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Original Articles

Circular RNA profile identifies circPVT1 as a proliferative factor and prognostic marker in gastric cancer

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ABSTRACT

Circular RNAs (circRNAs) comprise a novel class of widespread non-coding RNAs that may regulate gene expression in eukaryotes. However, the characterization and function of circRNAs in human cancer remain elusive. Here we identified at least 5500 distinct circRNA candidates and a series of circRNAs that are differentially expressed in gastric cancer (GC) tissues compared with matched normal tissues. We further characterized one circRNA derived from the PVT1 gene and termed it as circPVT1. The expression of circPVT1 is often upregulated in GC tissues due to the amplification of its genomic locus. circPVT1 may promote cell proliferation by acting as a sponge for members of the miR-125 family. The level of circPVT1 was observed as an independent prognostic marker for overall survival and disease-free survival of patients with GC. Our findings suggest that circPVT1 is a novel proliferative factor and prognostic marker in GC.

Introduction

Stomach adenocarcinoma, otherwise known as gastric cancer (GC), is one of the most common cancers worldwide and is thus a global cancer burden. According to the GLOBOCAN database, GC is the fourth most common cancer and the third-leading cause of cancer-related deaths worldwide [1]. Despite many advances in the diagnosis and treatment of this disease, the prognosis of patients with GC remains poor, with a 5-year overall survival of less than 30% in most countries [2]. Therefore, the discovery of new molecular mechanisms and therapeutic targets that may control the severity of GC and present a predictive value for prognosis are of great importance.

Circular RNAs (circRNAs) from back-spliced exons have been recently identified as a naturally occurring family of noncoding RNAs (ncRNAs) that is highly prevalent in the eukaryotic transcriptome [3–6]. circRNAs are characterized by a covalently closed loop structure with neither a 5′ cap nor a 3′ polyadenylated tail. They are highly stable, are found predominantly in the cytoplasm and can be sorted into exosomes [7]. The formation of circRNA involves the occurrence of back-splicing by the canonical spliceosome [8]. Such circularization is facilitated by surrounding complementary sequences and is regulated by specific RNA binding proteins [9–13]. It is becoming increasingly evident that circRNAs are not simply by-products of mis-spliced RNAs or splicing errors, but rather, they are the products of regulated back-spliced RNAs with distinct sets of cis-elements and/or trans-factors [14]. Accordingly, many circRNAs have been found to be upregulated during brain development and during epithelial–mesenchymal transition (EMT) in humans [13,15,16].

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Recently, circRNAs were shown to act as miRNA sponges that regulate gene expression [4,17]. Importantly, a growing number of studies have demonstrated that circRNAs are closely associated with disease and may play a significant role in the pathogenesis and diagnosis of disease [18–20]. However, the characterization and function of circRNAs in human cancer remain largely unknown.

In this study, we generated ribo-minus RNA sequencing data from gastric cancer tissues of three patients, and identified approximately 5500 circRNA candidates (at least two unique back-spliced reads). We characterized one circRNA derived from the PVT1 gene locus, termed circPVT1, which is frequently upregulated in patients with GC. Functional assays revealed that circPVT1 could promote cell proliferation by sponging members of miR-125 family. circPVT1 was further found to be an independent prognostic marker for survival in patients with gastric cancer.

Materials and methods

Human samples

We retrospectively collected paired cancer specimens and adjacent normal tissues from patients with gastric cancer who had surgically proven primary GC and who received a D2 radical gastrectomy (R0 resection) at Fudan University Shanghai Cancer Center (FUSCC) between December 2007 and December 2010. None of these patients received preoperative chemotherapy or radiotherapy. Clinic pathological features, which included age, gender, tumor site, tumor size, differentiation grade, TNM stage (American Joint Committee on Cancer classification, AJCC), lymphatic invasion and neural invasion, are shown in Table 1. The median follow-up time was 26.0 months (range: 1–85 months). The follow-up interval began on the date of surgery and ended on the date of disease progression, death or the last clinical investigation. This study was approved by The Clinical Research Ethics Committee of Fudan University Shanghai Cancer Center. Written informed consent was obtained from all participants.

RNA-seq analysis

Total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Approximately 3 μg of total RNA from each sample was subjected to the RiboMinus Eukaryote Kit (Qiagen, Valencia, CA) to remove ribosomal RNA prior to the construction of the RNA-seq libraries. Strand-specific RNA-seq libraries were prepared using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, Beverly, MA, USA). Briefly, approximately 50 ng of ribosome-depleted RNA samples was fragmented and then used for first- and second-strand cDNA synthesis with random hexamer primers. A dUTP mix was used for second-strand cDNA synthesis. An End-It DNA End Repair Kit was used to repair the ends of the double-stranded cDNA fragments, which were then modified by the Klenow fragment so that an A was added to the 3′ end of the DNA fragments and were finally ligated to adapters. The ligated products were purified and treated with uracil DNA glycosylase (UDG) to remove the second-strand cDNA. Purified first-strand cDNA was subjected to 13–15 cycles of PCR amplification, followed by analysis of the libraries with a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA); the cDNA was then sequenced in a HiSeq 2000 system (Illumina, San Diego, CA, USA) on a 100-bp paired-end run.

Identification and quantification of circRNAs

The RNA-seq fastq reads were first mapped to the human reference genome (GRCh37/hg19) obtained from the UCSC genome database (http://genome.ucsc.edu/) using TopHat2 [21]. The unmapped reads were then used to identify circRNAs as previously described [4]. Briefly, the unmapped reads were processed to 20-nucleotide anchors from both ends of the sequencing read. Anchors that aligned in the reverse orientation (head-to-tail) represent a back-spliced junction. Anchor alignment was extended such that the complete read alignment and the breakpoint were flanked by a GT/AG splice site. The total number of reads that spanned back-spliced junctions was used as an absolute measure of circRNA abundance. Counts of reads that mapped across an identified backsplice were normalized by read length and number of mapped reads (i.e., spliced reads per billion mapping [SRPBM]). The genomic regions that mapped to inferred circRNAs were annotated according to RefSeq. Gene coordinates were downloaded from the RefGene tables in the UCSC Genome Browser (downloaded on 03/28/2014). The host genes of circRNAs were determined using a custom script. For each circRNA, we searched for the longest transcript fragment whose boundaries (5′ end or 3′ end) exactly matched both ends

Table 1
Relationship between circPVT1/PVT1 expression and clinicopathologic factors of patients with gastric cancer.

Parameter	No. of patients	circPVT1 (high)	circPVT1 (low)	P value	PVT1 (high)	PVT1 (low)	P value
Sex				0.138			0.125
Male	144	86	58		90	54	
Female	43	21	22		22	21	
Age (yr)				0.551			0.169
<60	84	48	36		54	30	
≥60	103	59	44		58	45	
Tumor site				0.694			0.275
Upper	45	24	21		28	17	
Middle	65	41	24		43	22	
Lower	58	32	26		33	25	
Diffuse	19	10	9		9	11	
Tumor size (cm)				0.174			0.030*
≤5	109	66	43		72	37	
>5	78	41	37		40	38	
Differentiation grade				0.188			0.546
Well-moderate	47	30	17		84	56	
Poor-undifferen-tiation	140	77	63		28	19	
T stage				0.020*			0.105
T1–T3	40	29	11		20	20	
T4	147	78	69		92	55	
Lymph node status				0.544			0.201
Negative	24	14	10		12	12	
Positive	163	93	70		100	63	
Distant metastasis				0.494			0.077
M0	160	91	69		92	68	
M1	27	16	11		20	7	
TNM stage				0.194			0.400
I–II	37	24	13		21	16	
III–IV	150	83	67		91	59	
Lymphatic invasion				0.464			0.337
Negative	72	42	30		45	27	
Positive	115	65	50		67	48	
Nervous invasion				0.030*			0.398
Negative	69	48	21		40	29	
Positive	118	59	59		72	46	

The TNM Staging System is based on the tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M)*P < 0.05.

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of this circRNA in the same strand and then defined the corresponding gene of this transcript fragment as the host gene of this circRNA.

Cell culture and treatments

MGC-803 and AGS cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C and 5% CO_2. HEK-293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Transcription was blocked by the addition of 2 μg/ml Actinomycin D or DMSO (Sigma–Aldrich, St. Louis, MO, USA), which served as a control for the cell culture medium.

Nucleic acid preparation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Genomic DNA was isolated with a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA), and total RNA was isolated using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). The nuclear and cytoplasmic fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). Total RNA from the nuclear and cytoplasmic fractions was isolated with TRIzol. Complementary DNA was synthesized using the PrimeScript RT reagent kit (Takara Bio Inc., Dalian, China), and RT-PCR was performed using SYBR Premix Ex Taq (Takara Bio Inc.). The primers are listed in Supplementary Table S2.

RNase R treatment

Total RNA (2 μg) was incubated for 20 min at 37 °C with or without 3 U/μg of RNase R (Epicentre Technologies, Madison, WI, USA). The resulting RNA was purified with an RNeasy MinElute Cleanup Kit (Qiagen).

Vector construction

The circPVT1 genomic region and its wild-type flanking introns were amplified from the genomic DNA using PrimerSTAR Max DNA Polymerase Mix (Takara) and were subcloned into a pcDNA3.0 vector. In the luciferase reporter assay, circPVT1 was amplified from the genomic DNA and was inserted into the region directly downstream of a cytomegalovirus (CMV) promoter-driven firefly luciferase cassette in a pCDNA3.0 vector. Mutations in the miRNA binding sites in the circPVT1 sequence were generated using a Mut Express II Fast Mutagenesis Kit (Vazyme, NanJing, China). The constructs were verified by sequencing. The primers are listed in Supplementary Table S2.

Oligonucleotide transfection

siRNA and miRNA mimics were synthesized by RiboBio (Guangzhou, China). The sequences that were used are shown in Supplementary Table S2. The cells were transfected using Lipofectamine RNAiMax (Life Technologies).

CCK8 assay

Cell proliferation was assessed by Cell Counting Kit-8 assay (Dojindo Laboratories, Kumamoto, Japan). The cells (1×10^3) were seeded into 96-well plates. Then, 10 μl of CCK-8 solution was added to each well on days 1, 2, 3, 4 and 5. After 2 h of incubation at 37 °C, the absorbance at 450 nM was measured using an automatic microplate reader (Synergy4; BioTek, Winooski, VT, USA). The experiment was repeated three times.

Colony formation assay

For the colony formation assays, the cells were trypsinized, and 1×10^3 cells were plated in 6-well plates and incubated at 37 °C for 14 days. Colonies were dyed with dyeing solution containing 0.1% crystal violet and 20% methanol. Cell colonies were then counted and analyzed.

5-Ethynyl-2′-deoxyuridine (EdU) incorporation assay

The EdU assay was performed with a Cell-Light EdU DNA Cell Proliferation Kit (RiboBio, Shanghai, China). Cells (1×10^4) were seeded in each well of 96-well plates. After incubation with 50 μM EdU for 2 h, the cells were fixed in 4% paraformaldehyde and stained with Apollo Dye Solution. Hoechst 33342 was used to stain the nucleic acid within the cells. Images were obtained with an Olympus FSX100 microscope (Olympus, Tokyo, Japan), and the number of EdU-positive cells was counted.

Luciferase reporter assay

HEK-293T cells (5×10^3) were seeded into 96-well plates and were cotransfected with a mixture of 50 ng of firefly luciferase reporter, 5 ng of pRL-CMV Renilla luciferase reporter, and miRNA mimics. After 48 h of incubation, the firefly and Renilla luciferase activities were quantified with a dual-luciferase reporter assay (Promega, Madison, WI, USA).

Statistical analysis

Statistical analyses were performed using SPSS 20.0 (IBM, SPSS, Chicago, IL, USA) and GraphPad Prism. Student's t-test and one-way ANOVA were used to determine the statistical significance for comparisons of 2 or more groups. The Pearson correlation coefficient was used to analyze the correlations. Overall survival (OS) and disease-free survival (DFS) were measured from the date of surgery. For OS, patients known to be alive at the time of the last follow-up were censored on the last date of contact. DFS was defined as the time from the date of surgery to the date of progression (local and/or distal tumor recurrence) or to the date of death; those patients who were alive without disease at the time of the last follow-up were censored. DFS and OS curves were calculated with the Kaplan–Meier method and were analyzed with the log-rank test. Univariate analysis and multivariate models were constructed using a Cox proportional hazards regression model. Receiver operating characteristic (ROC) curves were generated using Medical statistical software version 12.2.2. All tests were 2-sided, and $P < 0.05$ was considered statistically significant.

Results

Identification of circular RNAs by RNA-seq analysis in gastric cancer

We first characterized circular RNA transcripts using RNA-seq analysis of ribosomal RNA-depleted total RNA from three paired normal and cancerous gastric tissues (Fig. 1A). Each sample was sequenced on an Illumina HiSeq and yielded ~60 million reads, which were mapped to the human reference genome (GRCh37/hg19) by TopHat2 [21]. A computational pipeline based on the anchor alignment of unmapped reads was used to identify circRNAs without reliance on gene annotations [4]. In all, 15623 distinct circRNA candidates were found in these tissues and 5568 of these circRNAs contained at least two unique back-spliced reads (Fig. 1B). We normalized the back-spliced reads (support for circRNA) by read length and the number of mapped reads (spliced reads per billion mapping, SRPBM), which permits quantitative comparisons between back-spliced events from different RNA-seq data [22]. Each normal and cancerous gastric tissue sample expressed approximately 3000 circRNAs with SRPBM that ranged from 0.1 to 62.6 (Fig. 1C). The expression analysis of these circRNA transcripts revealed that a series of circRNAs was differentially expressed in cancerous tissues compared with matched normal tissues (Fig. 1D, Supplementary Table S1). Among the 180 differentially expressed circRNAs, 82 were upregulated and 98 were downregulated in GC tissues compared with normal tissues. Approximately 80% of these circRNAs (145/180) are derived from 138 protein coding genes (Supplementary Table S1). These circRNAs and their host genes locate at diverse genomic regions (Fig. 1E). Notably, the expression levels of circRNAs were not correlated with their corresponding parent genes (Fig. 1E and Supplementary Fig. S1), which suggests post-transcriptional regulation. We further confirmed the RNA-seq results of four circRNAs in 20 paired normal and cancerous gastric tissues by quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis (Supplementary Fig. S2).

Characterization of circPVT1 circular RNA in gastric cancer

We noted that one of the most differentially expressed circRNAs (chr8:128902834- 128903244, Fig. 1E) is derived from a long non-coding RNA region within the PVT1 locus, which is located on chromosome 8q24, a cancer susceptibility locus. We termed this circRNA as "circPVT1". The genomic structure shows that a third exon from the PVT1 gene is flanked by long introns on each side (Fig. 2A). The distinct product of the expected size was amplified by outward-facing primers and was confirmed by Sanger sequencing (Fig. 2A). Resistance to digestion by RNase R exonuclease further confirmed that this RNA species exists in a circular form (Fig. 2B). qRT-PCR analysis of nuclear and cytoplasmic RNAs showed that circPVT1 was preferentially localized within the cytoplasm and that

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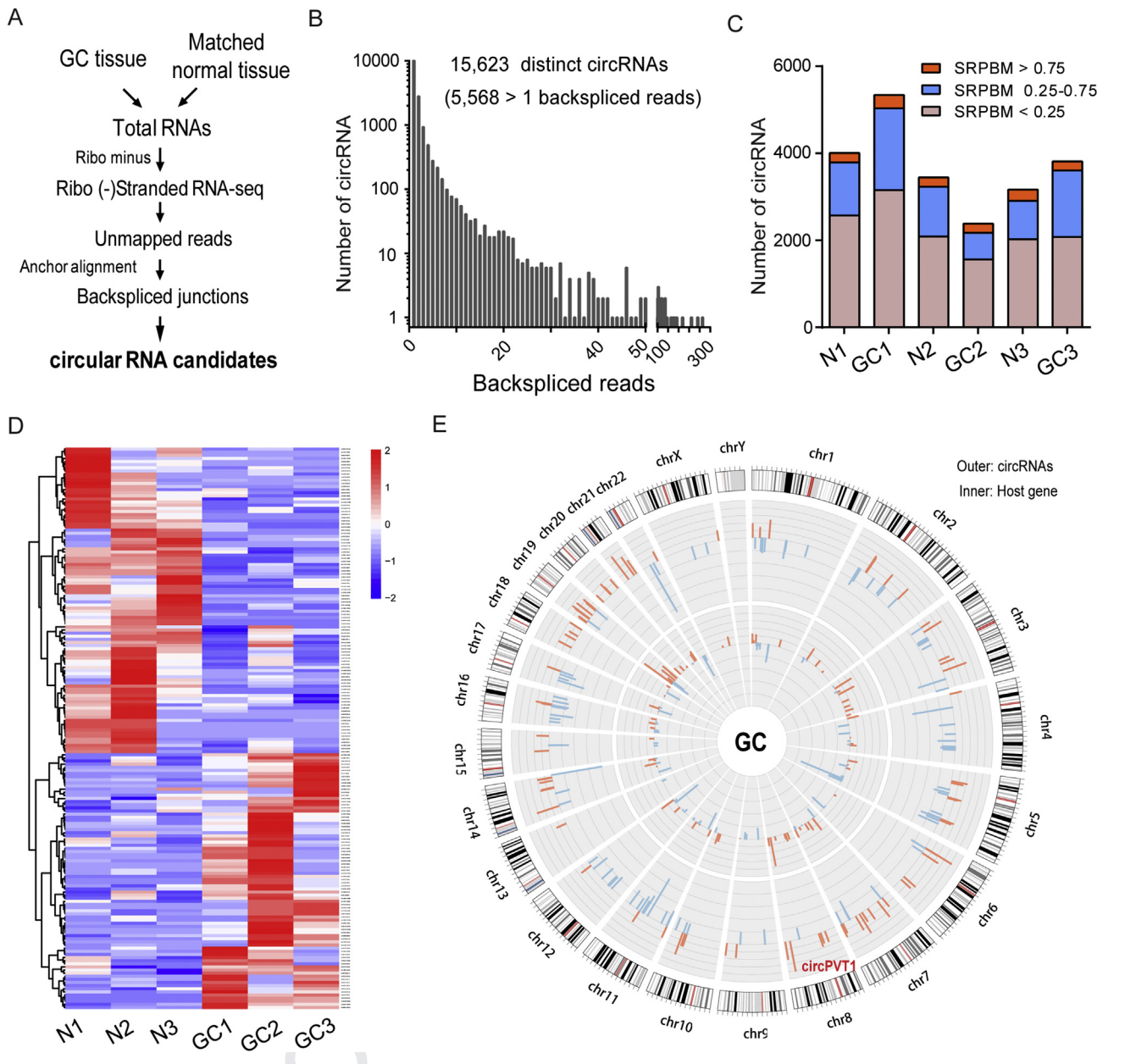

Fig. 1. Identification of circular RNAs by RNA-seq analyses in gastric cancer. **(A)** RNA-seq analysis of circular RNAs in three paired human gastric cancer tissues and matched normal tissues. **(B)** The total number of circRNAs and back-spliced reads that were identified in three paired gastric cancer tissues and matched normal tissues. **(C)** The number of circular RNAs that were identified from three paired gastric cancer tissues and matched normal tissues using different spliced reads per billion mapping (SRPBM) cut-off. **(D)** Clustered heatmap of the differentially expressed circRNAs in three paired human gastric cancer tissues and matched normal tissues. Rows represent circRNAs while columns represent tissues. The circRNAs were classified according to the Pearson correlation. **(E)** Circos plots showing the differentially expressed circRNAs and their host genes in GC tissues. Outer: the differentially expressed circRNAs; Inner: host genes. The values represent the log(Fold change) of cancer VS normal, Red: upregulated, Blue: downregulated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PVT1 was primarily localized within the nucleus in AGS and MGC-803 cells (Fig. 2C and Supplementary Fig. S3A). We next investigated the stability and localization of circPVT1. Total RNA was harvested at the indicated time points after treatment with Actinomycin D, which is an inhibitor of transcription. Analysis of circPVT1 and PVT1 RNA revealed that the circular RNA isoform circPVT1 is highly stable, as its transcript half-life exceeded 24 h, whereas the linear transcript of PVT1 RNA exhibited a half-life of <4 h in AGS and MGC-803 cells (Fig. 2D and Supplementary Fig. S3B). Taken together, these results indicate that circPVT1 is a stable and cytoplasmic circRNA derived from the PVT1 gene locus.

circPVT1 expression is frequently upregulated in gastric cancer in part due to DNA amplification

We further determined the expression level of circPVT1 by qRT-PCR analysis in an independent cohort of 187 patients with GC. 18s ribosomal RNA (rRNA) served as internal control for normalization. circPVT1 expression was markedly upregulated in GC tissues compared with matched noncancerous tissues (Fig. 3A). When primary cancers were compared with corresponding noncancerous tissues, the upregulation of circPVT1 (greater than a two-fold change) was observed in 67% (125/187) of patients with GC

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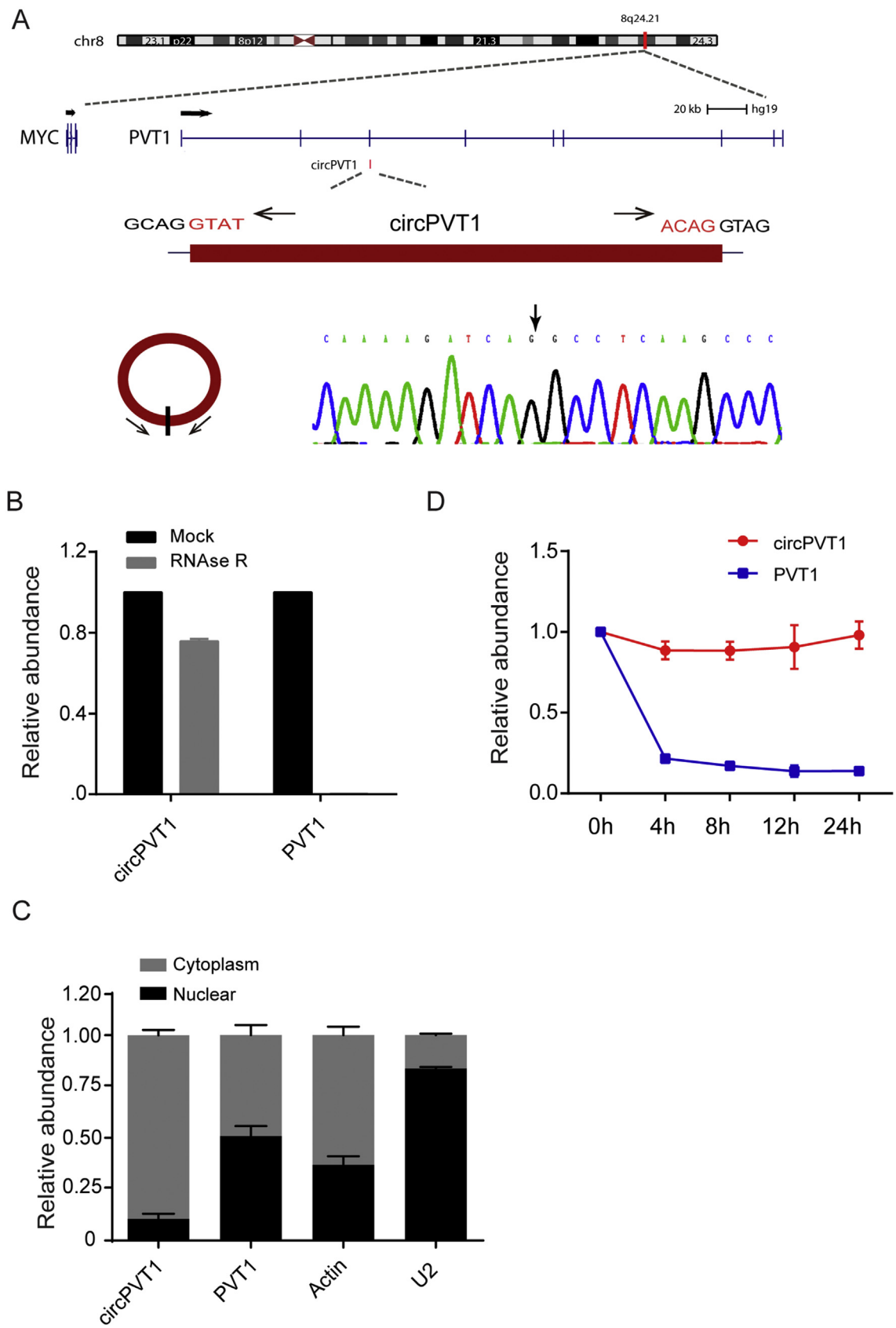

Fig. 2. The characteristics of the circular RNA circPVT1 in gastric cancer cells. **(A)** The genomic loci of the PVT1 gene and circPVT1. The expression of circPVT1 was detected by RT-PCR and was validated by Sanger sequencing. Arrows represent divergent primers that bind to the genomic region of circPVT1. **(B)** qRT-PCR analysis of circPVT1 and PVT1 RNA after treatment with RNase R in AGS cells. **(C)** qRT-PCR analysis of circPVT1 and PVT1 RNA in either the cytoplasm or the nucleus in AGS cells. **(D)** qRT-PCR analysis of circPVT1 and PVT1 RNA after treatment with Actinomycin D at the indicated time points in AGS cells.

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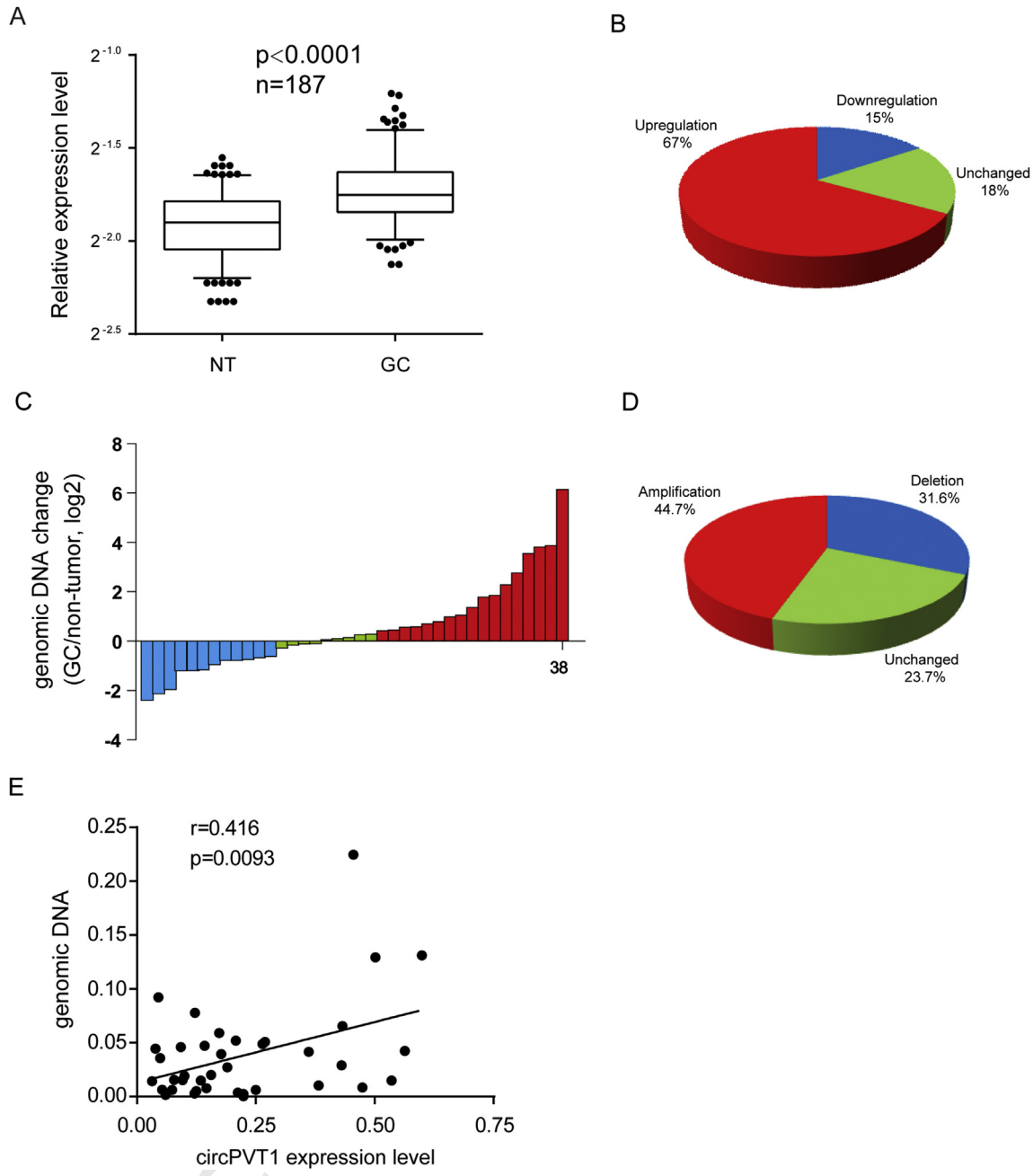

Fig. 3. circPVT1 is frequently up-regulated in gastric cancer due to DNA amplification. **(A)** qRT-PCR analysis of circPVT1 in 187 paired gastric cancer and noncancerous tissues. 18s rRNA served as an internal control. **(B)** Pie chart of the proportions of GC samples in which circPVT1 expression was upregulated (red), downregulated (blue), or showed no change (green). **(C)** qRT-PCR analysis of the DNA copy numbers in the circPVT1 genomic region in 38 GC and matched noncancerous tissues. β-actin was used as the internal control. Data are presented as the log2 fold change. **(D)** Pie chart of the proportions of chromosomal regions in GC samples that were amplified (red), unchanged (green), and deleted (blue). **(E)** Correlation between the circPVT1 expression level (x) and the genomic DNA content (y) in GC tissues. Statistical analysis was performed with Pearson's correlation analysis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3B). An analysis of the genomic region revealed that the circPVT1 locus was amplified in approximately 45% (17/38) of GC tissues (Fig. 3C and D). Upregulated circPVT1 expression was positively correlated with its genomic content (Fig. 3E), which suggests that increased expression of circPVT1 may result from the amplification of its corresponding chromosomal region. We also noted that PVT1 RNA was also significantly upregulated in GC tissues compared with normal gastric tissues (Supplementary Fig. S4A). However, the expression levels of circPVT1 and PVT1 RNA were poorly correlated in GC tissues (Supplementary Fig. S4B). We investigated the expression and correlation of circPVT1 and PVT1 in different GC cells and human gastric epithelium GES-1 cells. Consistent with the results from GC tissues, we found that the expression level of circPVT1 in GC cells is higher than that of normal gastric epithelium cells, and the expression levels of circPVT1 and PVT1 RNA were also poorly correlated (Supplementary Fig. S5). These results suggest the post-transcriptional regulation of either circPVT1, PVT1 RNA or both in GC cells.

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Silencing of circPVT1 inhibits the proliferation of gastric cancer cells

To evaluate the biological functions of circPVT1, we used RNA interference to silence the expression of circPVT1 in GC cells. We designed two siRNAs to target the backsplice sequence (Fig. 4A). A nonspecific control siRNA sequence was also used. As expected, siRNA directed against the backsplice sequence inhibited only the circular transcript of circPVT1 and did not affect the expression of the PVT1 linear species (Fig. 4B). A subsequent cell proliferation assay showed that the downregulation of circPVT1 significantly suppressed growth of MGC-803 and AGS gastric cancer cells (Fig. 4C and D). A colony formation assay also revealed compromised colony-forming ability of the two GC cell lines upon the downregulation of circPVT1 expression (Fig. 4E and F). Furthermore, a 5-ethynyl-2′-deoxyuridine (EdU) incorporation assay showed that the proliferation of GC cells was impaired upon the knockdown of circPVT1 expression (Fig. 4G and H). Collectively, these findings suggest that circPVT1 is a factor that affects proliferation of GC cells.

circPVT1 serves as a miRNA sponge for the miR-125 family

Given that circRNA has been shown to act as a miRNA sponge and circPVT1 is stable in the cytoplasm, we next explored whether circPVT1 could bind to miRNAs. We constructed a circPVT1 fragment and inserted it immediately downstream of the luciferase reporter gene. We hypothesized that circPVT1-associated miRNAs may potentially inhibit the luciferase activity, presumably via the miRNA-mediated activation of deadenylation and subsequent

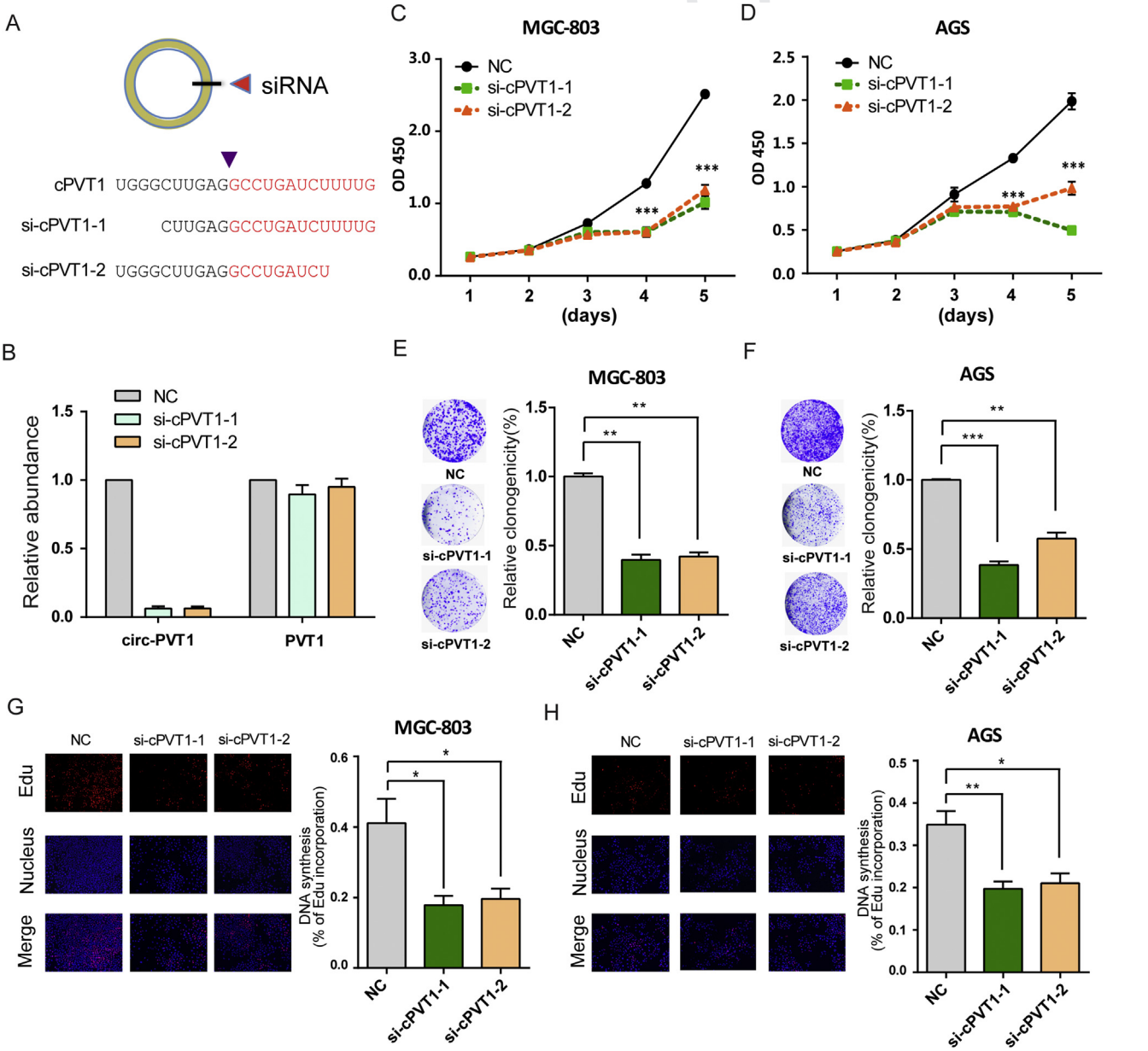

Fig. 4. Silencing of circPVT1 inhibits the proliferation of gastric cancer cells. **(A)** Schematic representation and target sequences of the siRNAs specific to the backsplice junction of circPVT1. **(B)** qRT-PCR analysis of circPVT1 and PVT1 RNA expression after treatment with two siRNAs. **(C and D)** Assessment of proliferation of MGC-803 and AGS cells transfected with control or circPVT1 siRNAs by CCK-8 assay. **(E and F)** Colony formation assay of MGC-803 and AGS cells transfected with control or circPVT1 siRNAs. **(G and H)** Assessment of DNA synthesis using an EdU (5-ethynyl-2'-deoxyuridine) assay in MGC-803 and AGS cells transfected with control or circPVT1 siRNAs. Micrographs represent at least three experiments. Scale bar = 200 μm. **(C–H)** Data are the means ± SEM of three experiments. *P < 0.05; **P < 0.01; ***P < 0.001 (Student's t-test).

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exonucleolytic degradation. We next used ComiR miRNA target prediction tool [23] to find 46 potential miRNAs that could bind to circPVT1. We then performed a luciferase assay using these miRNAs. Each miRNA mimic was co-transfected with the luciferase reporters into HEK-293T cells. Compared with the control RNA, miR-125a, miR-125b and let-7b reduced the luciferase reporter activity by at least 25% (Fig. 5A). miR-125a and miR-125b belong to the miR-125 family and share similar miRNA seed sequence. In addition, both of these miRNAs have been reported to be tumor suppressors [24,25]. Two potential binding sites of miR-125 family were found within the circPVT1 sequence (Fig. 5B and Supplementary Fig. S6A). Next, we mutated each miRNA target site with the inclusion of the circPVT1 sequence in the 3′UTR. We found that the transfection of MGC-803 cells with miR-125 had no significant effect on luciferase activity when either or both of the corresponding target sites were mutated from the luciferase reporter (Fig. 5C and Supplementary Fig. S6B). This result suggests that these two target sites function synergistically. qRT-PCR analysis showed that miR-125 could not significantly decrease the level of circPVT1 (Supplementary Fig. S7). This result indicates that circPVT1 may not be digested by miR-125. We also observed that the expression of E2F2, which is a target of mir-125b, was downregulated after the silencing of circPVT1. This result indicated that circPVT1 may facilitate E2F2 expression by acting as a sponge of miR-125b (Fig. 5D). Moreover, the ectopic expression of circPVT1 could attenuate the anti-proliferative effects of miR-125b (Fig. 5E). Taken together, these results suggest that circPVT1 could bind to miR-125 and inhibit its activity.

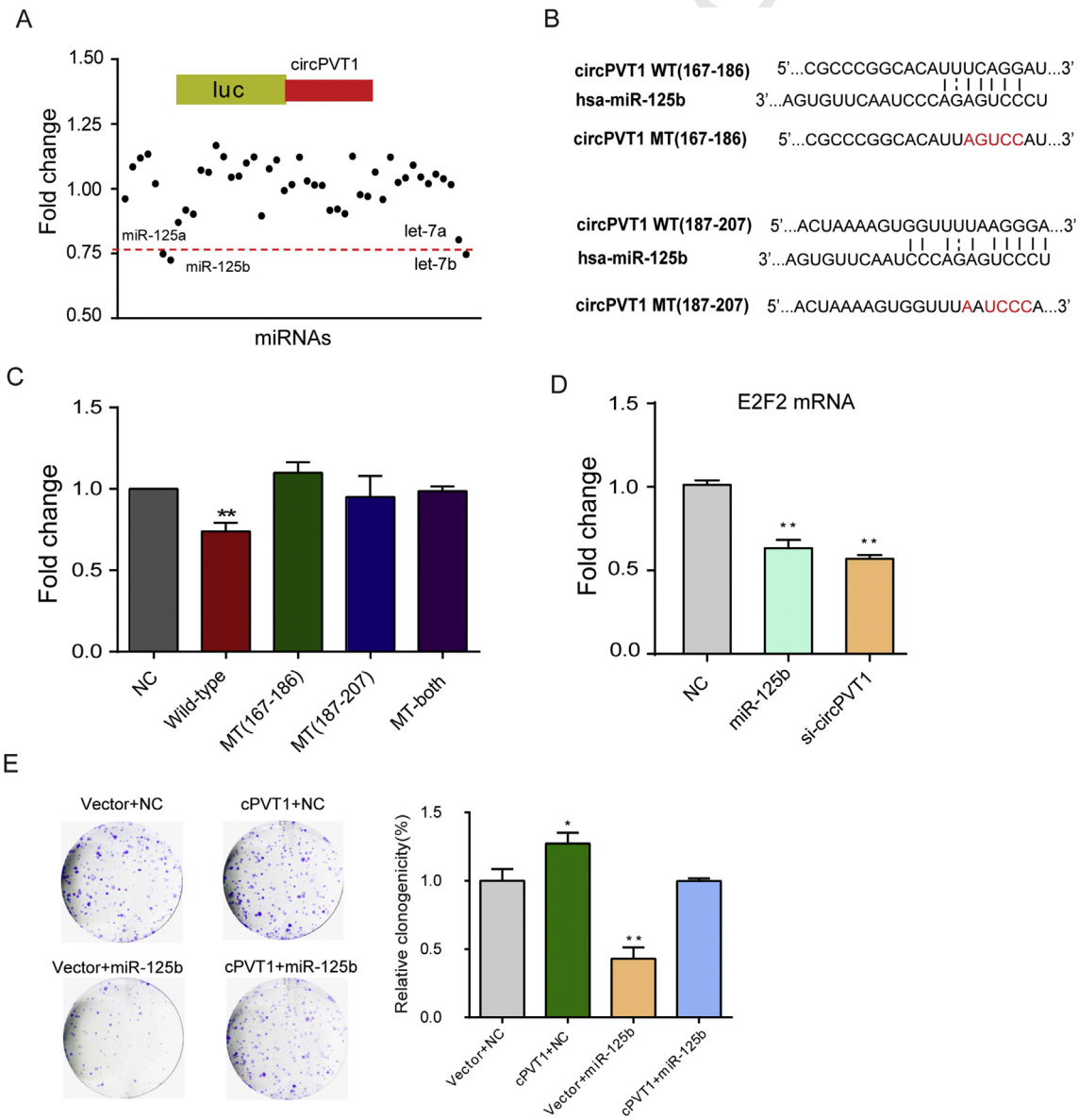

Fig. 5. circPVT1 serves as a miRNA sponge for the miR-125 family. **(A)** A luciferase reporter assay was used to detect the luciferase activity of LUC-cPVT1 in HEK-293T cells transfected with miRNA mimics to identify miRNAs that were able to bind to the circPVT1 sequence. Three miRNAs that inhibited luciferase activity by 25% are indicated by red dots. **(B)** A schematic drawing shows the putative binding sites of miR-125b with respect to circPVT1. **(C)** A luciferase reporter assay was used to detect the luciferase activity of LUC-cPVT1 or the LUC-cPVT1-mutant in SGC-803 cells co-transfected with miRNA mimics. Data are the means ± SEM of three experiments. **(D)** qRT-PCR analysis of E2F2 expression level after transfection with miR-125b mimics or circPVT1 siRNA in AGS cells. *P < 0.05; **P < 0.01; ***P < 0.001 (Student's t-test). **(E)** Colony formation assay in cells transfected with circPVT1 or miR-125b as indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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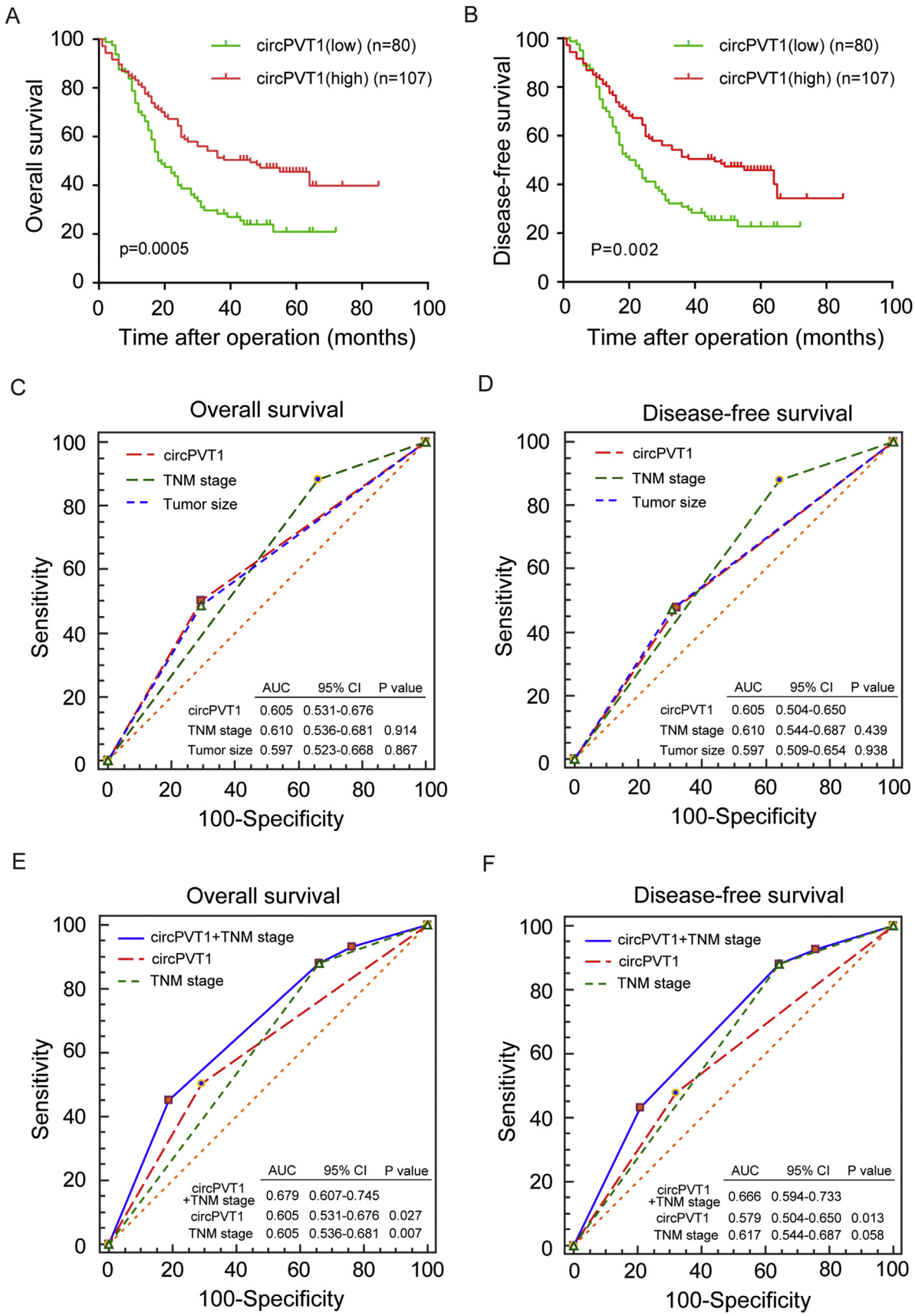

Fig. 6. circPVT1 is an independent prognostic marker of survival in patients with gastric cancer. **(A and B)** Kaplan–Meier analysis of the correlation between circPVT1 expression and overall survival (OS, **A**) and disease-free survival (DFS, **B**) in 187 patients with GC. Log-rank tests were used to determine statistical significance. **(C and D)** Receiver operating characteristics (ROC) curves for OS (**C**) and DFS (**D**). P values show the area under the ROC (AUROC) of the circPVT1 signature versus the AUROC of the TNM stage or tumor size. **(E and F)** ROC curves for OS (**E**) and DFS (**F**). P values show AUROC the combined circPVT1 expression and TNM stage model versus AUROC of circPVT1 expression alone or TNM stage alone.

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circPVT1 is an independent prognostic marker for survival of patients with gastric cancer

We further evaluated the association between the circPVT1 expression level and different clinicopathological features of 187 patients with GC. The patients were stratified according to circPVT1 expression levels with the cut-off using Youdeng's index. Significantly higher numbers of late T stage (T4) tumors and cases of positive neural invasion were found in the low-circPVT1 (low-cPVT1) group ($P < 0.05$, Table 1). No significant differences were observed in the other clinical and pathological characteristics between the high- and low-cPVT1 groups. To investigate whether circPVT1 could serve as a prognostic marker, overall survival (OS) and disease-free survival (DFS) curves were plotted by Kaplan–Meier method according to the circPVT1 expression level. Patients with low levels of circPVT1 had a significantly shorter OS (median survival of 20 months vs 46 months; $P = 0.0005$, log-rank test; Fig. 6A) and DFS (median survival of 17 months vs 36 months; $P = 0.002$, log-rank test; Fig. 6B) than those with the high levels of circPVT1.

To assess whether the ability of circPVT1 to predict survival is independent of other clinical or pathological factors of the patients with GC, univariate and multivariate Cox proportional hazards analyses were performed. The results showed that circPVT1 level, tumor size and TNM stage were independent prognostic factors for OS and DFS in patients with GC (Tables 2 and 3). The receiver operating characteristic (ROC) curves revealed that the expression levels of circPVT1 showed no differences compared with tumor size and TNM stage with regard to OS and DFS (Fig. 6C and D). Furthermore, the combination of circPVT1 expression and TNM stage gave a better prognostic value than did TNM stage alone (Fig. 6E and F).

Combination of circPVT1 and PVT1 expression is associated with a better prognosis of patients with gastric cancer

We also investigated the clinical significance of PVT1 expression. Unlike circPVT1, patients with low levels of PVT1 had a better OS (median survival of 38 months vs 24 months, $P = 0.006$, log-rank test; Fig. 7A) and DFS (median survival of 33 months vs 17 months, $P = 0.014$, log-rank test; Fig. 7B) than those with high levels of PVT1. Furthermore, when the combination of circPVT1 and PVT1 expression was considered, patients with low levels of circPVT1 and high levels of PVT1 had a significantly shorter OS (median survival of 17 months vs 48 months; $P < 0.0001$, log-rank test; Fig. 7C) and DFS (median survival of 14 months vs 46 months; $P < 0.0001$, log-rank test; Fig. 7D) than those with high levels of circPVT1 and low levels of PVT1. This indicates that the combined detection of circPVT1 and PVT1 expression predicted a more accurate prognosis of patients with GC.

Discussion

In this study, we identified a large number of circular RNAs in gastric cancer tissues. We also found that a substantial fraction of circRNAs were differentially expressed in cancer tissues compared with normal tissues, which suggests that these RNAs may be regulated and may exert a potential function. We further characterized one of the most differentially expressed circRNAs and provided the first evidence that a specific circRNA may play an important role as a prognostic marker in cases of gastric cancer.

The majority of circRNAs are generated by exon circularization from precursor RNAs, which indicates that additional transcript can be produced from one gene locus. We characterized one of the

Table 2
Univariate analysis identifies factors influencing the overall survival rate of gastric cancer patients.

Factors	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Sex	1.324 (0.883–1.986)	0.175		
Age	1.243 (0.864–1.790)	0.241		
Tumor size	1.630 (1.136–2.338)	0.008*	1.659 (1.146–2.401)	0.007*
Differentiation grade	0.600 (0.383–0.940)	0.026*		0.052
TNM stage	2.588 (1.478–4.534)	0.001*	2.723 (1.548–4.789)	0.001*
Lymphatic invasion	1.865 (1.256–2.771)	0.002*		0.153
Nervous invasion	1.998 (1.333–2.993)	0.001*		0.167
circPVT1 expression	0.536 (0.373–0.771)	0.001*	0.596 (0.413–0.859)	0.006*
PVT1 expression	1.631 (1.137–2.340)	0.008*	1.455 (1.005–2.105)	0.047*

HR, Hazard ratio; CI, confidence interval.
*$P < 0.05$.

Table 3
Univariate analysis identifies factors influencing the disease-free survival rate of gastric cancer patients.

Factors	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
Sex	1.136 (0.753–1.713)	0.544		
Age	1.203(0.843–1.716)	0.039		
Tumor size	1.548 (1.089–2.202)	0.015*	1.610 (1.120–2.313)	0.010*
Differentiation grade	0.595 (0.386–0.918)	0.019*		0.063
TNM stage	2.530 (1.471–4.350)	0.001*	2.487 (1.432–4.319)	0.001*
Lymphatic invasion	1.833 (1.250–2.689)	0.002*		0.140
Nervous invasion	1.749 (1.192–2.567)	0.004*		0.362
circPVT1 expression	0.615 (0.432–0.876)	0.007*	0.639 (0.443–0.921)	0.012*
PVT1 expression	1.523 (1.069–2.171)	0.020*	1.435 (0.998–2.061)	0.051

HR, Hazard ratio; CI, confidence interval.
*$P < 0.05$.

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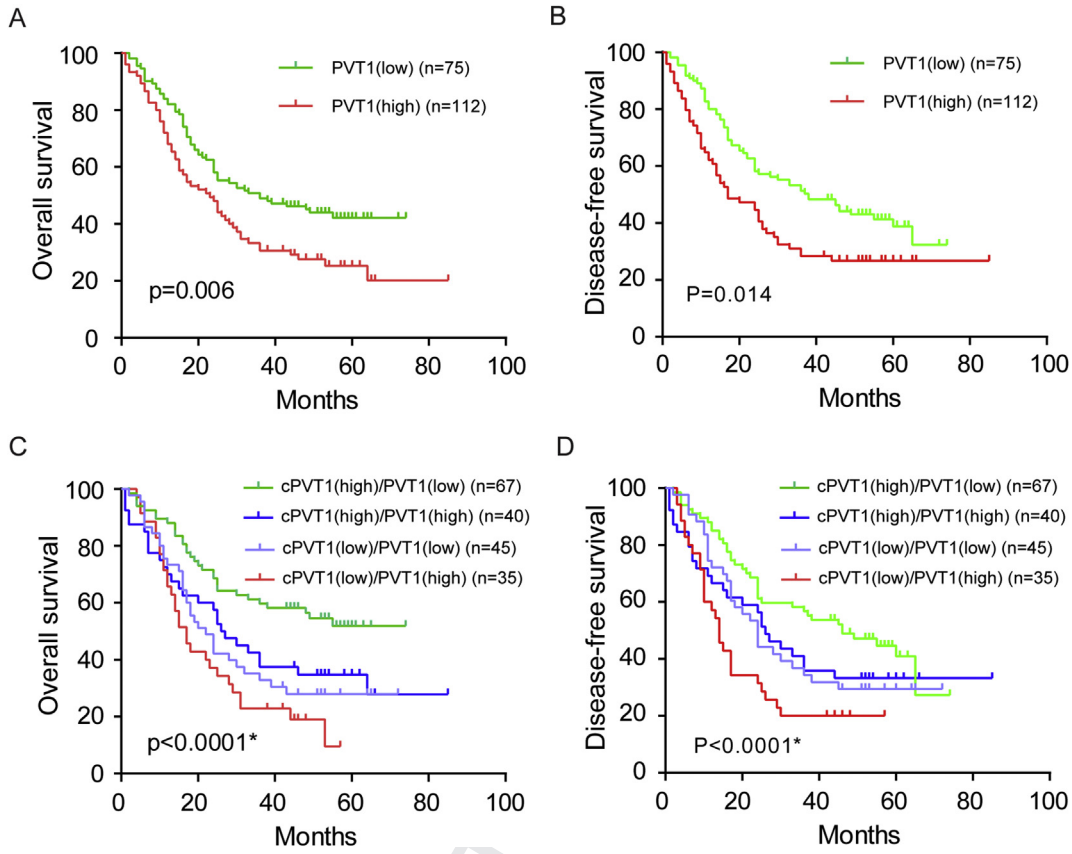

Fig. 7. Combination of circPVT1 and PVT1 expression predicts a better prognosis of patients with gastric cancer. **(A and B)** Kaplan–Meier analysis of the correlation between PVT1 expression and overall survival (OS, **A**) and disease-free survival (DFS, **B**) in 187 patients with GC. **(C and D)** Kaplan–Meier analysis of the correlation between circPVT1/PVT1 expression and overall survival (OS, **C**) and disease-free survival (DFS, **D**) in 187 patients with GC. The patients were divided into four groups according to the expression levels of circPVT1 and PVT1. Log-rank tests were used to determine statistical significance.

differentially expressed circRNAs derived from the PVT1 gene (termed circPVT1). The human PVT1 gene (also known as the Pvt1 oncogene) is a long intergenic noncoding RNA (lincRNA) that is homologous to the mouse plasmacytoma variant translocation gene (Pvt1) [26,27]. Recent studies have shown that PVT1 RNA plays an important role in human cancer via the regulation of protein stability of important oncogenes, primarily the c-Myc oncogene [28,29]. circPVT1 is derived from Exon 3 of the PVT1 gene and flanks two long introns (35269 bp and 41466 bp), which harbor many Alu repeats and may facilitate circPVT1 formation. The present results revealed that about two-thirds of GC tissues demonstrated a high level of circPVT1 expression. The upregulation of circPVT1 can be explained in part by genomic DNA amplification. Notably, PVT1 RNA is also upregulated in GC compared with matched normal tissues. However, the expression levels of circPVT1 and PVT1 RNA are poorly correlated, and they are localized to different areas of the cell. This suggests that they are post-transcriptionally regulated and may have distinct roles. We also investigated whether circPVT1 could influence the expression level of its host gene PVT1 and its neighboring gene c-Myc. We observed that circPVT1 did not significantly change the expression levels of PVT1 RNA. Notably, we found that circPVT1 could increase the c-Myc protein level in GC cells (Supplementary Fig. S8). Unlike PVT1 RNA, which interacts directly with c-Myc protein, circPVT1 may facilitate c-Myc expression by acting as a sponge for let-7b (Fig. 5A). Thus, circPVT1 and PVT1 RNA might function in different ways through the synergistic regulation of c-Myc expression. In addition, a gain of chromosome 8q24, where circPVT1 and PVT1 are located, is frequently observed in other types of cancer, which indicates that this may be a general mechanism of human cancer cells.

circRNAs are promising potential biomarkers for disease because of their unique structure, high stability and specific expression patterns. In this study, we showed that circPVT1 is an independent prognostic marker for survival in patients with GC. Surprisingly, patients with high levels of circPVT1 had a significantly better overall survival than those with low levels of circPVT1, which seems to contradict the upregulation of circPVT1 in GC. A possible explanation may be the association of circPVT1 with the tumor suppressor miR-125. Supporting with this notion, the expression level of circPVT1 was found to be positively correlated with that of miR-125 in GC cells (Supplementary Fig. S5). Unlike circPVT1, high expression of PVT1 RNA correlated with poor overall survival, as it interacted directly with the c-Myc oncoprotein.

In summary, our study revealed the circular RNA profile of GC tissues and characterized a differentially expressed circRNA derived from the PVT1 gene. We demonstrated that circPVT1 is a new proliferative factor and prognostic marker in gastric cancer. Our findings suggest that circRNAs may serve as a novel class of non-coding RNAs that may have potential functions and clinical significance in human cancer.

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Conflict of interest

Q4 The authors disclose no potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.canlet.2016.12.006.

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