European Review for Medical and Pharmacological Sciences 2017; 21: 3021-3027

Up-regulation of long non-coding RNA PCAT-1 correlates with tumor progression and poor prognosis in gastric cancer

W.-C. CUI[1], Y.-F. WU[2], H.-M. QU[3]

[1]Department of Gastrointestinal Surgery, Dongying People's Hospital, Dongying, Shandong, China
[2]Department of Gastroenterology, Binzhou People's Hospital, Binzhou, Shandong, China
[3]Department of Gastroenterology, Weifang People's Hospital, Weifang, Shangdong, China

Wei-chun Cui and Yan-fang Wu contributed equally to this work

Abstract. – OBJECTIVE: Long non-coding RNAs (lncRNAs), emerging non-coding RNAs, have been proved to serve as a critical role in the proliferation, metastasis apoptosis of gastric cancer. However, the potential biological role PCAT-1 in gastric cancer (GC) remains undefined. The present study aimed to investigate the expression and clinical significance of PCAT-1 in GC.

PATIENTS AND METHODS: The expression of PCAT-1 was detected with a quantitative Real-time PCR assay. The association between PCAT-1 expression and clinicopathological factors, as well as survival rates, was analyzed. Cox proportional-hazards regression analysis was applied in order to estimate univariate and multivariate hazard ratios for overall survival. Then, effects of PCAT-1 on the biological behavior of GC cells were investigated.

RESULTS: We found that PCAT-1 expression was elevated in GC tissues and cell lines, and PCAT-1 levels were highly positively correlated with invasion depth ($p < 0.001$), TNM stages ($p < 0.001$) and lymphatic metastasis ($p = 0.003$). The biological function of PCAT-1 was explored and the results showed silencing of PCAT-1 could suppress cell proliferation, migration and invasion in vitro. Kaplan-Meier analysis demonstrated that increased PCAT-1 expression contributed to poor overall survival (OS) ($p < 0.01$). Furthermore, in a multivariate Cox model, our results showed that PCAT-1 expression was an independent poor prognostic factor for OS in GC.

CONCLUSIONS: Our finding suggested that PCAT-1 may have potential roles as a biomarker and/or a therapeutic target in gastric cancer.

Key Words:
Long non-coding RNA, PCAT-1, Prognosis, Gastric cancer.

Introduction

Gastric cancer (GC) is the fourth most common malignancies worldwide. The incidence is much higher in male and in developing countries, including East Asian and Eastern European nations[1,2]. Despite recent advances in diagnostic method, surgical technique and new chemotherapy regimens, the long-term survival rate for GC is still quite low[3,4]. Most of gastric cancer are diagnosed in advanced stages and thus have lost the opportunity for radical surgery. Therefore, it is critical to identify promising prognostic biomarkers and develop novel therapeutic agents for GC. Long noncoding RNAs (lncRNAs) are types of transcriptional products of the eukaryotic genome comprising > 200 nt in length[5]. Previous studies[6-8] have revealed that lncRNAs are involved in many biological processes, and aberrant numbers of lncRNAs were found playing essential roles in carcinogenesis, progression or metastasis of various types of cancers. For example, long non-coding RNA HOTAIR promotes tumor cell invasion and metastasis by recruiting EZH2 and repressing E-cadherin in oral squamous cell carcinoma[9]. Yap et al[10] found that lncRNA ANRIL was significantly up-regulated in prostate cancer and involved in repressing of the p15/CDKN2B-p16/CDKN2Ap14 gene cluster in Cis by directly binding to the PRC. Hu et al[11] showed that high linc-UBC1 expression suppressed the proliferation, motility and invasion of gastric cancer cells. However, to our knowledge, the clinical significance and biological function of lncRNA PCAT-1 in GC remains unclear. In the present study we focused on the lncRNA PCAT-1, and examined the expression level of PCAT-1 in GC tissues and cell lines. The oncogenic

Corresponding Author: Hong-Mei Qu, MD; e-mail: holi5888@126.com

activity of PCAT-1 was investigated in gastric cancer cell lines. Finally, we investigated whether PCAT-1was a useful prognostic indicator in GC patients.

Patients and Methods

Patients

A total of 175 patients, who were diagnosed as GC in Cangzhou Central Hospital were selected in this study. The diagnosis of all samples was histopathologically confirmed by two pathologists. None of the patients received any kind of treatment prior to sample collection. Samples were flash frozen in liquid nitrogen until use. This study has been approved by the Ethical Committee of Cangzhou Central Hospital and has obtained written consents from all participants.

Cell Culture and Transfection

Human GC cell lines AGS, BGC-823, MKN-45, HGC-27 were obtained from the Academy of Military Medical Science (Haiding, Beijing, China), and human gastric mucosa cell line GES-1 were obtained from the Chinese Academy of Sciences (Pudong, Shanghai, China). Cells were maintained at 37°C in a fully humidified incubator and an atmosphere of 5% CO_2 in air. Cells were cultured in a 6-well for 24 hours and then were transfected with over expression plasmid vector or shRNAs. Cells were transfected with siRNAs using lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. PCAT-1 small hairpin RNA (shR-PCAT1) and negative control shRNA (shR-NC) were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA). The sequence of shR-PCAT1 was 5'-GAGAAAGCAUCUGUAC-CCUUACAAU-3'. The overexpression plasmid vector of PCAT-1 (PCAT-1-pcDNA3.1 + vector) was synthesized in Invitrogen Life Technology (Carlsbad, CA, USA).

Quantitative Real-time PCR Assay

Total RNA was isolated tissue using TRIZOL reagent according to the manufacturer's protocol (Invitrogen Inc., Carlsbad, CA, USA). cDNA was reversely transcribed using a first strand synthesis kit (Invitrogen, Carlsbad, CA, USA). The expression level of PCAT-1 was measured by quantitative Real-time PCR (qRT-PCR), which was performed using the Applied Biosystems 7900HT (Foster City, CA, USA) with 1.0 μl of cDNA and SYBR green Real-time PCR Master Mix (TaKaRa, Tokyo, Japan). The primers were as follows: PCAT-1 sense: 5'-AATGGCATGAACCTGGGAGG-CG-3'; PCAT-1 antisense: 5'-GGCTTTGGGAAG-TGCTTTGGAG-3'; GAPDH sense: 5'-AGAAGGCT-GGGGCTCATTTG-3'; GAPDH antisense: 5'-AG-GGGCCATCCACAGTCTTC-3'. The relative expression of lncRNA HOTTIP was calculated and normalized using the delta-delta CT ($2^{-\Delta\Delta Ct}$) method relative to β-actin. Independent experiments were done in triplicate.

Cell Proliferation Assay

The proliferative capacity of the gastric cancer cells was examined by MTT assay. After transfection with plasmid vector or shRNAs, HGC-27 cells were seeded in the 96-well plate and cultured for 24 h, at a density of 1×103 cells/well. Cells were cultured for 4 h at 37°C. Then, the medium was discarded and 150 μL dimethyl sulfoxide (DMSO) was added and oscillated for 15 min. Optical density (OD) was detected at a wavelength of 490 nm using an enzyme-labeled analyzer. Three independent experiments (three replicates in each) were performed.

Cell migration and Invasion Assay

Cell migration and invasion assays were performed using transwell assay kit (BD Biosciences, Bedford, MA, USA). The migration assay was conducted as follows: 2×104 cells in 0.1 ml of media added to the upper chamber (BD Biosciences, Bedford, MA, USA), followed by an addition of 0.6 ml medium in lower chamber; medium containing 20% fetal bovine serum (FBS) was used as a chemoattractant in the lower chamber. After incubation for 24 h, cells on the upper surface of the filter were removed using cotton buds. Fixed cells were stained with crystal violet and the number of invasive cells was counted. Five random fields in each chamber were analyzed. Assays were performed in triplicate.

Statistical Analysis

All statistical analyses were performed using the SPSS for Windows statistical software, version 16.0 (SPSS Inc., Chicago, IL, USA). Differences between groups were measured using the t-test. Patient survival and their differences were determined by Kaplan-Meier method and log-rank test. Cox regression (proportional hazard model) was adopted for multivariate analysis of prognostic factors. $p < 0.05$ was considered to indicate a significant difference.

Results

PCAT-1 is up-regulated in GC samples and cell lines

qRT-PCR was used to detect PCAT-1 expression levels in cell lines and clinical samples, which

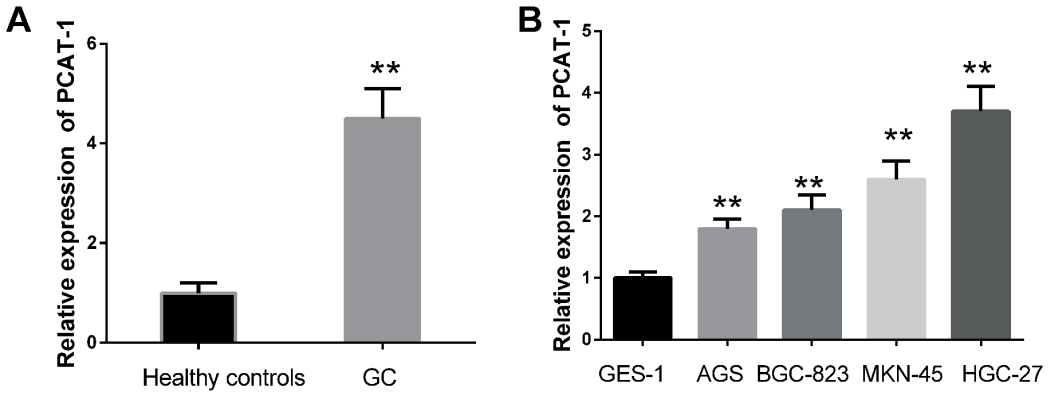

Figure 1. The expression levels of PCAT1 in gastric cancer tissues and GC cell lines. ***(A)*** PCAT1 expression was significantly up-regulated in tumor tissues compared with the paired adjacent normal tissues ($p < 0.001$). ***(B)*** Expression levels of PCAT1 in normal gastric mucous epithelium cell (GES-1) and four GC cells (AGS, BGC-823, HGC-27 and MKN-45). $^{*}p < 0.05$, $^{**}p < 0.01$.

were normalized to GAPDH. The results informed that the PCAT-1 expression level was significantly increased in GC tissues compared with matched non-tumor tissue samples (Figure 1 A, $p < 0.01$). Meanwhile, PCAT-1 expression was also increased in four GC cell lines, including AGS, BGC-823, MKN-45, HGC-27 (Figure 1 B, all $p < 0.01$).

The association between tissue PCAT-1 expression and clinical parameters of GC All patients with gastric cancer were assigned into 2 groups (high tissue PCAT-1 group and low PCAT-1 group) based on the median expression level of tissue PCAT-1 (0.98 fold). As shown in Table I, PCAT-1 levels were highly positively correlated with invasion depth ($p <$

Table I. Association of lncRNA PCAT-1 expression with clinicopathologic factors of GC patients.

			lncRNA PCAT-1		
Parameters	**Group**	**Total**	**High**	**Low**	***p*-value**
Gender	Male	98	48	50	0.697
	Female	77	40	37	
Age (years)	< 50	115	55	60	0.368
	≥ 50	60	33	27	
Location	Distal	76	37	39	0.845
	Middle	66	35	31	
	Proximal	33	16	17	
Size	> 5 cm	104	49	55	0.310
	< 5 cm	71	39	32	
Histologic differentiation	Well	48	20	28	0.143
	Moderately	66	35	31	
	Poorly	31	17	14	
	Undifferentiated	30	16	14	
Invasion depth	T1	48	9	39	<0.001*
	T2	39	16	23	
	T3	54	35	19	
	T4	34	28	6	
TNM Stages	I	36	10	26	<0.001*
	II	80	33	47	
	III	52	40	12	
	IV	34	28	6	
Lymphatic metastasis	Yes	84	52	32	0.003*
	No	91	36	55	
Distant metastasis	Yes	20	9	11	0.635
	No	155	79	76	

$^{*}p < 0.05$

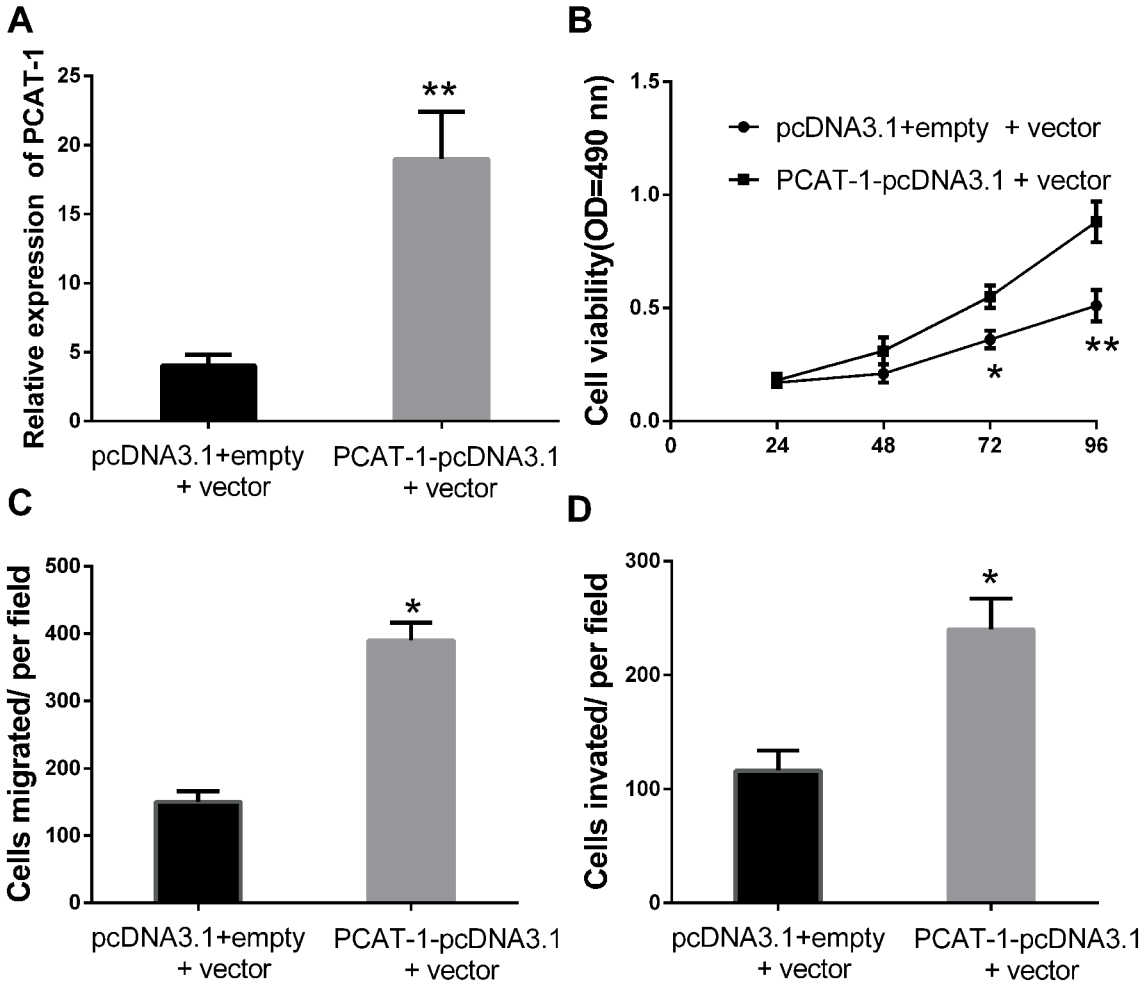

Figure 2. PCAT1 promotes the proliferation, migration and invasion of GC cells *in vitro*. ***(A)*** Relative PCAT1 expression in HGC-27 cells when transfected with shR-PCAT1 or its negative control (miR-NC) detected by qRT-PCR. ***(B)*** Proliferation of transfected HGC-27 cells evaluated by MTT assay. ***(C-D)*** migration and invasion abilities were determined in HGC-27 cells transfected with shR-PCAT1 or negative control (NC). $^{*}p < 0.05$, $^{**}p < 0.01$.

0.001), TNM stages ($p < 0.001$) and lymphatic metastasis ($p = 0.003$). However, there was no significant correlation of DANCR expression with other clinical features. These results indicated that PCAT-1 expression may play an oncogenic role in GC progression.

Effects of PCAT-1 on Cell Proliferation, invasion and Migration in GC Cells

In order to investigate the potential effect of PCAT-1 on the progression of GC, we transfected GC cell line HGC-27 cells with either shR-PCAT1 or PCAT-1-pcDNA3.1 + vector. PCAT-1 expression in HGC-27 cells was determined using qRT-PCR (Figure 2 A, Figure 3 A). Next, we explored the effect of lncRNA PCAT-1 on GC cell proliferation invasion and migration in GC cells. Our results showed up-regulation of PCAT-1 efficiently promoted cell proliferation of GC ($p < 0.05$; Figure 2 B), the cell migration ($p < 0.05$; Figure 2 C) and cell invasion ($p < 0.05$; Figure 2 D). In contrast, down-regulation of PCAT-1 efficiently inhibited cell proliferation of GC ($p < 0.05$; Figure 3 B), the cell migration ($p < 0.05$; Figure 3 C) and cell invasion ($p < 0.05$; Figure 3 D). The results indicated that PCAT-1 served as a tumor promoter in GC.

Prognostic Value of PCAT-1 in GC

Then, we evaluated the prognostic significance of PCAT-1expression in patients with GC (Figure 4). According to the Kaplan-Meier survival analysis, the patients with high PCAT-1 expression exhibited evidently poorer ove-

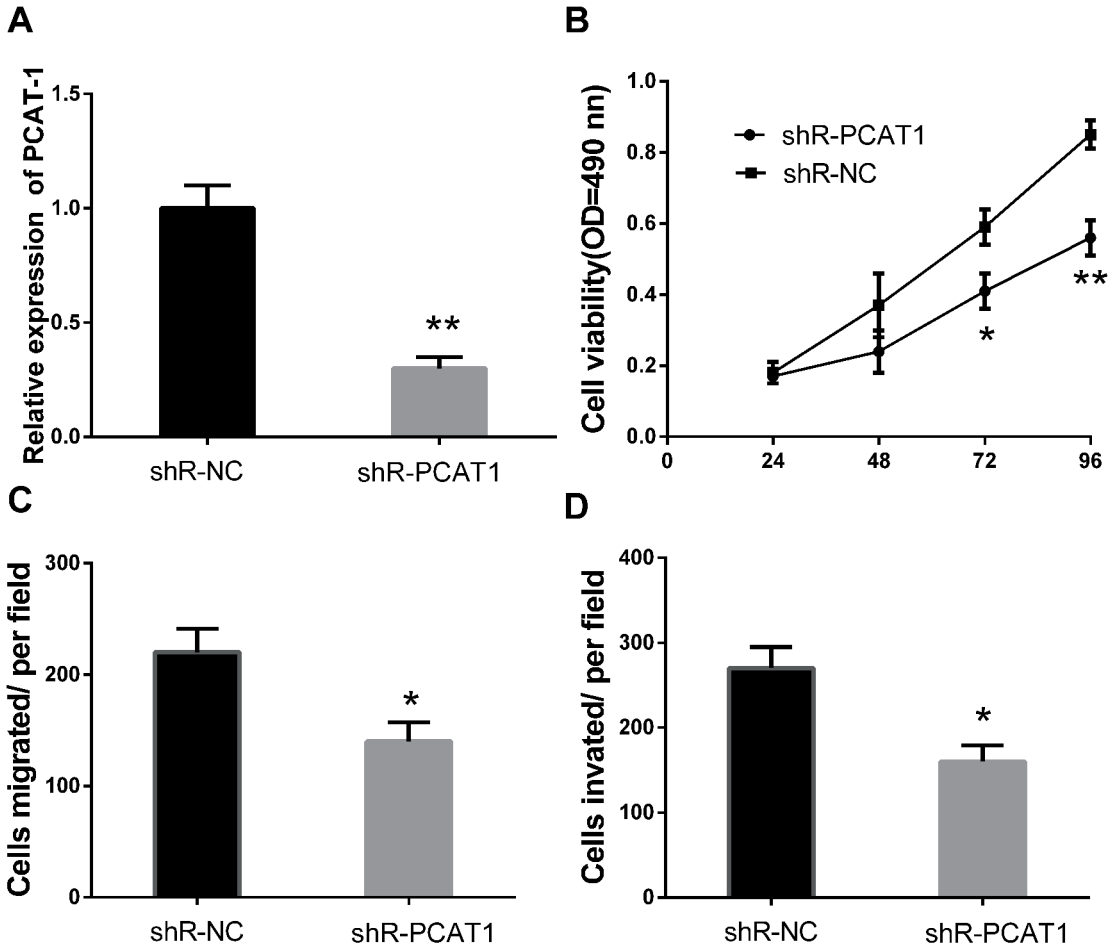

Figure 3. PCAT1 promotes the proliferation, migration and invasion of GC cells *in vitro*. ***(A)*** Relative PCAT1 expression in HGC-27 cells when transfected with PCAT-1-pcDNA3.1 + vector by qRT-PCR. ***(B)*** Proliferation of transfected HGC-27 cells evaluated by MTT assay. ***(C-D)*** migration and invasion abilities were determined in HGC-27 cells transfected with PCAT-1-pcDNA3.1 + vector. $^{*}p < 0.05$, $^{**}p < 0.01$.

Table II. Univariate analysis of OS in GC patients.

Prognostic variables	OS HR	95% CI	*p*-value
Age (< 50 years vs. > 50 years)	0.724	0.519-1.452	0.478
Gender (male vs. female)	0.791	0.438-1.165	0.559
Location (Distal vs. Middle + Proximal)	0.823	0.533-1.769	0.515
Tumor size(> 5 cm vs. < 5 cm)	0.845	0.414-1.773	0.793
Histologic differentiation (Well + Moderately vs. Poorly + Undifferentiated)	1.773	0.724-2.831	0.216
Invasion depth (T3+T4 vs. T1+T2)	1.817	1.034-3.775	0.009
TNM stage (III + IV vs. I+II)	1.563	1.466-5.651	< 0.001
Lymphatic metastasis (No vs. Yes)	0.583	0.447-1.467	0.093
Distant metastasis (No vs. Yes)	0.569	0.235-1.343	0.028
Expression of PCAT-1 (High vs. Low)	1.883	1.043-2.784	0.001

rall survival rates than those with low PCAT-1 expression ($p < 0.001$). In univariate analysis, high expression of PCAT-1 was evaluated to correlate closely with poor OS ($p = 0.001$, Table II). Multivariate analysis confirmed that PCAT-1 expression level was an independent

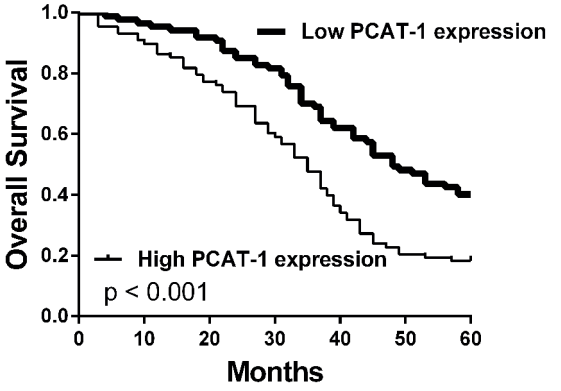

Figure 4. Kaplan-Meier curves for survival time in patients with cervical cancers divided according to PCAT1 expression: significantly shorter survival times for patients with high PCAT1 expression than for those with high PCAT1 expression ($^{*}p < 0.05$).

prognostic factor for overall survival (p = 0.005; Table III).

Discussion

Although the incidence of GC has rapidly declined over the past few decades, GC remains a leading cause of cancer death[12,13]. The overall 5-year survival rate of patients with GC and even resectable disease has a 50-90% risk of recurrence and death[14,15]. Therefore, it is important to explore the molecular events in GC, which would provide insights for improved diagnosis and prognosis of this deadly disease, thus improving clinical strategies and outcomes. As a new identified lncRNA, PCAT-1 is located in the chromosome 8q24 gene desert and contributes to cell proliferation in prostate cancer[16]. Recently, some studies reported that PCAT-1 plays an important role in progression of tumor. Zhao et al[17] found that over-expression of PCAT-1 could promote proliferation and metastasis in non-small cell lung cancer cells. Prensner et al[18] showed that PCAT-1 promoted prostate cancer cell proliferation through association with polycomb repressive complex 2 (PRC2) and cMyc protein. Liu et al[19] reported that PCAT-1 expression was down-regulated in human bladder cancer and silencing PCAT-1 inhibited bladder cancer cell growth and induced apoptosis. Furthermore, PCAT-1 was reported to serve as a novel biomarker of poor prognosis in hepatocellular carcinoma, and esophageal squamous carcinoma[20,21]. All those results informed that PCAT-1 function as a tumor promoter in different tumor. In the present study, we found that PCAT-1 is up-regulated in GC cell lines and tissues. Furthermore, PCAT-1 overexpression promotes cell proliferation, migration, and invasion in human GC cell lines. We also found that the relative expression level of PCAT-1 was associated with invasion depth, TNM stages and lymphatic metastasis. Moreover, Kaplan-Meier analysis showed that GC patients with high PCAT-1 expression tend to have shorter overall survival. Finally, in the multivariate analysis, high lncRNA PCAT-1 expression was an independent prognostic factor for OS. All those findings indicated the underlying clinical significance of PCAT-1 overexpression as a biomarker for predicting GC prognosis.

Conclusions

The present study, for the first time, suggested that PCAT-1 was increased in GC tissues and associated with advanced tumor progression. Our research has provided a better understanding of the PCAT-1 function in GC. PCAT-1 may be used as a new target for prognosis and treatment of GC.

Conflict of interest

The authors declare no conflicts of interest.

Table III. Multivariate analysis of OS in GC patients.

	OS		
Prognostic variables	**HR**	**95% CI**	***p*-value**
Invasion depth (T3+T4 vs. T1+T2)	0.724	0.581-2.993	0.451
TNM stage (III + IV vs. I+II)	2.893	1.231-5.231	0.004
Distant metastasis (No vs. Yes)	0.563	0.135-1.342	0.094
Expression of PCAT-1 (High vs. Low)	2.559	1.127-5.883	0.005

References

1) Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30.
2) Berretta S, Berretta M, Fiorica F, Di Francia R, Magistri P, Bertola G, Fisichella R, Canzonieri V, Tarantino G, Di Benedetto F. Multimodal approach of advanced gastric cancer: based therapeutic algorithm. Eur Rev Med Pharmacol Sci 2016; 20: 4018-4031.
3) GASTRIC (Global Advanced/Adjuvant Stomach Tumor Research International Collaboration) Group, Paoletti X, Oba K, Burzykowski T, Michiels S, Ohashi Y, Pignon JP, Rougier P, Sakamoto J, Sargent D, Sasako M, Van Cutsem E, Buyse M. Benefit of adjuvant chemotherapy for resectable gastric cancer: a meta-analysis. JAMA 2010; 303: 1729-1737.
4) Jiang J, Zhao JH, Wang XL, DI JI, Liu ZB, Li GY, Wang MZ, Li Y, Chen R, Ge RL. Analysis of mitochondrial DNA in Tibetan gastric cancer patients at high altitude. Mol Clin Oncol 2015; 3: 875-879.
5) Li CH, Chen Y. Targeting long non-coding RNAs in cancers: progress and prospects. Int J Biochem Cell Biol 2013; 45: 1895-1910.
6) Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155-159.
7) Yang L, Bai HS, Deng Y, Fan L. High MALAT1 expression predicts a poor prognosis of cervical cancer and promotes cancer cell growth and invasion. Eur Rev Med Pharmacol Sci 2015; 19: 3187-3193.
8) Song H, Han LM, Gao Q, Sun Y. Long non-coding RNA CRNDE promotes tumor growth in medulloblastoma. Eur Rev Med Pharmacol Sci 2016; 20: 2588-2597.
9) Wu Y, Zhang L, Zhang L, Wang Y, Li H, Ren X, Wei F, Yu W, Liu T, Wang X, Zhou X, Yu J, Hao X. Long non-coding RNA HOTAIR promotes tumor cell invasion and metastasis by recruiting EZH2 and repressing E-cadherin in oral squamous cell carcinoma. Int J Oncol 2015; 46: 2586-2594.
10) Yap KL, Li S, Muñoz-Cabello AM, Raguz S, Zeng L, Mujtaba S, Gil J, Walsh MJ, Zhou MM. Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell 2010; 38: 662-674.
11) Hu Y, Pan J, Wang Y, Li L, Huang Y. Long noncoding RNA linc-UBC1 is negative prognostic factor and exhibits tumor pro-oncogenic activity in gastric cancer. Int J Clin Exp Pathol 2015; 8: 594-600.
12) Shao Y, Ye M, Jiang X, Sun W, Ding X, Liu Z, Ye G, Zhang X, Xiao B, Guo J. Gastric juice long noncoding RNA used as a tumor marker for screening gastric cancer. Cancer 2014; 120: 3320-3328.
13) Ohtsu A. Chemotherapy for metastatic gastric cancer: past, present, and future. J Gastroenterol 2008; 43: 256-264.
14) He H, Chen W, Wang X, Wang C, Liu F, Shen Z, Xu J, Gu J, Sun Y. Snail is an independent prognostic predictor for progression and patient survival of gastric cancer. Cancer Sci 2012; 103: 1296-1303.
15) Wang CW, Fang XH. The role of targeted agents in the treatment of advanced gastric cancer: a meta-analysis of randomized controlled trials. Eur Rev Med Pharmacol Sci 2016; 20: 1725-1732.
16) Prensner JR, Iyer MK, Balbin OA, Dhanasekaran SM, Cao Q, Brenner JC, Laxman B, Asangani IA, Grasso CS, Kominsky HD, Cao X, Jing X, Wang X, Siddiqui J, Wei JT, Robinson D, Iyer HK, Palanisamy N, Maher CA, Chinnaiyan AM. Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. Nat Biotechnol 2011; 29: 742-749.
17) Zhao B, Hou X, Zhan H. Long non-coding RNA PCAT-1 over-expression promotes proliferation and metastasis in non-small cell lung cancer cells. Int J Clin Exp Med 2015; 8: 18482-18487.
18) Prensner JR, Chen W, Han S, Iyer MK, Cao Q, Kothari V, Evans JR, Knudsen KE, Paulsen MT, Ljungman M, Lawrence TS, Chinnaiyan AM6, Feng FY7. The long non-coding RNA PCAT-1 promotes prostate cancer cell proliferation through cMyc. Neoplasia 2014; 16: 900-908.
19) Liu L, Liu Y, Zhuang C, Xu W, Fu X, Lv Z, Wu H, Mou L, Zhao G, Cai Z, Huang W. Inducing cell growth arrest and apoptosis by silencing long non-coding RNA PCAT-1 in human bladder cancer. Tumour Biol 2015; 36: 7685-7689.
20) Yan TH, Yang H, Jiang JH, Lu SW, Peng CX, Que HX, Lu WL, Mao JF. Prognostic significance of long non-coding RNA PCAT-1 expression in human hepatocellular carcinoma. Int J Clin Exp Pathol 2015; 8: 4126-4131.
21) Shi WH, Wu QQ, Li SQ, Yang TX, Liu ZH, Tong YS, Tuo L, Wang S, Cao XF. Up-regulation of the long noncoding RNA PCAT-1 correlates with advanced clinical stage and poor prognosis in esophageal squamous carcinoma. Tumour Biol 2015; 36: 2501-2507.