Am J Cancer Res 2018;8(3):538-550
www.ajcr.us /ISSN:2156-6976/ajcr0048093

Original Article

A feedback loop consisting of RUNX2/LncRNA-PVT1/miR-455 is involved in the progression of colorectal cancer

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Received January 4, 2017; Accepted November 14, 2017; Epub March 1, 2018; Published March 15, 2018

Abstract: Long non-coding RNAs (lncRNAs) have been shown to participate in cancer progression. In the present study, we explored the potential roles of lncRNA-PVT1 in the development process of colorectal cancer (CRC) via miR-455. We found that PVT1 is up-regulated in human CRC tissues compared to adjacent normal tissues. A functional study showed that the silencing of PVT1 expression by siRNAs inhibited cell proliferation, migration and invasion, whereas the overexpression of PVT1 accelerated cell proliferation, migration and invasion *in vitro*. A mechanistic study indicated PVT1 regulated the growth of CRC tumors by acting as a competing endogenous RNAs (ceRNA) and negatively regulated miR-455. Furthermore, we discovered that RUNX2, a functional transcription factor in CRC, up-regulated PVT1 expression. Therefore, our study suggested that the RUNX2/PVT1/miR-455 regulatory axis plays an important role in CRC tumorigenesis and may be a therapeutic target for the treatment of CRC.

Keywords: Colorectal cancer, LncRNA-PVT1, MicroRNA-455, proliferation, migration and invasion

Introduction

The incidence of colorectal cancer (CRC), an important cause of morbidity and mortality worldwide, has rapidly increased in recent years [1]. The poor prognosis of CRC is attributed to the high rates of lymphatic and distant metastases [2], and most patients present with advanced CRC upon diagnosis due to a lack of effective biomarkers of early disease. Therefore, molecular and functional studies of CRC progression and development, such as non-coding RNA studies, are urgently needed.

Only 1.2% of the mammalian genome encodes proteins [3], and most of the mammalian genome consists of a vast amount of non-coding RNAs (ncRNAs) [4, 5]. To date, many ncRNAs have been shown to play significant roles in the development, progression and metastasis of cancer [6]. Recently, long non-coding RNAs (lncRNAs), non-coding RNAs longer than 200 nucleotides, have been shown to regulate gene expressions [7, 8] and be involved in tumorigenesis in various cancers, including liver cancer, lung cancer, breast cancer, and CRC [9-14]. LncRNA-PVT1 is a well-studied lncRNA in the context of cancer located near MYC at locus 8q24 of the human chromosome [15] that inhibits apoptosis in ovarian cancer and breast cancer [16]. However, the function and mechanism of PVT1 in CRC require further study.

MicroRNAs (miRNAs) are another type of non-coding RNA that are approximately 20 nucleotides in length and have been reported to regulated gene expression via post-transcriptional regulation by targeting the 3'-UTR of target genes [17-19]. An increasing number of studies have indicated that miRNAs are involved in the occurrence and development of various cancers, such as breast cancer [20], the stem cells of pancreatic cancer [21], endometrial cancer [22], lung cancer, and nervous system cancer [23]. They participate in various biological processes, such as proliferation, differentiation, apoptosis, migration and invasion [24-27], making them effective biomarkers for human cancer diagnostics. Among these miRNAs, microRNA-455 (miR-455) has been reported to play

important roles in the development progression of human cancers [28-30], but the function and mechanism of miR-455 in CRC is unclear.

In the present study, we measured the mRNA expression level of PVT1 in human CRC tissues and studied the functional effects of PVT1 on CRC cell proliferation, apoptosis, migration and invasion. In addition, we assessed the regulatory relationship between PVT1 and miR-455 and the miR-455-mediated roles of PVT1 in CRC. Furthermore, we examined the regulatory network of the RUNX2/PVT1/miR-455 axis in CRC. Consequently, we demonstrated that PVT1 is involved in the progression of CRC via miR-455.

Materials and methods

Clinical specimens

Twenty patients with CRC treated at the Shandong University Affiliated Shandong Cancer Hospital and Institute between 2014 and 2015 were included in this study. This study was also approved by the Ethics committee of Shandong University Affiliated Shandong Cancer Hospital and Institute, and each patient provided informed consent. CRC was histologically diagnosed based on the World Health Organization (WHO) criteria. All tissue samples were frozen at -80°C.

Cell lines

Normal human colon epithelial cells (FHC) and LOVO cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). SW480, SW620, HT29, and HCT116 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). FHC cells were cultured in DMEM/F12 medium (Invitrogen, Carlsbad, California); LOVO cells were cultured in F12-K medium (Life Technologies, Inc., Grand Island, NY). SW480 and SW620 cells were maintained in Leibovitz's L-15 medium (Gibco ® Life technologies Italia, Monza, Italy). HT-29 and HCT-116 cells were cultured in McCoy's 5A medium (HyClone, Logan, UT). All media were supplemented 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml), and all cells were cultured in an appropriate incubator containing 5% CO_2 at 37°C.

Vector construction, miRNAs and siRNA transfection

PVT1 overexpression vector and RUNX2 expression vector were purchased from GenePharma Co., Ltd. (Shanghai, China). To transfect miRNA inhibitors, 2×10^5 HT29 and SW480 cells were cultured in 6-well plates and then transfected with 200 μl of scrambled miRNA or miR-455 inhibitors (GenePharma Co., Ltd., Shanghai, China) for 72 hrs using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. For the siRNA transfection, PVT1 siRNA was purchased from GenePharma (GenePharma Co., Ltd., Shanghai, China). The specific sequence of the PVT1 siRNA was 5'-GCU UGG AGG CUG AGG AGU UTT-3' (sense) and 5'-AAC UCC UCA GCC UCC AAG CTT-3' (antisense). The cells were transfected with NC-siRNA or PVT1-siRNAs using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

Quantitative real-time reverse transcription PCR (qRT-PCR)

We extracted total RNA from CRC tissues and the treated HT29 and SW480 cells using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's introductions. Briefly, 1 μg of total RNA was used to synthesize first-strand cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher). qRT-PCR was performed with specific primers (Qiagen, Hilden, Germany) and SYBR-Green PCR Master Mix kit (Takara) on an ABI 7500 system (Applied Biosystems, Foster City, CA, USA). The primer sequences for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, internal loading control) were: 5'-CCT CGT CTC ATA GAC AAG ATG GT-3' (forward) and 5'-GGG TAG AGT CAT ACT GGA ACA TG-3' (reverse); the primer sequences for PVT1 were: 5'-TGA GAA CTG TCC TTA CGT GAC C-3' (forward) and 5'-AGA GCA CCA AGA CTG GCT CT-3' (reverse); the primer sequences for RUNX2 were: 5'-ACTA CCA GCC ACC GAG ACC A-3' (forward) and 5'-ACT GCT TGC AGC CTT AAA TGA CTC T-3' (reverse). The mRNA expression levels of PVT1 and RUNX2 were normalized to that of GAPDH. The primer sequences for U6 were: 5'-CTC GCT TCG GCA GCA CA-3' (forward) and 5'-AAC GCT TCA CGA ATT TGC GT-3' (reverse); the primer sequences for hsa-miR-455 were: 5'-ATG TGC CTT TGG ACT ACA TCG AA-3' (forward) and 5'-TTA CTA CGT CAT

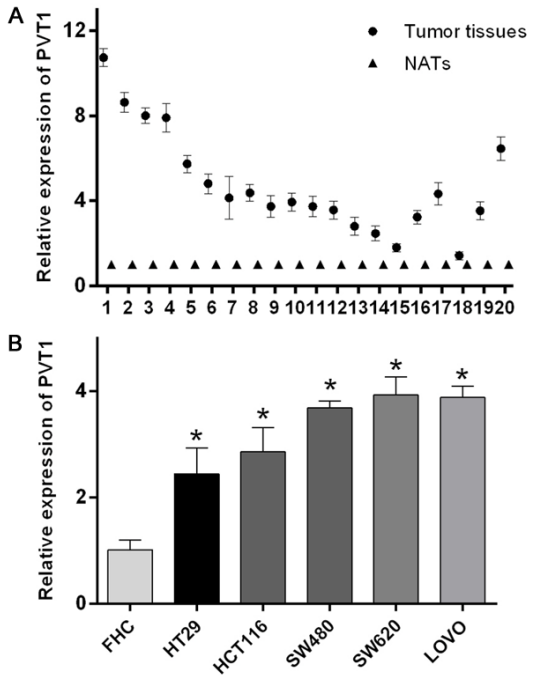

Figure 1. LncRNA-PVT1 is up-regulated in human CRC. Levels of PVT1 in CRC tissues and cells. A. Relative PVT1 expression in CRC tissues. The relative expression level of PVT1 was measured by qRT-PCR in 20 pairs of CRC tissues and paired adjacent normal tissues. Data were analyzed using Student's *t*-test and normalized to GAPDH ($P < 0.001$). B. Relative PVT1 expression in cells. qRT-PCR was used to analyze PVT1 expression in CRC cell lines (HT29, HCT116, SW480, SW620, and LOVO) and a normal human colon epithelial cell (FHC) line (*$P < 0.05$, vs. FHC cell).

GAC TAG TAA-3' (reverse). The mRNA expression level of miR-455 was normalized to that of U6.

Western blot

HT29 cells were transfected with RUNX2 vector or control vector and lysed using RIPA buffer (Pierce) containing a protease inhibitor cocktail (Sigma-Aldrich). Equivalent amounts of total protein were separated on 8% SDS/PAGE gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk and then incubated with primary antibodies at 4°C overnight. The next day, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Abcam), and signals were detected using an enhanced chemiluminescence (ECL) substrate kit (Amersham Biosciences) on an enhanced chemiluminescence detection system (Amersham Biosciences). The primary antibodies were anti-RUNX2 antibody (1:500 dilution, Santa Cruz Biotechnology, Inc.) and anti-GAPDH antibody (a loading control, 1: 4000 dilution, Beverly, MA, USA).

Luciferase reporter assay

HT29 and SW480 cells (5×10^4 cells/well) were cultured in 24-well plates and co-transfected with wild-type (PVT1-WT) or mutant (PVT1-Mut) PVT1 and scrambled miRNA or miR-455 using Lipofectamine 3000 (Invitrogen). The cells were also transfected with a Renilla plasmid (RL-SV40) (Internal control). The luciferase activities were measured with a Dual-Luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions.

Proliferation assay

For the CCK-8 assay, the transfected HT29 and SW480 cells (50000 cells/well) were seeded in a 96-well plate and incubated for 0, 12, 24, or 48 hrs. At specified time points, we added Cholecystokinin octapeptide (10 μl, CCK-8, Dojindo Molecular Technologies, Japan) to each well and incubated the cells for an additional 4 hrs. The absorbance at 570 nm was then measured using a micro-plate reader (Bio Tek Instruments, Inc., Winooski, VT, USA). For the clonal colony-forming assay, the transfected HT29 and SW480 cells (500 cells/well) were cultured in 6-well plates with complete medium at 37°C for 14 days (complete medium was changed every three days). The colonies were then fixed with methanol, stained with Giemsa dye solution, and counted.

Migration and invasion assay

For the migration assay, HT29 and SW480 cells were transfected for 48 hrs and then cultured in serum-free medium (HT-29 cells were cultured in McCoy's 5A medium; SW480 cells were maintained in Leibovitz's L-15 medium). The cells were seeded into the top of a 24-well Millipore Transwell chamber (Millipore Corporation, MA, USA), and complete medium containing 10% FBS was added to the lower chamber. After 24 hrs, migratory cells located on the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet solution (Sigma-Aldrich, Saint Louis, MO, USA). The migrated cells were photographed and counted. For the invasion assay, we coated the top of the 24-well Millipore Transwell

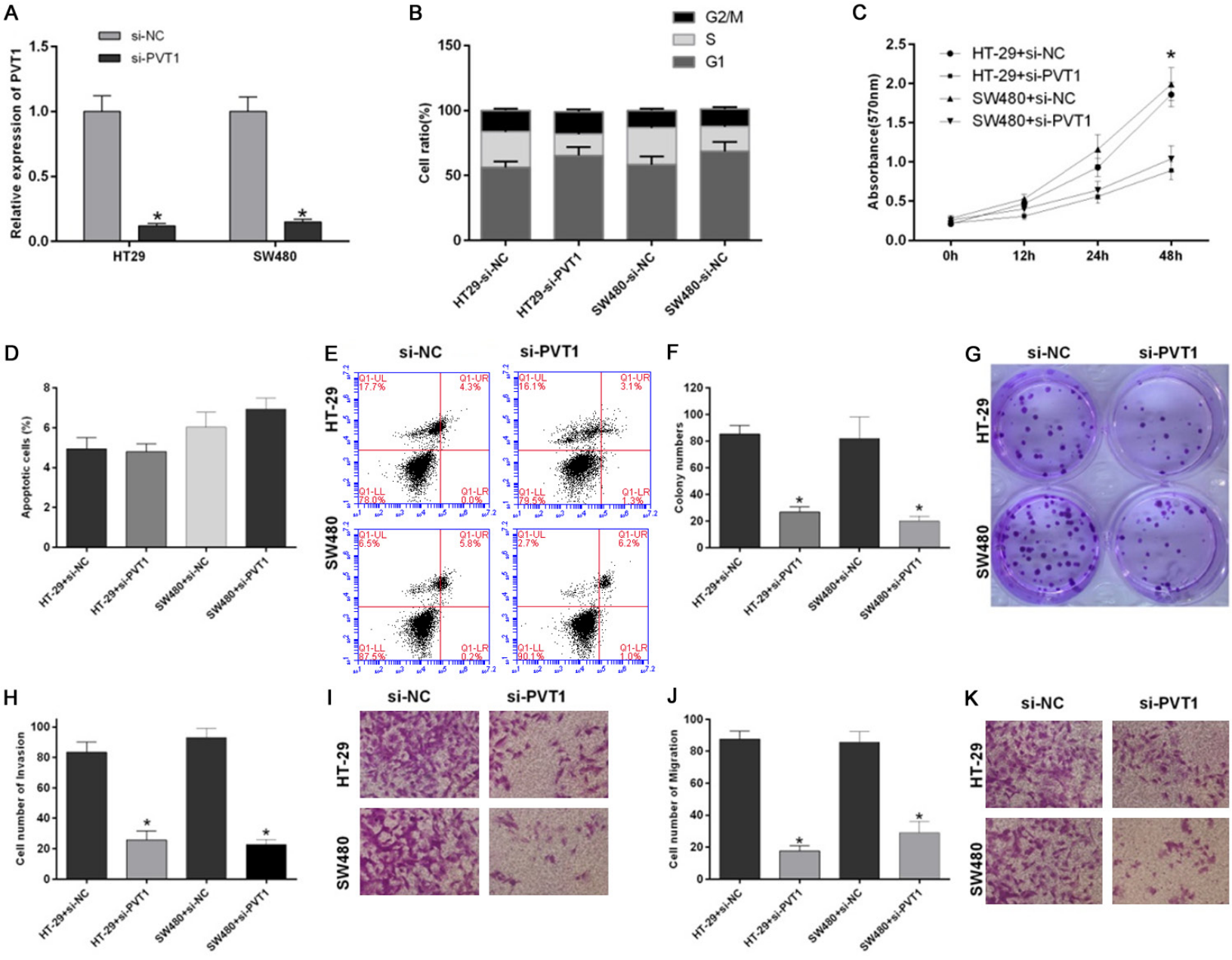
A
si-NC
si-PVT1
Relative expression of PVT1
HT29
SW480
B
G2/M
S
G1
Cell ratio(%)
HT29-si-NC
HT29-si-PVT1
SW480-si-NC
SW480-si-NC
C
HT-29+si-NC
HT-29+si-PVT1
SW480+si-NC
SW480+si-PVT1
Absorbance(570nm)
0h
12h
24h
48h
D
Apoptotic cells (%)
HT-29+si-NC
HT-29+si-PVT1
SW480+si-NC
SW480+si-PVT1
E
si-NC
si-PVT1
HT-29
SW480
F
Colony numbers
G
si-NC
si-PVT1
HT-29
SW480
H
Cell number of Invasion
I
si-NC
si-PVT1
HT-29
SW480
J
Cell number of Migration
K
si-NC
si-PVT1
HT-29
SW480

Figure 2. Silencing PVT1 expression with siRNA inhibits the progression of CRC. HT29 and SW480 cells were transfected with PVT1 siRNA or negative control (NC) for 48 hrs using Lipofectamine 3000. A. qRT-PCR was used to measure the expression level of PVT1 in the treated HT29 and SW480 cells (*P < 0.05, vs. NC group). B. The cycle distribution of the treated HT29 and SW480 cells was assessed by flow cytometry. C. The proliferation of the treated HT29 and SW480 cells was measured with a CCK-8 assay (*P < 0.05, vs. NC group). D, E. Apoptotic HT29 and SW480 cells were detected by Annexin V-FITC/PI staining (P > 0.05, vs. NC group). F, G. A colony-forming assay was performed to further assess cell proliferation (*P < 0.05, vs. NC group). H, I. The invasiveness of treated HT29 and SW480 cells was measured with Transwell assay, magnification 200 ×. The number of invasive cells was counted and calculated as a percentage of the total cells (*P < 0.05, vs. NC group). J, K. The migration of treated HT29 and SW480 cells was measured with a Transwell assay, magnification 200 ×. The number of migratory cells was counted and calculated as a percentage of the total cells (*P < 0.05, vs. NC group).

Chamber with diluted Matrigel (BD Biosciences) for 1 hr at 37°C. The remaining steps of the assay are similar to those of the migration assay.

Flow cytometric analysis of the cell apoptosis

The treated HT29 and SW480 cells were washed twice, treated with trypsin, and fixed. The cell pellet was stained with FITC-Annexin V and Propidium iodide (PI), and flow cytometry was conducted within 5 min. The images apoptotic cells were obtained using a FACS Calibur system (BD Biosciences), and the data were analyzed using the FlowJo software (Tree Star Corp, Ashland, OR).

Tumor formation in nude mice

All experiments were approved by the Institutional Committee and carried out based on the guidelines of the Institutional Animal Care and Use Committee. Treated HT29 and SW480 cells (1×10^7 cells in 100 µl) were subcutaneously injected into nude mice, and the tumor volumes and weights were measured and calculated 0, 8, 16, 24, 32, 40, and 48 days after injection; the mice were sacrificed after 48 days.

Statistical analysis

Differences between groups were analyzed with Student's *t*-test using the SPSS 20.0 software (SPSS; Chicago, IL, USA). All experiments were performed in triplicate, and all data are expressed as the mean ± S.D. P < 0.05 indicated a significant difference.

Results

LncRNA-PVT1 is up-regulated in human CRC

We collected 20 cases of human CRC tissues and adjacent noncancerous tissues. The relative expression level of PVT1 was measured by qRT-PCR, which showed that PVT1 was highly expressed in CRC tissues compared with adjacent noncancerous tissues (P < 0.001) (**Figure 1A**). To confirm this change in CRC cells, we measured the PVT1 expression level in five CRC cell lines (HT29, HCT116, SW480, SW620, and LOVO) and normal human colon epithelial cells (FHC) by qRT-PCR. We found that the expression level of PVT1 was dramatically higher in CRC cell lines (HT29, HCT116, SW480, SW620, and LOVO) than FHC cells (**Figure 1B**).

Silencing PVT1 expression inhibits the progression of CRC

To explore the effects of PVT1 expression on the progression of CRC, HT29 and SW480 cells were transfected with PVT1 siRNAs and negative control (NC) for 48 hrs using Lipofectamine 3000. qRT-PCR demonstrated that PVT1 was efficiently silenced in HT29 and SW480 cells by siRNAs (P < 0.05) (**Figure 2A**). The cell cycle distribution of the treated HT29 and SW480 cells was then assessed by flow cytometry. We discovered that silencing PVT1 expression with siRNAs inhibited growth by arresting cells at the G1-to-S-phase transition (**Figure 2B**). Similarly, a CCK8 assay revealed that silencing PVT1 expression with siRNAs inhibited the proliferation of HT29 and SW480 cells (P < 0.05) (**Figure 2C**). To further explore the effect of PVT1 on CRC, apoptosis was assessed using Annexin V-FITC/PI staining. To our surprise, transfection with PVT1 siRNA do not significantly affected cell apoptosis in HT29 and SW480 cells compared with the control group (**Figure 2D**, **2E**). Then, a colony forming assay was performed to further assess cell proliferation. We also found that silencing PVT1 with siRNA decreased the proliferation of HT29 and SW480 cells (P < 0.05) (**Figure 2F**, **2G**). These results revealed that silencing PVT1 inhibited cell proliferation but do not affected cell apoptosis.

To examine the effect of silencing PVT1 on the metastasis of CRC cells, the migration and

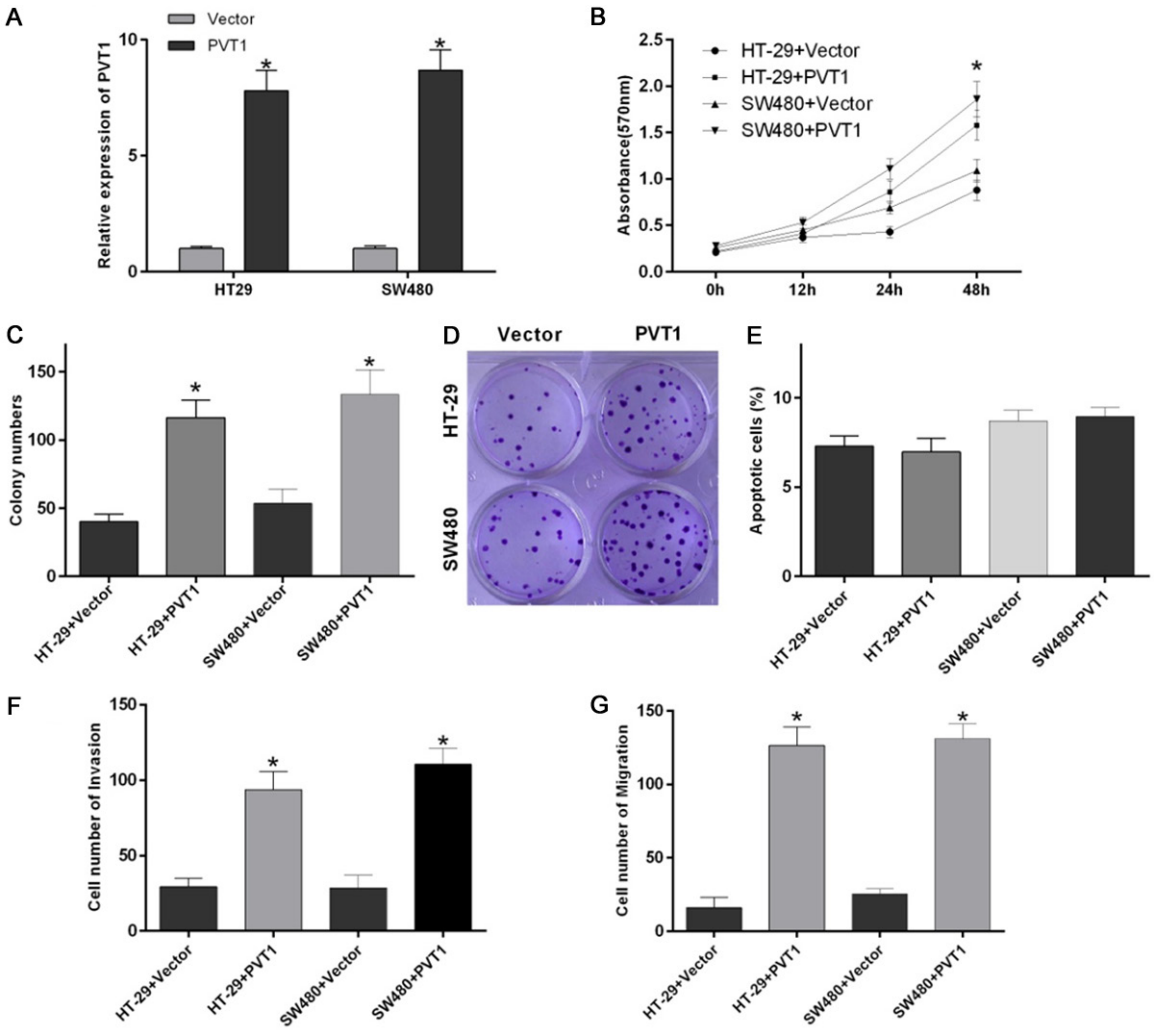

Figure 3. Overexpression of PVT1 promotes the progression of CRC. HT29 and SW480 cells were transfected with PVT1 overexpression vector or the control vectors. A. The expression level of PVT1 was measured by qRT-PCR. B. A CCK-8 assay was performed to assess cell proliferation. C, D. Cell proliferation was further analyzed with a colony-forming assay. E. Apoptosis was assessed using Annexin V-FITC/PI staining ($P > 0.05$, vs. vector group). F. Invasiveness was assessed with a Transwell invasion assay, magnification 200 ×. G. Migration was analyzed with a Transwell migration assay, magnification 200 ×. All data were analyzed with Student's *t*-test (*$P < 0.05$, vs. the control group).

invasion of HT29 and SW480 cells were assessed using a Transwell assay. The results indicated that the migration of HT29 and SW480 cells transfected with PVT1 siRNAs was significantly decreased compared to the control group (**Figure 2H**, **2I**). Likewise, the results also indicated silencing PVT1 with siRNA inhibited the invasion of HT29 and SW480 cells (**Figure 2J**, **2K**).

Overexpression of PVT1 promotes the progression of CRC

To further explore the effects of PVT1 on the progression of CRC, HT29 and SW480 cells were transfected with PVT1 vector or control vector. qRT-PCR indicated that PVT1 was efficiently over-expressed by the over-expression vector in HT29 and SW480 cells ($P < 0.05$) (**Figure 3A**). A CCK-8 assay was performed assess the proliferation of HT29 and SW480 cells, which showed overexpressing PVT1 promoted cell proliferation ($P < 0.05$) (**Figure 3B**). A colony-forming assay also demonstrated that the overexpression of PVT1 accelerated cell proliferation ($P < 0.05$) (**Figure 3C**, **3D**). However, the overexpression of PVT1 do not affected cell apoptosis in HT29 and SW480 cells (**Figure 3E**). To further evaluate the role of PVT1 in the metastasis of CRC cells, a Transwell

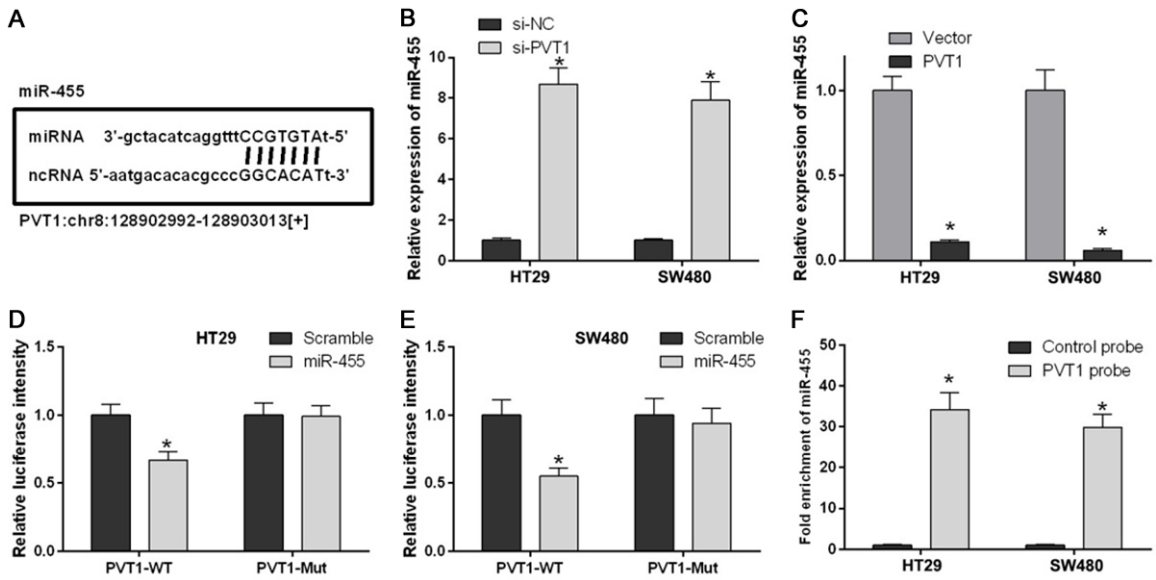

Figure 4. PVT1 serves as a ceRNA and negatively regulate miR-455 expression in CRC. A. The WT PVT1 binding sequence of the miR-455 gene was predicted by TargetScan, miRDB and microrna.org. B. qRT-PCR was used to measure the expression of miR-455 in HT29 and SW480 cells transfected with PVT1 or NC siRNA for 48 hrs (*P < 0.05, vs. NC group). C. qRT-PCR was used to measure the expression of miR-455 in HT29 and SW480 cells transfected with PVT1 or the control vector (*P < 0.05, vs. the control group). D. Normalized luciferase intensity of HT-29 cells. The wild-type PVT1 or mutant PVT1 vector was co-transfected into HT29 cells with miR-455. The luciferase activity was measured with a luciferase report gene assay and normalized to the Renilla luminescence (*P < 0.05). E. Normalized luciferase intensity of SW480 cells as described in C (*P < 0.05). F. miR-455 was significantly enriched by PVT1 in HT29 and SW480 cells transfected with a PVT1 probe (*P < 0.05, vs. the control group).

assay was used to assess the migration and invasion of HT29 and SW480 cells, which demonstrated that the overexpression of PVT1 promoted cell invasion (P < 0.05) (**Figure 3F**) and migration (P < 0.05) (**Figure 3G**). These results indicate that PVT1 overexpression promotes cell proliferation, migration and invasion.

PVT1 serves as a ceRNA and negatively regulates miR-455 expression in CRC

A bioinformatics analysis predicted miR-455 as a putative target of PVT1 (**Figure 4A**). To confirm this prediction, qRT-PCR was first used to measure the expression level of miR-455 in HT29 and SW480 cells transfected with PVT1 or NC siRNA and PVT1 overexpression or the control vector. The results indicated that silencing PVT1 with siRNA increased the expression level of miR-455 (P < 0.05) (**Figure 4B**), and the overexpression of PVT1 decreased the expression level of miR-455 (P < 0.05) (**Figure 4C**). Second, a luciferase reporter assay suggested that PVT1 directly interacts with miR-455 in CRC cells (P < 0.05) (**Figure 4D**, **4E**). Furthermore, we constructed a PVT1 probe to mimic cellular PVT1 in CRC cells, and we found that miR-455 was significantly enriched by the PVT1 probe in HT29 and SW480 cells (P < 0.05) (**Figure 4F**).

Blockage of miR-455 rescued the si-PVT1 induced CRC cell carcinogenesis inhibition

qRT-PCR was used to assess the effect of miR-455 inhibitor on CRC cells. Specifically, miR-455 was upregulated in HT29 and SW480 cells transfected with PVT1 siRNA compared to the NC group, and this upregulation was rescued by miR-455 inhibitor (P < 0.05) (**Figure 5A**). In terms of function, we discovered that silencing PVT1 with siRNA markedly inhibited the proliferation of CRC cells (P < 0.05), which was rescued by transfection with miR-455 inhibitor (P < 0.05) (**Figure 5B**, **5C**). Moreover, our results also showed that miR-455 inhibitor reversed PVT1 siRNA-mediated CRC invasion and migration (P < 0.05), but PVT1/miR-455 axis do not affected cell apoptosis (**Figure 5D-F**).

Silence of PVT1 expression suppresses the growth of CRC tumor by miR-455 in vivo

To explore the effect of PVT1 on tumorigenesis *in vivo*, HT29 cells were transfected with PVT1

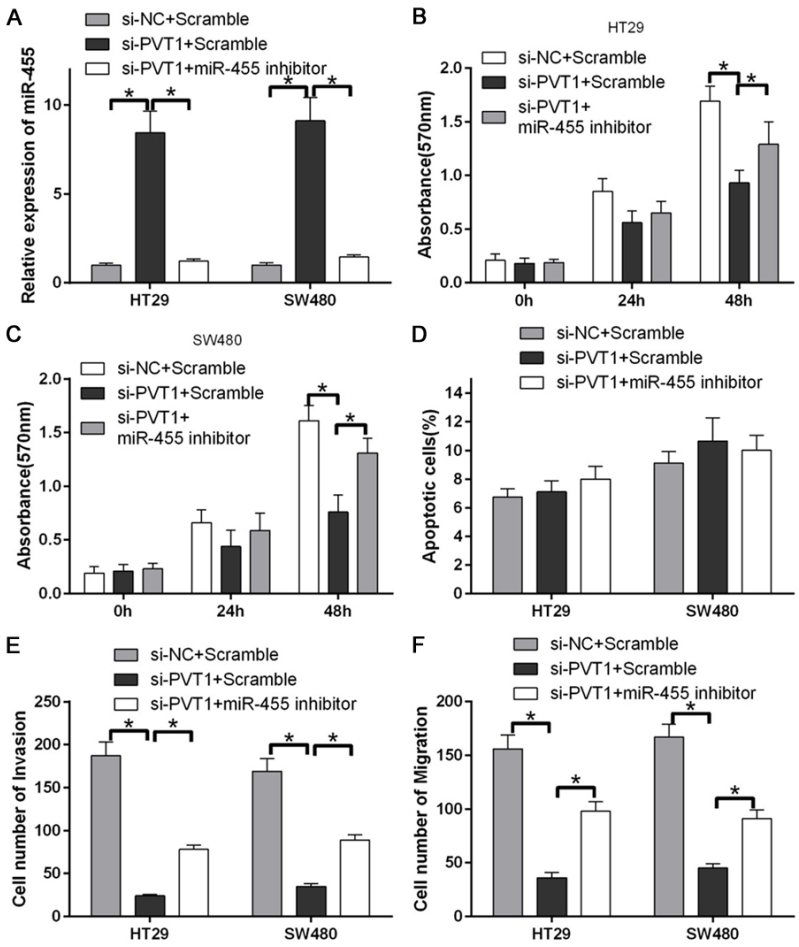

Figure 5. Silencing PVT1 with siRNA inhibits proliferation, migration and invasion via miR-455 in CRC cells. HT29 and SW480 cells were transfected with PVT1 or NC siRNA for 48 hrs and then transfected with miR-455 inhibitor using Lipofectamine 3000. A. The relative miR-455 expression level was measured by qRT-PCR, $*P < 0.05$ compared with the control group. B. The proliferation of transfected HT29 cells was assessed with a CCK-8 assay at 0, 24, and 48 hrs ($*P < 0.05$, vs. NC group). C. The proliferation of transfected SW480 cells was assessed with a CCK-8 assay at 0, 24, and 48 hrs ($*P < 0.05$, vs. NC group). D. Apoptotic transfected HT29 and SW480 cells were detected with Annexin V-FITC/PI staining ($P > 0.05$, vs. NC group). E. A Transwell invasion assay was performed to examine the effect of PVT1 and miR-455 on the invasiveness of HT29 and SW480 cells ($*P < 0.05$). F. A Transwell migration assay was used to analyze the effect of PVT1 and miR-455 on the migration of HT29 and SW480 cells ($*P < 0.05$).

siRNA and NC for 48 hrs and then transfected with miR-455 inhibitor. The transfected HT29 cells were subcutaneously implanted into nude mice, and the dissected tumor volumes and weights were measured 0, 8, 16, 24, 32, 40, and 48 days after injection. The results showed that silencing PVT1 with siRNA significantly suppressed the growth of CRC tumors, and this suppression was reversed by miR-455 inhibitor ($P < 0.05$) (**Figure 6A**, **6B**). Dissected tumors were photographed 48 days after injection (**Figure 6C**).

RUNX2 up-regulates PVT1 expression

To further investigate the regulatory relationship between RUNX2 and PVT1, HT29 cells were transfected with RUNX2 vector and control vector, and qRT-PCR was used to measure the relative RUNX2 mRNA expression level (**Figure 7A**). Western blotting was used to measure the relative RUNX2 protein expression level (**Figure 7B**). Importantly, our data show that the overexpression of RUNX2 significantly up-regulated the mRNA expression level of PVT1 ($P < 0.05$) (**Figure 7C**).

miR-455 directly targets RUNX2 in CRC cells

Moreover, we found that the 3'UTR of RUNX2 contains a potential binding site for miR-455. Thus, we constructed a wild-type and mutant 3'UTR of RUNX2 to perform a luciferase reporter assay. The results show that miR-455 directly targeted RUNX2 (**Figure 8A**), and the overexpression of miR-455 inhibited RUNX2 protein and mRNA expression (**Figure 8B**, **8C**). We also previously identified RAF-1 as a direct target of miR-455. Taken together, these results indicate a feedback loop and a complex regulatory network among RUNX2/PVT1/miR-455 (**Figure 9**) that implicates this axis as a potential therapeutic target for the treatment of CRC.

Discussion

Several studies have discovered lncRNAs that serve as oncogenes or cancer suppressor genes, are involved in tumorigenesis and metastasis, and can help diagnose and predict the prognosis of cancer [31, 32]. Moreover, PVT1

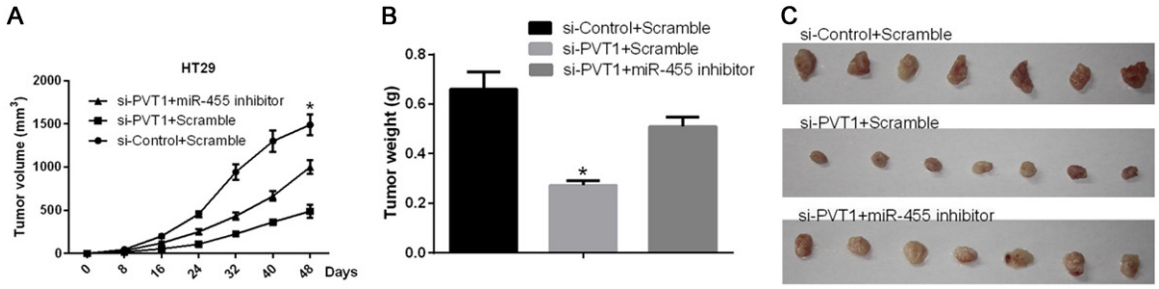

Figure 6. Silencing PVT1 expression suppresses the growth of CRC tumors via miR-455 *in vivo*. HT29 cells were transfected with PVT1 or NC siRNA for 48 hrs and then transfected with miR-455 inhibitor before being subcutaneously injected into nude mice. A. The dissected tumor volume was measured 0, 8, 16, 24, 32, 40, and 48 days after injection (*$P < 0.05$). B. The tumors were weighed 0, 8, 16, 24, 32, 40, and 48 days after injection (*$P < 0.05$). C. Dissected tumors were photographed 40 days after injection.

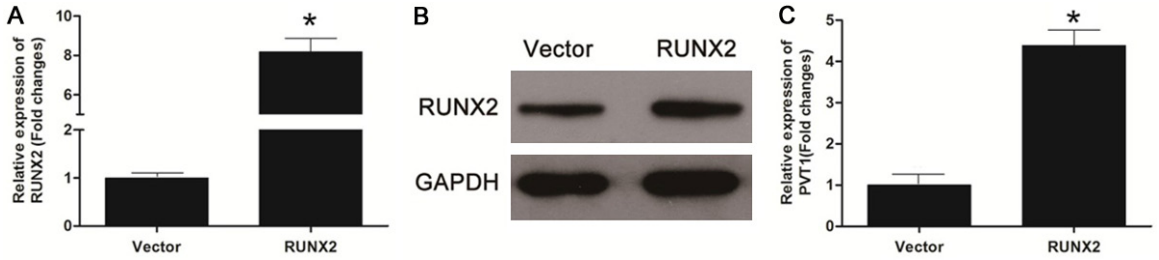

Figure 7. RUNX2 up-regulates PVT1 expression. HT29 cells were transfected with RUNX2 vector and control vector using Lipofectamine 3000. A. The relative RUNX2 mRNA expression level was detected by qRT-PCR, *$P < 0.05$ compared with the control group. B. The relative RUNX2 protein expression level was detected by Western blotting; GAPDH served as an internal reference. C. The mRNA expression level of PVT1 was measured by qRT-PCR (*$P < 0.05$, vs. the control group).

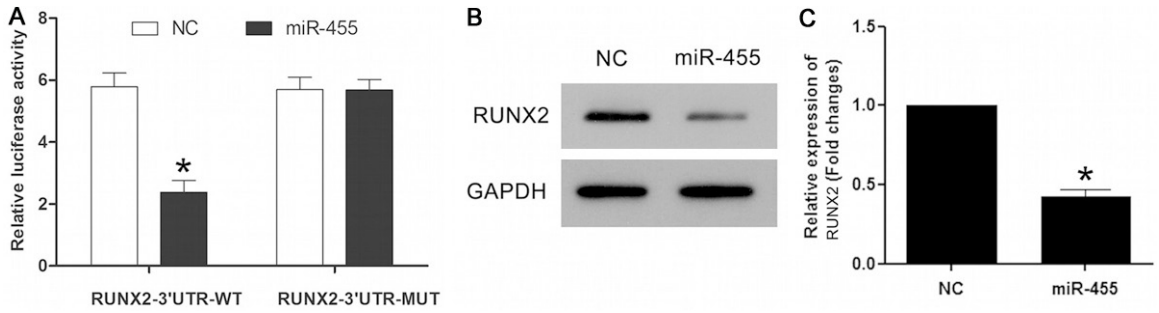

Figure 8. miR-455 directly targets RUNX2 in HT-29 cells. A. Normalized luciferase intensity of HT-29 cells. The wild-type RUNX2 or mutant RUNX2 vector was co-transfected into HT29 cells with miR-455. The luciferase activity was detected with the luciferase report gene assay and normalized to the Renilla luminescence (*$P < 0.05$). B. Western blot to detect the RUNX2 protein level in miR-455-transfected HT29 cells. C. qRT-PCT assay to detect the RUNX2 mRNA level in miR-455-transfected HT29 cells (*$P < 0.05$).

has been identified as a variant translocation in plasmacytomas [33], and recent studies also revealed that the overexpression of PVT1 accelerates invasion by promoting epithelial-to-mesenchymal transition in esophageal cancer [34]. Furthermore, PVT1 affects the cell cycle and apoptosis of prostate cancer and may serve as a non-invasive biomarker in men of African descent [35]. Our results indicated that PVT1 is up-regulated in human CRC, and silencing PVT1 expression with siRNA inhibits cell proliferation, migration and invasion but not affected apoptosis *in vitro*. Conversely, the overexpression of PVT1 accelerates cell proliferation, migration and invasion *in vitro*. Therefore, we speculated that PVT1 significantly affects the development and progression of CRC.

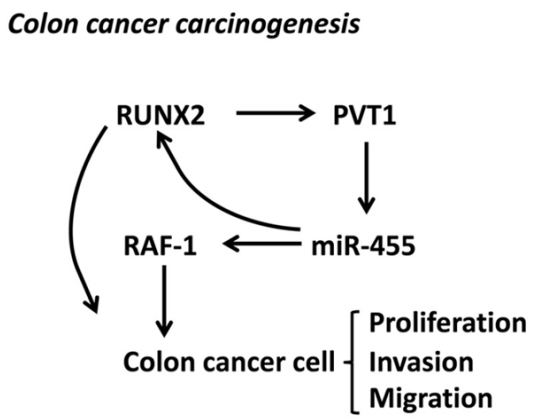

Figure 9. The regulatory network involving PVT1 in CRC. RUNX2 increased the expression level of PVT1, then PVT1 decreased the expression level of miR-455, miR-455 inhibits proliferation, migration and invasion by targeting RAF-1 and RUNX2.

miRNAs are ncRNAs that are approximately 20 nucleotides in length and regulate gene expression by inhibiting translation or degrading mRNA transcripts [36]. Numerous studies revealed that miRNAs are closely related to oncogenesis [37], and recent studies have shown that miR-455 specifically serves as a tumor suppressor in gastric cancer [28]. Moreover, miR-455 has a crucial function in mesenchymal cell differentiation and osteoarthritis [38], and it inhibits non-small cell lung cancer by targeting ZEB1 [39]. A growing body of evidence also indicates that lncRNAs act as competitive endogenous RNAs (ceRNAs) and participate in the occurrence and development of various diseases [40-42]. Some studies have characterized the lncRNA-associated ceRNA network in human ovarian cancer [43] and lung adenocarcinoma [44], and the role of lncRNAs as ceRNAs has also been analyzed in pulmonary fibrosis [45]. For example, lncRNA H19 and PTCSC3 have been found to affect the occurrence and development of cancers by acting as miRNA sponges [46, 47]. Accordingly, we revealed that PVT1 serves as a ceRNA that negatively regulates miR-455 expression in CRC. Meanwhile, silencing PVT1 with siRNA inhibited proliferation, migration and invasion via miR-455 in CRC cells. Moreover, silencing PVT1 expression suppressed the growth of CRC tumors via miR-455 *in vivo*.

Runt-related transcription factors (Runxs), one of the metazoan genes, is involved in various biologic processes, such as tumor suppression, bone development, and neurogenesis [48]. The Runx family consists of three members (Runx1, Runx2, and Runx3). Specifically, Runx has been shown to inhibit c-Myc expression in a DNA-binding and C-terminally dependent manner [49], and Runx2 has been related to the risk of asthma [50]. Furthermore, miR-455 has been shown to inhibit the migration and invasion of hepatocellular carcinoma by targeting Runx2 [51]. Our data indicated that RUNX2 increases the expression level of PVT1 in CRC, whereas miR-455 inhibited RUNX2, which constitutes a feedback loop among RUNX2/PVT1/miR-455.

RAF-1 is a serine/threonine-specific kinase that participates in the MAPK/ERK signal transduction pathway. Specifically, RAF-1 can activate the protein kinases MEK1 and MEK2, which also activate the serine/threonine-specific protein kinases ERK1 and ERK2 [52]. Moreover, activated RAF-1 affects the regulation of genes involved in cell differentiation, cycle, apoptosis, and migration [53]. We reported that miR-455 suppresses the proliferation and invasion of colorectal cancer via RAF [54]. Taken together, our data construct a complex regulatory network involved in CRC tumorigenesis that includes lncRNA PVT1, the transcription factor RUNX2, miR-455 and its target gene RAF-1 (**Figure 9**). In summary, our study identified the RUNX2/PVT1/miR-455/RAF-1 axis as a new potential target for CRC therapy.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 8160-2578), Shandong Province Natural Science Foundation (No. ZR2017MH129), China Pstdoctoral Science Foundation (No. 2017M611177) and the Medical Science and Technology Development Plans of Shandong (No. 2015W-S0152).

Disclosure of conflict of interest

None.

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