithelial-mesenchymal transition (EMT) [10]. Oliver A. Kent demonstrated that genetic perturbation of the RREB1-SIN3A-KDM1A complex represents a new category of RASopathy-like syndromes arising through epigenetic reprogramming of MAPK pathway genes [11].

Recently, many studies have demonstrated that RREB1 plays an important role in the development of various cancers [12]. However, the role of RREB1 in gastric cancer has not yet been reported. In this study, we demonstrated that knockdown of RREB1 expression inhibited gastric cancer cell proliferation via enhanced p16 expression both in vivo and in vitro.

## 2. Materials and methods

### 2.1. Clinical samples and cell culture

We collected 60 paired gastric cancer tissues and adjacent normal tissues from the First Affiliated Hospital of Xi'an Jiaotong University from October 2019 to December 2019. Patients who received preoperative chemotherapy or radiotherapy were excluded from this study. This study was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University. All subjects were fully informed of the nature of the study, and all subjects provided written consent for their participation.

The human gastric cancer cell lines SGC7901, BGC823, AGS, MGC803 and MNK45 were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. These cells were cultivated in DMEM (Gibco BRL, Carlsbad, CA, USA) supplemented with 10% FBS

(Gibco BRL, Carlsbad, CA, USA) at 5% CO<sub>2</sub> at 37°C.

### 2.2. Immunohistochemistry staining

Tissues obtained from patients or mice were soaked in formalin for 24 h and sliced into continuous pathological sections at a thickness of 4  $\mu$ m with paraffin embedding. For each specimen, at least three slices were randomly selected for IHC. The experiment was performed using an IHC kit (SA1023, Bausch & T organisms, Wuhan, China) according to the manufacturer's instructions. Then, the tissue was dewaxing and dehydration by xylene and alcohol and inhibit endogenous peroxidase by placed in 3% hydrogen peroxide for 30 min. After that sections were antigen retrieval was carried out by soaking the slides in 0.01 M citrate buffer (pH 6.0) and heating them for 30 min in a microwave. Next the slice was incubated with a primary antibody overnight at 4°C. The concentrations of the primary antibodies for each antibody were as follows: RREB1 primary antibody (ab64168, Abcam, Cambridge, UK, 1:200 dilution), Ki67 primary antibody (ab156014, Abcam, Cambridge, UK, 1:200 dilution), and p16 primary antibody (ab156014, Abcam, Cambridge, UK, 1:200 dilution). After wash for twice, the slice was incubated with biotinylated secondary antibody at 37°C for one hour. 3-Diaminobenzidine tetrahydrochloride (DAB) was applied as a chromogen, and the sections were counterstained with hematoxylin. We selected at least five high-magnification fields from each section to determine the results. The IHC score was evaluated as the intensity and positively stained cell ratio. The degree of intensity was defined as 0 for no staining, 1 for weak staining, 2 for moderate staining, 3 for intense staining and 4 for strongly intense staining. The grade for positively stained number of cells was 0 for 0%, 1 for 1–25%, 2 for 26–50%, 3 for 51–75% and 4 for 76–100%. The immunoreactivity score (IRS) was the product of the area and intensity. Samples with IRS $\leq$ 3 were assigned to the negative group, and others were assigned to the positive group. Two independent pathologists who were blinded to the experimental design performed this process by using Image J software.

### 2.3. Establishment of stably transfected cell lines

The overexpression vectors were purchased from GeneChem Co., Ltd. (Shanghai, China). For overexpression vectors,  $5 \times 10^4$  cells were cultured in six-well plates for 24 h, and transfection was performed according to the manufacturer's instructions. The MOIs for AGS were 50 and 20 for MGC-803. The knockdown shRNA was purchased from GenePharma Co., Ltd. (Shanghai, China). The transfections were performed according to the manufacturer's instructions, and the MOI was 20 for both SGC-7901 and BGC-823 cells. After transfection for 24 h, the cell was cultured in a fresh medium for two days, then treated by 2  $\mu$ g/mL puromycin until it establishes stable cell lines.

### 2.4. Western blotting

Cell protein extraction was performed using the RIPA Protein Extraction Kit (Heart, Xi'an, China) following the manufacturer's instructions. Western blotting was performed as described in the literature [13]. The extracted proteins were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membrane was blocked with 5% milk for 1 h and incubated with the primary antibody at 4°C overnight. The primary antibodies included anti-RREB1 (ab64168, Abcam, Cambridge, 1:1000 dilution, 20,280-1-AP, Proteintech, USA, 1:500 dilution), anti-cyclin D1 (16,396-1-AP, Proteintech, Boston, USA, 1:1000 dilution), anti-PCNA (10,205-2-AP, Proteintech, USA, 1:1000 dilution), anti-Bax (27,282-1-AP, Proteintech, USA, 1:1000 dilution), anti-p16 (27,282-1-AP, Proteintech, USA, 1:1000 dilution), and anti-GAPDH (10,494-1-AP, Proteintech, USA, 1:1000 dilution). After incubation, the PVDF membrane was rinsed and incubated with the secondary antibody for 1 h at room temperature. Protein expression was detected using a chemiluminescence detection system and the intensity of protein was analyzed by the own software of the machine (Millipore, Billerica, MA, USA).

### 2.5. Cell proliferation assay

Two thousand cells were seeded in 96-well plates for the MTT assay, then the cell was cultivated for

different time. At each time point the cell was treated by 20  $\mu$ L 5 mg/mL MTT and cultivated at 37°C for 4 hours, then the cells were dissolved by 150  $\mu$ L dimethyl sulfoxide. And cell viability was measured in a microplate reader (Thermo Fisher, Waltham, USA) every day for one week. Six parallel wells were used for each experiment, which was repeated three times. The data are presented as the mean  $\pm$  standard deviation (SD).

### 2.6. Colony-forming assay

Single-cell suspensions were seeded in 6-cm culture dishes at 800 cells/well. Fourteen days later, these cells were fixed with 4% paraformaldehyde and stained with 1% crystal violet. The number of visible colonies was calculated. The experiment was performed at least three times.

### 2.7. Cell cycle and cell apoptosis assays

The cell cycle assay was performed using a kit according to the manufacturer's instructions (Kaiji Biotechnology Co., Ltd., Hangzhou, China). For detection of the cell cycle,  $1 \times 10^5$  cells were cultured in six-well plates for 24 h and harvested. After three washes with PBS, the cells were fixed with cold 75% anhydrous ethanol overnight at -20°C. Then, the cells were washed twice with PBS and stained with RNase A (100  $\mu$ L) for 30 min at 37°C, followed by propidium iodide (400  $\mu$ L) staining for 30 min at 4°C in the dark. Then, the cells were detected by a FAC sorter (BD, Franklin Lakes, USA) and calculated using Cell Quest software (BD, Franklin Lakes, USA). Each experiment was repeated for fifth.

Cell apoptosis was assessed using a kit (Lianke Biotechnology Co., Ltd.). For detection of apoptosis,  $2 \times 10^5$  cells were cultured in six-well plates for 24 h and harvested. Next, the cells were resuspended in binding buffer at a concentration of  $1 \times 10^6$  cells/ml and stained with Annexin V-APC and PE. Then, the apoptotic rate was determined and calculated using the FAC sorter and Cell Quest software (BD, Franklin Lakes, USA). Each experiment was repeated for fifth.

## 2.8. Cancer xenograft models in nude mice

Twenty 4-week-old female BALB/c-nude mice (Shanghai SLAC Laboratory

Animal Co., Ltd., Shanghai, China) were randomly divided into four groups. Approximately  $5 \times 10^6$  stable BGC823 RREB1 knockdown cells or MGC803 RREB1 overexpression cell line and parallel control cells were injected into the right axillary side. The length (a) and width (b) of the tumors were measured every 3 days after injection using calipers. The volume (V) was calculated as  $V = ab^2/2$ . After approximately three weeks, the mice were sacrificed. The tumors of the mice were measured and fixed in 10% formalin for subsequent experiments.

## 2.9. Statistical analysis

The data analysis was performed using SPSS 18.0 (Chicago, IL, USA), and the results are expressed as the mean  $\pm$  standard deviation. Normally distributed data were compared using a t test or one-way ANOVA (comparisons between two groups used the LSD). Data that failed to meet this standard were analyzed using the Kruskal–Wallis test. The chi-squared test or Fisher's exact test was used to compare categorical data. *P*-values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. RREB1 is highly expressed in gastric cancer

RREB1 is a Ras-associated gene that promotes the proliferation of prostatic cancer [14,15]. Here, we demonstrated that RREB1 was expressed at higher levels in gastric cancer. By IHC staining of gastric cancer and its adjacent normal gastric tissue, we found that RREB1 was mainly expressed in the nucleus and cytoplasm (Figure 1(a)), and the IHC score of RREB1 was higher in the cancer tissue than in the normal tissue (Figure 1(b)). Then, by searching the GEPIA database (<http://gepia.cancer-pku.cn/>), we found that the level of RREB1 was higher in cancer tissue than normal tissue (Figure 1(c)). However, there was no significant relationship between RREB1 expression and the overall or disease-free survival time of gastric cancer patients in this database (Figure 1(d,e)).

Finally, we analyzed the clinic pathological characteristics of patients with different levels of RREB1 expression in IHC-stained samples. We found that the patients with higher RREB1 expression had a larger tumor size and more lymphovascular invasion than those with negative expression, as shown in Table 1.

### 3.2. Knockdown of RREB1 inhibits cell proliferation in vivo and in vitro

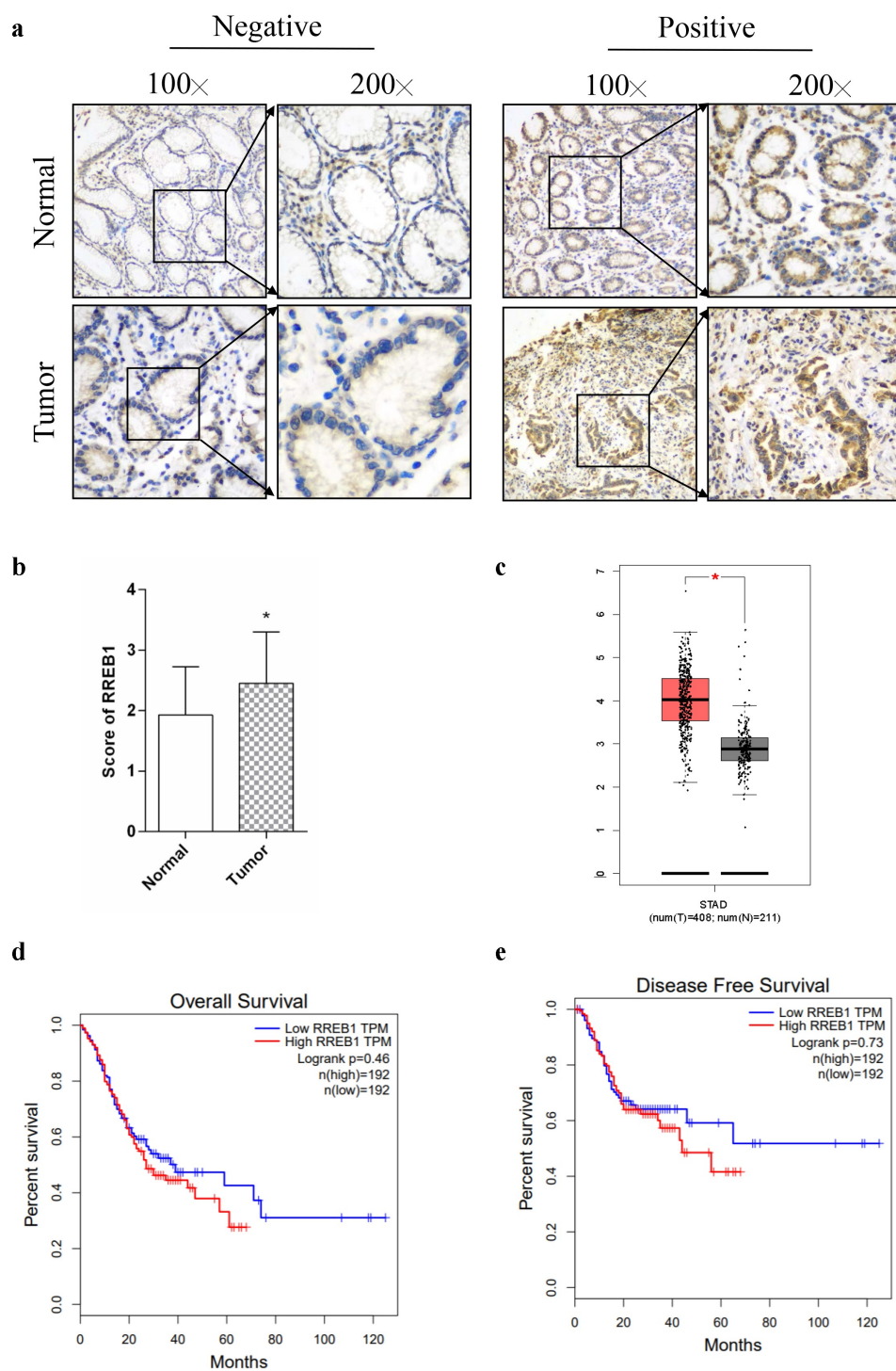
To elucidate the function of RREB1, we detected the expression of RREB1 in gastric cell lines and found that RREB1 was expressed in all cell lines. SGC7901 and BGC823 had higher RREB1 expression, and AGS, MGC803 and MNK45 had lower expression (Figure 2(a)). We established RREB1 overexpression and knockdown cell lines, as shown in Figure 2(b-e). MTT assays showed that the wild-type SGC7901 and BGC823 cell lines had a proliferation advantage over the knockdown RREB1 cell line on days 4 and 5. Colony formation assays also showed that the wild-type SGC7901 and BGC823 cells had a higher colony forming ability than the RREB1 knockdown cells. The RREB1-overexpressing cell line was not different from the wild-type cell line in the MTT and colony formation assays (Figure 2(f,g)).

To further verify the effect of RREB1 on cell proliferation, we detected the cell cycle by using flow cytometry. The results showed that knockdown of RREB1 induced G0 to G1 cell arrest, as shown in Figure 3(a). We detected the apoptosis of the wild-type and RREB1 knockdown cell lines to determine

**Table 1.** Association between the clinicopathological features of CRC patients and RREB1 expression.

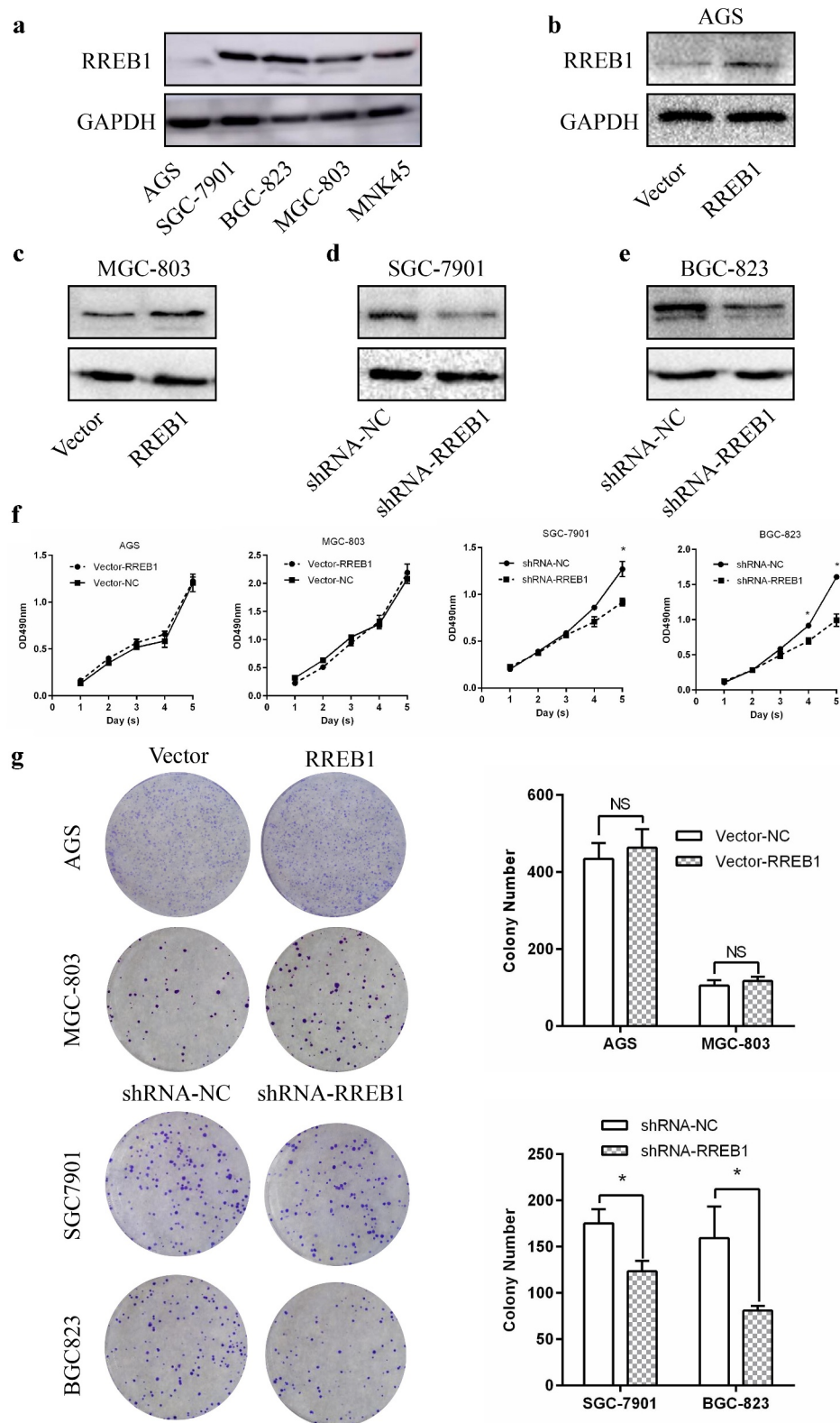
Indicators	RREB1 expression		$\chi^2/t$	<i>P</i>
	Negative (n = 16)	Positive (n = 44)		
Age (years, $x \pm s$ )	61.30 $\pm$ 8.77	60.80 $\pm$ 10.02	0.119	0.907
Sex			0.682	0.409
Male	12	26		
Female	4	18		
BMI (kg/m <sup>2</sup> , $x \pm s$ )	20.27 $\pm$ 2.53	21.75 $\pm$ 4.22	-0.948	0.356
Tumor size (cm, $x \pm s$ )	3.09 $\pm$ 2.25	4.45 $\pm$ 1.86	-2.352	<b>0.022</b>
TNM stage (I/II, III/IV)	10/6	22/22	0.737	0.391
T stage (1/2, 3/4)	8/7	17/27	0.990	0.320
N (-, +)	7/9	18/26	0.039	0.844
Differentiation (well, moderate)	8/8	10/34	2.959	0.085
Lymphovascular invasion (yes, no)	1/15	16/28	3.862	<b>0.049</b>





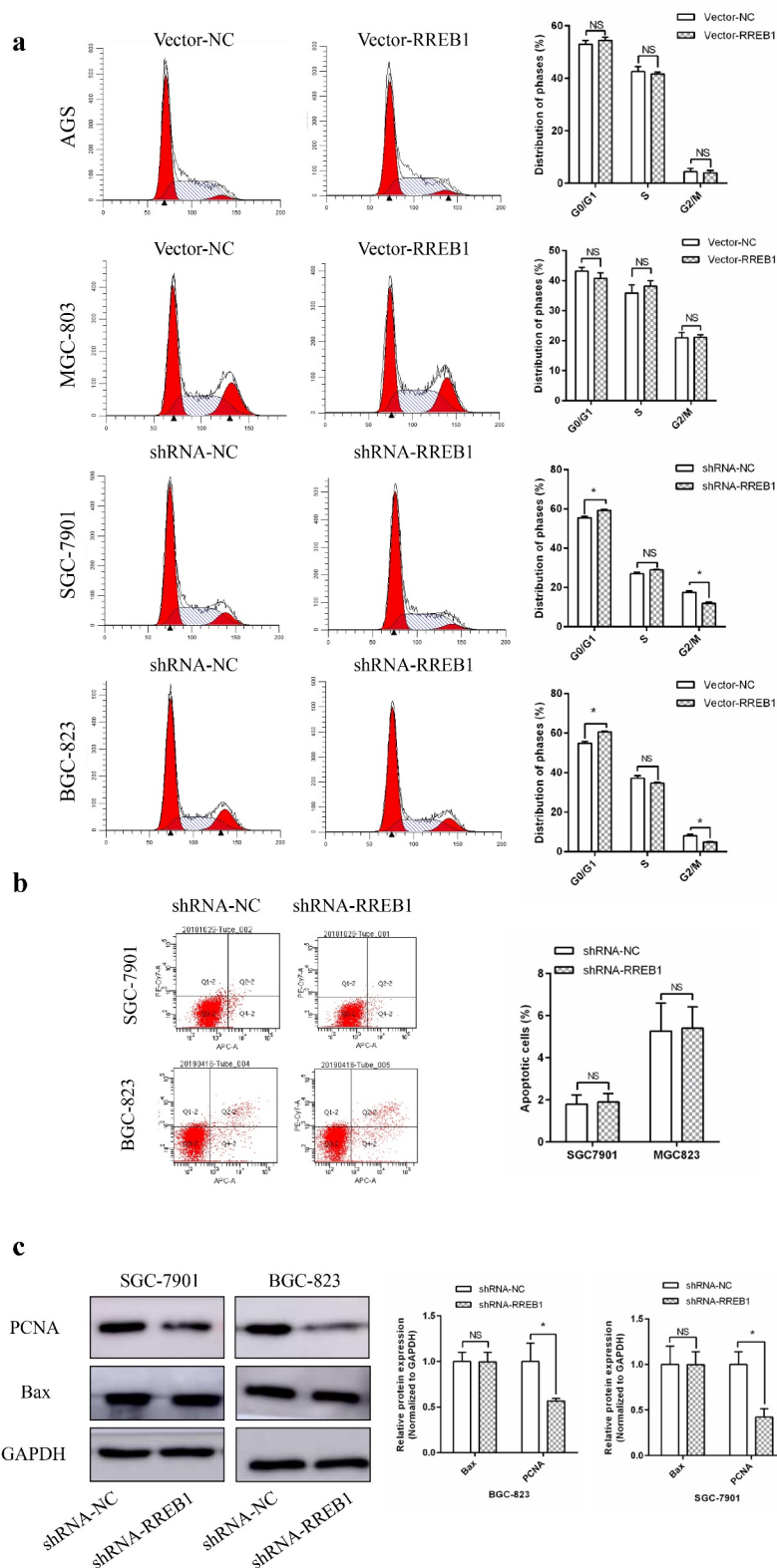
**Figure 1.** RREB1 is highly expressed in gastric cancer tissue but has no significantly with the prognosis of patients.

a. Typical IHC staining of RREB1 in normal and gastric cancer tissues. 100x and 200x magnification. RREB1 expressed both in the nucleus and cytoplasm.  $N = 60$ . b. The IHC score of RREB1 in gastric cancer is higher than the paired normal gastric tissues,\* represents  $P < 0.05$ , t test.  $N = 60$ . c. RREB1 expression is higher in gastric cancer tissue than the normal gastric tissue by searching the GEPIA database; there were 408 gastric cancer tissues (the red bar) and 211 normal gastric mucosa tissues (the gray bar), and the \* represents  $P < 0.05$ , t test. d. The overall survival time of the high RREB1 expression patients has no difference compared to the low RREB1 expression patients by searching the GEPIA database.compared to the low RREB1 expression group,  $P= 0.45$ , log-rank.  $N = 408$ . e. The disease-free survival time of the high RREB1 expression patients has no difference compared to the low RREB1 expression patients by searching the GEPIA database.compared to the low RREB1 expression group.  $P= 0.73$ , log-rank.  $N = 408$ .



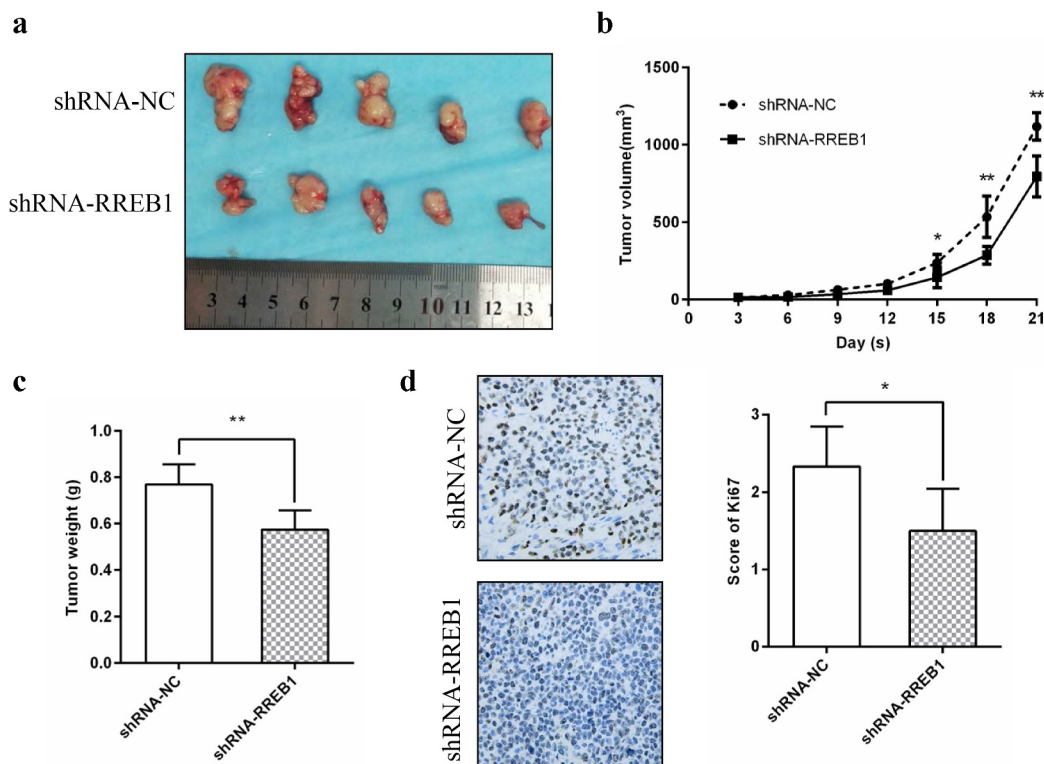
**Figure 2.** Knockdown of RREB1 inhibit gastric cancer cell proliferation.

a. RREB1 expression in different gastric cancer cell lines. We can see RREB1 is high expressed in SGC-7901 and BGC-823 cell line, but low expressed in AGS and MGC-803 cell line.  $N = 3$ . b. Western blotting detection of RREB1 expression in AGS-overexpressing and control cell lines.  $N = 3$ . c. Western blotting detection of RREB1 expression in MGC-803 overexpression and control cell lines.  $N = 3$ . d. Western blotting detection of RREB1 expression in SGC-7901 knockdown and control cell lines.  $N = 3$ . e. Western blotting detection of RREB1 expression in BGC-823 knockdown and control cell lines.  $N = 3$ . f. MTT assays show the proliferation of different RREB1 cell lines compared with the control, we found that knockdown RREB1 can inhibit cell proliferation on days 4 and 5 for both SGC7901 and BGC823 cell lines. \* represents  $P < 0.05$ , t test.  $N = 6$ . g. Colony formation assays show the ability of cell lines with different RREB1 expression levels and the statistical chart, the result demonstrated that wild-type SGC7901 and BGC823 cells had a higher colony forming ability than the RREB1 knockdown cells.\* represents  $P < 0.05$ , t test.  $n = 3$ .



**Figure 3.** Cell cycle and apoptosis of RREB1 overexpression and knockdown cell lines.

a. Flow cytometric detection of the cell cycle in cell lines with different RREB1 expression levels, there were no difference between overexpression RREB1 and control cell line, but knockdown of RREB1 can lead to a G0 to G1 and G2 to M induce growth arrest. \* represents  $P < 0.05$ , one-way ANOVA, LSD compared between two groups.  $N = 5$ . b. Flow cytometric detection of apoptosis in cell lines with different RREB1 expression levels, there were no difference between both overexpression and knock down RREB1 cell line when compared to control group, t test.  $n = 5$ . c. Representative Western blot detection of PCNA/Bax in cell lines with different RREB1 expression levels, PCNA was expressed higher in the control group when compared with the RREB1 knockdown group, \* represents  $P < 0.05$ , t test.  $N = 3$ .



**Figure 4.** RREB1 promotes cell proliferation in vivo (n = 5).

a. Tumor gross pathological specimens derived from the control and RREB1 knockdown cell lines. b. The tumor growth curve of tumors derived from the BGC-823 control and RREB1 knockdown cells, \* represents  $P < 0.05$ , \*\* represents  $P < 0.01$ , t test. c. The tumor weights of tumors derived from the BGC-823 control and RREB1 knockdown cells, the result demonstrated that the wild type BGC-823 cell line had a growth advantage over the control group, \*\* represents  $P < 0.01$ , t test. d. Ki67 staining and its IHC scores of control and RREB1 knockdown cell lines, the wild type cell line had a higher Ki67 score than the control group. \* represents  $P < 0.05$ , t test. 200x magnification.

whether the RREB1 knockdown-mediated inhibition of cell proliferation resulted in apoptosis and found no difference between the cell lines with different RREB1 expression levels (Figure 3(b)). We detected cell cycle- and apoptosis-related proteins and found that PCNA, but not Bax, expression was lower in the RREB1 knockdown cell line than the wild-type cell line (Figure 3(c)).

We confirmed the effect of RREB1 on cell proliferation in vivo. The results demonstrated that knockdown of RREB1 inhibited tumor growth in vivo (Figure 4(a-c)). Tumor Ki67 IHC staining also demonstrated a lower expression of Ki67 in the knockdown RREB1 tumors (Figure 4(d)).

### 3.3. Knockdown of RREB1 inhibits cell proliferation via enhanced p16 expression

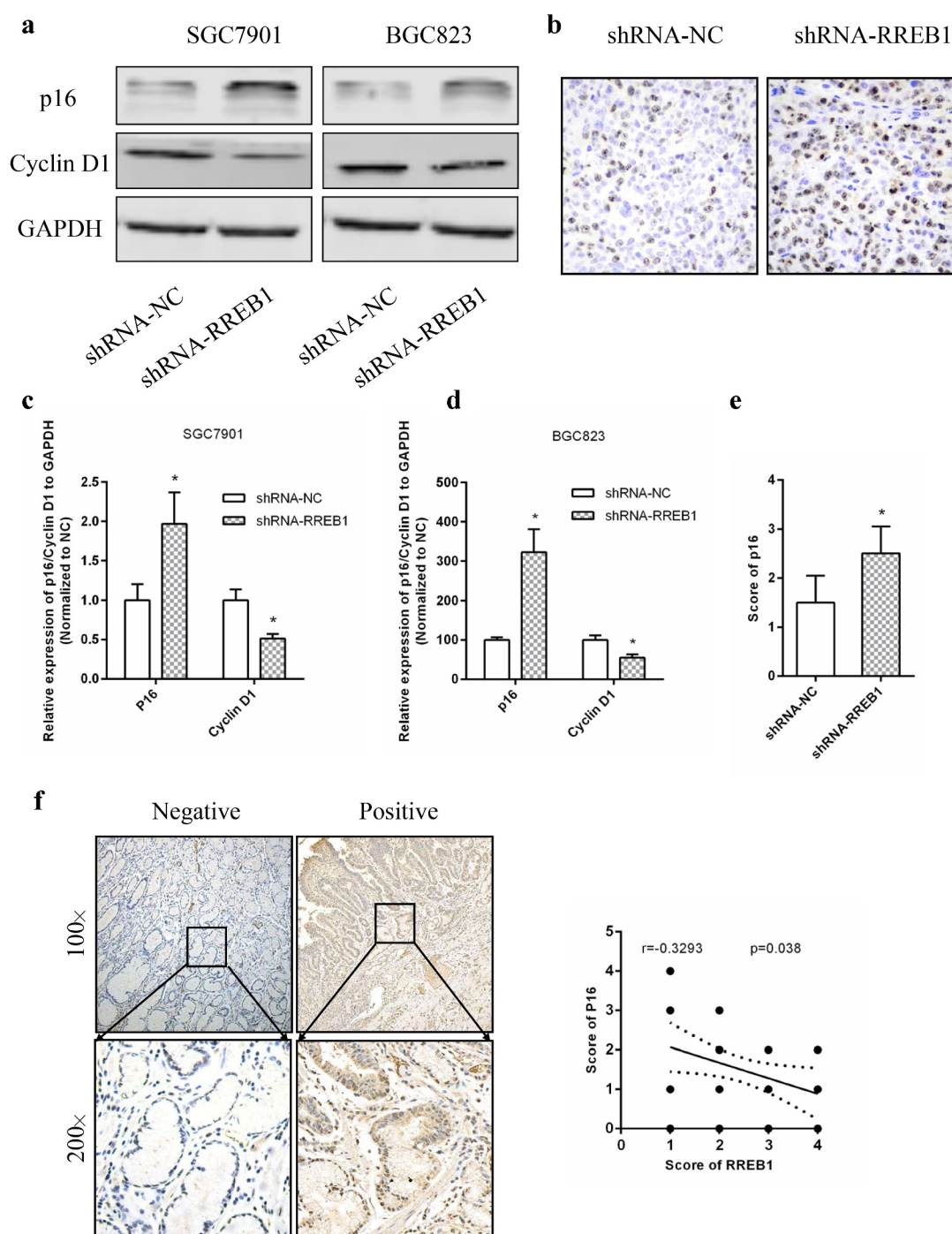
RREB1 inhibited cell proliferation by reducing p16 expression in thyroid cancer<sup>5</sup>. Therefore, we

detected p16 expression in different RREB1 expression cell line. Western blotting showed that the expression of p16 was higher and cyclin D1 was lower in the RREB1 knockdown cell line (Figure 5(a,c,d)), and p16 expression was higher in tumor tissue from the RREB1 knockdown mice (Figure 5(b,e)). We detected the expression of p16 using IHC and analyzed its relationship with RREB1. As shown in Figure 5(f), the expression of RREB1 was negatively related to p16.

## 4. Discussion

Gastric cancer is one of most common cancers in China and a major threat to human health [16,17]. One of the major characteristics of cancer is uncontrolled cell proliferation, and the Ras-related pathway plays an important role in this process [3]. The present study demonstrated that the Ras-related gene RREB1 was highly expressed





**Figure 5.** Knockdown of RREB1 inhibits cell proliferation via enhanced p16 expression.

a/c/d. Knockdown RREB1 can promote P16 expression but inhibit cyclin D1 expression compared to control cell lines and the statistical graph, \* represents  $P < 0.05$ .  $n = 3$ . b/e. Knockdown of RREB1 can enhance p16 expression in vivo, the typical image and IHC staining score of p16 in the mice tumor tissues, \* represents  $P < 0.05$ .  $n = 6$ , for each group. f. The typical images of IHC staining of p16 in gastric cancer tissue and its expression was correlated with RREB1 expression. Pearson test,  $P < 0.05$ ,  $r = 0.3293$ .  $n = 60$ .

in gastric tissue, and knockdown of RREB1 expression inhibited cell proliferation via enhanced p16 expression both in vivo and in vitro. This may give a new sight for development new drug or discover Ras related mechanism for development of cancer.

Ras is one of the most studied oncogenes and plays a crucial role in tumor development. While there have been only a few drugs targeting Ras by now. Discovering Ras-related genes and their functions is very useful. RREB1 is associated with

Ras and highly expressed in prostate cancer tissue and thyroid carcinomas [15,18]. This study demonstrated that RREB1 was highly expressed in gastric cancer and that RREB1-positive patients had a larger tumor size and a higher rate of lymphovascular invasion than RREB1-negative patients. However, due to the small number of patients involved, we did not demonstrate the relationship between the long-term prognosis of patients and different levels of RREB1 expression. To our knowledge, this may be the first study to report RREB1 expression in gastric cancer.

Previous studies demonstrated that RREB1 was necessary for UMUC-3 cell proliferation [14], and it activated various Ras-related molecules [15,19]. The present study demonstrated that RREB1 knockdown inhibited cell proliferation, and this effect may rely on p16. However, we demonstrated that RREB1 overexpression had no significant effect on cell proliferation, which may be attributed to the complex role of RREB1. Previous studies demonstrated that RREB1 promoted Ras-related protein expression and bound to the p53 promoter to increase p53 transcription [20]. Rahrman [21] also demonstrated that in B-cell lymphoma, knockdown of RREB1 inhibited cell proliferation, but the overexpression of RREB1 had no effect on cell growth. In our study, we showed the same effect of RREB1 on cell proliferation as Rahrman. However, the underlying mechanism may need further exploration.

The tumor suppressor proteins p16 and RB1 were well studied in a previous study and are deregulated in most human cancers [22]. The role of p16 in gastric cancer is complex [23,24], and p16 serves as a tumor suppressor by inhibiting the induction of G0/G1 cell cycle arrest [25]. In our study we found that p16 is negatively related with RREB1 and knockdown of RREB1 inhibit cell proliferation maybe relay on increasing p16 expression.

The present study demonstrated that RREB1 is an oncogene in the development of gastric cancer and is associated with cell proliferation. These results provide new insight into the treatment of gastric cancer and may be useful for finding drugs that can target Ras-related pathways.

## Disclosure statement

The authors declare that they have no competing interests.

## Funding

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## Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

Qi Gao and Yunhua Wu designed the study and wrote the manuscript. Chaoxiang Lu, Wang Kai and Weike Xie primarily performed the experiments, wrote the manuscript and prepared the figures. Qi Wang, Li Wang and Shiqi Liu made substantial contributions to the acquisition and analysis of data. Yongkang Pan made contributions to the analysis and interpretation of data, and revised the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Ethical approval for this study was obtained from the Medical Ethics Committee of the Jiaotong University. Patients provided written informed consent prior to sample collection. Animal experiments were approved by the Animal Committee of Xi'an Jiaotong University Health Science Center and all protocols were performed in accordance with Institutional Animal Care and Use Committee guidelines.

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