

ORIGINAL ARTICLE

TRIM11 stimulates the proliferation of gastric cancer through targeting CPEB3/EGFR axis

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Summary

Purpose: To explore the relationship between TRIM11 (Tripartite motif-11) level and clinical pathology of gastric cancer (GC), as well as its regulatory role in the development of GC.

Methods: Differential expression of TRIM11 in GC and paracancer tissues was determined. The relationship between TRIM11 level and clinical pathology of GC patients was assessed. After knockdown of TRIM11, changes in the proliferative potentials of AGS and SGC-7901 cells were examined by cell counting kit-8 (CCK-8), colony formation and 5-Ethynyl-2'-deoxyuridine (EdU) assay. The cytoplasmic polyadenylation element-binding protein 3 (CPEB3) level in GC species was tested and its regulatory role in viability of GC cells was explored as well. The involvement of CPEB3/EGFR axis in TRIM11-regulated proliferative ability of GC was detected by Western blot and rescue experiments.

Results: TRIM11 was upregulated in GC species and its high level was related to poor prognosis, advanced pathological stage and large GC tumor size. Knockdown of TRIM11 attenuated the proliferative potential of GC cells. Protein level of CPEB3 was upregulated, while EGFR and AKT were downregulated in GC cells with TRIM11 knockdown. Moreover, CPEB3 was lowly expressed in GC samples and notably, knockdown of CPEB3 abolished the inhibitory effect of silenced TRIM11 on the proliferative potential of GC.

Conclusions: TRIM11 is upregulated in GC and correlated to prognosis, pathological stage and GC tumor size. TRIM11 triggers the proliferative potential of GC through regulating CPEB3/EGFR axis.

Key words: TRIM11, CPEB3, GC, proliferative potential

Introduction

Gastric cancer (GC) derives from gastric mucosal epithelium and accounts for 95% of malignant tumors in the stomach [1,2]. Globally, the incidence of GC ranks fifth (accounting for 7%) in high-grade malignant tumors, and its mortality ranks third (9%) [3,4]. At present, GC is frequent in China, and it has become one of the leading causes of death [5,6]. The onset of GC is insidious with atypical symptoms in the early disease phases. It is also easy to be misdiagnosed as gastritis or ulcer when seeking medical treatment [6,7]. In addition, strong invasion and metastatic trend of GC lead to infiltration of tumor cells to distant organs, es-

pecially liver, lungs, bones and lymph nodes [8,9]. Therefore, most patients are already diagnosed as advanced GC at the initial diagnosis, and their 5-year survival rate is often less than 10% [10]. The pathogenesis of GC is complicated, involving both genetic and environmental factors [10,11]. In-depth study of the molecular mechanisms of the development, invasiveness and metastasis of GC is of particular significance, that could improve the therapeutic outcomes [12,13].

TRIM11 (Tripartite motif-11) is a member of the TRIM protein family located on human chromosome 1q42.13. It contains an open reading

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frame and 6 exons. TRIM11 is 2715 bp long and encodes a 55 kD protein [14,15]. As a newly discovered antiviral factor, TRIM11 exerts an antiretroviral effect by interfering with the transduction of HIV-1, and an anti-inflammatory role by negatively regulating interferon IFN β [16]. The zinc finger domain presents an E3 ubiquitin ligase activity and participates in the ubiquitin proteasome pathway by recruiting E2 and substrate proteins. Serving as an E3 ubiquitin ligase, TRIM11 is extensively involved in many life processes [17,18]. In addition, dysregulated TRIM11 is linked to tumorigenesis and tumor development through activating relevant pathways to further influence tumor cell phenotypes [15,18,19].

CPEB3 is predicted by bioinformatics analysis to be the downstream target that specifically binds TRIM11. Members in CPBE family are highly conserved RNA-binding proteins that are able to extend polyA of mRNAs [20,21]. CPEB family can not only activate the translation of the target RNA but also act as an inhibitory signal to damage the histological barrier that blocks tumor cell invasion [22,23]. In this paper, we mainly discussed the role of TRIM11 and CPEB3 in the malignant proliferative potential of GC.

Methods

Tumor species

Forty-five paired GC and paracancer tissues were surgically resected and stored at -80°C. GC cases were independently diagnosed by two experienced pathologists.

Clinical data of each subject were recorded. This study got approval by Ethics Committee of our hospital and conducted after each patient provided signed informed consent.

Cell culture

GC cell lines (AGS, BGC-823 and SGC-7901) and immortalized normal gastric epithelial cell line (GES-1) were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μ g/mL streptomycin in a 5% CO₂ incubator at 37°C. When cells reached 80-90% confluence trypsin and EDTA were used for digestion and passage.

Transfection

TRIM11 shRNAs, sh-NC, si-CPEB3 and si-NC were constructed by GenePharma (Shanghai, China). Cells were grown to 30-40% confluence and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Twenty-four h later, cells were digested at 1:3, and Puromycin was added until cell adherence. Two weeks later, monoclonal stably transfected si-CPEB3 and si-NC cells were collected and cultured in DMEM medium with 4 μ m/mL Puromycin.

Table 1. Primer sequence

Gene	Primer sequence
TRIM11	forward: 5'-GTGCCTATGGAGCTGAGGAC-3' reverse: 5'-CAGGATCAGCTCAGGGTTG-3'
CPEB3	forward: 5'-GAAAGGTAAACACTACCCTCCCA-3' reverse: 5'-CCAGGAAGGCATTGTTAAGTGC-3'
β -actin	forward: 5'-TACCTCATGAAGATCCTCACC-3' reverse: 5'-TTTCGTGGATGCCACAGGAC-3'

Table 2. Association of TRIM11 expression with clinicopathologic characteristics of gastric cancer.

Parameters	Number of cases	TRIM11 expression		p value
		Low	High	
Age (years)				0.273
<60	18	7	11	
\geq 60	27	15	12	
Gender				0.652
Male	23	12	11	
Female	22	10	12	
T stage				0.042
T1-T2	28	17	11	
T3-T4	17	5	12	
Tumor size (cm)				0.013
<3	31	19	12	
\geq 3	14	3	11	
Distant metastasis				0.420
No	28	15	13	
Yes	17	7	10	

Cell proliferation assay

Cells were inoculated in a 96-well plates with 2×10^5 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Colony formation assay

Cells were inoculated in 6-well plates with 100 [ONLY?] cells per well and cultured for 2 weeks. DMEM was replaced once in the first week and twice in the second week. Visible colonies were washed in phosphate buffered saline (PBS), fixed in methanol for 20 min and

died in 0.1% crystal violet for 20 min, and pictures were captured and calculated.

5-Ethynyl-2'- deoxyuridine (EdU) assay

Cells in 96-well plates (6×10^5 cells/well) were incubated with EdU solution in the dark, and 30 min later, they were dyed with DAPI for another 30 min. EdU-positive ratio was calculated by the number of EdU-positive cells to that of 4',6-diamidino-2-phenylindole (DAPI)-labeled nuclei.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Extracted RNAs by TRIZol reagent (Invitrogen,

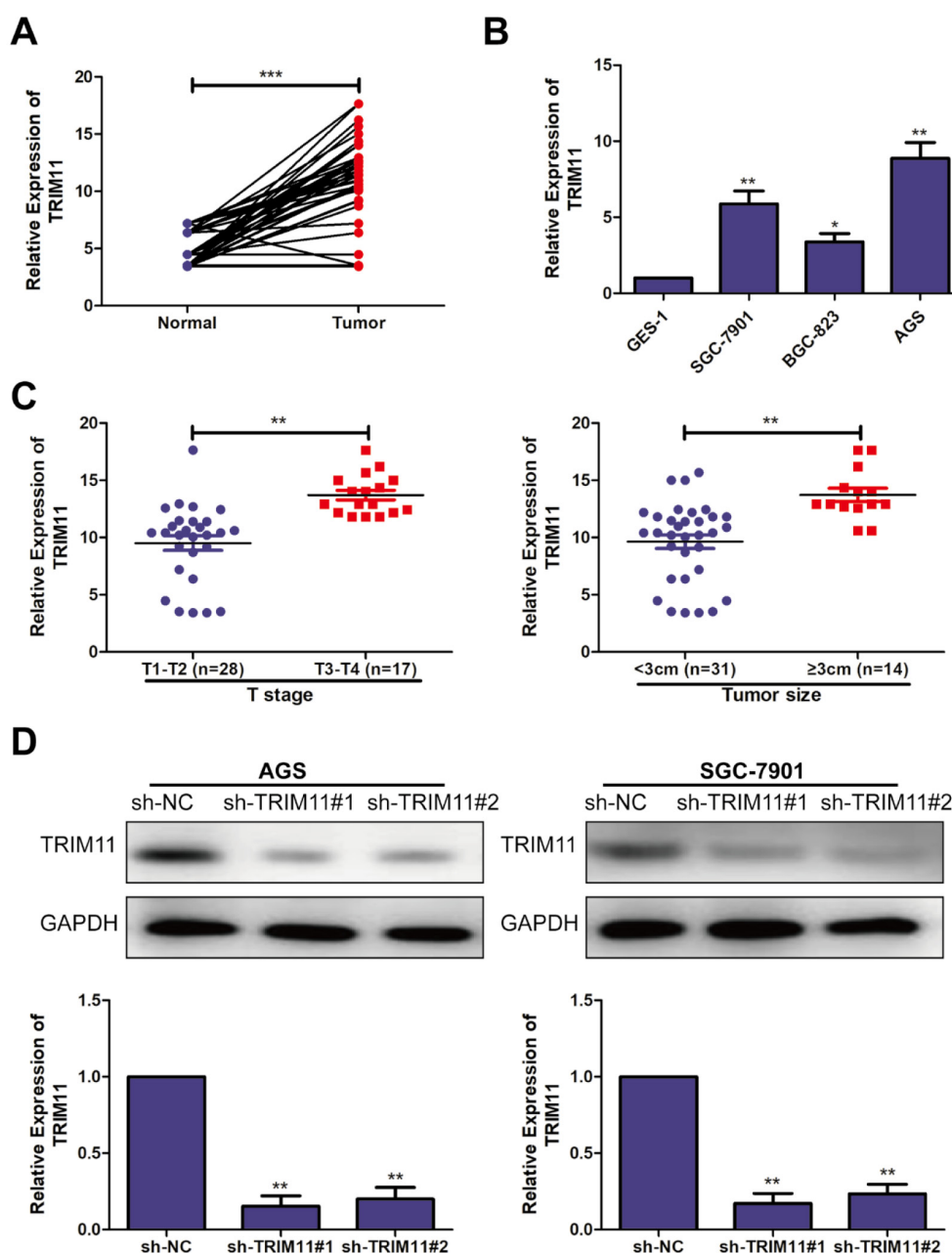


Figure 1. TRIM11 was highly expressed in GC species. **A:** Differential expressions of TRIM11 in GC and paracancer species. **B:** TRIM11 level in GES-1 and GC cell lines. **C:** TRIM11 levels in GC species with different T stages (T1-T2 and T3-T4) or tumor sizes (<3 cm and ≥ 3 cm). **D:** Transfection efficacy of sh-TRIM11#1 and sh-TRIM11#2 in AGS and SGC-7901 cells. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Japan). The obtained cDNAs underwent qRT-PCR using SYBR[®]Premix Ex Taq[™] (TaKaRa, Otsu, Japan). β -actin was used as internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$ (Table 1).

Western blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Bill-

erica, MA, USA). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Luciferase assay

AGS cells inoculated in 24-well plates were co-transfected with NC/pcDNA-TRIM11 and NC/pcDNA-CPEB3, respectively. Luciferase assay was conducted 48h after transfection by Dual Luciferase Reporter assay system (Promega, Madison, WI, USA). All the experiments were performed in triplicate.

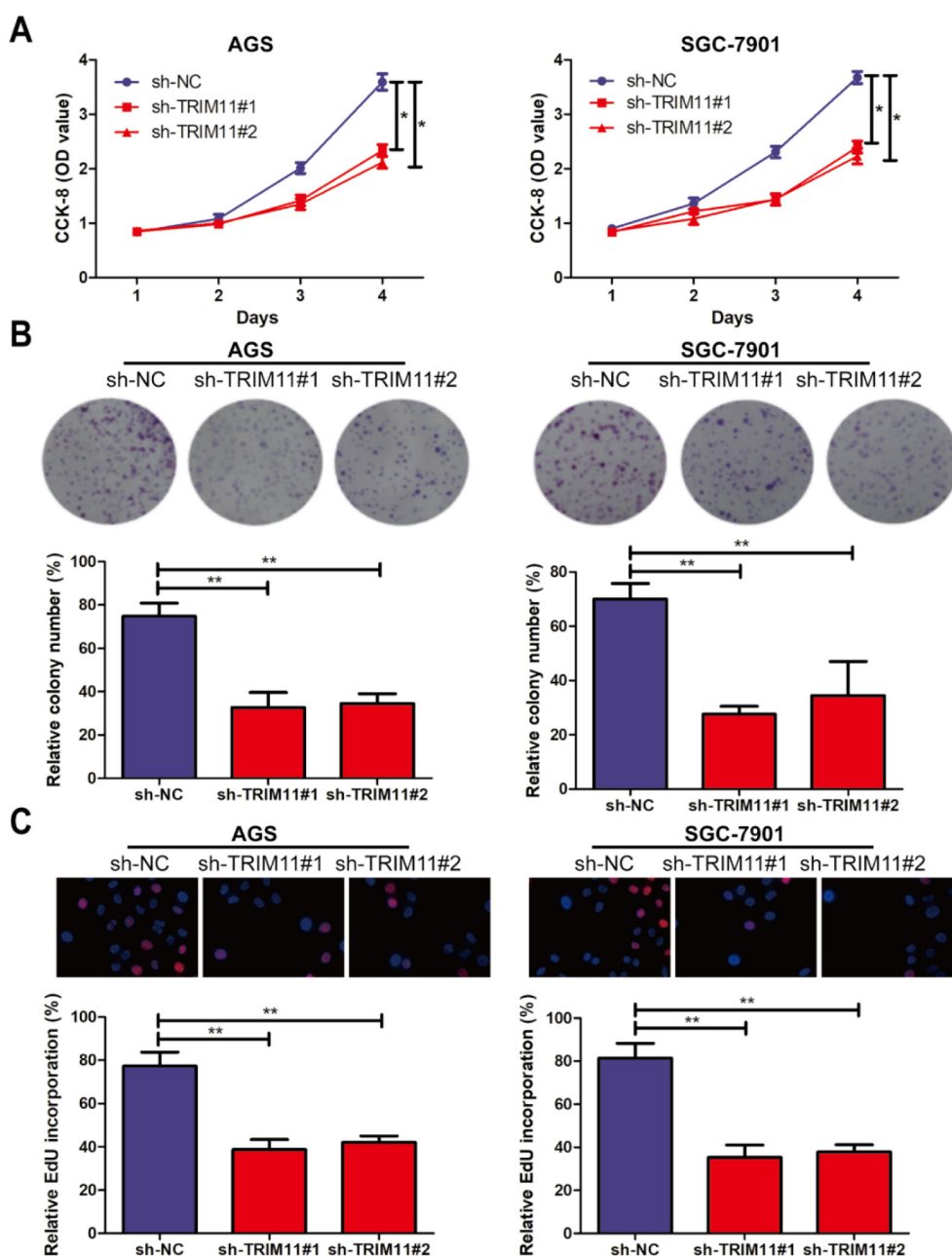


Figure 2. Silence of TRIM11 attenuated the proliferative potential of GC. **A:** Viability in AGS and SGC-7901 cells transfected with sh-NC, sh-TRIM11#1 or sh-TRIM11#2. **B:** Colony number in AGS and SGC-7901 cells transfected with sh-NC, sh-TRIM11#1 or sh-TRIM11#2 (magnification: 20 \times). **C:** EdU-positive rate in AGS and SGC-7901 cells transfected with sh-NC, sh-TRIM11#1 or sh-TRIM11#2 (magnification: 40 \times). Data were expressed as mean \pm SD. * p <0.05, ** p <0.01.

Statistics

SPSS 22.0 (IBM, Armonk, NY, USA) statistical package was used for data analyses. Data were expressed as mean \pm standard deviation. Differences between two groups were analyzed by the t-test. Pearson's correlation test was applied for evaluating the relationship between expression levels of TRIM11 and CPEB3 in GC samples. $P < 0.05$ was considered as statistically significant.

Results

TRIM11 was highly expressed in GC samples

We collected 45 matched GC and paracancer tissues. QRT-PCR data showed higher abundance of TRIM11 in GC tissues than the paracancer

tissues (Figure 1A). Identically, TRIM11 was highly expressed in GC cell lines as well (Figure 1B).

TRIM11 was correlated with pathological stage, tumor size and overall survival in GC patients

Based on the cut-off value of TRIM11 in 45 GC tissue samples, GC patients were classified into high or low-level group, respectively. Through analyzing their clinical data, it was shown that TRIM11 level was positively correlated to pathological stage and tumor size of GC patients, while it was unrelated with age, sex and distant metastasis (Table 2). Moreover, higher level of TRIM11 was observed in GC patients with T3-T4 or tumor size

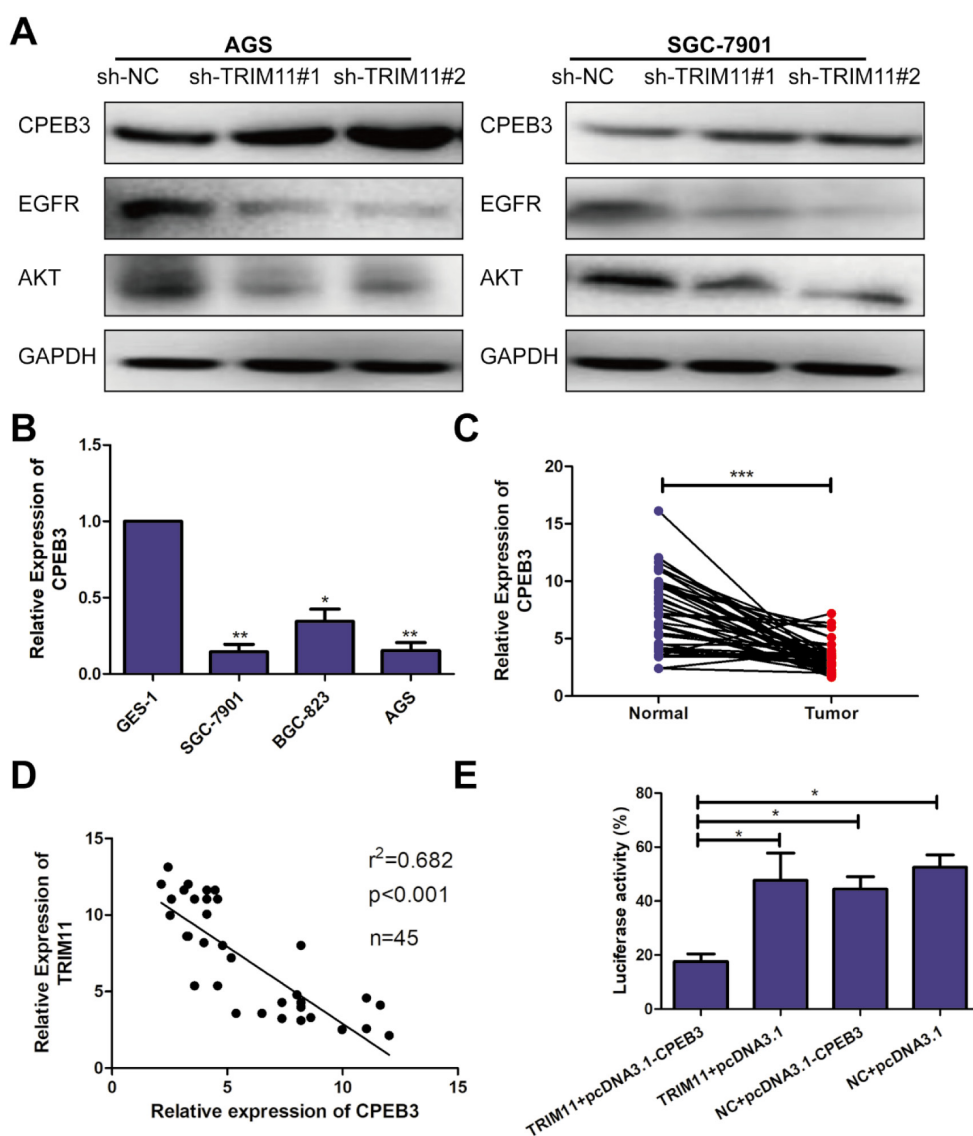


Figure 3. TRIM11 regulated CPEB3/EGFR axis. **A:** Protein levels of CPEB3, EGFR and AKT in AGS and SGC-7901 cells transfected with sh-NC, sh-TRIM11#1 or sh-TRIM11#2. **B:** CPEB3 level in GES-1 and GC cell lines. **C:** Differential expressions of CPEB3 in GC and paracancer tissues. **D:** A negative correlation between expression levels of CPEB3 and TRIM11 in GC species. **E:** Luciferase activity in co-transfected AGS cells. Data were expressed as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

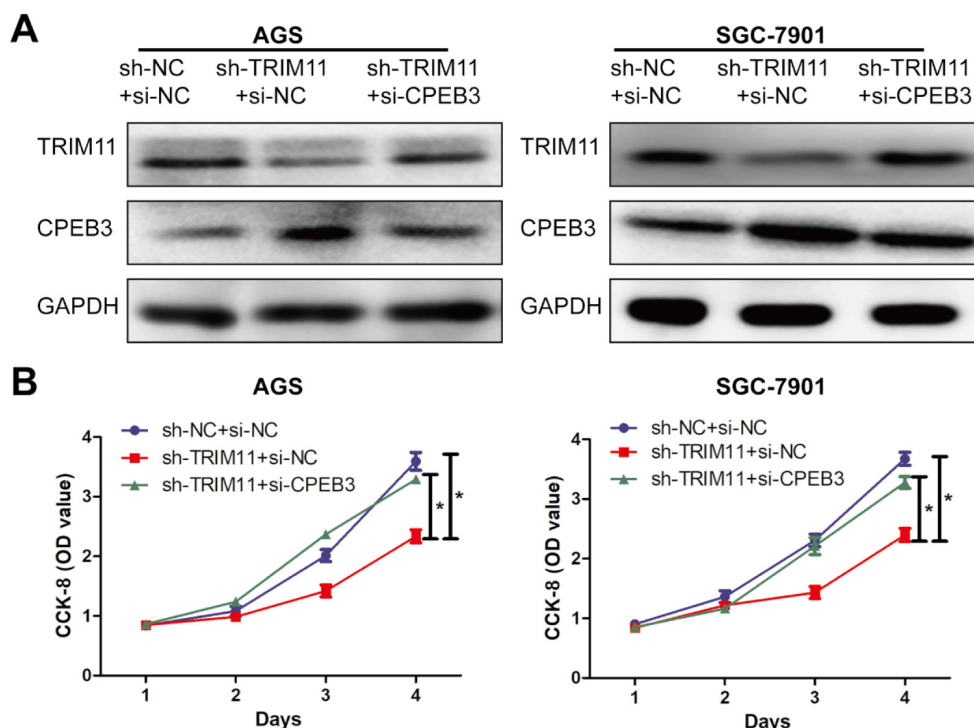


Figure 4. TRIM11 regulated the proliferative potential of GC through targeting CPEB3. **A:** Protein levels of TRIM11 and CPEB3 in AGS and SGC-7901 cells transfected with sh-NC+si-NC, sh-TRIM11+si-NC or sh-TRIM11+si-CPEB3. **B:** Viability in AGS and SGC-7901 cells transfected with sh-NC+si-NC, sh-TRIM11+si-NC or sh-TRIM11+si-CPEB3. Data were expressed as mean \pm SD. * p <0.05.

≥ 3 cm compared with those with T1-T2 or tumor size < 3 cm (Figure 1C).

Silence of TRIM11 attenuated the proliferative potential of GC

Two TRIM11 shRNAs were constructed, and both sh-TRIM11#1 and sh-TRIM11#2 presented an excellent transfection efficacy in AGS and SGC-7901 cells (Figure 1D). Subsequently, decreased viability (Figure 2A) and colony number (Figure 2B) were seen in GC cells transfected with sh-TRIM11#1 or sh-TRIM11#2 compared with controls. Similarly, knockdown of TRIM11 in GC cells also reduced EdU-positive rate (Figure 2C). The above data demonstrated the promotive role of TRIM11 in the proliferative potential of GC.

TRIM11 regulated CPEB3/EGFR axis

CPEB3/EGFR axis is reported to be linked to tumor cell proliferation. Here, the protein level of CPEB3 was markedly upregulated, while EGFR and AKT were downregulated in GC cells with TRIM11 knockdown (Figure 3A).

CPEB3 was lowly expressed in GC specimens

Compared with GES-1 cells, CPEB3 was lowly expressed in GC cells (Figure 3B). CPEB3 was identically downregulated in GC species (Figure 3C).

A negative correlation was identified between the expression levels of CPEB3 and TRIM11 in GC (Figure 3D). Furthermore, luciferase assay confirmed the bioinformatics prediction that CPEB3 was the target gene binding TRIM11 (Figure 3E).

TRIM11 regulated the proliferative potential of GC through targeting CPEB3

To explore the involvement of CPEB3 in GC development, we designed rescue experiments. Co-silence of TRIM11 and CPEB3 downregulated CPEB3 and upregulated TRIM11 in GC cells with TRIM11 knockdown (Figure 4A). Moreover, decreased viability in GC cells with TRIM11 knockdown was partially reversed by co-transfection of si-CPEB3 (Figure 4B). Hence, CPEB3 was responsible for TRIM11-regulated proliferative potential of GC.

Discussion

Tumor burden is persistently increasing owing to aging, smoking, stress and lifestyle changes world over [24,25]. Tumor incidence in developing countries is only 50% to that in developed countries, while tumor mortality is similar because of limited screening and treatment [25]. In China, the morbidity and mortality of GC are in high place, ranking fourth and second in the world, respective-

ly [2-4]. Abnormally expressed genes in GC greatly influence tumor cell behaviors and treatment sensitivity [6-8]. Clarifying certain gene functions involved in GC development contributes to unveiling potential therapeutic targets [9-12].

TRIM family is closely linked to apoptosis, virus response, malignancies and hereditary diseases [14-16]. As a vital member of TRIM family, TRIM11 has been identified to be a tumor-associated gene [15,18,19]. TRIM11 is highly expressed in hepatocellular cancer and glioma, exerting different tumorigenesis mechanisms [15,18,19]. In this paper, TRIM11 was upregulated in GC and its level was positively correlated to prognosis, pathological stage and tumor size. *In vitro* evidence showed that knockdown of TRIM11 attenuated the proliferative potential of GC cells. It is concluded that TRIM11 was able to stimulate the development of GC.

To further explore the biological function of TRIM11, CPEB3 was predicted to be the target gene binding TRIM11 and further confirmed by luciferase assay. Subsequently, protein level of

CPEB3 was found to be upregulated in GC cells with TRIM11 knockdown. In addition, a negative correlation was identified between the expression levels of TRIM11 and CPEB3 in GC samples. Rescue experiments uncovered that knockdown of CPEB3 abolished the inhibitory effect of silenced TRIM11 on the proliferative potential of GC. The above evidence indicated that the transcriptional activity of the gene locus at TRIM11 may be regulated by CPEB3, thereby promoting the proliferative potential of GC.

Conclusions

TRIM11 is upregulated in GC and correlated to prognosis, pathological stage and size of GC. TRIM11 triggers the proliferative potential of GC through regulating CPEB3/EGFR axis.

Conflict of interests

The authors declare no conflict of interests.

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