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PCAT6 participates in the development of gastric cancer through endogenously competition with microRNA-30

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Abstract. – OBJECTIVE: To investigate the role of lncRNA PCAT6 in the progression of gastric cancer and its underlying mechanism.

PATIENTS AND METHODS: Expression levels of PCAT6 in 72 gastric cancer tissues and paracancerous tissues were detected by qRT-PCR (Quantitative Real-Time Polymerase Chain Reaction). The correlation between PCAT6 expression and clinical data of gastric cancer patients was analyzed by the chi-square test. After lentivirus transfection of PCAT6 in gastric cancer cells, proliferation, apoptosis, and invasion were detected by CCK-8 (cell counting kit-8), flow cytometry, and transwell assay, respectively. Western blot was utilized to detect protein expressions of apoptosis-related and EMT-related (epithelial-mesenchymal transition) genes in gastric cancer cells. Furthermore, target genes of PCAT6 were predicted via bioinformatics method and verified by luciferase reporter gene assay. The effects of target genes on biological functions of gastric cancer cells were determined as well.

RESULTS: PCAT6 was overexpressed in gastric cancer tissues than those of paracancerous tissues. PCAT6 expression was negatively correlated to prognosis, tumor size, TNM (tumor node metastasis) stage and metastasis of gastric cancer. For in vitro experiments, overexpression of PCAT6 increased proliferation, migration, and invasion, whereas decreased apoptosis of gastric cancer cells. MicroRNA-30 was predicted as the target gene of TCAT6. Furthermore, microRNA-30 was found to bind to TCAT6 via targeting MKRN3. Either microRNA-30 knockdown or PCAT6 overexpression could remarkably promote MKRN3 expression.

CONCLUSIONS: PCAT6 is overexpressed in gastric cancer, which promotes the development of gastric cancer by endogenously competition with microRNA-30 via targeting MKRN3.

Key Words:
Gastric cancer, MicroRNA-30, MKRN3, EMT.

Introduction

Gastric cancer (GC) is one of the most significant causes of tumor death worldwide. It is also the most common gastrointestinal cancer in East Asia[1]. Despite continuous advances have been made in surgical treatment, chemotherapy, and targeted drug treatment, the 5-year survival rate of GC remains unsatisfactory. Most of GC patients have already been in advanced stage accompanied by lymph node metastasis when first diagnosed, which seriously restricts the effective treatment[2]. More importantly, the molecular mechanisms underlying the progression and metastasis of GC have not been fully elucidated[3]. Therefore, it is of great clinical significance to explore new biomarkers, so as for better prediction and treatment of GC.

Non-coding RNAs have now been recognized as the key factors in the regulation of gene expressions. Long non-coding RNAs (lncRNAs) are a class of ncRNAs with over 200 nucleotides in length. Functionally, lncRNAs could not encode proteins. LncRNAs are involved in a variety of biological processes, including embryonic stem cells[4,5], differentiation of helper T cells[6], autophagy, myocardial infarction, cell senescence, apoptosis, metastasis of cancer cells, and resistance to chemotherapy drugs[7]. Various studies have shown that lncRNA expression is tissue-specific and dysregulated in many types of tumors. Some lncRNAs are found to be associated with recurrence and poor prognosis of cancer via silencing tumor suppressor genes and activating proto-oncogenes. The specific mechanisms of regulating tumor development by lncRNAs mainly include epigenetic modifications, alternative splicing, RNA attenuation, and post-translational regulation.

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At present, several lncRNAs have been well elucidated in regulating the occurrence and progression of GC, such as HOTAIR, ANRIL, H19, GAPLINC, PVT1, and MEG38-10. For instance, overexpression of H19 promotes proliferation and invasion of gastric cancer cells by actively binding to ISM1[11]. LncRNA prostate cancer-associated transcript 6 (PCAT6) was initially recognized to be able to promote keratinocyte proliferation and colony formation of prostate cancer cells in an androgen-independent manner[12]. So far, the specific role of PCAT6 in GC, however, has not been reported yet.

Patients and Methods

Sample Collection

72 GC tissues and paracancerous gastric mucosa tissues that were resected over 5 cm away from the tumor tissues (NC) were collected in Yantai Yuhuangding Hospital from July 2012 to August 2017. Samples were immediately preserved in liquid nitrogen for the following experiments. GC patients were all pathologically diagnosed and GC tissues were confirmed by H&E (hematoxylin and eosin) staining. This investigation was approved by the Yantai Yuhuangding Hospital Ethics Committee, and all patients signed the informed consent.

Cell Culture and Transfection

GC cell lines (BGC-823, SGC-7901, HGC-27, and MKN45) and gastric mucosal epithelial cell line (GES-1) were cultured in DMEM (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), and maintained in a 5% CO2 incubator at 37°C. Cell transfection was performed according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The overexpression lentivirus of PCAT6, microRNA-30 negative control, microRNA-30 mimics, and microRNA-30 inhibitor were constructed by GenePharma (Shanghai, China). The sequences were as follows: microRNA-30 mimics: 5'-UGUAACCAUCCUCGACUGGAAG-3'; microRNA-30 inhibitor: 5'-CUUCCAGUCGAGGAUGUUUACA-3'; microRNA-30 negative control: 5'-TTACAAGCCTATCCAAATGAG-3'.

RNA Extraction and qRT-PCR (Quantitative Real-Time Polymerase Chain Reaction)

Total RNA was extracted from the tissues and cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), respectively, followed by measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScriptTM RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by 2-ΔΔCt. Primer sequences used in this study were as follows: PCAT6, F: 5'-CAGGAACCCCCTCCTTACTC-3', R: 5'-CTAGGGATGTGTCCGAAGGA-3'; microRNA-30, F: 5'-GGGGTGTAAACATCCTCGACTG-3', R: 5'-ATTGCGTGTCGTGGAGTCG-3'; MKRN3, F: 5'-AGCAGCGGCATTTGGACAA-3', R: 5'-CGTGCGAATAGCGACAGTTCT-3'.

CCK-8 (Cell Counting Kit-8) Assay

The cells were seeded in 96-well plates at a density of 2×103/well with 6 replicates in each group. 10 μL of CCK-8 (Dojindo, Kumamoto, Japan) solution was added to each well and incubated for another 2 h. The absorbance at a wavelength of 450 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA).

Bioinformatics Prediction

The target gene of PCAT6 was predicted through Starbase and microRNA-30 was screened out. Subsequently, the target gene of microRNA-30 was predicted through TargetScan, mirBase, and mirDB.

Luciferase Reporter Gene Assay

3'UTR sequences of PCAT6 and MKRN3 were downloaded from NCBI. Wild-type PCAT6 (PCAT6 WT), mutant-type PCAT6 (PCAT6 MUT), wild-type MKRN3 (MKRN3 WT), and mutant-type MKRN3 (MKRN3 MUT) were constructed, respectively. GC cells were co-transfected with 50 pmol/L microRNA-30 mimics or negative control, PCAT6 WT or PCAT6 MUT and MKRN3 WT or MKRN3 MUT, respectively. Luciferase activity was accessed after transfection for 48 h.

Western Blot

The RIPA (radioimmunoprecipitation assay) protein lysate (Beyotime, Shanghai, China) was used to extract the total protein in each group of cells and tissues. The BCA (bicinchoninic acid) method was performed to quantitate the protein concentration. Protein samples were electropho-

resed on polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibody (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. The membrane was incubated with the secondary antibody after rinsing with the buffer solution (TBST). Chemiluminescence was used to expose the protein bands on the membrane.

Transwell Assay

Fibronectin (FN) was diluted at the dose of 100 μg/mL. Matrigel was diluted with serum-free DMEM at the ratio of 1:9. Subsequently, 50 μL of FN and 100 μL of Matrigel were added into the lower and upper chamber, respectively. Cells were seeded in the upper chamber at a density of 1×106 cells per well. 24 h later, transwell chamber was removed from the incubator and placed in a 24-well plate with 500 μL of methanol for fixation overnight at 4°C. After washing with phosphate-buffered saline (PBS) for three times, images were captured using an inverted microscope (Nikon, Tokyo, Japan).

Cell Apoptosis

For cell apoptosis, cells were digested, washed twice with 4°C pre-cooled PBS and centrifuged at 1000 rpm for 5 min. Totally 500 μL of PBS was added to resuspend the collected cells. The cells were then resuspended in 100 μL of Annexin V binding buffer, mixed with 10 μL of Annexin-V and 10 μL of PI (propidium iodide). Finally, cells were incubated at 4°C for 15 min in the dark, followed by the flow cytometric analysis.

Statistical Analysis

SPSS19.0 (Statistical Product and Service Solutions) statistical software package (IBM, Armonk, NY, USA) was used for data analysis. Kaplan-Meier was introduced for analyzing the prognosis of GC patients. The *t*-test was used to analyze the difference between two groups and the chi-square test was used to analyze the classification data. $p<0.05$ was considered statistically significant.

Results

LncRNA PCAT6 Promoted GC Development

LncRNA PCAT6 expressions in 72 GC tissues and NC tissues were detected by qRT-PCR. The data showed that PCAT6 was overexpressed in GC tissues than that of paracancerous tissues (Figure 1A). Furthermore, GC patients were assigned to high and low expression group based on their PCAT6 expressions. Our results illustrated that PCAT6 expression was negatively correlated to the prognosis of GC patients (p=0.0239, Figure1B). Further analysis demonstrated that PCAT6 expression was correlated to tumor size, TNM stage and metastasis, whereas not correlated to age and gender of GC patients (Table I). For *in vitro* experiments, PCAT6 was overexpressed in GC cell lines (BGC-823, SGC-7901, HGC-27, and MKN45) than that of gastric mucosal epithelial cell line (GES-1). In particular, HGC-27 cells expressed the highest expression of PCAT6, which was selected for the following experiments (Figure 1C).

Effects of PCAT6 on Cellular Phenotypes of GC Cells

We thereafter constructed the overexpression lentivirus of PCAT6, and the transfection efficacy was verified by qRT-PCR (Figure 2A). The CCK-8 assay showed that the proliferative ability of HGC-27 cells was remarkably increased after PCAT6 overexpression (Figure 2B). Apoptosis in HGC-27 cells was decreased after overexpression of PCAT6 (Figure 2C). Western blot results also demonstrated that caspase-3, caspase-9, and Bax were downregulated, whereas Bcl-2 was overexpressed after PCAT6 overexpression, further indicating that overexpressed PCAT6 could regulate apoptosis of GC cells (Figure 2D). Subsequently, increased migration of HGC-27 cells was observed after transfection of PCAT6 overexpression lentivirus (Figure 2E). EMT-related genes were also found to be upregulated except for E-cadherin (Figure 2F). The above data elucidated that overexpressed PCAT6 promotes GC development.

Downregulated MicroRNA-30 Promoted GC Development

We speculated whether PCAT6 could bind to some microRNAs to exert its role as ceRNA. MicroRNA-30 was then screened out to be the target gene of PCAT6. We found that microRNA-30 was downregulated in GC cell lines, especially in HGC-27 cells (Figure 3A). Hence, microRNA-30 inhibitor was constructed and its transfection efficacy was verified (Figure 3B). After transfection with microRNA-30 inhibitor in HGC-27 cells, proliferation and migration were remarkably in-

Table I. Relationship between PCAT6 expression and basic characteristics of GC patients.

Clinicopathologic features	Number of cases	LncRNA PCAT6 expression		*p*-value
		Low (n=36)	High (n=36)	
Age (years)		36	36	0.0926
≤55	29	18	11	
>55	43	18	25	
Gender				0.3061
Male	50	27	23	
Female	22	9	13	
Tumor size (cm)				0.0322*
≥5	31	11	20	
<5	41	25	16	
TNM stages				0.0001*
N0-2	28	22	6	
N3-N4	44	14	30	
Metastasis				0.0285*
YES	45	18	27	
NO	27	18	9	

creased, whereas apoptosis was decreased (Figure 3C-3E). These data showed that downregulated microRNA-30 could promote GC development.

PCAT6 Regulated MKRN3 Expression Through Endogenously Competition With MicroRNA-30

Target genes of microRNA-30 were then predicted by bioinformatics and MKRN3 was screened out. To further verify whether microRNA-30 could bind to MKRN3, MKRN3 WT and MKRN3 MUT were constructed, respectively (Figure 4A, left). Lower luciferase activity was found in GC cells co-transfected with microRNA-30 mimics and MKRN3 WT than that of MKRN3 MUT (Figure 4A, right). Besides, MKRN3 expression was upregulated after transfection with microRNA-30 inhibitor (Figure 4B), indicating that MKRN3 is the target gene of microRNA-30. Furthermore, PCAT6 WT and PCAT6 MUT were constructed to detect the binding condition of PCAT6 with microRNA-30 (Figure 4C, left). Lower luciferase activity was found in GC cells co-transfected with microRNA-30 mimics and PCAT6 WT than that of PCAT6 MUT (Figure 4C, right). Similarly, overexpression of PCAT6 remarkably led to downregulated microRNA-30 and upregulated MKRN3 (Figure 4D and 4E). Our data fully elucidated that PCAT6 regulates MKRN3 expression through endogenous competition with microRNA-30.

Discussion

GC is one of the common malignancies in the digestive system. Globally, the incidence and mortality rate of GC are relative high, which threatens human health. More seriously, the disease

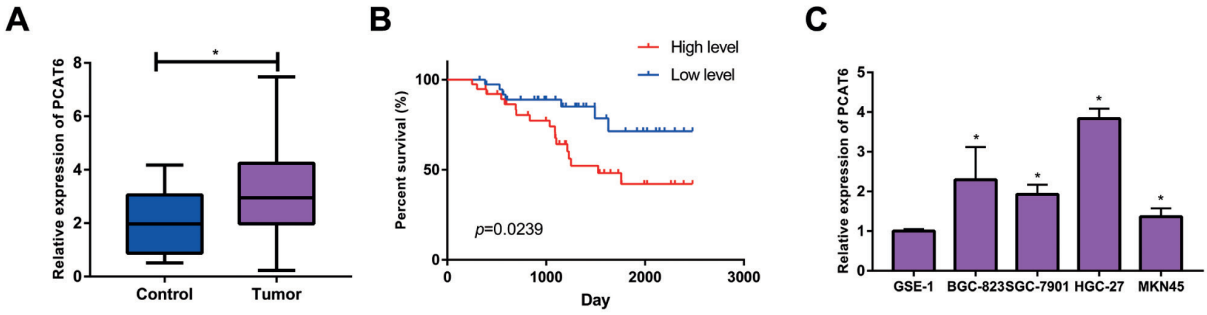

Figure 1. PCAT6 was overexpressed in GC. A, PCAT6 was overexpressed in GC tissues. B, GC patients with higher expression of PCAT6 presented worse prognosis. C, PCAT6 was overexpressed in GC cell lines (BGC-823, SGC-7901, HGC-27 and MKN45) than that of gastric mucosal epithelial cell line (GES-1).

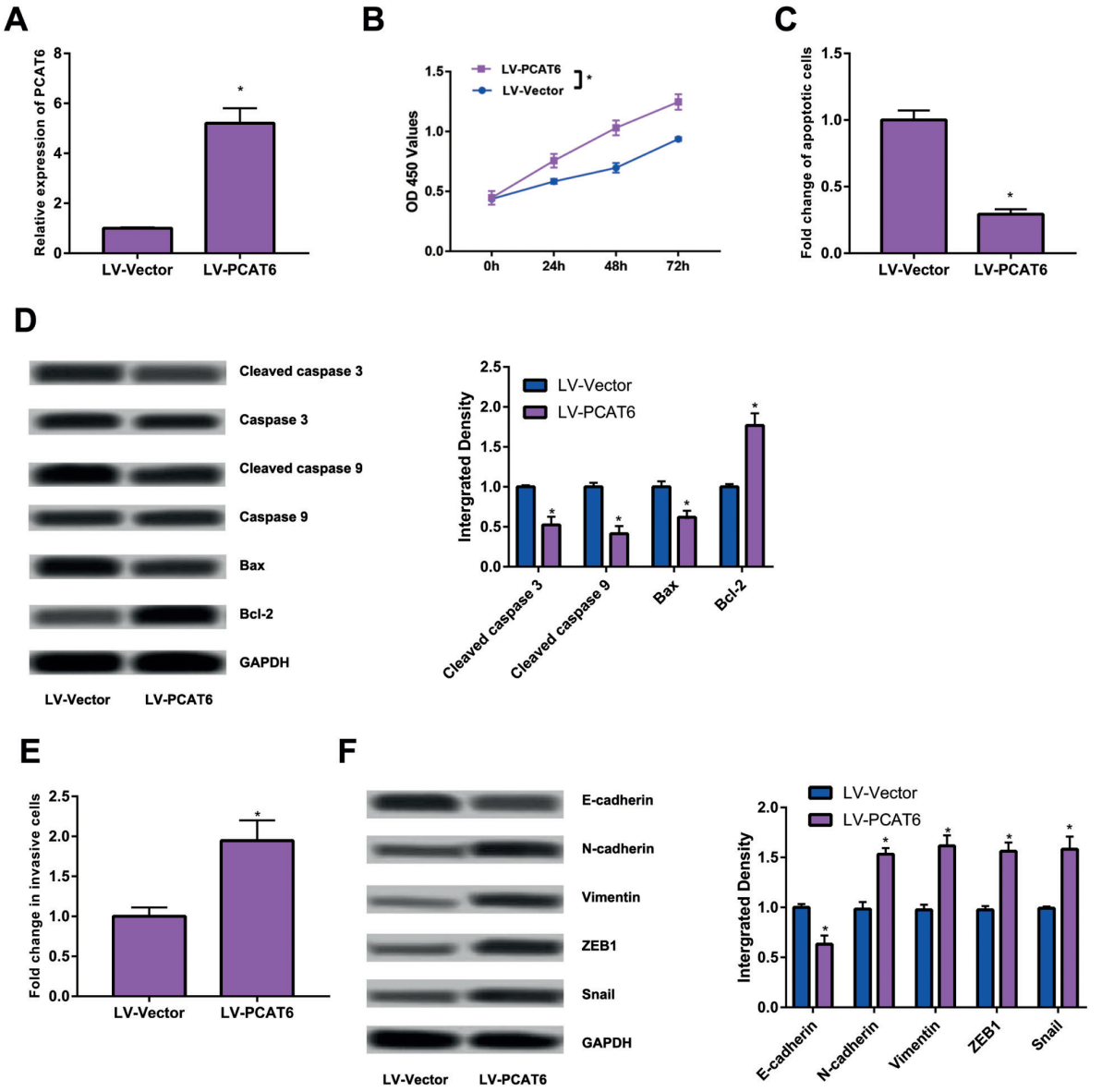

Figure 2. Overexpressed PCAT6 promoted proliferation of GC cells. A, Transfection efficacy of overexpression lentivirus of PCAT6. B, Overexpressed PCAT6 promoted proliferation of GC cells. C, Overexpressed PCAT6 inhibited apoptosis of GC cells. D, Protein expressions of apoptosis-related genes after overexpression of PCAT6 in GC cells. E, Overexpressed PCAT6 promoted migration of GC cells. F, Protein expressions of EMT-related genes after overexpression of PCAT6 in GC cells.

progress of GC is very fast. Surgical efficacy of advanced GC is worse than that of early GC, which is manifested as the high recurrence and metastatic rate13. It is believed that GC is a result of the multi-gene interaction. Therefore, molecular mechanisms underlying the incidence and development of GC need to be urgently explored[14].

LncRNAs are a kind of small RNAs with over 200 nucleotides in length. LncRNAs could not encode proteins. They were initially considered as meaningless transcripts for a long time. However, some studies[15,16] have reported that lncRNAs participate in the regulation of many biological processes, including tumor development. The data showed that about 18% of lncRNAs are associated with tumors. PCAT6 is overexpressed in lung cancer tissues than that of normal tissues[17], which is associated with the metastasis of lung cancer. A retrospective analysis18 suggested that serum level of PCAT6 in patients with non-small

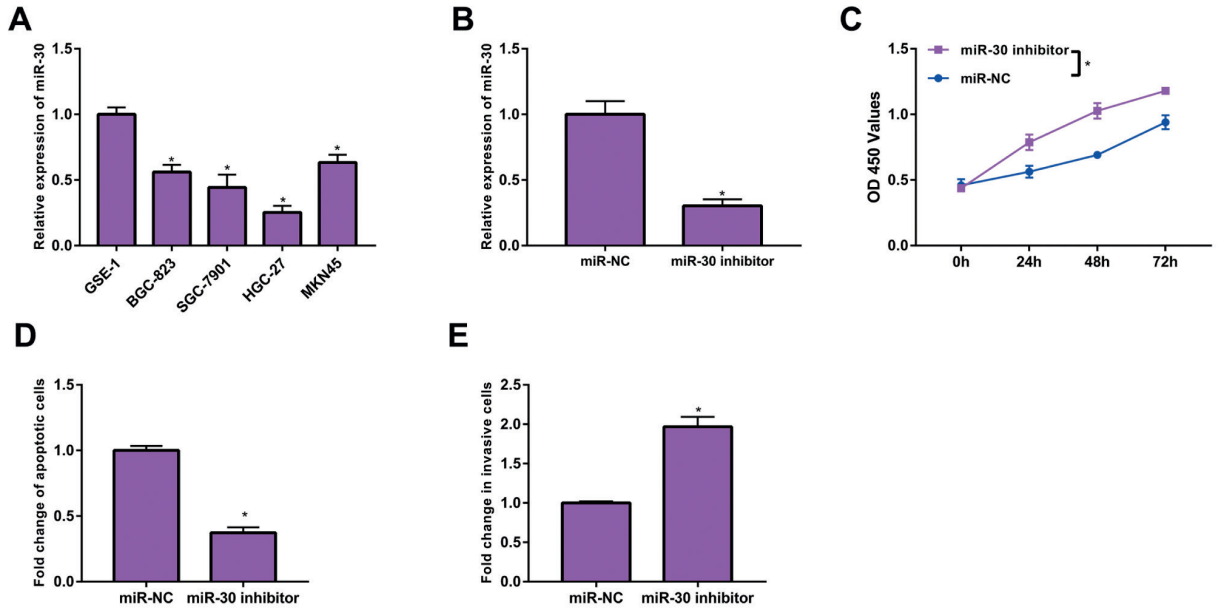

Figure 3. Downregulated microRNA-30 promoted GC development. A, MicroRNA-30 was downregulated in GC cell lines. B, Transfection efficacy of microRNA-30 inhibitor. C, Downregulated microRNA-30 promoted proliferation of GC cells. D, Downregulated microRNA-30 inhibited apoptosis of GC cells. E, Downregulated microRNA-30 promoted migration of GC cells.

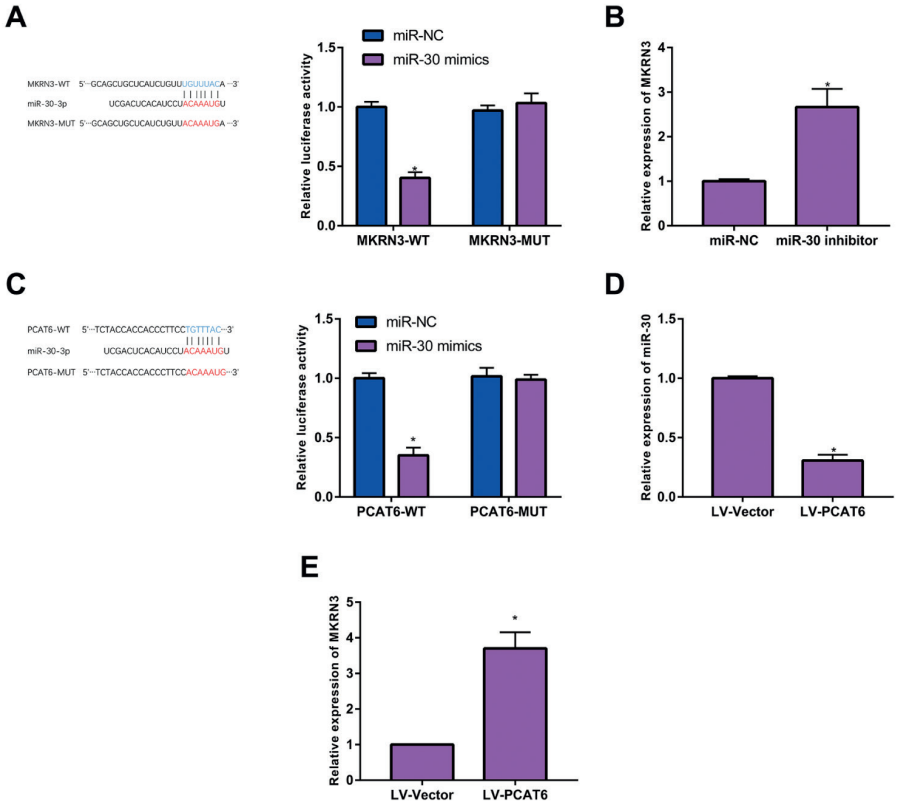

Figure 4. PCAT6 regulated MKRN3 expression through endogenously competition with microRNA-30. A, Luciferase reporter gene assay showed that microRNA-30 could bind to MKRN3. B, Downregulated microRNA-30 promoted MKRN3 expression. C, Luciferase reporter gene assay showed that microRNA-30 could bind toPCAT6. D, Overexpressed PCAT6 inhibited microRNA-30 expression. E, Overexpressed PCAT6 promoted MKRN3 expression.

cell lung cancer was remarkably elevated. In this study, PCAT6 was overexpressed in GC. PCAT6 expression was negatively correlated with the overall survival of GC patients. Overexpressed PCAT6 promoted the proliferation and migration, but inhibited apoptosis of GC cells.

Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells lose cell polarity and cell-cell junction. EMT acquires the characteristics of mesenchymal cells via altering cell morphology and structure, so as to improve cell migration and invasiveness. Scholars[19,20] have shown that EMT is closely related to tumor invasion, metastasis, tumor drug resistance, and tumor stem cell characteristics. In this study, invasive ability of GC cells was enhanced when PCAT6 was overexpressed. Protein expressions of EMT-related genes were also remarkably increased.

Both microRNAs and lncRNAs exert very important roles in tumorigenesis[21]. LncRNAs are closely interacted with microRNAs to exert their regulatory roles in modulating cellular functions[22]. MicroRNA-30 is confirmed to be able to regulate different types of tumors. MicroRNA-30b and microRNA-30c inhibit tumor growth and metastasis in human glioblastoma progenitor cells, lung cancer cells, and hepatocytes[23]. Studies have found that microRNA-30a is negatively correlated with lymph node and lung metastasis in breast cancer patients by directly targeting MTDH[24]. In this work, microRNA-30a expression was remarkably reduced in GC cells, which is capable of promoting GC development.

Bioinformatics predicted that MKRN3 is the target gene of microRNA-30. MKRN3 encodes proteins that contain C3HC4 and C3H zinc finger motifs. Our data showed that both PCAT6 overexpression and microRNA-30 knockdown could lead to MKRN3 upregulation. The binding condition of PCAT6, microRNA-30, and MKRN3 was further verified by luciferase reporter gene assay.

Conclusions

We found that PCAT6 is overexpressed in gastric cancer, which promotes the development of gastric cancer by endogenous competition with microRNA-30 via targeting MKRN3.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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