Upregulated plasmacytoma variant translocation 1 promotes cell proliferation, invasion and metastasis in colorectal cancer

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Abstract. Emerging evidence indicates that the long non-coding RNA (lncRNA) plasmacytoma variant translocation 1 (PVT1) is associated with tumourigenesis in various types of cancer. However, its specific effects on the proliferation, invasion and metastasis of colorectal cancer (CRC) are still poorly understood. The present study aimed to investigate PVT1 expression in CRC and explore its role in CRC pathogenesis. The reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) technique was used to assess PVT1 expression in CRC cell lines. Gene Expression Omnibus (GEO) database analysis and measurement of clinical samples was used to analyse the correlation between PVT1 expression, CRC metastasis and overall survival (OS). In addition, knockdown of PVT1 expression was performed using short interfering RNA (siRNA) and RT-qPCR, western blotting, CCK-8 assays, tumour cell clone-formation and Matrigel invasion assays were used to observe its biological functions in HCT116 cells. The present study demonstrated that the expression of PVT1 in CRC cell lines was higher than that in normal colon mucosal cell lines. Using GEO database analysis and the measurement of clinical samples, it was revealed that CRC patients with high PVT1 expression demonstrated poor OS. Multivariate analysis indicated that high PVT1 expression is an independent risk factor for patients with CRC. In addition, PVT1 knockdown suppressed the proliferation, invasion and metastasis of CRC cells in vitro, which were associated with decreasing vimentin, cyclin D1 and cyclin-dependent kinase 4 expression and enhanced E-cadherin expression. The results of the present study suggest that PVT1 may serve a critical role in CRC progression and metastasis and may serve as a potential prognostic biomarker for CRC.

Introduction

Colorectal cancer (CRC) has a substantial effect on human health; it is the third most prevalent type of cancer worldwide, causing an estimated 693,900 deaths in 2012 (1,2). Despite the development of new therapeutic approaches and treatments for CRC in recent decades, there has been little change in the overall mortality rate (3,4). CRC development involves a multistep process, which can be due to genetic or environmental factors, leading to mutations in a series of molecules associated with cancer cell proliferation, apoptosis, and differentiation (5,6). Owing to the rising popularity of molecular therapies, various studies have investigated the molecular pathogenesis of CRC by analysing the molecular abnormalities in CRC progression (7-9).

Long non-coding RNAs (lncRNAs) are a subset of RNAs first identified in eukaryotes. They have a transcript length of 200-100,000 nt and lack a complete functional open reading frame (ORF). Rarely, they may encode a short functional peptide and are located in the nucleus or cytoplasm (10,11). Recently, more focus has been placed on lncRNAs for their effect on biological cell behaviour, especially in tumour cells. Increasing number of studies have revealed that lncRNAs are abnormally expressed in many different cancers, such as gastric cancer (12), cervical cancer (13), non-small cell lung cancer (NSCLC) (14), and CRC (15). These abnormally expressed IncRNAs have been used as biomarkers for cancer therapies and diagnoses.

The lncRNA plasmacytoma variant translocation 1 (PVT1) is located on chr8q24.21 and is 1,716 nt in length. The gene region of PVT1 contains the myelocytomatosis (myc) oncogene; the MYC protein can result in the accumulation of PVT1 in primary human cancers (16). Emerging evidence indicates that PVT1 is associated with tumourigenesis in various cancers, including gastric cancer (17), NSCLC (18), and hepatocellular cancer (19); however, the specific effects of PVT1 on the proliferation, invasion, and metastasis of CRC are still poorly understood. In the present study, we first demonstrated that PVT1 is overexpressed in CRC tissues and cell lines. We then determined that CRC patients with high PVT1 expression showed poor overall survival (OS), by analysing Gene Expression Omnibus (GEO) datasets. PVT1 knockdown was also shown to suppress the proliferation, invasion, and metastasis of CRC cells in vitro. These results suggest that PVT1...
plays a significant role in CRC tumourigenesis and tumour progression.

Materials and methods

Bioinformatics analysis. All microarray expression dates, containing primary CRC data and their correlated clinic data, were deposited in the GEO database: GSE9348 (20), GSE23878 (21), GSE22598 (22), and GSE17536 (23) (Affymetrix Human Genome U133 Plus 2.0 platform) and GSE50760 (24) (Illumina HiSeq 2000 platform). GSE9348 has 70 primary CRC samples and 12 normal colon samples; GSE23878 has 70 primary CRC samples and 24 normal colon samples; GSE22598 contains 17 pairs of CRC and adjacent non-tumour tissues; GSE17536 is divided into the low PVT1 expression group (n=83) and high PVT1 expression group (n=60); GSE50760 has 17 metastasis CRC samples and 37 non-metastasis CRC samples.

Cell culture and transfection. The human colorectal cancer cell lines used in this study were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HCT116 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and other cell lines (SW480, HT29, NCM460, SW620, CaCO2) were cultured in RPMI-1640 media (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. HCT116, SW480, HT29, NCM460, SW620 and CaCO2 are all human colorectal cancer cell lines, while NCM460 is a normal colonic epithelial cell line from the tissue of a patient with gastric cancer. Transfection was conducted with. When cell densities were approximately 60%, 50 nM short interfering RNA (siRNA) oligos were transfected by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The sequences of the PVT1 targeting siRNAs were as follows: PVT1-si-1: 5'-CUGGACCUUAUGGCUCCA-3'; PVT1-si-2: 5'-CACUGAGGCUACUGCAUCU-3'; sequences of non-target scramble controls were provided by RiboBio (Guangzhou, China).

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR). Tissue RNA isolation and amplification were performed as described previously (25). RNA was isolated from the cells, using Trizol reagent (Invitrogen, The Netherlands). For the RT-qPCR, RNA was reverse transcribed to cDNA, using a Revert Aid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.), RT-qPCR was performed using a SYBR Premix ExTaq II kit (Takara Biotechnology Co., Ltd., Dalian, China) in the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to determine the relative expression of target genes. The following program was used for qPCR: 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec and then 60°C for 30 sec. The sequences of RT-qPCR primers were as follows: PVT1 forward 5'-TTCAGCACTCTG GACGGACTT-3', reverse 5'-TATGGCATGGGGCAGGTA G-3'; human cyclin D1 forward 5'-TCGGTGCCTGCTTG G-3', reverse 5'-CCATCAAGGGACAA CATGGG-3'; human cyclin D1 forward 5'-AACGGATTT GGTCCAATGGC-3', reverse 5'-TTGATTTTGGAGGA GTCTG-3'.

Western blotting. Cell lysis, cell lysate electrophoresis, and target protein visualisation were performed as described previously (25). Firstly, the cells were resuspended in lysis buffer [1% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 50 mM NaF, 2 mM EDTA, 400 mM NaCl, 10% glycerol plus Complete protease inhibitor mixture (Merck KGaA, Darmstadt, Germany)]. Then, 50 μg of cell lysates were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto Nitrocellulose membrane (Bio-Rad Laboratories, Inc.). After the membranes were blocked in Tris-buffered saline/Tween-20 (25 mM Tris-HCl, 150 mM NaCl, pH 7.5 and 0.05% Tween-20) with 5% defatted milk for 1 h at 37°C, the membranes were incubated overnight at 4°C with the primary antibodies, including E-cadherin (cat. no. 3195, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), vimentin (cat. no. 5741, 1:500; Cell Signaling Technology, Inc.), cyclin D1 (cat. no. 2978, 1:1,000; Cell Signaling Technology, Inc.), CDK4 (cat. no. 12790, 1:500; Cell Signaling Technology, Inc.), and GAPDH (cat. no. 5174, 1:1,000; Cell Signaling Technology, Inc.). After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and visualized using the ECL detection system. Densitometric analysis of immunodetected bands was performed using Image Analysis software (Bio-Rad Laboratories, Inc.).

Cell proliferation assay. The transfected cells were seeded in 96-well flat-bottom plates at a density of 2x10^3 cells/well in 200 μl of medium, and cultured for the CCK-8 (Dojin Laboratories, Kumamoto, Japan), with the operating steps carried out as described previously (26).

Tumour cell clone-formation assay. The tumour cell clone-formation assay was carried out as described previously (26). Briefly, 1x10^4 cells were seeded into each well of a 6-well culture plate and incubated for 14 days, after which the cell colonies were stained with haematoxylin and counted.

Matrigel invasion assays. Colorectal cell invasiveness was determined in a 24-well transwell plate (8 μp pore size; Costar), as described previously (26). Briefly, 5x10^4 cells were placed in the upper chamber of each insert coated with 200 mg/ml of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). After 48 h, the invaded cells were stained with haematoxylin and counted. Each experiment was repeated three times independently.

Statistical analysis. All statistical analyses were carried out using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA) and presented with Graphpad prism software (GraphPad Biotechnology Co., Ltd., Dalian, China).
Data are expressed as the mean ± standard error of the mean. Differences between two independent groups were tested using Student’s t-test and analysis of variance was performed for comparisons among multiple groups. OS was calculated using the Kaplan-Meier method, and the results of the analysis were considered significant in a log-rank test if $P<0.05$.

Results

PVT1 is upregulated in CRC tissues and cell lines. To determine the expression of PVT1 in CRC tissues, we first analysed three previously published datasets (nos. GSE9348, GSE23878, and GSE22598), using Affymetrix HG_U133 Plus 2 arrays to identify dysregulated lncRNAs in CRC tissues. We found that the lncRNA PVT1 was significantly upregulated in GSE9348, GSE23878, and GSE22598 ($P<0.001$, Fig. 1A-C). Next, PVT1 expression was also determined by RT-qPCR in five CRC cell lines (SW480, HT29, Caco-2, HCT116, and SW620), and the normal colon mucosal cell line NCM460. The results showed that PVT1 expression was higher in the CRC cell lines than in NCM460 ($P<0.05$, Fig. 1D), and that PVT1 expression was the highest in HCT116 cells. Therefore, we selected HCT116 cells for further studies.

Knockdown of PVT1 inhibits cell proliferation and invasion in CRC. To verify the function of PVT1 in colon cancer cells, we first measured the efficiency of the siRNA si-PVT1. Compared with the siPVT1-1 and siPVT1-2 groups, the siPVT1-1+2 group showed the highest efficiency in HCT116 cells (Fig. 3A). Therefore, we chose siPVT1-1+2 for in vivo knockdown of PVT1 expression to assess the biological function of PVT1 in CRC tissues. We investigated the effect of PVT1 knockdown on CRC cell proliferation by performing CCK-8 proliferation assays. PVT1 knockdown expression significantly inhibited HCT116 cell proliferation compared to that of the control group in the 96 h ($P<0.001$, Fig. 3B). PVT1 knockdown also inhibited HCT116 cell clone formation compared to that of the control group ($P<0.05$, Fig. 3C).

We also explored the effect of PVT1 knockdown on CRC cell invasion. A transwell invasion assay was performed to assess the effect of PVT1 on the invasiveness of CRC cells. PVT1 knockdown significantly inhibited HCT116 cell invasion compared to that of the control group ($P<0.01$, Fig. 4). These results demonstrated that PVT1 knockdown suppressed

Figure 1. PVT1 is highly expressed in CRC tissues and cell lines. PVT1 expression, as measured by Affymetrix microarray, was upregulated in CRC tissues compared with that in normal colon mucosal tissues in (A) GSE9348 (containing 12 normal colorectal tissues and 70 CRC tissue biopsies), (B) GSE23878 (containing 24 normal colorectal tissues and 35 CRC tissue biopsies) and (C) GSE22598 (containing 17 pairs of CRC tissues and corresponding normal colorectal tissues) from the GEO database. (D) PVT1 expression significantly increased in CRC cell lines (SW480, HT29, Caco-2, HCT116, and SW620) compared with that in NCM460, a normal colon mucosal cell line. Data are shown as mean ± standard error of the mean. *P<0.05, **P<0.01, ***P<0.001 vs. NCM460 cells. PVT1, plasmacytoma variant translocation 1; GEO, Gene Expression Omnibus; CRC, colorectal cancer.
the proliferation, invasion, and metastasis of CRC cells in vitro.

**Knockdown of PVT1 suppresses proliferation and EMT markers in CRC.** To confirm that PVT1 knockdown suppresses the proliferation, invasion, and metastasis of CRC cells in vitro, RT-qPCR and western blotting were used to assess the mRNA and protein level of the epithelial marker E-cadherin, mesenchymal markers vimentin, and proliferation markers cyclin D1 and CDK4 in HCT116 cell lines. PVT1 knockdown significantly decreased vimentin and enhanced E-cadherin expression (P<0.05, Fig. 5A-B), thereby inhibiting the progression of EMT. Meanwhile, PVT1 knockdown significantly inhibited cyclin D1 and CDK4 (P<0.01, Fig. 5C and D). This indicates that PVT1 regulates proliferation and EMT markers expression in CRC cell lines.

**Discussion**

CRC is one of the most common causes of cancer-associated mortality worldwide (29), especially in developed countries. It was estimated in 2015 that there were 777,987 new cases
and 352,589 deaths from CRC in developed countries (30). Surgery is currently the primary method of treatment for CRC, along with adjuvant radio-chemotherapy treatments. Although substantial progress has been made in the diagnosis and treatment of CRC, it retains a high morbidity and mortality rate owing to frequent recurrence and metastasis after treatment. Therefore, the treatment of CRC requires a novel therapeutic target to better control recurrence and metastasis.

LncRNAs are emerging as pivotal regulators in various biological processes. They are modulators of gene expression at the epigenetic, transcriptional, and post-transcriptional levels (31,32), controlling the fate of cellular processes including cell proliferation, apoptosis, and differentiation (33). Recent studies have revealed that disrupting or disabling lncRNA expression strongly correlates with a decrease in the incidence and development of malignant tumours, because lncRNAs have roles in cancer cell proliferation, the epithelial-mesenchymal transition (EMT), and drug resistance (34,35). Because lncRNAs are easier to extract, can be detected with higher specificity and sensitivity, and exist steadily in the blood and tissue (36), they have great potential to be a novel biomarker for cancer diagnosis, predicting recurrence, and chemosensitivity. Several lncRNAs have been shown to be differentially expressed in CRC and indicators of a poor prognosis, including MEG3 (37), GAS5 (38), MALAT1 (39), TUG1 (40), HOTAIR (41), and PVT1 (42).

The lncRNA PVT1 is reported to be overexpressed in many diseases, including several cancers. PVT1 overexpression has recently been identified as an independent predictor for OS in various human cancers, such as gastric cancer (17), NSCLC (18), and hepatocellular cancer (19). However, there has been insufficient research on the association of PVT1 expression with the OS of CRC patients. Takahashi et al. (42) demonstrated that the location of PVT1 was similar to that of MYC, which were mapped to chromosome 8q24. They also showed that 8q24 copy-number amplification promoted
MYC and PVT1 expression-prognostic indicators for CRC in patients. Similarly, Li et al (43) reported that the higher levels of AFAP1-AS1, MALAT1, H19, HOXA-AS2, BCAR4 or PVT1 in CRC tissues might predict the poor prognosis of CRC patients. In our study, we aimed to explore this lncRNA, which has the potential to be developed into a novel biomarker for CRC diagnosis and prognosis. We reported that PVT1 expression was significantly higher in CRC tissues than in normal colon mucosal tissues by GEO database analysis. Furthermore, multivariate analysis showed that CRC patients with PVT1 overexpression had a poorer OS time, which indicates that overexpression of PVT1 may be an independent indicator of poor prognosis in CRC patients.

Evidence strongly suggests that PVT1 plays a critical role in the development and progression of cancer by regulating cancer cell proliferation, metastasis, cell cycle, apoptosis, stemness, and drug resistance (44). Huang et al (45) demonstrated that PVT1 was overexpressed in small cell lung cancer (SCLC) tissues, and that knocking down PVT1 expression with siRNA significantly suppressed SCLC cell migration and invasion in vitro. Additionally, Kong et al (17) revealed that upregulation of PVT1 promotes cell proliferation in gastric cancer by epigenetically regulating p15 and p16. Shen et al (46) also showed that PVT1 could decrease miR-195 expression by enhancing histone H3K27me3 in the miR-195 promoter region and by direct sponging of miR-195 to modulate EMT and chemo-resistance in cervical cancer cells. However, the effects of PVT1 on CRC proliferation, invasion, and metastasis are poorly understood. Guo et al (47) reported that PVT1 may be a new oncogene co-amplified with c-Myc in CRC tissues and functionally correlated with the proliferation and apoptosis of CRC cells. Our results demonstrated that inhibition of PVT1 suppressed CRC cell proliferation, invasion, and metastasis in HCT116 cell lines, which was associated with decreased vimentin, cyclin D1, and CDK4 expression, but enhanced E-cadherin expression. This indicates that PVT1 contributes to the regulation of proliferation and EMT marker expression in CRC cell lines.

In summary, the results presented in this study indicate that PVT1 expression is upregulated in CRC patients, and that patients with high PVT1 expression show poor OS. Multivariate analysis indicated that high PVT1 expression is an independent risk factor for CRC patients. We also demonstrated that PVT1 expression mediates the proliferation, invasion, and metastasis of CRC cells. PVT1 knockdown significantly suppressed the proliferative and invasive capabilities of CRC cells. However, our study exists two limitations: 1) our study was limited by the use of only one CRC cell line; 2) our study was not further investigate the regulatory mechanism underlying PVT1’s promotion of the proliferation, invasion, and metastasis of CRC cells. Taken together, our study demonstrated the oncogene role of PVT1 in tumour progression of CRC, and shows potential as a target for development of novel CRC therapies after further investigation.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus datasets (ncbi.nlm.nih.gov/gds/).

Authors' contributions
CW and XS developed the concept and designed the study. CW, XZ and CP collected the data. CW, XZ and CP analysed and interpreted the data. All authors contributed to the writing of the manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors confirm that they have no competing interests.

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