Long Noncoding RNA PVT1 Predicts Poor Prognosis and Promotes the Progression of Colorectal Cancer Through the MiR-24-3p/NRP1 Axis in Zebrafish Xenografts

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Research Article

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Abstract

Background

Long noncoding RNAs (lncRNAs) play important roles in the progression of human cancer. It is reported lncRNA plasmacytoma variant translocation 1 (PVT1) is involved in colorectal cancer (CRC), however, the underlying mechanism remains to be explored deeply, especially by *in vivo* models.

Methods

The expression level of PVT1 in CRC tissues was analyzed in TCGA and GEO database. The prognosis of PVT1 in CRC was assessed by analyzing the correlation between the expression level of PVT1 and overall survival in CRC patients by Kaplan-Meier survival analysis. The CCK-8 and colony formation assays were performed to study the proliferation of CRC cells, and transwell assays was used to assess the migration of CRC cells *in vitro*. The targeting relationships among PVT1, miR-24-3p and Neuropilin 1 (NRP1) was predicted by StarBase, and examined by dual-luciferase reporter assays. The expression levels of PVT1, miR-24-3p and NRP1 were quantified by quantitative real time PCR and/or western blotting. The zebrafish CRC xenograft models were used to study the function of PVT1, miR-24-3p and NRP1 *in vivo*.

Results

Bioinformatics analysis showed that PVT1 was upregulated in CRC tissues and highly associated with poor prognosis. Knockdown of PVT1 inhibited cell proliferation and migration of CRC cells, while overexpression of PVT1 promoted the progression of CRC cells *in vitro*. In zebrafish xenografts, silencing of PVT1 also suppressed the growth and metastasis of CRC cells. miR-24-3p, binding with PVT1, negatively regulated the progression of CRC cells. Inhibition of miR-24-3p counteracted the effects of PVT1 knockdown. In addition, miR-24-3p could regulate CRC progression by targeting NRP1 directly. Moreover, the expression of NRP1 was regulated by PVT1, and NRP1 overexpression could partially rescue the inhibition effects of PVT1 knockdown on CRC progression *in vitro* and *in vivo*.

Conclusions

Our study reveals that PVT1 promoted the proliferation and metastasis of CRC via regulating miR-24-3p/NRP1 axis, and it could be a prognosis biomarker of CRC. This study provides a potential therapeutic target for CRC patients.

Background

Colorectal cancer (CRC) is one of common malignant cancers with high morbidity and mortality, it is the second cause of cancer death in the world [1, 2]. In recent years, although early diagnosis and new therapeutic approaches for CRC patients are developed rapidly, their poor prognosis and overall survival (OS) rate have not been improved significantly [3–5]. Therefore, understanding the molecular mechanisms in the development and progression of CRC is critical for changing such situations.

Long non-coding RNAs (lncRNAs), a class of non-coding transcripts more than 200 nucleotides in length, have been thought to be transcriptional “noise” before [6–8]. In recent years, it is proved that lncRNAs can regulate gene expression both at transcriptional and post-transcriptional levels [9, 10]. Many lncRNAs also have been identified to play important roles in human diseases, especially in cancer [11–16]. It is reported that lncRNAs regulate tumor proliferation, apoptosis, migration and invasion, some of them have been identified as a potential prognostic biomarker of cancer [17–19]. The lncRNA plasmacytoma variant translocation 1 (PVT1) is located on human chromosome 8q24, which is the star genomic region for human cancer studies. The oncogene c-Myc locates in this region [20, 21], and it has been verified which plays oncogenic roles in various human cancers, including lung cancer, breast cancer, and colorectal cancer [22]. Similar to c-Myc, PVT1 is also involved in multiple tumors and correlated with poor prognosis [21, 23, 24]. However, the precise molecular mechanism of PVT1 in CRC needs to investigate furtherly.

Many of lncRNAs, including PVT1, act as competing endogenous RNAs (ceRNAs) which competitively absorb miRNAs and indirectly regulate the expression of miRNA targeting genes [25–27]. It has been reported PVT1 can participate the progression of CRC via ceRNA mechanisms, such as PVT1/miR-455/RUNX2 axis, PVT1/miR-106b-5p/FJX1 axis, PVT1/miR-216a-5p/YBX1 axis [28]. PVT1 also can act as a sponge of miR-24-3p in LPS-induced cardiac fibroblasts and ischemic stroke [29, 30], while such regulation in tumor progression is still unclear.

Due to the difficulty, most of ceRNA network studies have limited *in vivo* experiments, especially for tumor metastasis. Zebrafish xenograft model has been developed as a rapid and promising model for human cancers in recent years [31–33]. So far, more and more zebrafish cell line derived xenograft (zCDX) models have been established successfully, such as colorectal cancer, breast cancer, prostate cancer, lung cancer [32, 34], and they also have been applied to study the roles of lncRNA in human cancers [35]. Compared to mouse xenograft models, zebrafish xenografts can be used to evaluate the proliferation and metastasis of tumor cells simultaneously by direct observation *in vivo* [32, 36–38]. Taking these advantages, zebrafish xenograft provides an easy *in vivo* animal model for studying ceRNA network.

In this study, we found that PVT1 was upregulated in CRC tissues and correlated with poor prognosis by analyzing the public database of patients. Functional experiments showed that PVT1 promoted the proliferation and metastasis of CRC cells *in vitro* and *in vivo*. Furthermore, molecular mechanism studies showed that PVT1 up-regulated the expression of NRP1 through competitively sponging to miR-24-3p, and inhibition of miR-24-3p or overexpression of NRP1 could rescued the suppression effects of PVT1 silence partially in CRC cells.

Methods

TCGA and GEO database analysis

The expression levels of lncRNA PVT1 in tumor tissue samples was analyzed through Gene Expression Profiling Interactive Analysis (GEPIA) (<http://gepia.cancer-pku.cn/>) using The Cancer Genome Atlas

(TCGA) database, which contains 275 colon adenocarcinoma tissue samples and 41 normal tissue samples, 92 rectum adenocarcinoma tissue samples and 10 normal tissue samples. The dataset GSE18105 from Gene Expression Omnibus (GEO) database was used to analyze the expression levels of PVT1 in CRC tissues by InCAR software (<https://Incar.renlab.org/>), which contains 94 tumor samples and 17 normal samples. And the dataset GSE41258 was used to compare the expression of PVT1 in primary and metastasis CRC tissues, which includes 67 metastasis tumors and 186 primary tumors samples. The OS of CRC patients was analyzed by Kaplan-Meier method (<https://kmplot.com/analysis/>).

Cell Culture

The human CRC cell lines were obtained from Institute of Biochemistry and Cell Biology of Chinese Academy of Science (Shanghai, China). HCT116, SW620 and FHC cells were cultured in 1640 medium, SW480 cells were cultured in DMEM medium, and LoVo cells were cultured in F-12K medium. All culture media were supplemented with 10% fetal bovine serum (FBS), and all cells were cultured in a humidified atmosphere with 5% at 37 °C.

Rna Exaction And Quantitative Real-time Pcr

Total RNA was exacted from cultured cells using TRIzol reagent (Invitrogen, CA, USA). Then the total RNA was reverse transcribed to cDNA using random primers according to instructions of PrimeScript RT kit (Takara, Dalian, China). Then, the expression levels of different genes were detected by quantitative real-time PCR (qRT-PCR) using SYBR Green Master Mix kit (Takara, Dalian, China). And glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as endogenous control. The specific primers were used for reverse transcription of miRNA, and the sequences were: 5'–GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCTGTTC-3' (miR-24-3p), 5'–GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACCGATGT-3' (miR-455-5p), 5'–GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAGGAGA-3' (miR-605-5p), 5'–GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGAGCTA-3' (miR-143-3p), 5'–GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACACAAAC-3' (miR-497-5p). U6 was used as internal control for detecting miRNA expression. All data were analyzed by $2^{-\Delta\Delta Ct}$ methods. The primer sequences for qRT-PCR were listed in Table 1.

Table 1
Primer sequences for qRT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
PVT1	TTGGCACATACAGCCATCAT	CAGTAAAAGGGGAACACCA
NRP1	cgggacccattcaggatcac	gctgatcgtactcctctggc
GAPDH	GGGAGCCAAAAGGGTCAT	GAGTCCTTCCACGATACCAA
miR-24-3p	GTTTGGCTCAGTTCAGCAG	GTGCAGGGTCCGAGGT
miR-455-5p	GGGTATGTGCCTTTGGACT	GTGCAGGGTCCGAGGT
miR-605-5p	GGTAAATCCCATGGTGCCT	GTGCAGGGTCCGAGGT
miR-143-3p	GTGGTGAGATGAAGCACTG	GTGCAGGGTCCGAGGT
miR-497-5p	GTTTCAGCAGCACACTGTG	GTGCAGGGTCCGAGGT
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT

Cell Transfection

A small interfering RNA (siRNA) targeting PVT1 (si-PVT1) (5'-cagccatcatgatggtact-3'), negative control (NC) siRNA (5'-TTCTCCGAACGTGTCACGT-3'), miR-24-3p mimics (5'-TGGCTCAGTTCAGCAGGAACAg-3') or inhibitor (5'-CTGTTCTGCTGAACTGAGCCA-3') were all purchased from General Biosystems (China). The control plasmid (pcDNA3.1), overexpression plasmids (pcDNA3.1-PVT1 or pcDNA3.1-NRP1) were synthesized by General Biosystems (China). The cells were cultured into six-well plates, and the siRNAs or plasmids were transfected into the cells using lipofectamine 2000 reagents (Invitrogen, USA) according to the manufacturer's protocol. After 24 h, the silencing efficiencies were examined by qRT-PCR.

Cck-8 Assay

Cell proliferation was evaluated using Cell Counting Kit-8 (CCaK-8, DOJINDO, Japan). The transfected cells were seeded at a density of 2000 cells /well in 96-well plates. 10 μ L CCK-8 reagent containing 100 μ L medium was added into each well, and then cultured for 2 h. Cell proliferation was quantified by measuring the optical density at 450 nm on a microplate reader (BioTek Elx800, USA) every 24 hours from 0 to 96 hours according to the manufacturer's instructions.

Colony Formation Assay

The transfected cells were seeded into six-well plates with a density of 600 cells /well. The culture media, containing 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin, were replaced every three days.

After 2 weeks, the colonies were washed twice with PBS, fixed with methanol for 10 min and then stained with 0.1% crystal violet for 10 min. The stained colonies were photographed and counted.

Transwell Assay

Cell migration was evaluated by transwell assay using 24-well plates with 8 μm pore size chamber inserts. The transfected cells of 5×10^4 diluted with 200 μL serum-free medium were seeded into the upper chambers of transwell plates. Then the upper chambers were placed into the lower chambers in 24-well plates containing 800 μL medium with 10% FBS. After 24 h, the cells were fixed using methanol for 10 min and stained using 0.1% crystal violet for 10 min. Then the cells were imaged under the inverted microscope (10X).

Western Blot

The transfected cells were lysed by Radio Immunoprecipitation Assay Lysis buffer (RIPA, Beyotime, China) for total protein extraction. The protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electro-transferred on polyvinylidene fluoride membranes (Millipore, Schwalbach, Germany), then were blocked with 5% skim milk. Subsequently, the membranes were incubated with primary antibody (BOSTER, China) which for neuropilin-1 (1:500) or GAPDH (1:10000) at 4 °C overnight. Then the membranes were put into the secondary antibody (1:5000, BOSTER, China) for 1 hours at room temperature. After washing, the proteins were visualized with a BeyoECL plus kit (Beyotime, China).

Luciferase Reporter Assay

The potential binding site of PVT1 or NRP1 3' UTR with miR-24-3p was predicted by StarBase (<https://starbase.sysu.edu.cn/>), miRDB (<http://mirdb.org/>) and DIANA (<http://diana.imis.athena-innovation.gr/>). The wild-type and mutant-type fragments of PVT1 and NRP1 3' UTR were synthesized and cloned into pGL3-basic vector. The reporter plasmids were co-transfected with miR-24-3p mimics or NC mimics into HEK-293T cells using Lipofectamine 2000 reagents. After 48 h, the luciferase activity was detected by a dual-luciferase assay kit (Promega, UAS) according to the manufacturer's protocols.

Zebrafish Xenograft Model

The adult Tg(fli1a:EGFP) zebrafish were maintained at 28 °C and 14 h-10 h light-dark cycle in a fish auto culture system (Haishen, China). The embryos of zebrafish were cultured into 10% Hank's solution, which was composed of (in mM) 140 NaCl, 5.4 KCl, 0.25 Na_2HPO_4 , 0.44 KH_2PO_4 , 1.3 CaCl_2 , 1.0 MgSO_4 and 4.2 NaHCO_3 (pH 7.2). The 48-hpf (hours post-fertilization) Tg(fli1a:EGFP) zebrafish embryos were used for

cancer cell transplant experiments in our study [39]. Zebrafish handling procedures were approved by Lianshui County People's Hospital, Kangda College of Nanjing Medical University.

Before injection, the tumor cells, transfected with siRNA or/and plasmid, were harvested and stained by CM-Dil (Invitrogen, USA) [35]. The 48-hpf zebrafish larvae were mounted with 1.2% low-melting agarose (Promega, USA), then approximately 300–400 labeled cells were injected into the perivitelline space (PVS) of zebrafish larvae under a microinjector (Picosprizer III, USA). Then the injected zebrafish larvae were cultured at 34 °C in light incubator. At 1 day post injection (dpi), the larvae with similar sizes of fluorescence area were selected for further research and cultured at 34 °C until the end of experiments. At 4 dpi, the zebrafish larvae were mounted with 1.2% low-melting agarose, then the images were acquired by stereotyped microscope (MVX10, Olympus, Japan) or confocal microscope using 20X objective lens (Fluoview 3000, Olympus, Japan). The resolution of the images was 1600×1200 (MVX10) or 1024×1024 pixels (Fluoview 3000).

Statistical analysis

All the statistical data were analyzed using unpaired Student's t-test and their figures were generated by GraphPad Prism 7.0. The values of $P < 0.05$ were considered to be statistically significant. All results were presented as the mean $\pm$ SD.

Results

PVT1 is upregulated in CRC tissues and associated with poor prognosis of CRC patients

Firstly by analyzing TCGA database, we found the expression of PVT1 was significantly upregulated both in colon adenocarcinoma (COAD) and rectum adenocarcinoma (READ) comparing to the normal tissues (Fig. 1A, $P < 0.05$). The data of GEO dataset GSE18105 also showed that the expression of PVT1 was upregulated in CRC tissues (Fig. 1B, $P < 0.001$). To study the relationship between PVT1 and the prognosis of CRC, we then analyzed another GEO dataset GSE41258 and found the expression of PVT1 was much higher in metastasis tumors than that in primary tumors of CRC patients (Fig. 1C, $P < 0.001$). Furthermore, the OS of CRC patients was also analyzed and showed high PVT1 expression level was correlated with low survival rate of CRC patients (Fig. 1D, $P < 0.05$). These results present that the expression of PVT1 is upregulated in CRC tissues, and also associated with poor prognosis of CRC patients.

PVT1 promotes the proliferation and migration of CRC cells in vitro

We examined the expression levels of PVT1 in four CRC cell lines (HCT116, SW480, SW620 and LoVo) and human normal colon epithelial cell (FHC). Compared with FHC, and found that PVT1 was highly expressed in CRC cells, especially in HCT116 and SW480 cell lines (Fig. 2A, $P < 0.05$). To verify the

biological functions of PVT1 in CRC cells, we transfected small interference RNA (siRNA) or overexpression plasmid to downregulate or upregulate the expression of PVT1 in CRC cells. Compared with negative control siRNA (NC), PVT1 siRNA (si-PVT1) transfection efficiently decreased the expression of PVT1, which the knockdown efficiency was 79.7% and 89.4% in HCT116 and SW480 cells, respectively (Fig. 2B, $P < 0.01$). Overexpression plasmid (pcDNA3.1-PVT1) transfection increased the expression level of PVT1 by 128 times compared to control (pcDNA3.1 empty vector transfection) (Fig. 2C, $P < 0.001$). Then we used CCK-8 to assess the proliferation of CRC cells, and found PVT1 knockdown inhibited the proliferation of HCT116 (Fig. 2D, $P < 0.001$) and SW480 cells (Fig. 2E, $P < 0.001$), but overexpression PVT1 increased the proliferation of LoVo cells (Fig. 2F, $P < 0.001$). And colony formation assays also verified the proliferation role of PVT in CRC (Fig. 2G-I, $P < 0.05$). Subsequently, we used transwell assays to assess the migration of CRC cells, and found silence of PVT1 significantly suppressed the cell migration in HCT116 (Fig. 2J, $P < 0.01$) and SW480 cells (Fig. 2L, $P < 0.05$). On the contrary, the migration of LoVo cells was enhanced when overexpressing PVT1 (Fig. 2L, $P < 0.05$). Taken together, these data shows that PVT1 promotes the proliferation and migration of CRC cells *in vitro*.

Knockdown of PVT1 inhibits the growth and metastasis of CRC cells *in vivo*

To verify the functional roles of PVT1 in CRC cells *in vivo*, the zebrafish xenograft models were used. HCT116, transfected with si-PVT1 or NC and labeled by CM-Dil, were transplanted into the PVS of 48-hpf (hours post fertilization) Tg(fli1a: EGFP) transgenic zebrafish larvae, which the vascular endothelial cells were labeled by EGFP. At 4 days post injection (dpi), we quantified the area of CM-Dil positive signals in yolk representing the cell growth (Fig. 3A, B), and found silencing PVT1 inhibited the growth of HCT116 compared with NC groups (Fig. 3C, $P < 0.05$). We then quantified the area of CM-Dil positive in trunk (Fig. 3D, E) representing cell metastasis, and found PVT1 knockdown also inhibited the metastasis of HCT116 cells (Fig. 3F, $P < 0.05$). Similarly, knocking-down PVT1 also inhibited the growth and metastasis of SW480 cells (Fig. 3G-L, $P < 0.05$). These results demonstrate that PVT1 regulates the growth and metastasis of CRC cells *in vivo*.

PVT1 negatively regulates the expression of miR-24-3p as a sponge in CRC cells

Previous studies have revealed that PVT1 can act as a miRNA sponge to regulate the progression of various tumors [22, 40]. We used three online tools to predict candidate miRNAs which could bind with PVT1, including StarBase, miRDB and DIANA Tools. By cross comparison, we found miR-24-3p, miR-143-3p, miR-455-5p, miR-497-5p and miR-605-5p were predicted by all the three online tools (Fig. 4A). Then we examined the expressions of these miRNAs when knocking-down PVT1, and screened out three miRNAs (miR-24-3p, miR-455-5p, miR-605-5p) whose expression levels were upregulated when PVT1 silence in SW480 cells (Fig. 4B, $P < 0.05$). It is reported that miR-24-3p plays regulation roles in proliferation, migration and invasion of CRC cells [41], and our analysis also showed its potential association with the poor prognosis of CRC (Fig. 4C). Therefore, we chose miR-24-3p for the further studies. According to the predicted binding site between PVT1 and miR-24-3p by StarBase (Fig. 4D), dual luciferase reporter assays were performed for the verification *in vitro*. Compared with control, we found luciferase activity was suppressed when co-transfecting PVT1 reporter plasmid and miR-24-3p mimics in HEK-293T cells

(Fig. 4E, $P < 0.01$), while such regulation was totally blocked when co-transfecting the mutant PVT1 reporter plasmid and miR-24-3p mimics (Fig. 4D, E). These results suggest that PVT1 negatively regulate the expression of miR-24-3p through binding with miR-24-3p directly in CRC cells.

Knockdown of miR-24-3p promotes the proliferation and metastasis of CRC cells *in vitro* and *in vivo*

To study the roles of miR-24-3p in CRC, we efficiently silenced the expression of miR-24-3p by transfecting miR-24-3p inhibitor (Fig. 4F, $P < 0.001$). CCK-8 and transwell assays showed that miR-24-3p inhibition promoted the proliferation and migration of SW480 cells *in vitro* (Fig. 4G, H). By using zebrafish xenograft models, knockdown of miR-24-3p also promoted the growth and metastasis of CRC cells *in vivo* (Fig. 4I, J). These data show that miR-24-3p negatively regulates the progression of CRC cells *in vitro* and *in vivo*.

Knockdown Of Mir-24-3p Rescues The Progression Of Crc Cells Which Repressed By Pvt1 Silence

To determine whether miR-24-3p mediated the regulation of PVT1 in CRC progression, we studied it by cotransfecting si-PVT1 and miR-24-3p inhibitor into SW480 cells simultaneously. Similar to our previous results, knockdown of PVT1 suppressed the proliferation of CRC cells, but miR-24-3p inhibition counteracted the suppression effects which caused by PVT1 knockdown (Fig. 5A, $P < 0.05$). Transwell assay also showed that silence of miR-24-3p partly restored the ability of cell migration of CRC cells which suppressed by PVT1 knockdown (Fig. 6B, $P < 0.05$). These results indicate that PVT1 promotes the progression of CRC cells via regulating the expression of miR-24-3p.

Nrp1 Is Directly Target Of Mir-24-3p In Crc Cells

To find the direct downstream target genes of miR-23-3p, we also used StarBase, miRDB and DIANA tools to predict. A serious of tumor-related genes were predicted by cross comparison, such as NRP1, PAK4, MMP14, KLF4, FSCN1 (Fig. 6A). We selected NRP1 for further research because it was reported that NRP1 involved in the progression of CRC [42]. Then we overexpressed miR-24-3p by transfecting its mimics into SW480 cells, and found the expression of NRP1 were decreased both in transcriptional and translational expression level (Fig. 6B, C, $P < 0.01$). According to the binding site between 3'UTR of NRP1 and miR-24-3p which was also predicted by StarBase tool (Fig. 6D), we did the dual luciferase reporter assays and found miR-24-3p could bind with 3'UTR of NRP1 directly (Fig. 6E, $P < 0.001$). These data show that miR-24-3p can downregulate the expression of NRP1 in CRC cells by binding the 3'UTR of NRP1 directly.

Overexpression Nrp1 Rescues The Progression Of Crc Cells Which Suppressed By Mir-24-3p Overexpression

To determine whether NRP1 mediated the regulation of miR-24-3p in CRC progression, we tried to perform similar experiments by cotransfecting NRP1-overexpressing plasmid (pcDNA3.1-NRP1) and miR-24-3p mimic into SW480 cells simultaneously. First, we transfected pcDNA3.1-NRP1 into SW480 cells and examined the overexpression efficiency by qRT-PCR and western blot. We found NRP1-overexpressing plasmid transfection could efficiently upregulated the expression level of NRP1 (Fig. 6F, G, $P < 0.01$). NRP1 overexpression not only promoted the proliferation of CRC cells, but also rescued the CRC proliferation which suppressed by miR-24-3p overexpression (Fig. 6H, $P < 0.05$). The data of transwell assays also showed the similar rescue roles when overexpressing NRP1 (Fig. 6I, $P < 0.05$). These results suggest miR-24-3p regulates the progression of CRC cells via targeting NRP1.

Knockdown of PVT1 suppressed the growth and metastasis of CRC cells by regulating NRP1 expression *in vitro* and *in vivo*

To investigate whether PVT1 functioned through NRP1 pathway in CRC cells, we firstly detected the expression level of NRP1 when knocking-down PVT1 in SW480 cells. We found PVT1 knockdown reduced the expression level of NRP1 (Fig. 7A, B, $P < 0.001$). Then we cotransfected si-PVT1 and pcDNA3.1-NRP1 in CRC cells, and we also found the suppression effects which caused by PVT1 knockdown were partially counteracted by NRP1 overexpression both in proliferation and migration levels (Fig. 7C, D, $P < 0.01$). NRP1-related OS analysis in CRC patients indicated its clinical significance (Fig. 7E, $P < 0.01$), and we then examined the PVT1/NRP1 regulatory pathway in zebrafish xenografts. *In vivo* experiments also showed that NRP1 overexpression could rescue the CRC progression which suppressed by PVT1 knockdown (Fig. 7F, G, $P < 0.05$). The results indicate that PVT1 regulates the progression of CRC cells through NRP1 pathway.

Discussion

It is reported that PVT1 is elevated in multiple cancers, such as gastric cancer, breast cancer, lung cancer [43]. PVT1 has been identified which plays oncogenic roles through various pathways and is associated with poor prognosis of CRC patients [23]. In the present study, we found that PVT1 was upregulated in CRC tissues and predicted the poor prognosis by analyzing public database. Functional studies demonstrated that PVT1 could promote the proliferation and metastasis of CRC cells *in vitro* and *in vivo*. These results verify the oncogenic roles of PVT1 in CRC.

PVT1 has been identified that it can function as miRNA sponges to regulate miRNA-targeting genes in CRC cells [22, 26, 30, 43]. In this study, we screened the miRNAs which could be absorbed by PVT1 in CRC cells. And we found miR-24-3p could bind with PVT1 directly, and its expression level was regulated by PVT1. It is also reported that miR-24-3p functions as a tumor suppressor, which is downregulated in CRC tissues and inhibits the proliferation, migration and invasion of CRC cells [41]. In previous study, PVT1 has been proved that can sponge miR-24-3p in LPS-induced cardiac fibroblasts and ischemic stroke, while thus regulation has not been proved in cancers. In this study, bioinformatics analysis indicated the expression level of miR-24-3p and OS of CRC patients were negatively correlated. Moreover, we also

found miR-24-3p knockdown not only promoted the progression of CRC, but also prevented the inhibition effects of PVT1 silence. These data demonstrate that PVT1 can functions as an oncogene by sponging miR-24-3p in CRC.

NRP1 was proved to promote the cell viability, migration, and invasion in CRC [42], and it was also a target gene of miR-24-3p 7 [44, 45]. Bioinformatics analysis indicated that the expression level of NRP1 predicted the poor prognosis of CRC (Fig. 7E). Based of it, we furtherly studied whether miR-24-3p/NRP1 axis played roles in the progression of CRC cells. Our data showed miR-24-3p could directly bind with the 3'UTR of NRP1 and regulate the proliferation and migration of CRC cells through NRP1. Then we investigated whether PVT1 functioned through NRP1 in CRC. And we also found PVT1 knockdown decreased the expression of NRP1, NRP1 overexpression could rescued the tumor progression which inhibited by PVT1 silence in CRC cells both *in vitro* and *in vivo*. These results suggest PVT1 can function as a ceRNA by regulating miR-24-3p/NRP1 axis in CRC cells.

Mouse xenograft models are widely applied for human cancer research, which is also used for verification of PVT1 in the proliferation of CRC, however the metastasis mouse model for studying the function of PVT1 is very few [46, 47]. Compared with mouse models, zebrafish xenografts have multiple advantages in tumor biology. First, we can access the proliferation and metastasis of tumor cells in 96 hours after transplantation. Second, the proliferation and metastasis can be analyzed simultaneously by monitoring the behavior of tumor cell *in vivo* due to the transparent zebrafish larvae. Third, the genetic operation was relatively easy. Instead of shRNA plasmid transfection, siRNA transfected tumor cells can be used for the verification in 4 days. Using mouse models, He and his colleagues reported PVT1 overexpression promoted the metastasis of CRC cells from spleen parenchyma to liver during 28 days post-injection [46]. In this study, we showed that silencing PVT1 by siRNA transfection also inhibited the proliferation and metastasis of CRC cells at 4 dpi in zebrafish xenografts, which was consisted with that in mouse models. By taking advantage of zebrafish xenografts, we furtherly not only verified the relationship between miR-24-3p and NRP1 in CRC progression, but also demonstrated the regulatory mechanism between PVT1 and NRP1 *in vivo*. These data suggest that zebrafish xenograft could be a reliable and feasible model for human cancer research, especially for ceRNA mechanism verification *in vivo*.

Conclusions

In summary, our results demonstrated the oncogenic roles of PVT1 which also predicted the poor prognosis of CRC patients. Additionally, we revealed a novel signaling pathway of PVT1 in CRC, which functioned as a ceRNA by regulating miR-24-3p/NRP1 axis. Our results indicate PVT1 could be a molecular marker of diagnostic and prognosis, and also be a potential treatment target for CRC patients.

Abbreviations

LncRNA: long noncoding RNA; PVT1: plasmacytoma variant translocation 1; CRC: colorectal cancer; OS, overall survival; ceRNA: competing endogenous RNAs; COAD: colon adenocarcinoma; READ: rectum adenocarcinoma; TCGA: The Cancer Genome Atlas; GEPIA: Gene Expression Profiling Interactive Analysis; GEO: Gene Expression Omnibus; qRT-PCR: quantitative real-time PCR; CCK-8: Cell Counting Kit-8; RIPA: Radio Immunoprecipitation Assay Lysis buffer; hpf: hours post-fertilization; dpi: days post injection; PVS: perivitelline space; FBS: fetal bovine serum; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; NC: negative control; NRP1: Neuropilin 1.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

HY: Conceptualization; Data curation; Formal analysis; Visualization; SG: Data curation; Formal analysis; Visualization; Funding acquisition; Writing - original draft; GC: Investigation; Methodology; Resources; HY and XZ: Software; Resources; Validation;YZ: Supervision; Funding acquisition; Project administration; Validation; Writing - review & editing; all authors have read and approved the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Lianshui County People's Hospital, Kangda College of Nanjing Medical University. All participants obtained written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

PVT1 is upregulated in CRC tissues and associated with poor prognosis. **A** The expression of PVT1 in CRC tissues (COAD and READ, T) was analyzed byTCGA database comparing to normal tissues. (N).**B** The expression of PVT1 in CRC tissues was analyzed by GEO dataset GSE18105 comparing to normal tissues. **C** The expression of PVT1 in metastasis CRC tissues was analyzed byGSE41258 comparing to primary CRC tissues. **DOS** of CRC patients with high PVT1 expression was analyzed by Kaplan-Meier analysis comparing to that with low PVT1 expression. *: P <0.05, ***: P <0.001.

Figure 2

PVT1 promotes the proliferation and migration of CRC cells *in vitro*. **A** The expression level of PVT1 was measured by qRT-PCR in human CRC cell lines (HCT116, SW480, SW620, LoVo) comparing to the normal colon epithelial cell (FHC). **B** The knockdown efficiency of PVT1 siRNA was examined by qRT-PCR in HCT116 and SW480 cells. **C** The overexpression efficiency of PVT1 plasmid was examined by qRT-PCR in LoVo cells. **D-F** CCK-8 assays were performed to evaluate the cell proliferation of HCT116 (**D**), SW480 (**E**) and LoVo cells (**F**) when downregulating or upregulating PVT1 expression. **G-I** Colony formation assays were performed to evaluate the cell proliferation in HCT116 (**G**), SW480 (**H**) and LoVo cells (**I**). **J-K** Transwell assays were performed to evaluate the cell migration in HCT116 (**J**), SW480 (**K**) and LoVo cells (**L**). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

Figure 3

PVT1 knockdown inhibits the growth and metastasis of CRC cells in zebrafish xenografts. **A,B** The CM-Dil-positive signals in the yolk of zebrafish represented the proliferation of HCT116 when transfecting NC (**A**) or si-PVT1 (**B**). **C** Quantification of cell proliferation in zebrafish xenograft when transfecting NC or si-PVT1 in HCT116 cells. **D, E** The CM-Dil-positive signals in the trunk of zebrafish represented the metastasis of HCT116 when transfecting NC (**D**) or si-PVT1 (**E**). **F** Quantification of cell proliferation in

zebrafish xenograft when transfecting NC or si-PVT1 in HCT116 cells. **G-L** Cell proliferation (**G-I**) and metastasis (**J-L**) in zebrafish xenograft when transfecting NC or si-PVT1 in SW480 cells. Arrows represent the metastasis CRC cells. Scale: 100 μ m. *: $p<0.05$, **: $p<0.01$.

Figure 4

miR-24-3p binds with PVT1 and negatively regulates CRC progression *in vitro* and *in vivo*. **A** Venn diagram for miRNA candidates that might bind with PVT1. **B** The expression levels of five miRNAs was examined when PVT1 knockdown. **C** The correlation between the expression levels of miR-24-3p and overall survival of CRC patients. **D** The schematic diagram of potential binding site between miR-24-3p and PVT1 which was predicted by StarBase. **E** Dual luciferase activities of PVT1-WT or PVT1-MT were examined when cotransfecting with miR-24-3p mimic. *: $p < 0.05$, **: $p < 0.01$. **F** The knockdown efficiency of miR-24-3p when transfecting its inhibitor. **G, H** The proliferation and migration of CRC cells when silencing miR-24-3p. **I, J** Quantification of the growth and metastasis of CRC cells when silencing miR-24-3p by zebrafish xenografts. *: $P < 0.05$, **: $p < 0.01$, ***: $P < 0.001$.

Figure 5

Knockdown of miR-24-3p rescues the progression of CRC cells which is suppressed by PVT1 silence. **A** The cell proliferation was evaluated by CCK-8 assay when cotransfecting miR-24-3p inhibitor and si-PVT1. **B** The cell migration was evaluated by transwell assay when cotransfecting miR-24-3p inhibitor and si-PVT1.*: $P < 0.05$, **: $p < 0.01$.

Figure 6

miR-24-3p negatively regulates CRC progression via targeting NRP1 directly. **A** Venn diagram for the potential target genes of miR-24-3p. **B, C** The expression of NRP1 was examined by qRT-PCR and western blot when miR-24-3p over expression in CRC cells. **D** The schematic diagram of potential binding site between miR-24-3p and NRP1. **E** Dual luciferase activities of NRP1-WT and NRP1-MUT were examined when cotransfecting with miR-24-3p mimic. **F, G** The expression of NRP1 was measured by qRT-PCR and western blot when transfecting NRP1 over expression plasmid. **H, I** The proliferation (**H**) and migration (**I**) of CRC cells were examined when cotransfecting miR-24-3p mimic and NRP1 over expression plasmid. *: $p < 0.05$, **: $p < 0.01$, ***: $P < 0.001$.

Figure 7

PVT1 regulates the progression of CRC cells via NRP1 *in vitro* and *in vivo*. **A, B** The expression of NRP1 was examined when silencing PVT1 in CRC cells by qRT-PCR (**A**) and western blot (**B**). **C, D** The proliferation (**C**) and migration (**D**) of CRC cells when cotransfecting si-PVT1 and NRP1 overexpression plasmid. **E** The correlation between the expression levels of NRP1 and overall survival of CRC patients. **F, G**

Quantification of the growth and metastasis of CRC cells when cotransfecting si-PVT1 and NRP1 overexpression plasmid by zebrafish xenografts. *: $p < 0.05$, **: $p < 0.01$, ***: $P < 0.001$.