

ORIGINAL ARTICLE

Long non-coding RNA PVT1 functions as an oncogene in human colon cancer through miR-30d-5p/RUNX2 axis

Xiuguo Yu¹, Jianpei Zhao², Yaguang He³

¹Department of General Surgery, Ningbo Ninth Hospital, Ningbo, China; ²Department of Anorectal Section, Ningbo Second Hospital, Ningbo, China; ³Department of Anorectal Section, Ningbo Ninth Hospital, Ningbo, China

Summary

Purpose: Recently, long noncoding RNAs (lncRNAs) have caught more attention for their role in tumor progression. Colon cancer is one of these ordinary malignant tumors. This study aimed to identify how lnc RNA PVT1 affects the progression of colon cancer.

Methods: PVT1 expression of both colon cancer cell tissue and 60 paired cancer and peri-tumoral tissue samples was detected by real-time quantitative polymerase chain reaction (RT-qPCR). The associations between lnc RNA PVT1 expression level and clinicopathological characteristics and patients' disease-free survival rate were evaluated. Furthermore, function assays containing cell proliferation assay, colony formation and transwell assay were conducted. Mechanism-associated experiments included western blot assay, luciferase assay and RNA immunoprecipitation assay.

Results: PVT1 expression was significantly higher in tumor tissues than in peritumoral tissues, and was associated

with lymph node metastasis, tumor stage and survival time of these patients. Moreover, knockdown of PVT1 promoted tumor growth and invasion in vitro. In addition, further experiments revealed that miR-30d-5p was a direct target of PVT1 and its expression in tumor tissues negatively correlated to PVT1 expression. Moreover, RUNX2 was identified as the direct target spot of miR-30d-5p according to the mechanism experiments. Besides, RUNX2 expression was positively correlated with PVT1 in cancer tissues and cells.

Conclusions: These results indicate that PVT1 could promote metastasis and proliferation of colon cancer via suppressing miR-30d-5p/RUNX2 axis, which may offer a new way for interpreting the mechanism of colon cancer development.

Key words: colon cancer, miR-30d-5p, PVT1, RUNX2

Introduction

Colon cancer ranked third common cancer among human malignant tumors in American Cancer Statistics in 2015 [1]. The incidence rate of colon cancer remains high both in males and in females worldwide [2]. In the last decades, although molecular targeted therapies are available for laparoscopy assisted colectomy (LAC) patients, only a small proportion of patients benefits from discovered driver mutations. Therefore, further exploration of the mechanisms of these genomic changes in colon cancer is urgently required.

Latest studies discovered that lnc RNA PVT1 is upregulated in many cancers and participates in tumor progression. For example, PVT1 promotes cell invasion and correlates with patients' prognosis in small cell lung cancer [3]. PVT1 induces epithelial-mesenchymal transition (EMT) and further promotes invasion of esophageal cancer [4]. Moreover, serum PVT1 can be used as a biomarker for diagnosis of cervical cancer [5]. A recent study revealed the association between the expression level of PVT1 and poor prognosis of colorectal

cancer patients [6]. However, it remains unknown how PVT1 exerts its function in colon cancer.

Recently, a large number of studies have revealed that the interaction between lncRNAs are identified in many kinds of cancers and probably associated with many known oncogenes. PVT1 can be a sponge and inhibits miR-186, which further promotes cell growth and invasion in gastric cancer [7]. Moreover, by sponging miR-203, PVT1 can promote tumor progression in esophageal squamous cell carcinoma [8]. Yang et al. performed bioinformatic analysis to demonstrate whether PVT1 acted as an endogenous miRNA sponge in colon cancer [9]. We discovered that PVT1 contained several binding sites fitting to miRNAs MiR-30d-5p, as one of these miRNAs is reported to inhibit tumor development in many tumors including colon cancer [9-12].

This study revealed that lncRNA PVT1 was upregulated in colon cancer tissues and promoted the proliferation and invasion *in vitro*. In addition, we further discovered the interaction between lncRNA PVT1 and miR-30d-5p as well as the possible mechanism.

Methods

Clinical samples and cell lines

Human tissues came from 60 colon cancer patients who were operated at the Ningbo Ninth Hospital. No patient received radiotherapy or chemotherapy preoperatively. All colon cancer tissues were stored at -80°C . An experienced pathologist assessed and confirmed the diagnosis of colon cancer. Written informed consent was provided by all of the patients and the study conformed to the requirements of the Ethics Committee of Ningbo Ninth Hospital and Helsinki Declaration.

The American Type Culture Collection (ATCC) provided us HCT116, HT29, SW620, SW480 and Caco2 colon cancer cell lines, normal human colonic epithelial cells (NCM460) and 293T embryonic kidney cell line. Culture medium was consisted of penicillin, 10% fetal bovine serum (FBS; Invitrogen Life Technologies; Carlsbad, CA, USA) and RPMI-1640 medium (Thermo Fisher Scientific, USA). Besides, cells were cultured in humidified incubator, which contained 5% CO_2 and was set at 37°C .

Cell transfection

Lentiviral small hairpin RNA (shRNA) targeting PVT1 was synthesized and then cloned into the pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc, San Diego, CA, USA). Then 293T cells were used for the packaging the viruses, the PVT1 lentiviruses (sh-PVT1) and the empty vector (sh-ctrl). The miRNA mimics and inhibitor provided by Genepharma Co., Ltd. (Shanghai, China) were used for transfection of colon cancer cells. Negative control was transfected non-specific siRNA.

RNA extraction and qRT-PCR

Total RNA was separated using TRIzol reagent (Invitrogen, CA, USA) and was reverse-transcribed to cDNAs via reverse Transcription Kit (Takara Biotechnology Co., Ltd., Dalian, China). SYBR Green real-time PCR was applied. Available primers were as follows: PVT1, forwards 5'-GCCCCTTCTATGGAATCACTA-3' and reverse 5'-GGGGCAGAGATGAAATCGTAAT-3'; miR-30d-5p, forward 5'-UGUAAACAUCGCCGACUGGAAG-3' and reverse 5'-TGTAACATCCCCGACTGGAAGA-3'; U6, forward 5'-CTCGCTTCGGCAGCACATATACT-3' and reverse 5'-ACGCTTCACGAATTTGCGTGTC-3'; β -actin, forward 5'-TTGTTACAGGAAGTCCCTTGCC-3' and reverse 5'-ATGCTATCACCTCCCCTGTGTG-3'. The performance of RT-qPCR was conducted on ABI 7500 system (Applied Biosystems, MA, USA). Thermal cycle was as follows: 95°C for 30 sec, 95°C for 5 sec for 40 cycles, and 60°C for 35 sec.

Western blot analysis

Reagent RIPA (Beyotime, Shanghai, China) was utilized to extract protein from cells. BCA protein assay kit (Takara, Dalian, China) was chosen for quantifying protein concentrations. The target proteins were separated by SDS-PAGE. Then they were replaced to polyvinylidene fluoride (PVDF) membrane, which was then incubated with antibodies. Cell Signaling Technology (CST, MA, USA) provided us rabbit anti-RUNX2 and rabbit anti- β -actin, as well as goat anti-rabbit secondary antibody. Chemiluminescent film was applied for assessment of protein expression with Image J software.

Luciferase assays and RNA immunoprecipitation assay (RIP)

In this study, pGL3 vector (Promega, WI, USA) was used for colony of 3'-UTR of RUNX2 or PVT1, wild-type (WT) 3'-UTR. Quick-change site-directed mutagenesis kit (Stratagene, Cedar Creek, CA, USA) for site-directed mutagenesis of miR-30d-5p binding site in RUNX2 or PVT1 3'-UTR, mutant (MUT) 3'-UTR. WT-3'-UTR or MUT-3'-UTR and miR-ctrl or miR-30d-5p mimics was used for cell transfection. 48 hrs later, the luciferase assays were performed on the dual Luciferase reporter assay system (Promega, WI, USA).

Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, NJ, USA) was used for RIP assay. Co-precipitated RNAs were detected by qRT-PCR.

Cell counting Kit-8 (CCK8) assay

Cell viability of the treated cells in 96-well plates was monitored every 24 hrs by CCK8 assay following the protocol (Dojindo Molecular Technologies, Inc. Kumamoto, Japan). The absorbance was then measured at 450 nm on the spectrophotometer (Thermo Scientific, Rockford, IL, USA).

Colony formation assay

Almost 200 cells were cultured in a 6-well plate with FBS for 14 days. Then cells were stained with 0.1% crystal violet after fixed with methanol. For the comparison, the number of colonies were counted.

Matrigel assay

5×10^4 cells in 200 μ L serum-free RPMI-1640 were transformed to the top chamber of an insert (8 μ m pore size; Millipore, NJ, USA) coated with 50 μ g Matrigel (BD Biosciences, CA, USA). And the bottom chamber was added RPMI-1640 and FBS. Forty-eight hrs later, the top surface of chambers was wiped by cotton swab and immersed for 10 min with precooled methanol. Then, the chambers were stained with crystal violet for 30 min. The data for invasion was counted from 3 fields per membrane.

Statistics

Statistical analysis was performed with SPSS 17.0 (SPSS Inc, Chicago, IL, USA). Chi-square test, Student's t-test and Kaplan-Meier method were used when appropriate. Results were presented as mean \pm SD and a value of $p < 0.05$ was considered statistically significant.

Results

PVT1 level in tissues and cells of colon cancer

First, RT-qPCR was conducted for detecting PVT1 expression in 60 patients' tissues and 5 colon cancer cells. As the result, PVT1 was significantly upregulated in tumor tissue samples (Figure 1A). PVT1 level was higher in colon cancer cells than that in normal colonic epithelial cells (Figure 1B). Analysis of clinicopathological features in those patients demonstrated that upregulated PVT1 obviously correlated to lymph node metastasis and tumor stage (Table 1). Kaplan-Meier analysis revealed that patients with low PVT1 level had a better disease-free survival (Figure 1C).

PVT1 knockdown inhibits cell proliferation and invasion in vitro

According to PVT1 expression in cancer cells, we chose HCT116 and SW480 colon cells for knockdown of PVT1. The PVT1 lentiviruses (sh-

PVT1) and the empty vector (sh-ctrl) were synthesized and transduced into these cells. Then the PVT1 expression was determined by qRT PCR (Figure 2A). Furthermore, results of CCK8 assay showed that cell proliferation of colon cancer cells was inhibited after PVT1 was silenced (Figure 2B and 2C). Meanwhile, colony formation assay also showed that the numbers of colonies were less in sh-PVT1 cells than in sh-ctrl cells (Figure 2D). Then we performed transwell assay and found that silenced PVT1 suppressed colon cancer cell invasion (Figure 2E).

Table 1. Relationship between lncRNA PVT1 expression and clinicopathological characteristics in colon cancer patients

Characteristics	Expression of lncRNA PVT1		p value
	High expression	Low expression	
Age (years)			0.835
≤ 60	15	13	
> 60	18	14	
Sex			0.436
Male	15	15	
Female	18	12	
TNM stages			0.040
I-II	12	17	
III-IV	21	10	
Lymphatic metastasis			0.006
No	9	17	
Yes	24	10	
Histological differentiation			0.397
Well	15	8	
Moderate	11	10	
Poor	7	9	

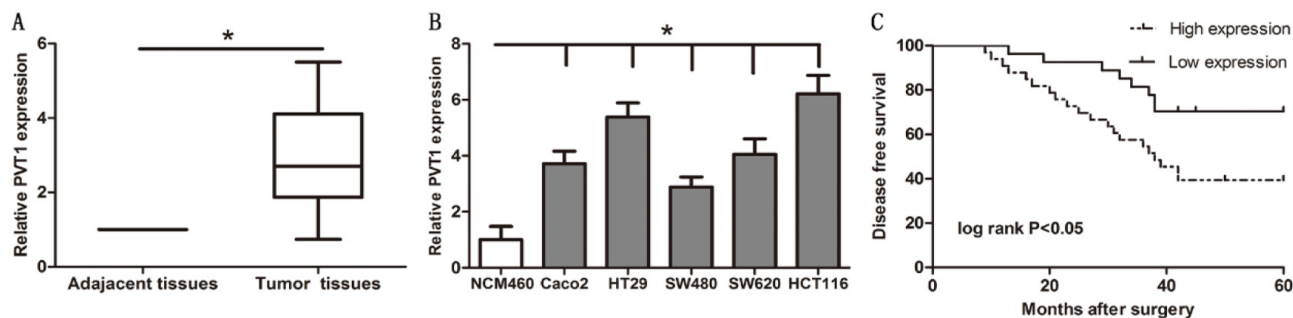


Figure 1. Expression levels of PVT1 increased in colon cancer tissues and cell lines, and were associated with short disease-free survival of colon cancer patients. (A) PVT1 expression was significantly increased in colon cancer tissues compared with adjacent tissues. (B) Expression levels of PVT1 relative to GAPDH were determined in the human colon cancer cell lines and normal colonic epithelial cells (NCM460) by RT-qPCR. (C) High level of PVT1 was associated with short disease-free survival of patients with colon cancer. Data are presented as the mean \pm standard error of the mean. * $p < 0.05$.

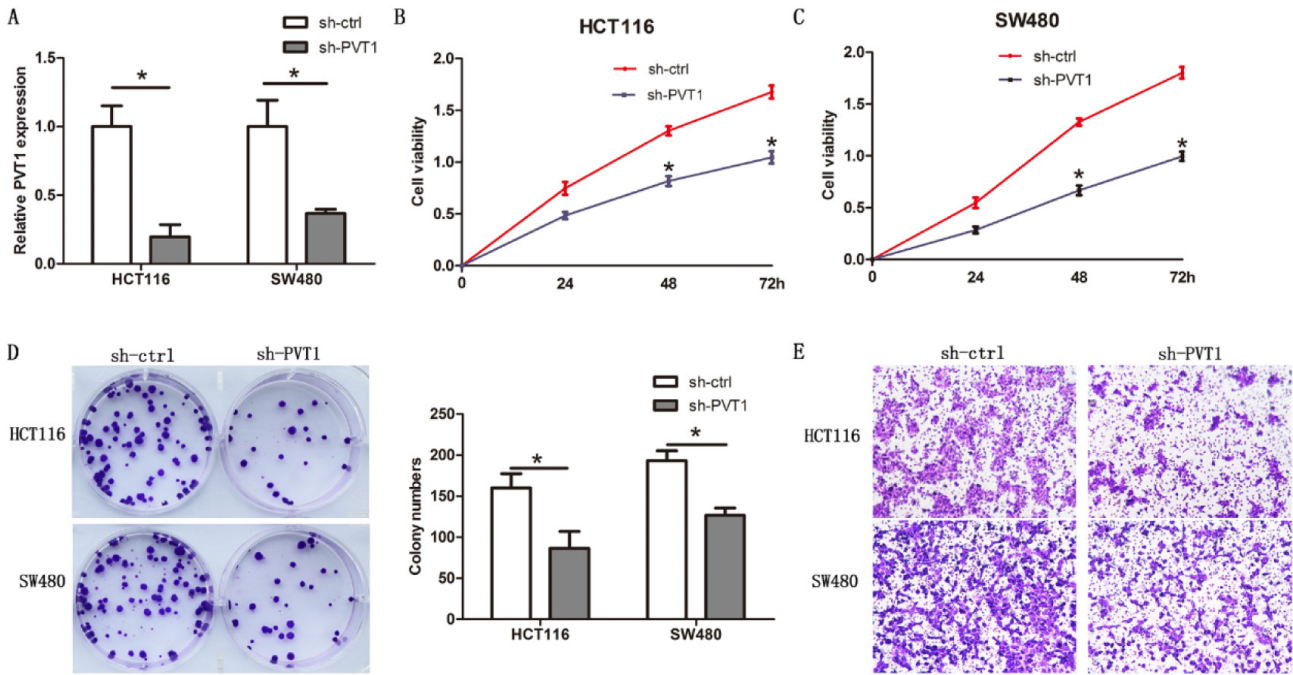


Figure 2. Knockdown of PVT1 decreased colon cancer cell proliferation and invasion. **(A)** PVT1 expression in colon cancer cells transfected with control shRNA vector (sh-ctrl) or PVT1 shRNA (sh-PVT1) was detected by RT-qPCR. β -actin was used as an internal control. **(B)** CCK8 assay showed that knockdown of PVT1 significantly decreased cell proliferation in HCT116 colon cells. **(C)** CCK8 assay showed that knockdown of PVT1 significantly decreased cell proliferation in SW480 colon cells. **(D)** Colony formation assay demonstrated that oncogenic survival of colon cells in sh-PVT1 group was significantly decreased compared with sh-ctrl group. **(E)** Transwell assay showed that the number of invaded cells in sh-PVT1 group was obviously reduced compared with sh-ctrl group. The results represent the average of three independent experiments (mean \pm standard error of the mean). * $p < 0.05$, as compared with the control cells.

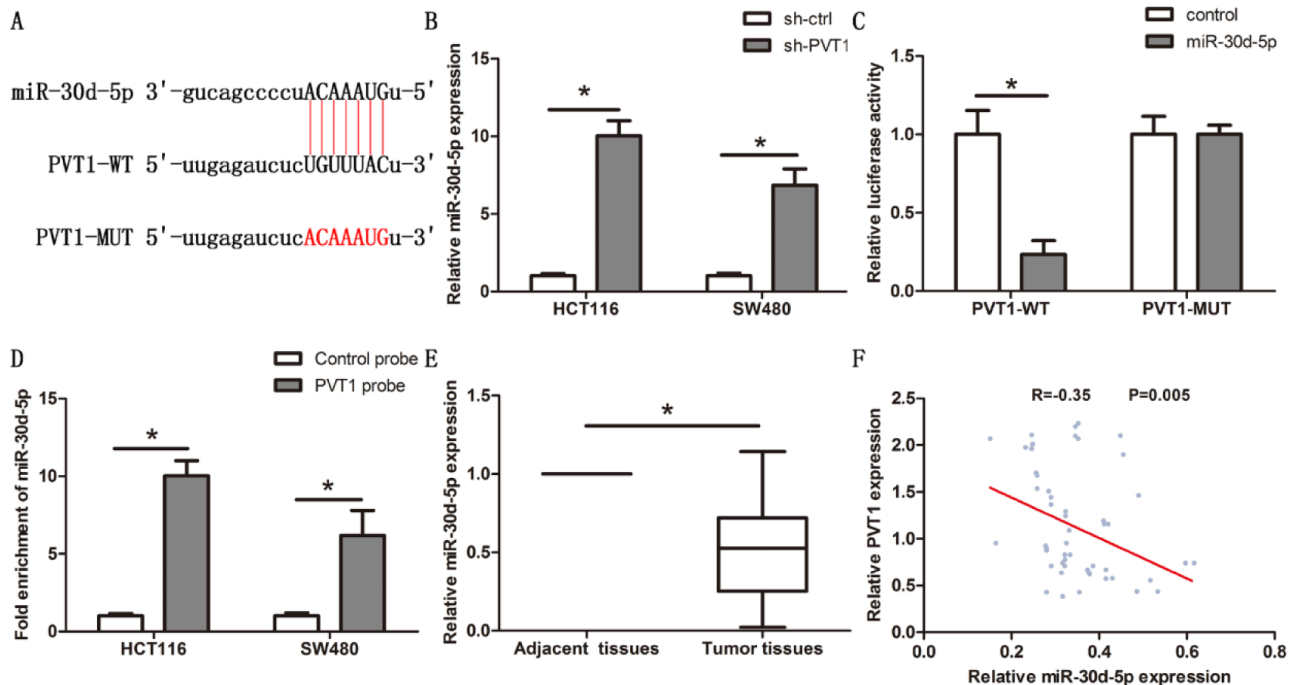


Figure 3. Interaction between PVT1 and miR-30d-5p. **(A)** The binding sites of miR-30d-5p on PVT1. **(B)** MiR-30d-5p expression was increased in sh-PVT1 group compared with sh-ctrl group. **(C)** Co-transfection of miR-30d-5p and PVT1-WT in HCT116 cells strongly decreased the luciferase activity, while co-transfection of miR-30d-5p and PVT1-MUT did not change the luciferase activity either. **(D)** MiR-30d-5p was significantly enriched by RNA immunoprecipitation (RIP) assay in the PVT1 group compared with control. **(E)** MiR-30d-5p was significantly downregulated in colon cancer tissues compared with adjacent tissues. **(F)** The linear correlation between the expression level of miR-30d-5p and PVT1 in colon cancer tissues. The results represent the average of three independent experiments. Data are presented as the mean \pm standard error of the mean. * $p < 0.05$.

The interaction between miR-30d-5p and PVT1 in colon cancer

DIANA LncBASE Predicted v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lnbasev2%2Findex-predicted) was used to find the miRNAs that contained complementary base with PVT1. PVT1 was predicted to harbor miR-30d-5p binding sites (Figure 3A). Besides, RT-PCR results showed that miR-30d-5p level of colon cancer cells was higher in sh-PVT1 group compared with that in sh-ctrl group (Figure 3B). Furthermore, the luciferase assay revealed that the luciferase activity of PVT1-WT cells with miR-30d-5p mimics was reduced, while luciferase activity of PVT1-MUT cells with miR-30d-5p was not obviously changed (Figure 3C). Meanwhile, RIP assay results demonstrated that miR-30d-5p could be remarkably enriched in the PVT1 group compared with the control group, suggesting that PVT1 might work as a miR-30d-5p sponge (Figure 3D). We further detected miR-30d-5p expression in colon cancer tissues, and found it was obviously lower in tumor tissues than in adjacent tis-

sues (Figure 3E). Correlation analysis demonstrated that miR-30d-5p expression level negatively correlated to PVT1 expression in colon tissues (Figure 3F).

PVT1 promotes colon cancer tumorigenesis via miR-30d-5p/RUNX2 axis

MicroRNAs participate in biological processes through their downstream targets. RUNX2, as a downstream target of miR-30d-5p, contributes to tumor development. The luciferase assay revealed that co-transfection of RUNX2-WT and miR-30d-5p reduced luciferase activity, which could be partly restored by PVT1-WT (Figure 4A). RT-PCR results further demonstrated that the RUNX2 expression was downregulated in colon cancer cells transfected with miR-30d-5p mimics (Figure 4B). Western blot assay showed that RUNX2 could be suppressed at protein level by miR-30d-5p mimics (Figure 4C and 4D). To explore the interaction between PVT1 and RUNX2, the expression level of RUNX2 was detected in cells and tissues. As the result, RUNX2 expression of sh-PVT1 cells was

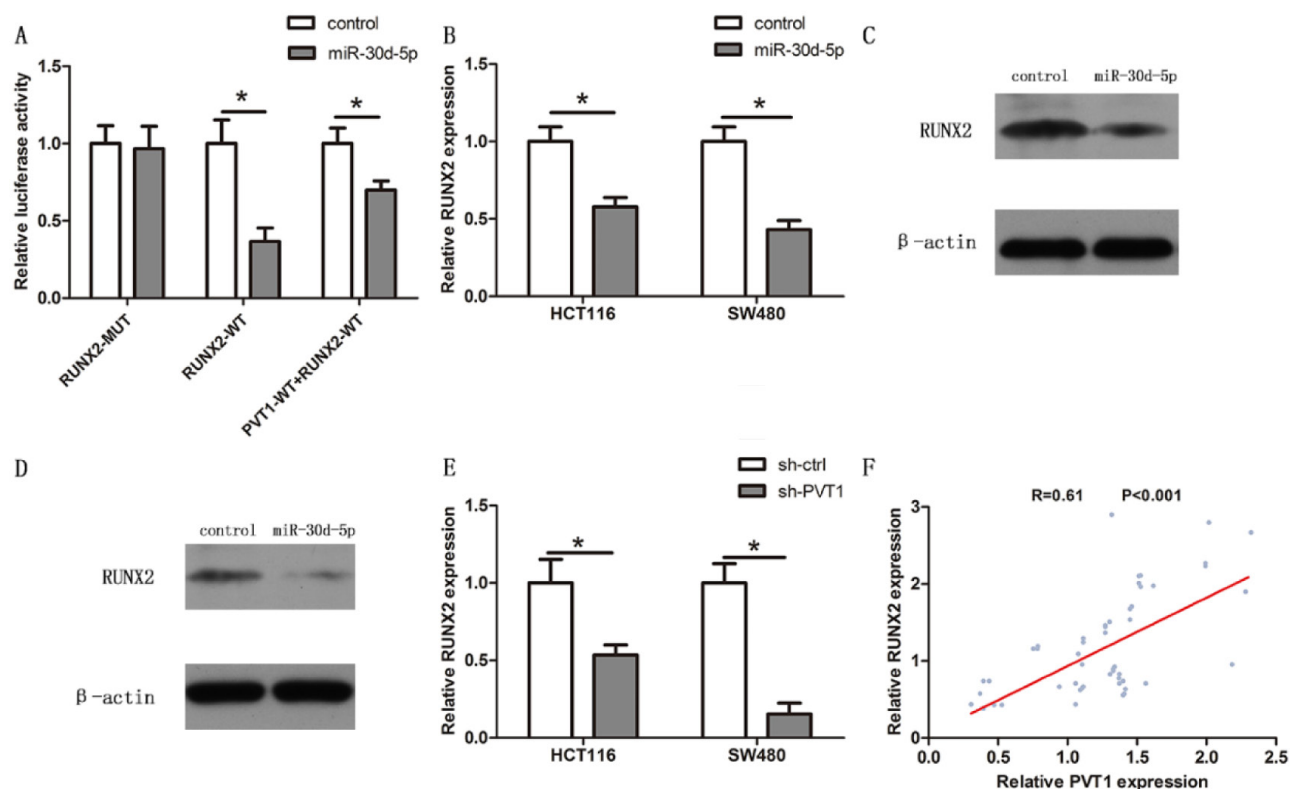


Figure 4. Mir-30d-5p/RUNX2 axis mediated the effect of PVT1 on colon cancer cells. **(A)** The luciferase reporter plasmids containing the wild type 3'UTR region or mutant 3'UTR region of RUNX2 were co-transfected into HCT116 cells with miR-30d-5p or in parallel with the luciferase reporter vector PVT1-WT. **(B)** RUNX2 expression of colon cells was decreased in cells transfected with miR-30d-5p mimics compared with control cells. **(C)** MiR-30d-5p mimics repressed RUNX2 protein expression in HCT116 cells. **(D)** MiR-30d-5p mimics repressed RUNX2 protein expression in SW480 cells. **(E)** The expression level of RUNX2 in sh-PVT1 cells was significantly decreased compared with sh-ctrl cells. **(F)** The linear correlation between the expression level of RUNX2 and PVT1 in colon cancer tissues. The results represent the average of three independent experiments. Data are presented as the mean \pm standard error of the mean. * p <0.05.

lower than that of sh-ctrl cells (Figure 4E). The linear correlation analysis revealed that the RUNX2 expression positively correlated to PVT1 expression in colon cancer tissues (Figure 4F).

Discussion

Evidence has proved that lncRNAs participate in tumorigenesis and growth development. In the present study, PVT1 was found upregulated in tissue samples and colon cancer cells and significant correlation was seen between tumor stage, lymph node metastasis and patients' prognosis. Furthermore, cell growth and invading ability was inhibited in colon cancer cells after PVT1 was knocked down. These data indicate that PVT1 serves as an oncogene and promotes the tumorigenesis of colon cancer.

Recent studies reveal that lncRNAs function in tumor progression by binding to miRNAs. For example, lncRNA XIST regulates its target miR-34a-5p and plays an oncogenic role in nasopharyngeal carcinoma [13]. LncRNA TUSC7, as a miR-211 sponge, was reported to suppress the cell growth of colorectal cancer [14]. The interaction between lncRNA MALAT1 and miR-205 was discovered in the progression of aggressive renal cell carcinoma [15]. MiR-18a sponged by lncRNA CASC2 inhibits the proliferation and metastasis in colorectal cancer [16]. Our study revealed that miR-30d-5p could directly bind to PVT1 through the luciferase assay. And miR-30d-5p was significantly enriched by PVT1 RIP assay. In addition, miR-30d-5p expression could be upregulated with knockdown of PVT1. In colon cancer tissues, the

miR-30d-5p expression was negatively correlated with PVT1 expression. All these results suggest that PVT1 might promote tumorigenesis of colon cancer via sponging miR-30d-5p.

RUNX2, a novel oncogene discovered recently, is reported to function in tumor proliferation and metastasis and can be regulated by several noncoding RNAs [17-20]. In our study, luciferase assay demonstrated that RUNX2 was directly targeted by miR-30d-5p. What's more, RUNX2 was downregulated through miR-30d-5p mimics in colon cancer cells. Furthermore, RUNX2 is a crucial regulator of cell growth, metastasis and apoptosis through a few important signal transduction pathways. In addition, PVT1 knockdown suppressed RUNX2 expression, and positive correlation between RUNX2 and PVT1 expression was discovered in tumor tissues. The results above suggest that PVT1 may realize its function by means of the miR-30d-5p/RUNX2 axis.

Conclusions

The data presented herein identified that PVT1 was overexpressed in colon cancer tissue samples and was related with worse disease-free survival of colon cancer patients. Besides, PVT1 could promote cell proliferation through miR-30d-5p/RUNX2 axis. These findings suggest that lncRNA PVT1 may contribute to therapy of colon cancer as a candidate target.

Conflict of interests

The authors declare no conflict of interests.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin* 2015;65:5-29.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015;65:87-108.
3. Huang C, Liu S, Wang H, Zhang Z, Yang Q, Gao F. LncRNA PVT1 overexpression is a poor prognostic biomarker and regulates migration and invasion in small cell lung cancer. *Am J Transl Res* 2016;8:5025-34.
4. Zheng X, Hu H, Li S. High expression of lncRNA PVT1 promotes invasion by inducing epithelial-to-mesenchymal transition in esophageal cancer. *Oncol Lett* 2016;12:2357-62.
5. Yang JP, Yang XJ, Xiao L, Wang Y. Long noncoding RNA PVT1 as a novel serum biomarker for detection of cervical cancer. *Eur Rev Med Pharmacol Sci* 2016;20:3980-6.
6. Takahashi Y, Sawada G, Kurashige J et al. Amplification of PVT-1 is involved in poor prognosis via apoptosis inhibition in colorectal cancers. *Br J Cancer* 2014;110:164-71.
7. Huang T, Liu HW, Chen JQ et al. The long noncoding RNA PVT1 functions as a competing endogenous RNA by sponging miR-186 in gastric cancer. *Biomed Pharmacother* 2017;88:302-8.
8. Li PD, Hu JL, Ma C et al. Upregulation of the long non-coding RNA PVT1 promotes esophageal squamous cell carcinoma progression by acting as a molecular sponge of miR-203 and LASP1. *Oncotarget* 2017;8:34164-76.

9. Yang JP, Yang XJ, Xiao L, Wang Y. Long noncoding RNA PVT1 as a novel serum biomarker for detection of cervical cancer. *Eur Rev Med Pharmacol Sci* 2016;20:3980-6.
10. Wu C, Jin B, Chen L et al. MiR-30d induces apoptosis and is regulated by the Akt/FOXO pathway in renal cell carcinoma. *Cell Signal* 2013;25:1212-21.
11. Chen D, Guo W, Qiu Z et al. MicroRNA-30d-5p inhibits tumour cell proliferation and motility by directly targeting CCNE2 in non-small cell lung cancer. *Cancer Lett* 2015;362:208-17.
12. Kobayashi N, Uemura H, Nagahama K et al. Identification of miR-30d as a novel prognostic maker of prostate cancer. *Oncotarget* 2012;3:1455-71.
13. Song P, Ye LF, Zhang C, Peng T, Zhou XH. Long noncoding RNA XIST exerts oncogenic functions in human nasopharyngeal carcinoma by targeting miR-34a-5p. *Gene* 2016;592:8-14.
14. Xu J, Zhang R, Zhao J. The novel long noncoding RNA TUSC7 inhibits proliferation by sponging MiR-211 in colorectal cancer. *Cell Physiol Biochem* 2017;41:635-44.
15. Hirata H, Hinoda Y, Shahryari V et al. Long noncoding RNA MALAT1 promotes aggressive renal cell carcinoma through ezh2 and interacts with miR-205. *Cancer Res* 2015;75:1322-31.
16. Huang G, Wu X, Li S, Xu X, Zhu H, Chen X. The long noncoding RNA CASC2 functions as a competing endogenous RNA by sponging miR-18a in colorectal cancer. *Sci Rep* 2016;6:26524.
17. Gokulnath M, Partridge NC, Selvamurugan N. Runx2, a target gene for activating transcription factor-3 in human breast cancer cells. *Tumour Biol* 2015;36:1923-31.
18. Tandon M, Chen Z, Pratap J. Runx2 activates PI3K/Akt signaling via mTORC2 regulation in invasive breast cancer cells. *Breast Cancer Res* 2014;16:R16.
19. Wen C, Liu X, Ma H, Zhang W, Li H. MiR3383p suppresses tumor growth of ovarian epithelial carcinoma by targeting Runx2. *Int J Oncol* 2015;46:2277-85.
20. Qin L, Zhang Y, Lin J, Shentu Y, Xie X. MicroRNA-455 regulates migration and invasion of human hepatocellular carcinoma by targeting Runx2. *Oncol Rep* 2016;36:3325-32.