European Review for Medical and Pharmacological Sciences 2019; 23: 4173-4184

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Long noncoding RNA PVT1-214 enhances gastric cancer progression by upregulating TrkC expression in competitively sponging way

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Abstract. – OBJECTIVE: Long noncoding RNA plasmacytoma variant translocation 1 (lncRNA PVT1) is aberrantly expressed and involved in the promotion of various cancers. However, the vital epigenetic function of [illegible] 214, a transcript isoform of PVT1, in gas[illegible] cancer (GC) remains unknown. We aimed to [illegible]gate the dysregulation and detailed mecha[illegible] underlying the involvement of lncRNA PVT1[illegible] in GC.

PATIENTS AND METHODS: [illegible]ession [illegible] PVT1-214 in GC tissues an[illegible] cell li[illegible] was detected by qRT-PCR. The [illegible]ationsh[illegible] between increased PVT1-214 lev[illegible]nd th[illegible]vanced clinicopathological f[illegible]ture[illegible] [illegible] was analyzed usin[illegible] Chi-sq[illegible]est. The influence of PVT1-[illegible] on the su[illegible]ate of GC cell lines was e[illegible] by the log-[illegible]est. Cell lines were us[illegible] to [illegible]e the carci[illegible]genic effects of PVT1-[illegible]14 *in vi*[illegible]d *in vivo*, and specific tests i[illegible]ded cell ap[illegible]is determined by flow cyt[illegible]etry, cell prolifera[illegible]ssayed by Cell Coun[illegible] Kit-8 (CCK-8) and [illegible]olony formation, and [illegible] use o[illegible] these cells for mice xenograft mo[illegible] [illegible]ire[illegible]omplementary binding was predicted [illegible]formati[illegible]nd verified by dual luciferase [illegible]r ass[illegible] RNA transfection, quan[illegible]e poly[illegible]ain reaction (qPCR), and We[illegible]rn blotti[illegible] [illegible]pearman's correlation coefficie[illegible]was adopte[illegible] to evaluate the correlation betw[illegible]iR-128 and PVT1-214 levels.

[illegible]: [illegible]VT1-214 expression in GC tissues [illegible]d cell lines is markedly elevated. In GC pa[illegible]s, high expression of PVT1-214 is associated [illegible] late tumor stage, increased tumor size, and [illegible]oor survival. PVT1-214 silencing represses cell proliferation and enhances apoptosis of GC cells both *in vivo* and *in vitro*. Additionally, [illegible]T1-214 functi[illegible] as a competing endogenous R[illegible](ceRNA) by [illegible]ding to miR-128. Inhibition of mi[illegible] releas[illegible] Tropomyosin receptor kinase C (Tr[illegible]e complementary binding complex, subsequently increasing the protein level [illegible]TrkC in GC cells.

[illegible]USIONS: PVT1-214-induced miR-128 pr[illegible]on regulates TrkC to further the progression of GC, indicating that this process will provide a promising therapeutic target in GC.

Key Words:
Gastric cancer, Plasmacytoma variant translocation 1-214 transcript, Tropomyosin receptor kinase C, Competing endogenous RNA.

Introduction

Gastric cancer (GC) persists as the third leading cause of cancer-related deaths worldwide, despite the fact that it ranks sixth in overall incidence[1]. Clinically, gastric cancer is often diagnosed late in disease development due to limited screening techniques[2]. This leads to a lack of curative therapeutic options, ultimately resulting in poor prognosis[3]. Excessive cellular proliferation occurs prominently in the progression of cancers, and this is associated with relapse and low survival rates in patients suffering from GC[4]. The genetic investigation into the cellular proliferation underlying GC progression will provide novel insights into GC tumorigenesis, ultimately aiding in the identification of potential diagnostic and

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therapeutic targets that in turn may further the improvement of patient survival.

Human genome sequencing data revealed that the majority of the human genome produces non-coding RNA molecules[5]. Long noncoding RNAs (lncRNAs) are transcripts that possess no definitive protein-coding competence and exceed 200 nucleotides in length[5]. An increasing number of studies have indicated that lncRNAs are involved in several biological behaviors underlying cancer progression, including proliferation[6], invasion[7], migration[7], and chemoresistance[8]. Thus, examining the interesting role of lncRNAs in the process of tumorigenesis may be of profound value for identifying potential molecular targets in GC development.

lncRNA plasmacytoma variant translocation 1 (PVT1) is known as an activator of the c-myc gene[9] located on chromosome 8q24.21[10,11]. Since c-myc is involved in the formation of several types of gastrointestinal tumors[12-14], PVT1 manifests its oncogenic impact on the biological properties of cancer cells by influencing proliferation, migration, and invasion in gastric cancer[15], hepatocellular carcinoma[16], pancreatic ca[illegible], and colorectal cancer[18]. Interestingly, 14 d[illegible] PVT1 transcripts encoded by the PVT1 g[illegible] are differentially upregulated in colorectal ca[illegible]. The transcript PVT1-214 was found to be sig[illegible]icant in stabilizing Lin28, an o[illegible]nic prot[illegible] that is associated with prolif[illegible] invasio[illegible] of colorectal cancer[19]. Ho[illegible]er, no [illegible]iminary data were available reg[illegible] the [illegible]inogenic effects of PVT1-214 i[illegible] the [illegible]xt[illegible], the findings of our s[illegible]y reve[illegible] PVT1-214 is significantly over[illegible]essed in GC[illegible]s, and the epigenetic regu[illegible] PVT1-214 i[illegible] context of GC develo[illegible]ent [illegible]es that it possesses a pathogeni[illegible]ole in GC pa[illegible]

Mat[illegible]ials and Methods

Gastri[illegible]cer Pat[illegible]ts and [illegible]respo[illegible] T[illegible]ues

[illegible]tal of [illegible] patients were enrolled at Fujian [illegible]ovincial [illegible]ncer Hospital, Fujian Medical U[illegible] Cancer Hospital (Fuzhou, Fujian, Chi[illegible] [illegible] to 2018. All patients were quali[illegible] by pathological diagnosis, whose cancerous [illegible]on-tumorous were obtained by surgery. An[illegible]ubsequent tissues were all rapidly frozen at -80°C. The study was approved by the Ethics Committee of Fujian Provincial Cancer Hospital. All patients were informed to sign the consents before this study.

Cell Culture

SGC-7901, MKN-45 cells, BGC-[illegible] and nor[illegible]mal human gastric mucosal epithe[illegible] cells (GES-1) were cultured in Dulbecco's [illegible]ed Eagle's Medium (Thermo Fisher Scient[illegible]altham, MA, USA). 10% fetal bo[illegible]e serum [illegible]rmo Fisher Scientific, Waltha[illegible]MA, USA) wa[illegible] into medium. All of th[illegible] were i[illegible] an atmosp[illegible] with 5% CO_2 at 37 °C[illegible]hase[illegible]om the ATCC.

RNAs Constr[illegible]ion an[illegible] Transfecti[illegible]

Two pa[illegible] [illegible]rt hairpin R[illegible]s (shRNAs) targeting [illegible]VT1-21[illegible] synthesized (Invitrogen, Waltham, MA, USA[illegible] expressed using the pL[illegible]uo RNAi exp[illegible]n vector (Addgene, W[illegible]ertown, MA, USA). GC cells were cultured [illegible] six-well plate[illegible] with 2 × 10^6 per well. When t[illegible] cell density [illegible]ched 80%, RNAi expression v[illegible] was trans[illegible]ted into harvested cells using Lip[illegible]mine [illegible]00 (Thermo Fisher Scientific, Waltha[illegible] USA). The sequences of final [illegible]uble-strand oligo DNAs for PVT1-214 stable [illegible]n and the vector control are presented [illegible] S[illegible]ementary Table I.

For microRNA repression, miR-128 inhibitor (RiboBio, Guangzhou, Guangdong, China) or negative control were transfected into GC cells utilizing Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). All the efficiency for interfering must be evaluated by qRT-PCR.

Nucleus/Cytoplasm Fractionation

Based on the manufacturer's instructions, PVT1-214 fractions in cytosolic and nuclear were isolated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA). RNU6-1 was applied to analyzed nuclear PVT1-214 fraction levels as a control transcript, GAPDH as a cytoplasmic control transcript.

Total RNA Extraction and Quantitative PCR

Based on the manufacturer's protocol, total RNA was extracted from tissues and cultured cells using Trizol solution (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was transcribed to cDNA using SuperScript IV Reverse Transcript (Thermo Fisher Scientific, Waltham, MA, USA). Briefly, the following components

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were combined in a reaction tube: 2 μM reverse primer 1 μL, 10 mM dNTP mix 1 μL, total RNA 5 μg, RNAase-free water up to 13 μL. The mixture was briefly centrifuged and heated at 65°C for 5 min, followed by incubation on ice for at least 1 min. Then, reverse transcript Buffer 4 μL, 100 mM DTT 1 μL, RNase Inhibitor 1 μL, SuperScript IV reverse transcriptase (200 U/μL) 1 μL were added into previous mixture. Incubating the combined reaction mixture at 50-55°C for 10 min, then at 80°C for 10 min. The prepared DNA was measured by a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, quantitative PCR was performed on the Applied Biosystems 7500 Sequence Detection system. Each 20 μL reaction contained 5 ng of cDNA, 500 nM of each primer and 10 μL 2×PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in a 96-well PCR plate. The following reaction conditions have been applied: 2 min at 50°C, 2 min at 95°C, and 45 cycles of 15 sec at 95°C and 1 min at 60°C. All expression levels were calculated by the $2^{-\Delta Ct}$ method with GAPDH as the control. The primer sequences are presented in Su[illegible]mentary Table I.

Proliferation Ability Assay

The proliferation ability of GC cells was [illegible]sessed by Cell Counting Kit-8 [illegible]CK-8; Me[illegible]chembio, USA) assay as well as colony formation assay. Cell viability was measured every 24 h according to the manufacturer's instructions of the CCK-8. The absorbance at each time [illegible] adopted to sharp the proliferation cur[illegible]. GC cel[illegible] were cultured in 96-well plates wi[illegible]× 10^4 cells/well for 2 weeks, followed by an[illegible]nt with 500 cells/well for colony formation ass[illegible]hen, 4% paraformaldehyde (5 min) [illegible] 1% cry[illegible]iolet (10 min) were used to fi[illegible]nd stain the c[illegible]. All colonies were obse[illegible]d with a microscop[illegible]

Dual-Luciferas[illegible] Rep[illegible]ssays

To assess the [illegible]ect inte[illegible] betw[illegible] PVT1-214 and miR[illegible] wild-type [illegible]nt PVT1-214 targeti[illegible]lementary ba[illegible] of miR-128 were con[illegible]ucted. [illegible] we fused them to the pGL3-luciferase repo[illegible]ector (Promega, Madiso[illegible]SA). Lucifera[illegible]smids and miR-128 o[illegible]ontrols were transfected into SGC-7901 cells. [illegible]ciferase and re[illegible]lla signals (the endogenic con[illegible] were tested [illegible]out 48 h hours after transfecti[illegible] the Dual[illegible]ciferase Reporter Assay System [illegible]ega, [illegible]dison, WI, USA).

[illegible]NA Binding Protein [illegible]oprecipitation (RIP)

A[illegible]ding to the manufacturer's protocol, RIP assays of the interaction between PVT1-214 and miR-128 were performed using the Imprint® RNA

Table I. Relationship betwee[illegible]NA PVT[illegible]4 expression and clinicopathological features of GC patients.

	N[illegible]	PVT1-214 expression		*p*-value
		Low (24)	High (24)	
Gender				
Male	23	14	9	0.149
Female	[illegible]25	10	15	
Age (ye[illegible]				
≥ 60[illegible]	[illegible]2	13	9	0.369
<[illegible]	26	11	13	
Tu[illegible]				
≥ 5 c[illegible]	24	8	16	0.021*
[illegible]cm	24	16	8	
[illegible]				
I-[illegible]	19	13	6	0.039*
I[illegible]V	29	11	18	
[illegible]astasis				
No	23	14	9	0.149
[illegible]es	25	10	15	
[illegible]t metastasis				
[illegible]	36	19	17	0.505
Yes	12	5	7	

*p<0.05 represents statistical difference.

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Supplemental Table I. The primers for genes detected by real-time PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
PVT1-214	GGACATGATACCTGGATGTG	CCTGAGTCTCAAGATGCAGTA[illegible]
miR-128	CCACCTCTACGCATCATTCA	CCAAGCTCGTCTGGTT[illegible]C
PVT1-214 shRNA1	GATCCGCGGATCTTGGACATGATA CCTTCAAGACGGGTATCATGTCCAA GATCCGCTTTTTT GTCGACA	AGCTTGTCGACAAAAAAG[illegible]TCT TGGACATGATACCCGTCTTG[illegible]T ATCATGTCCA[illegible]TCCGCG
PVT1-214 shRNA2	GATCCGCAGCAGCCATCTGGTAATT ATTCAAGACGTAATTACCAGATGG CTGCTGCTTTTTT GTCGACA	GCTTGTCGACA[illegible]AAAAGCAGCAGCC ATCTGGTAA[illegible]GTCTT[illegible]ATAATT ACCAGA[illegible]TC[illegible]GCG
shRNA	GATCCGCAGCAGCCATACAAGAAT TATTCAAGACGTAATTCTTGTATGG CTGCTGCTTTTTT GTCGACA	AGCTT[illegible]GACAA[illegible]CAG[illegible]C CAT[illegible]AGAATTACG[illegible]AA [illegible]GTATGGCTG[illegible]G
Rnu6-1	CTCGCTTCGGCAGCACA	AACGC[illegible]CGAATTTGCGT
β-actin	TGACGTTGACATCCGTAAAGACC	CTCAGGAG[illegible]ATGATCTTGA
GAPDH	CCCTTCATTGACCTCAACTACA	ATGACAAGC[illegible]CCGTTCTC

*p<0.05 represents statistical difference.

Immunoprecipitation RIP Kit (Sigma-Aldrich, St. Louis, MO, USA). Briefly, for anti-AGO2 [illegible]P, SGC-7901 cells were transfected with [illegible] mimics or miR-NC. After 48 h, a lysis buff[illegible]on-taining RNase inhibitor and protease inhibit[illegible]s used to lyse the cells. Then the material was i[illegible]bated with magnetic beads conju[illegible]to a hum[illegible] anti-Ago2 antibody (Abcam, [illegible]sco, CA, USA), or negative control I[illegible] Finally[illegible]P-qPCR assay with respective targ[illegible]mers w[illegible]tilized to identify the antibody bindin[illegible]

Flow Cytometr[illegible]

The apopto[illegible]ysis of the[illegible]sfected GC cells (af[illegible] 48 [illegible]ture) was performed by an Ap[illegible]ptosis Dete[illegible] Kits (eBiosciences, Wal[illegible]n, MA, USA). [illegible] thousand cells staine[illegible]ith Annexin V/FITC [illegible]ere measured by FAC[illegible]alibur [illegible]ow Cytometry (BD Biosciences, F[illegible]in[illegible]kes, NJ, USA).

[illegible] Mo[illegible]d[illegible]mor Growth In vivo

[illegible]ymic [illegible] mice (5-week-old) were pur[illegible]sed from [illegible]e Laboratory Animal Center of [illegible] Medical University. All mice were fed [illegible] pathogen-free conditions on a 12 [illegible]ight/dark cycle. The procedures of animal exp[illegible]nts attained approval from the Institutional An[illegible]l Care and Use Committee of Fujian Provincial Cancer Hospital. PVT1-214 shRNA and shvector control-transfected SGC-7901 cells (2 × 1[illegible] 100 [illegible] were respectively injected subcutane[illegible]o mice (n = 5). The tumor sizes [illegible]ere recorded every week. Five weeks after in[illegible] the bioluminescence of tumor burden was [illegible]se[illegible] by Xenogen IVIS 200 Imaging System (PerkinElmer, Waltham, MA, USA). Then, the mice were sacrificed and the weight of tumor nude was measured.

Western blot

RIPA buffer with 1 mmol/L protease inhibitors was used to resolve tested cells. Following the manufacturer's protocol, concentration of the protein in RIPA buffer was measured by a BCA Protein Assay Kit (Beyotime, Shanghai, China). The nitrocellulose PVDF membrane (Bioss Antibodies, Beijing, China) containing the proteins separated by 10% SDS-PAGE were incubated with primary antibodies (anti-TrkC, 1:2000, β-actin, 1:5000, Abcam, San Francisco, CA, USA) 4°C overnight. Then the secondary antibody with HRP (1:4000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to incubate the studied blot. Blotting was photographed by an ECLTM chemiluminescence detection system (Pierce, Waltham, MA, USA). The Western blot band intensity was quantificationally measured by Image J software.

Statistical Analysis

All data were presented as the mean ± standard deviation and analyzed statistically by GraphPad

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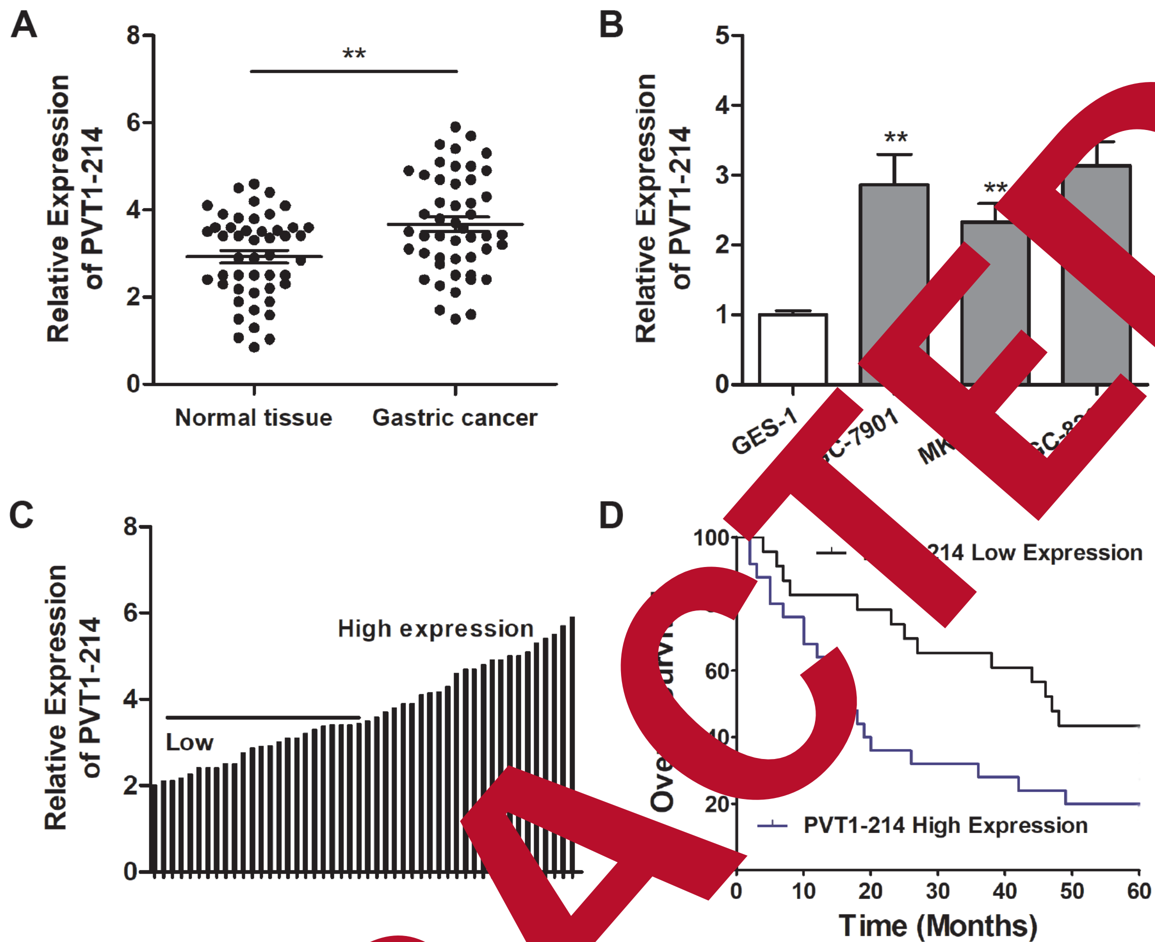

Figure 1. Increased PVT1-214 ex[illegible]sion [illegible] is associ[illegible]ith poor prognosis. ***A,*** Evaluation of lncRNA PVT1-214 expression in GC tissues and adja[illegible]normal ti[illegible]s by RT-PC[illegible]*B,* Difference of lncRNA PVT1-214 expression level between in GC cells (SGC-7901, MKN[illegible]GC-823) [illegible] GES-1 by RT-PCR. ***C,*** Division of lncRNA PVT1-214 expression by median value (high group n = 24, low gr[illegible]24)[illegible]ival analysis of GC patients between high and low lncRNA PVT1-214 expression. $^{**}p<0.01$[illegible]<0.05 [illegible] to the co[illegible]ol group.

Prism 5 softw[illegible]e (Gr[illegible]d Software, La Jolla, CA, USA). Comparison[illegible]he differences between th[illegible]wo groups was c[illegible]d out by the Student's [illegible]est. Further, the Spea[illegible]an's correlation coe[illegible]nt wa[illegible]dopted to evaluate the correlation [illegible]en [illegible]R-128 a[illegible]d PVT1-214 levels. Each experim[illegible]as perf[illegible]ed independently three [illegible]s. The[illegible]re[illegible] was deemed statistically sig[illegible]cant wh[illegible]alue was less than 0.05.

Results

[illegible]ased PVT1-214 Expression in GC Is As[illegible]iated with Poor Prognosis

To identify the role of lncRNA PVT1-214 in GC development, the expression levels of the PVT1-214 transcript were measured in GC tumor tissues and adjacent normal tissues. Our results demonstrated that the PVT1-214 transcript was more likely to be upregulated in GC tissues compared to levels observed in adjacent normal tissues (Figure 1A, $^{**}p < 0.01$). Additionally, compared to normal human gastric mucosal epithelial cells (GES-1), lncRNA PVT1-214 expression levels are significantly increased in GC cells (SGC-7901, MKN-45 and BGC-823) (Figure 1B, $^{**}p < 0.01$). To further examine the role of lncRNA PVT1-214 in GC carcinogenesis, we divided the GC patients into a high-level group (n = 24) and a low-level group (n = 24) (Figure 1C) according to the median value of lncRNA PVT1-214 expression. The associations between the clinicopathological characteristics of GC patients and lncRNA PVT1-214 expression were evaluated and are

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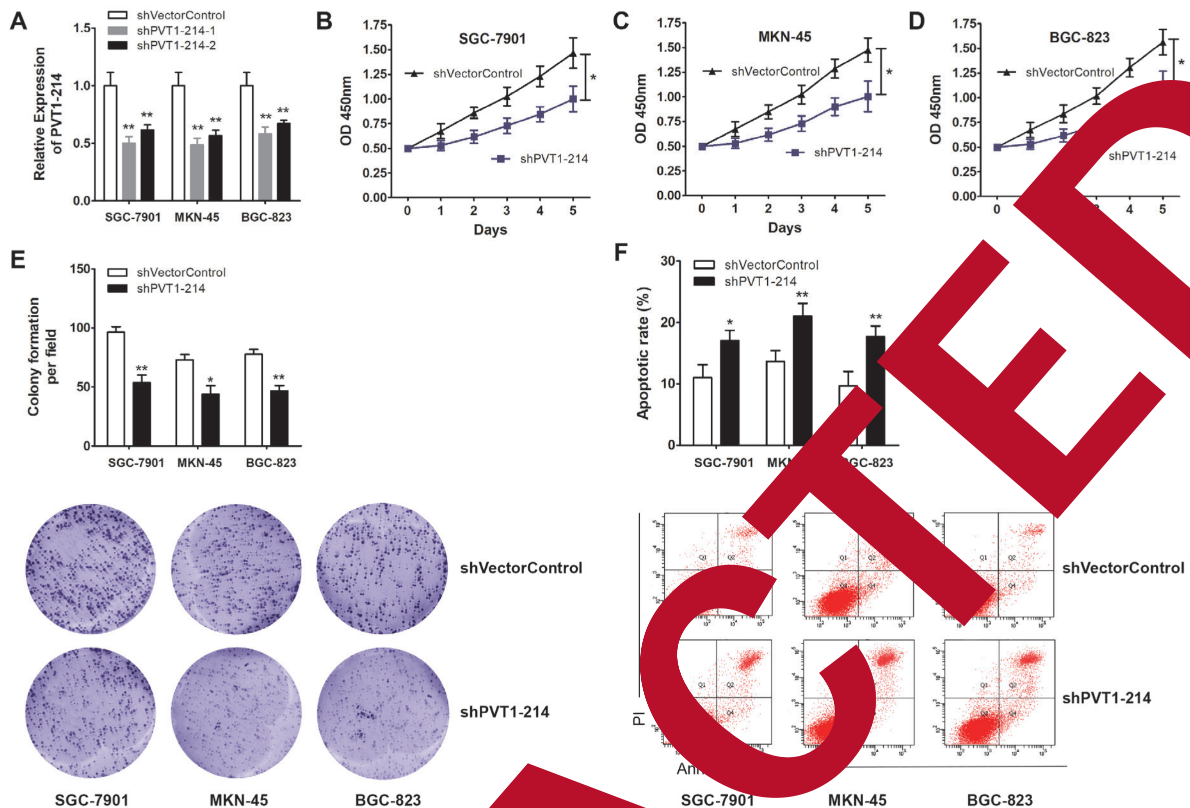

Figure 2. Lnc RNA PVT1-214 furthers GC cells ... feration ... *in vitro*. ***A,*** Two synthesized shRNAs targeting PVT1-214 was transfected to GC cells to knockdown ...1-214 ...ess... ***B-D,*** Proliferative ability of GC cells (SGC-7901, MKN-45, BGC-823) measured by CCK-8 assay after ... knockdown. ***E,*** Colony formation ability of GC cells after PVT1-214 knockdown. ***F,*** The apoptotic cells rate of GC ...ansfected with shRNA or controls showed by flow cytometry. $*p<0.05$, $**p<0.01$ compared to the c...p.

shown in Table I. Higher PV...4 ...s strongly associated w... large tu...ze ($p = 0.021$) and advanced TNM ...age ($p = 0.03$...plan-Meier analysis between ...wo groups fu... indicated that high e...ession ...VT1-214 was related to poor overal... survival (Fig... D, $p < 0.05$). Collectively, th... data suggested t...cRNA PVT1-214 is high... expressed in the GC ti...ues, and it can be con...ed as a...ong predictor for poor prognosis in G...nt...

...RNA ...1-2... Furthers GC Cells ...eration ... Growth In Vitro

T... verify the impact of lncRNA PVT1-214 on ...liferation of GC cells, we synthesized ...RNA to knock down the expression ... lncRNA PVT1-214 (two pair of oligonucle...) with a significant efficiency confirmed by qP... 48 h after transfection (Figure 2A, $**p < 0.01$). A CCK-8 assay then revealed that PVT1-214 restrained by short hairpin RNA inhibited the proliferation of GC cells (SGC-7901, MKN-45, BGC-823) (Figure 2B, C, D, $*p < 0.05$). Additionally, PVT1-214 knockdown decreased the number of clones in GC cells compared to those observed in cells treated with vector control, and this was indicated by the colony formation assay (Figure 2E, $**p < 0.01$, $*p < 0.05$). In addition, the apoptotic rate of GC cells increased after PVT1-214 was silenced compared to levels of apoptosis observed in cells treated with vector controls (Figure 3F, $** p < 0.01$, $*p < 0.05$). Thus, these results indicate that lncRNA PVT1-214 may regulate the proliferative abilities of GC cells *in vitro*.

Lnc RNA PVT1-214 Promotes GC Cell Growth In Vivo

We next utilized MKN-45 GC cells transfected with sh-PVT1-214 or sh-VectorControl to construct xenograft tumor models in nude mice to observe the carcinogenic effects of PVT1-214 dysregulation *in vivo*. The mouse models revealed

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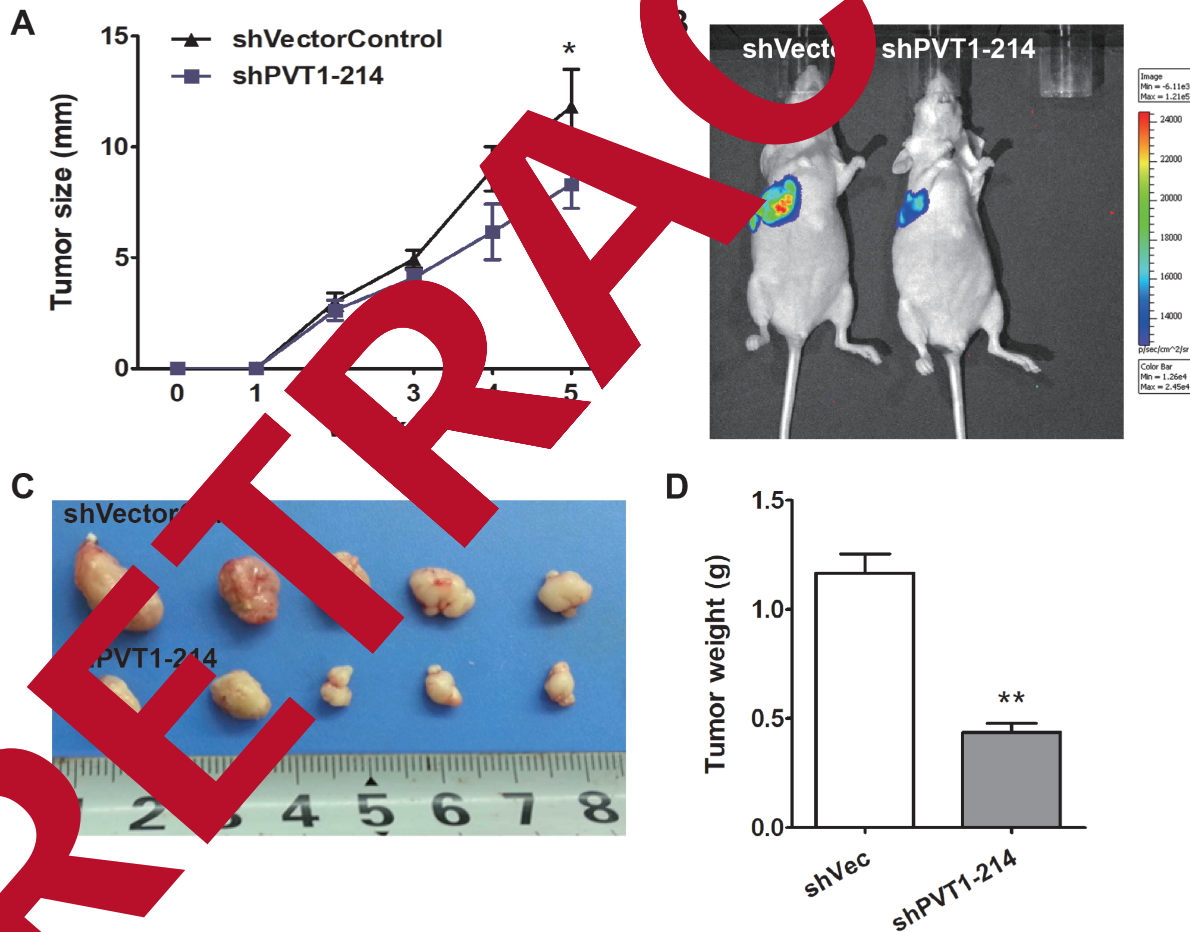
that stable knockdown of PVT1-214 resulting from the lentivirus suppressed the tumor growth of GC cells compared to that observed in control mice, and these factors included tumor size (11.9 ± 3.2 mm *vs.* 8.8 ± 2.4 mm; p = 0.043; Figure 3 A and B) and tumor weight (1.16 ± 0.24 g *vs.* 0.44 ± 0.14 g; p = 0.032; Figure 3 C and D). These data indicate that the expression of PVT1-214 could promote GC cell growth *in vivo.*

PVT1-214 Modulates GC Development by Interacting with MiR-128

Increasing numbers of lncRNAs have been found to endogenously compete with microRNA to promote the upregulation of target genes within the cytoplasm during tumorigenesis. A subcellular localization assay revealed that PVT1-214 is mainly located within the cytoplasm (Figure 4A), suggesting a potential role for PVT1-214 as a ceRNA. We utilized bioinformatics analyses to predict that miR-128 may be the target for the complementary binding sites within PVT1-214 (Figure 4B). Specifically, luciferase activity of [illegible] was initially found to be inhibited si[illegible]icantly b[illegible] wild-type PVT1-214 and not by m[illegible]t PVT1-214 (Figure 4C, **p <0.01). Next, a[illegible]O2-dependent immunoprecipitation performe[illegible]C cells confirmed that PVT1-214 di[illegible]tly targe[illegible]-128 (Figure 4D, **p <0.01)[illegible]dditionally, [illegible] expression was increa[illegible] in GC [illegible]ells transf[illegible] with sh-PVT1-214 co[illegible] to l[illegible]s found in control cells (Figure [illegible]E, **[illegible] and the[illegible]levels were likely low[illegible]n GC ce[illegible]GC-7[illegible], MKN-45, BGC-823[illegible] those prese[illegible]nal gastric mucosa ce[illegible] 4F, **p <0.[illegible] Our results also revea[illegible]d a neg[illegible] correlation between miR-128 and PVT1-214 exp[illegible]n in human GC tissue (Fi[illegible], r = -0.4157, p [illegible]0021). In conclusion,



F[illegible]3. Lnc RNA PVT1-214 furthers GC cell growth *in vivo*. ***A-B,*** Tumor size (**A**) and burden (**B**) between PVT1-214 stab[illegible]knockdown group and the negative control showed by tumorigenesis assay. ***C-D,*** The tumor growth of GC cells *in vivo* mice indicated by macrography ***(C)*** and weight ***(D)*** of tumor nudes after stable knockdown of PVT1-214. *p<0.05, **p<0.01 compared to the control group.

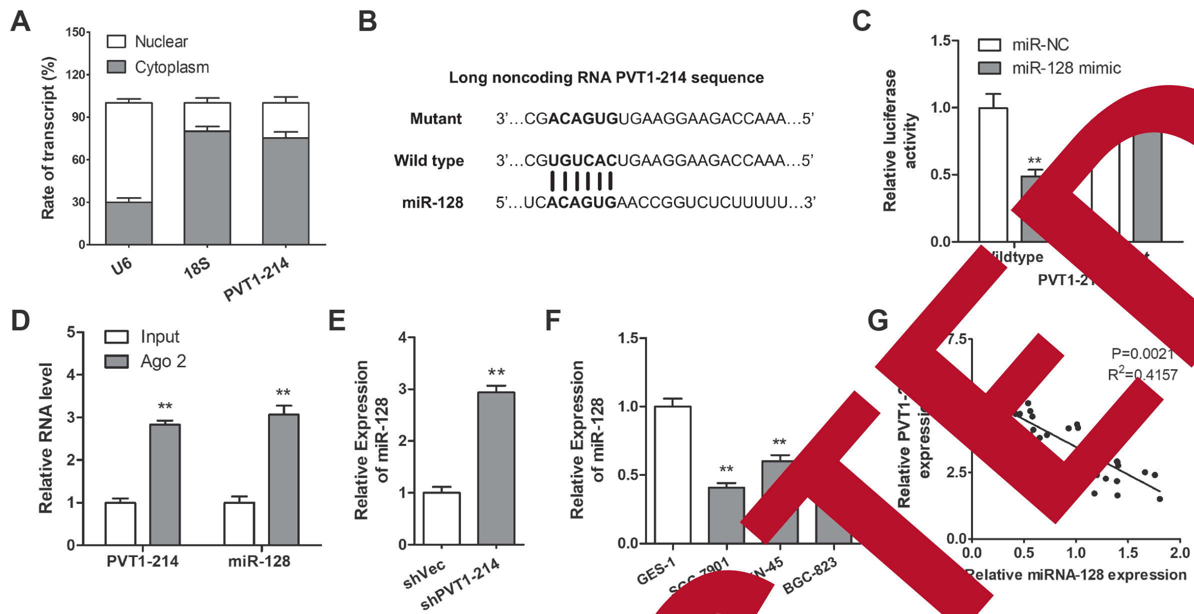


Figure 4. PVT1-214 modulated GC development by interacting [illegible]th miR-128. ***A,*** The subcellular localization assay for PVT1-214. ***B,*** The schematic diagram presents the complementary [illegible]ding sites withi[illegible]VT1-214 and miR-128. ***C,*** Luciferase reporter assay confirmed the molecular binding between PVT1-2[illegible]nd miR-128. [illegible]P and then PCR was conducted to measure the enrichment of PVT1-214 and miR-128 in Ago2 immun[illegible]ipitate and I[illegible]pellet. ***E,*** RT-PCR showed the miR-128 expression in GC cells transfected with shPVT1-214 or controls. [illegible]128 expr[illegible]on in GC cells (SGC-7901, MKN-45, BGC-823). ***G,*** A significant negative correlation betw[illegible] the levels of [illegible]VT1-214 in GC tissues. $^{**}p<0.01$ compared to the control group.

these results suggested that lncRNA PVT1[illegible] could form a complementary b[illegible]airing w[illegible] miR-128 as a ceRNA.

MiR-128 Directly Targ[illegible] TrkC i[illegible]C Cells

lncRNA PVT1-214 has b[illegible] cogenic gene in GC t[illegible] targets [illegible]8. Therefore, we attempted to id[illegible]fy the poten[illegible]ector protein of PVT1-21[illegible]iR-128. Using[illegible]getScan, bioinformatics [illegible]redic[illegible]evealed that miR-128 shares com[illegible]lementary bi[illegible]ites with Tropomyosin rece[illegible] kinase C (TrkC[illegible]ed mRNA (Figure 5A[illegible]nd this finding was ve[illegible]ified by luciferase rep[illegible]assay ([illegible]ure 5B, $^{**}p<0.01$). In SGC-7901/MK[illegible]23 cells, the level of TrkC was significant[illegible]gulated [illegible]he repression of miR-128 [illegible]ared [illegible]obs[illegible]ved in control cells (Figure 5[illegible] $^{**}p<0.$[illegible]<0.05). Collectively, these resul[illegible]dicate that [illegible]rkC is the functional protein targe[illegible] PVT1-214/miR-128 in GC cells.

Discussion

Recently, an increasing number of lncRNA transcripts have been demonstrated to be highly overexpressed in cancers. However, the biological effects of this overexpression remain unclear. Further investigations examining the regulatory mechanisms of these transcripts may help discover important targets for tumor diagnosis and therapy. lncRNA PVT1 is significantly associated with the initiation and migration of various cancers[20-22], including gastric cancer[23-25]. High expression of lncRNA PVT1 can be used to predict regional lymph node metastasis, poor overall survival, and disease-free survival in gastric cancer[23,24]. lncRNA-PVT1 overexpression in gastric cancer cells not only promotes angiogenesis within tumor stroma by initiating STAT3/VEGFA activation[25], but it also promotes the proliferation and invasion of cancer cells by cooperating with FOXM1[15]. The oncogenic role of lncRNA PVT1 in cancers has been widely confirmed; however, subtypes of lncRNA PVT1 still remain undefined. Investigation of the downstream targets of transcript isoforms from lncRNA PVT1 and the elaborate regulatory mechanisms controlling their function will facilitate a clearer understanding of their roles in tumorigenesis. Using microarray analysis, He et al[19] found 11 PVT1 transcripts possessing lengths of more than 500

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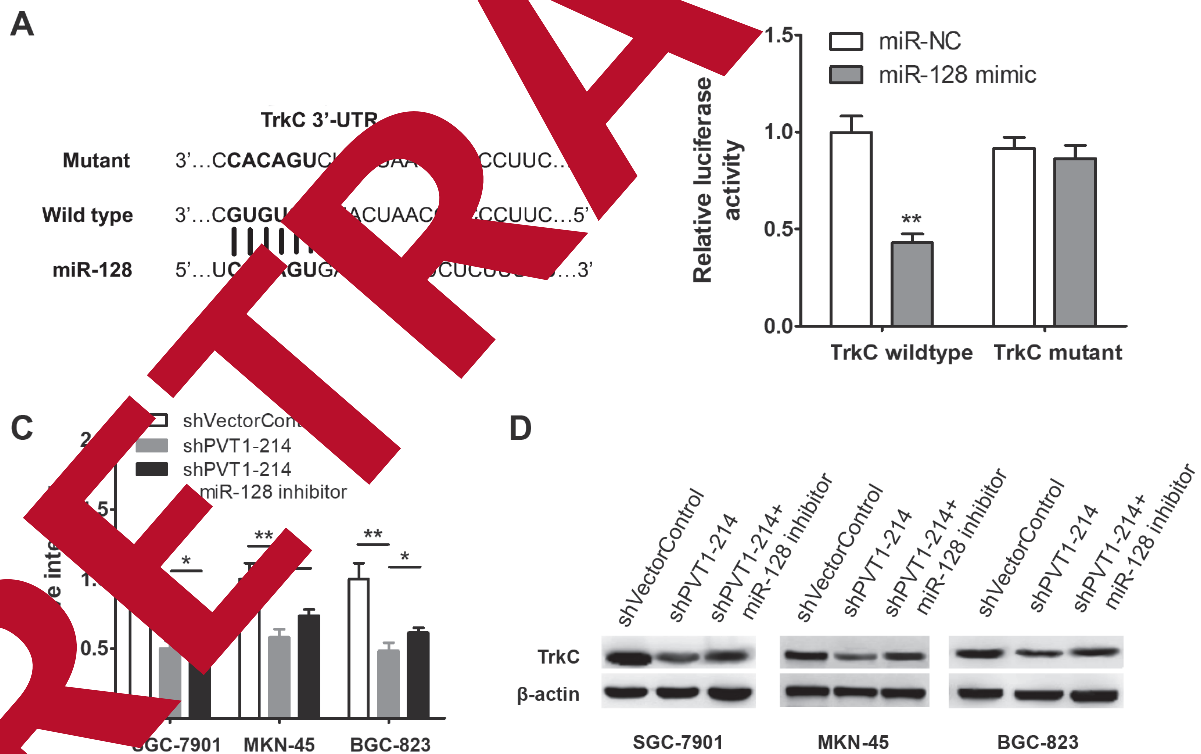
nt that were highly expressed in CRC tissues relative to non-tumor tissues, and this was verified by quantitative PCR. Interestingly, the fold change of over-expression in transcript PVT1-214 was the highest among the 11 PVT1 transcripts[19]. In the present study, elevated PVT1-214 was detected in GC clinical samples associated with advanced stage, large tumor size, and poor prognosis (Table I). Knockdown of lncRNA PVT1-214 reversed the oncogenesis of GC (Figures 2 and 3), suggesting an important role for lncRNA PVT1-214 in the regulation of tumorigenesis.

LncRNAs have been demonstrated to act as microRNA sponges to repress the regulatory function of miRNAs, which results in post-transcriptional modification on target messenger RNAs[26,27]. Various lncRNAs act as the competing endogenous RNA (ceRNA) of miR-128 in various cancers[28,29]. Our study revealed the existence of an interaction between PVT1-214 and miR-128 by dual-luciferase reporter assay. In accordance with the complementary binding sites on miR-128 and PVT1- 214, luciferase activity of miR-128 was inhibited by wildtype PVT1-214 (Figure 4C). Additionally, our data also indicated that miR-128 expression was significantly recovered by silencing PVT1- 214 expression in GC cells (Figure 4E) and was negatively corre[illegible] PVT1-214 levels in GC tissues (Figu[illegible]G). Thus these findings indicated that miR[illegible]s acted as a potential tumor suppressor gen[illegible]C. Consistent with this, Yu et al[30] found tha[illegible]128 restricted the expression of B[illegible]1, which [illegible] promote epithelial-to-mesen[illegible]mal transitio[illegible] Moreover, Liu et al[31] rep[illegible] that miR-128 in coopera[illegible] with miR-27b and m[illegible] do[illegible]regulated vascular endothelial [illegible]owth [illegible] (VEGF) t[illegible]hibit angiogenesis in [illegible]C.

The impo[illegible] role of TrkC [illegible] development was i[illegible] [illegible]served in the [illegible]text of intracranial ca[illegible]cer. Hi[illegible]pression of TrkC is a useful predictor for imp[illegible] prognosis in patients suf[illegible] from neurobla[illegible]as[32] or medulloblast[illegible]as[33]. Various studies[34,3[illegible]] have also shown that [illegible] regression of the TrkC gene could be detected [illegible] examining th[illegible]roliferation and migration of co[illegible]ctal cance[illegible] However, different expression patt[illegible] and di[illegible]se contributions to tumorigen-



F[illegible]5. miR-128 directly targets TrkC in GC cells. ***A,*** Bioinformatics tools reveal the complementary binding sites within miR-[illegible]8 and TrkC 3'-UTR. ***B,*** Luciferase reporter assay validated the molecular binding between miR-128 and TrkC 3'-UTR. ***C-D,*** Western blot assay showed the TRKC protein expression in GC cells transfected with miR-128 inhibitor or the other controls. *$p<0.05$, **$p<0.01$ compared to the control group.

esis indicated that the role of TrkC in cancers is still unclear. TrkC enhances epithelial-mesenchymal transition in breast cancer by regulating the JAK2/STAT3 activation, ultimately contributing to the formation of pulmonary metastasis[36]. In contrast, TrkC expression in breast cancer was indicative of tumor relapse and poor disease-free survival in an observational study[37]. In the current study, TrkC expression was elevated in gastric cell lines and upregulated by lncRNA PVT1-214 (Figure 5C). These results suggested that TrkC functioned as a tumor suppressor gene in gastric cancer, which was consistent with another representative study recently published by Bu et al[38].

Conclusions

We have demonstrated that lncRNA PVT1-214 was vital for the proliferation of GC cells *in vitro* and for tumor growth *in vivo*. Additionally, we have identified the complementary binding site of lncRNA PVT1-214, and we found that the repression of miR-128 was attributed to a direct interaction with lncRNA PVT1-214. Furthermore, [illegible] 128 inhibition increased the expression [illegible] to promote tumorigenesis. These findings s[illegible]est that lncRNA PVT1-214 may provide a prom[illegible]g molecular target for GC therapy.

Conflict of interest

The authors declare no confli[illegible]nterest.

Data availability [illegible]tement

The datasets analy[illegible] the current stu[illegible]available from the corresp[illegible]ing a[illegible]n reasonable request.

Disclo[illegible]e of Financial Arrangements

The [illegible]rch and [illegible]nuscript preparation were funded by the [illegible] Pro[illegible]al Health Technology Project (2016-CX-12), [illegible] Science F[illegible]dation of Fujian Province (2018J012[illegible]J01199)[illegible]ence and Techology Program [illegible]an Pro[illegible]01[illegible]003).

A[illegible]tributions

[illegible]ormed the animal experiment and molec[illegible] biological analysis, and made a major contribution in [illegible]g the manuscript. Xiaohui Chen assisted to perform th[illegible]formatics prediction. Changhua Zhuo helped Shen Zha[illegible]o finish the animal experiment. Chunwei Xu contributed to the assessment of clinicopathological data. Nanfeng Fan analyzed the survival data from GC patients. Rongbo Lin directed all these researches and reviewed this manuscript. All authors read and approved the final manuscript.

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