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LncRNA PCAT-1 regulated cell proliferation, invasion, migration and apoptosis in colorectal cancer through targeting miR-149-5p

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Abstract. – OBJECTIVE: Emerging studies suggested that lncRNA plays an important role in cell progression of multiple cancers. In CRC, the function of some lncRNAs has been verified to be related to cell proliferation, apoptosis, migration and invasion, providing a crucial theoretical basis for the treatment of colorectal cancer. Due to the complexity of the regulation mechanism of cell growth, the regulation mechanism related to lncRNA still needs to be further studied in CRC.

PATIENTS AND METHODS: The qRT-PCR assay was used to carry out the expression of prostate cancer-associated ncRNA transcripts 1 (PCAT-1) and miR-149-5p. The Western blots were used to measure the protein expression of CDK4, Cyclin D1, MMP-2, MMP-9, Bcl-2, Bax and β-actin. Additionally, flow cytometry and MTT assay were used to assess cell apoptosis and cell proliferation, respectively. Moreover, transwell assay was applied to measure the ability of cells migrated and invasion in CRC. Luciferase reporter assay was performed to detect luciferase activities.

RESULTS: In this study, lncRNA PCAT-1 expression was significantly upregulated in CRC cells and tissues. More than that, knockdown of lncRNA PCAT-1 inhibited cell proliferation, migration and invasion while promoted cell apoptosis in CRC cells. Of note, lncRNA PCAT-1 directly targeted miR-149-5p and miR-149-5p expression was significantly downregulated in CRC cells and tissues. Moreover, miR-149-3p reversed the suppressive effects of PCAT-1 on the cell growth of CRC cells.

CONCLUSIONS: Our study demonstrated that LncRNA PCAT-1 regulated cell proliferation, invasion, migration and apoptosis in colorectal cancer through targeting miR-149-5p and provided a new regulatory mechanism of CRC.

Key Words:
Colorectal cancer, LncRNA PCAT-1, MiR-149-5p, Cell growth, Apoptosis.

Introduction

Colorectal cancer (CRC) is a common malignant tumor with a second leading cause of cancer[1,2]. Therefore, improving the understanding of molecular regulation of CRC is an essential role in the treatment of CRC.

lncRNA is a member of ncRNA that is more than 200 nt in length[3]. The function of lncRNA, usually as a ceRNA, is targeting miRNA involved in the regulation of cancer cell progression[4-6]. miRNAs are 18-22 nt in length of ncRNAs[7]. Therefore, ncRNAs play a crucial role in tumor formation and cancer development. Accumulating studies have shown that differentially expressed lncRNAs in cancer are closely related to the proliferation, differentiation and apoptosis of cancer cells[8-10]. For example, overexpression of lncRNA PVT1 promoted cell growth and tumorigenesis in lung cancer[11]. Promotion of lncRNA BANCR regulated clinical progression and poor prognosis of gastric cancer cells[12]. Prostate cancer-associated ncRNA transcripts 1 (PCAT-1) is

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a lncRNA with a length of ~1900 nt and has been revealed as an oncogenic in cancers[13]. A number of reports indicate that PCAT-1 is highly expressed in a variety of cancer cells, including non-small cell lung cancer, prostate cancer, hepatocellular carcinoma and colorectal cancer[14-17]. However, the function and potential regulatory network of PCAT-1 in colorectal cancer has been not fully explored.

Consistent with previous studies, in this paper, we found that PCAT-1 is highly expressed in CRC tissues and cells. Furthermore, we further studied the biological functions and potential regulatory mechanisms of PCAT-1 in CRC. We found that PCAT-1 regulates cell growth and apoptosis of CRC by modulating the expression of miR-149-5p. Therefore, PCAT-1 plays an essential role in the pathogenesis of CRC.

Patients and Methods

Patients and Tissues

55 pairs of colorectal cancer tissues and adjacent tissues were collected from 55 patients, who underwent radical resection at the Department of Biochemistry and Molecular Biology, Medical College of Yan'an University. All fresh tissues were stored at -80°C for RNA extraction. All patients were not treated with radiotherapy or chemotherapy. This study was approved by the Research Ethics Committee of the Department of Biochemistry and Molecular Biology, Medical College of Yan'an University. Informed consent was acquired from all patients.

Cell Culture and Transfection

Human CRC cell lines (SW480, SW620, LOVO and HT29) and normal cell line (NCM460) were purchased from Cell Bank of Chinese Academy of Sciences. All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 5% CO_2.

Si-con, si-PCAT-1, pcDNA, pcDNA-PCAT-1, anti-miR-con and anti-miR-149-3p were purchased from Genepharma (Shanghai, China). The vectors and oligos were transfected into SW480 and LOVO cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Quantitative Real-Time PCR (qRT-PCR) Assay

Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The TaqMan® MicroRNA Real-time PCR Assay reagents and primers (Applied Biosystems; Foster City, CA, USA) were used to detect the expression of miR-149-5p. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used to make RNA reversely transcribed into cDNA and then SYBR® Premix Ex Taq™ reagent (TaKaRa, Otsu, Shiga, Japan) was used to detect the expression of PCAT-1. The primers used in this study were: PCAT-1 forward: 5'-AATGGCATGAACCTGGGAGGCG-3' and reverse: 5'-GGCTTTGGGAAGTGCTTTGGAG-3', GAPDH forward: 5'-CACCCACTCCTCCACCTTTG-3' and reverse: 5'-CCACCACCCTGTTGCTGTAG-3', miR-149-5p forward: TCTGGCTCCGTGTCTTCACTCCC and reverse: TATGGTTGTTCTGCTCTCTGTGTC, U6 forward: CTCGCTTCGGCAGCACA and reverse: AACGCTTCACGAATTTGCGT.

Western Blot

Total proteins were lysed in RIPA buffer (Beyotime, Shanghai, China). The proteins were loaded onto the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to run and then the gel was transferred onto the polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with the primary antibodies against CDK4, Cyclin D1, MMP-2, MMP-9, Bcl-2, Bax and β-actin (Abcam, Cambridge, MA, USA) at 4°C overnight. After that, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 37°C. Finally, the protein blots were detected using the Pierce™ ECL A and analyzed by Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

MTT Assay

Cells (10^4 cells/well) were seeded in 96-well cell culture plates (Corning Inc., Corning, NY, USA) and incubated for 24 h at room temperature. After that, 20 ul MTT was added into each well and incubated for 4 h at 37°C. Next, 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) were also added into each well at 37°C with 5% of CO_2 for 3 h. Then, cell viability was measured using a spectrophotometric microplate reader (Beyotime Institute of Biotechnology, Haimen, China) at wavelength of 490 nm.

Cell Migration and Invasion

Transwell assay (Corning, Corning NY, USA) was applied to assess cell migration and invasion of CRC. For invasion of CRC, Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was needed, while cell migration didn't need Matrigel. Transfected cells (1×10^6) were seeded into the upper chamber. DMEM medium with 10% FBS was added into the lower chamber. After 24 h, migrated and invasion cells in the lower chamber were fixed with 75% methanol, and stained with crystal violet. The Leica DM3000 microscope (Leica, Wetzlar, Germany) was performed to calculate the number of migrated and invasion cells

Luciferase Reporter Assay

The sequences of PCAT-1 3' UTR containing binding sites of miR-149-5p or PCAT-1 3' UTR containing mutate sites of miR-149-5p were inserted into psiCHECK-2 luciferase vector (Promega, Madison, WI, USA) to constructed PCAT-1-WT or PCAT-1-MUT luciferase reporter, respectively. Next, PCAT-1-WT or PCAT-1-MUT was cotransfected with miR-con or miR-149-5p into SW480 and LOVO cells. After transfection for 48 h, luciferase activities were measured using a Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA).

Cell Apoptosis

Cell apoptosis was detected using flow cytometric assay with Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Briefly, SW480 and LOVO cells transfected with si-con or si-PCAT-1 were collected and re-suspended in binding solution (200 µl). Then, Annexin V-FITC (10 µl) and propidium iodide (PI) (10 µl) were used to stain cells at 37°C for 15 min. The apoptosis rate of SW480 and LOVO cells was analyzed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical Analysis

The data were performed as mean ± SD (standard deviation) as indicated. Student's *t*-test was applied to compare the difference between two groups, and one-way analysis of variance followed by Tukey's post-hoc test was employed to perform the difference analysis among multiple groups (at least 3 groups). The statistical analyses were displayed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). A p-value <0.05 was considered as statistically significant.

Results

PCAT-1 Was Upregulated and miR-149-5p was Downregulated in CRC Tissues and Cell Lines

To explore the function of PCAT-1 and miR-149-5p, qRT-PCR analysis was used to determine the expression of PCAT-1 and miR-149-3p in 55 pairs of CRC tissues and adjacent tissues. The results showed that PCAT-1 expressed high while miR-149-5p expressed low in CRC tissues (Figure 1A and 1B). Additionally, correlation analysis determined that the expression of PCAT-1 was significantly negative related to miR-149-5p expression (Figure 1C). More than that, high expression of PCAT and low expression of miR-149-5p were measured in SW480, SW620, LOVO and HT29 cell lines as compared with NCM460 cell line (Figure 1D and 1E). These data indicated that the expression of PCAT-1 was higher, while the expression of miR-149-5p was lower in CRC tissues and cells than that in normal tissues and cells.

Knockdown of PCAT-1 Inhibited Cell Viability in CRC Cells

To explore the effect of PCAT-1 on cell proliferation in CRC, the si-lnc PCAT-1 and si-con were transfected into SW480 and LOVO cell lines and si-lnc PCAT-1 significantly inhibited PCAT-1 expression (Figure 2A and 2B). The MTT assay demonstrated that inhibition of PCAT-1 decreased the viability of SW480 and LOVO cells (Figure 2C and 2D). In addition, Western blot assay showed that CDK4 and Cyclin D1 protein was inhibited by knockdown of PCAT-1 in SW480 and LOVO cells (Figure 2E to 2H). Thus, knockdown of PCAT-1 inhibited the viability of CRC cells.

Cell Migration and Invasion Were Decreased by Silencing PCAT-1 Expression in CRC Cells

To determine whether PCAT-1 has any effect on cell migration and invasion in CRC, transwell assay was used to detect cell number of migrated and invasive of CRC cells. As shown in Figure

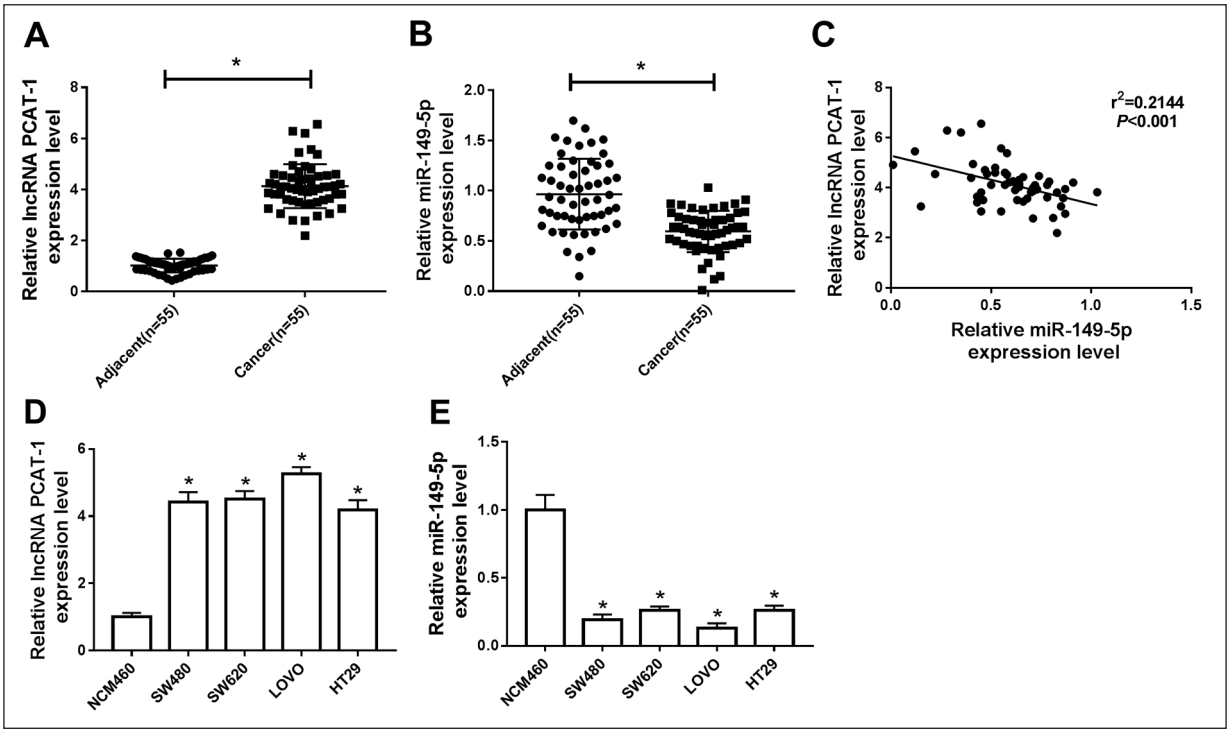

Figure 1. PCAT-1 was upregulated and miR-149-5p was downregulated in CRC tissues and cell lines. **(A-B)** The expression of PCAT-1 and miR-149-5p in CRC tissues and adjacent tissues was detected using qRT-PCR. **(C-D)** Pearson's correlation analysis was used to determine the relationship between PCAT-1 and miR-149-5p in CRC tissues. **(D-E)** The expression of PCAT-1 and miR-149-5p in CRC cell lines (SW480, SW620, LOVO and HT29) and normal cell line (NCM460) was detected using qRT-PCR. *$p<0.05$.

3A to 3D, cell migration and invasion in si-lnc PCAT-1 of SW480 and LOVO cells were remarkably lower than that in si-con and NC of SW480 and LOVO cells. Moreover, the protein expression of MMP-1 and MMP-9 was significantly reduced by downregulating PCAT-1 expression (Figure 3E and 3F). Taken together, knockdown of PCAT-1 attenuated cell migration and invasion in CRC.

Inhibition of PCAT-1 Promoted Cell Apoptosis in CRC Cells

Then, we used flow cytometry to detect cell apoptosis in transfected SW480 and LOVO cells. The results showed that si-lnc PCAT-1 transfection remarkably contributed to apoptosis rates of SW480 and LOVO cells (Figure 4A to 4D). In addition, Bcl-2 protein expression was inhibited while Bax protein expression was induced in si-lnc PCAT-1 group compared with NC and si-con groups (Figure 4E to 4H). Therefore, si-lnc PCAT-1 transfection could promote cell apoptosis in CRC.

MiR-149-3p Was a Target miRNA of PCAT-1

To further investigate the regulatory network of PCAT-1, miR-149-3p was predicted to be a target of PCAT-1 using DIANA tools (Figure 5A). The analysis of luciferase reporter assay determined that when the lncRNA PCAT-1-WT binds to miR-149-5p, luciferase activities were significantly reduced; however, lncRNA PCAT-1-MUT has no effect on luciferase activities in SW480 and LOVO cells (Figure 5B and 5C). In SW480 and LOVO cells, the expression of miR-149-5p was significantly reduced in cells transfected with si-lncRNA PCAT-1 compared with cells transfected with si-con. In addition, miR-149-5p expression induced by cells transfected pcDNA-lncRNA PCAT-1 in SW480 and LOVO cells (Figure 5D and 5E). These data indicated that PCAT-1 directly targeted miR-149-5p in CRC cells.

MiR-149-3p Reversed the Suppressive Effects of PCAT-1 on the Cell Growth of CRC Cells

To further explore whether PCAT-1 exerted its function by targeting miR-149-5p in CRC cells,

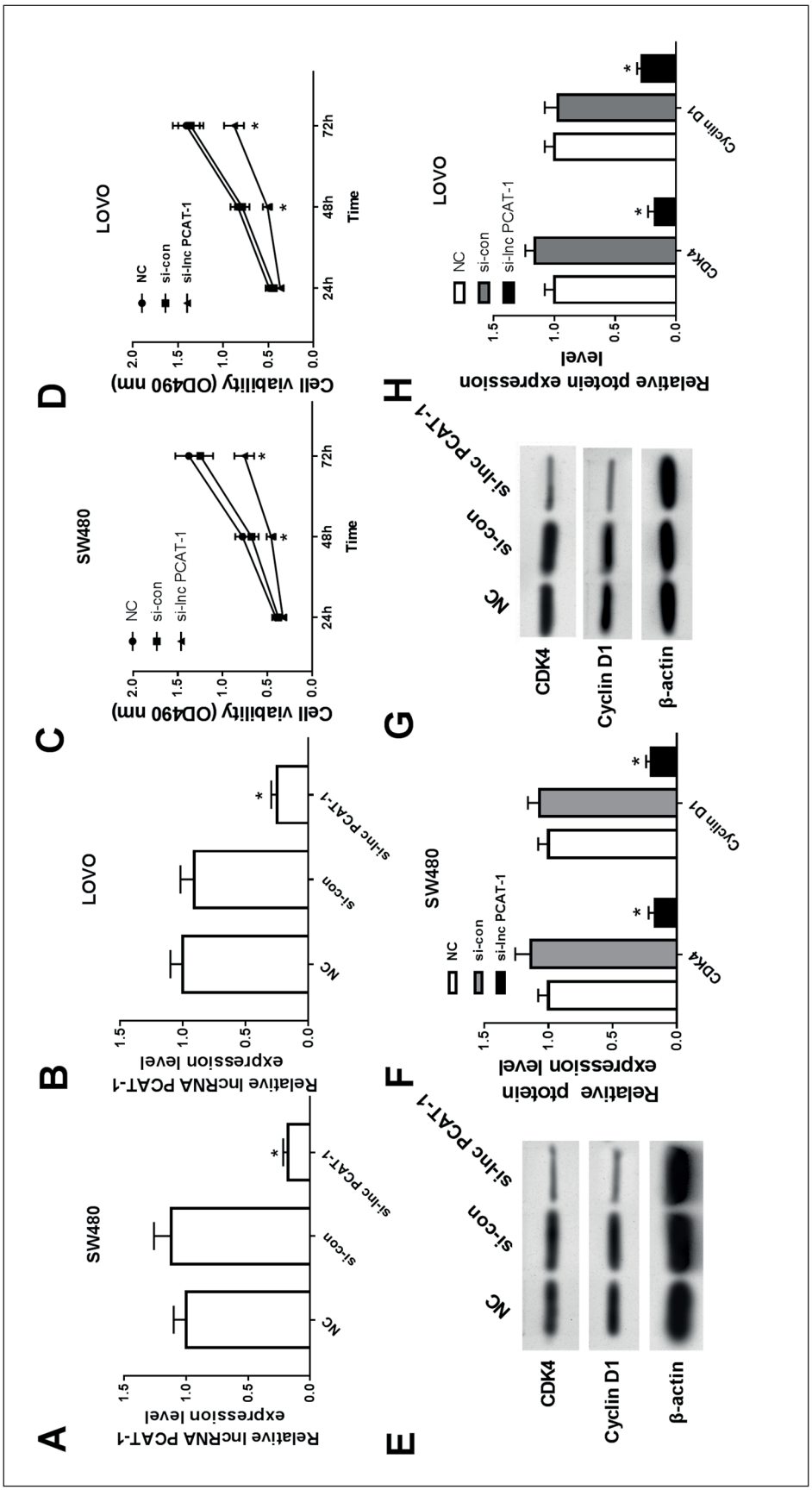

Figure 2. Knockdown of PCAT-1 inhibited cell viability in CRC cells. **(A-B)** The expression of miR-149-5p in SW480 and LOVO cells transfected with si-con and si-lncPCAT-1 was detected using qRT-PCR. **(C-D)** Cell proliferations were detected in SW480 and LOVO cells transfected with si-con and si-lncPCAT-1 using MTT assay. **(E-H)** The CDK4 and Cyclin D1 protein expression were measured in SW480 and LOVO cells transfected with si-con and si-lncPCAT-1 using western blot. *$p<0.05$.

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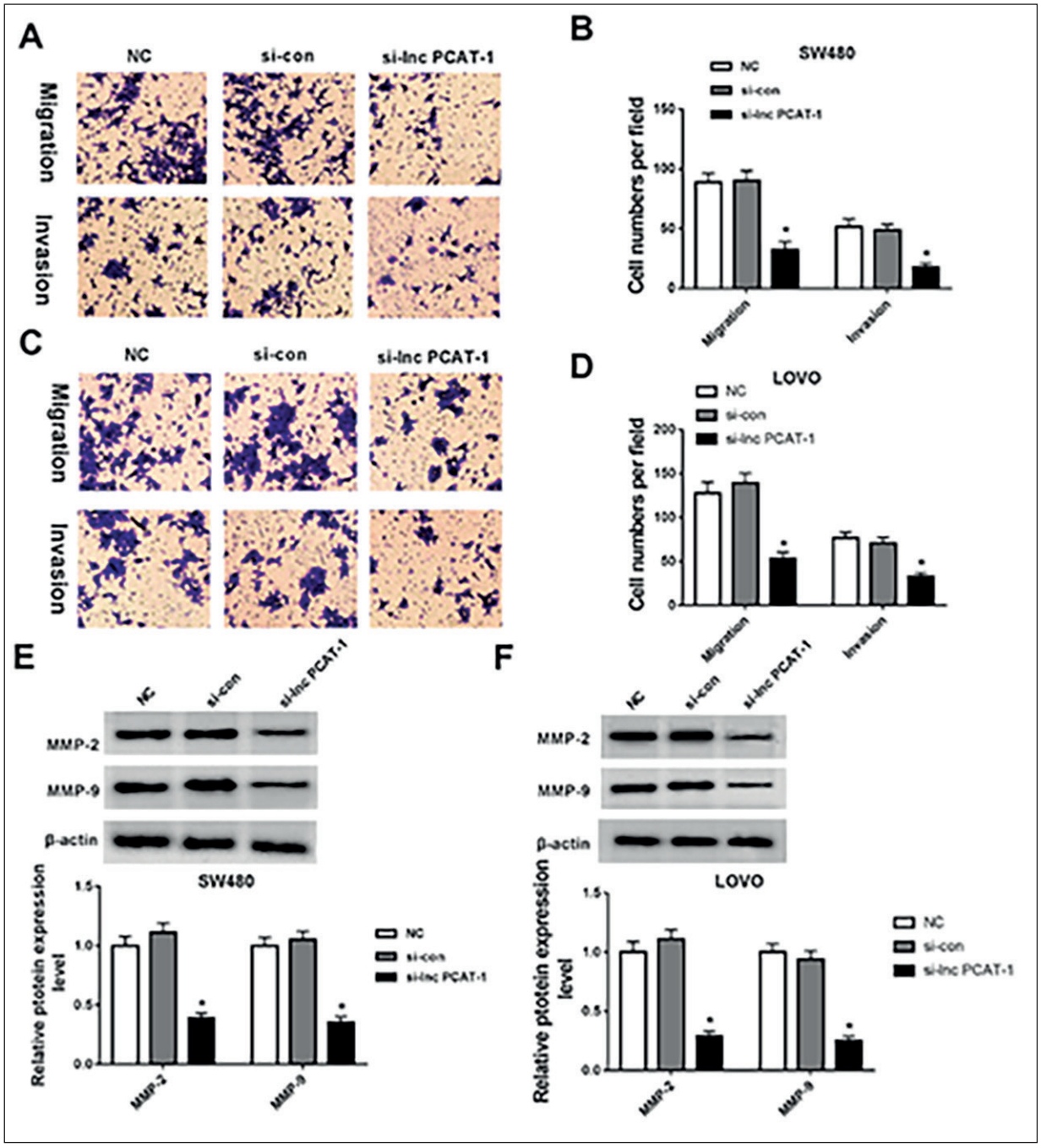

Figure 3. Cell migration and invasion were decreased by silencing PCAT-1 expression in CRC cells. **(A-D)** Cell migration and invasion were assessed in SW480 and LOVO cells transfected with si-con and si-lncPCAT-1 using transwell assay (magnification: ×300). **(E-F)** The MMP-2 and MMP-9 protein expression were measured in SW480 and LOVO cells transfected with si-con and si-lncPCAT-1 using Western blot. *$p<0.05$.

we used rescue experiments to verify the regulatory relationship between PCAT-1 and miR-149-5p. Inhibition of PCAT-1 induced miR-149-3p expression and cell apoptosis in CRC cells; however, suppression of miR-149-3p blocked these effects induced by si-lnc PCAT-1 transfection in SW480 and LOVO cells (Figure 6A to 6D). Moreover, cell proliferation, migration and invasion were significantly reduced by downregulating PCAT-1, which were weakened by decreasing

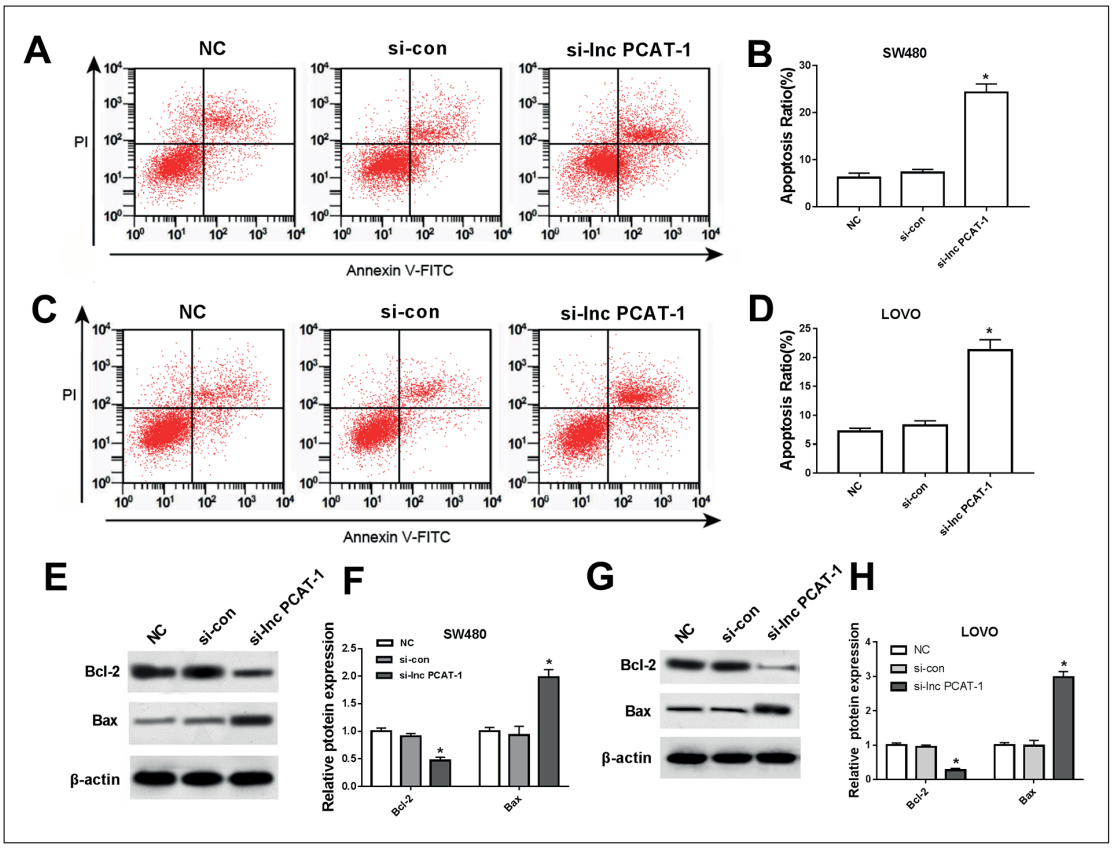

Figure 4. Inhibition of PCAT-1 promoted cell apoptosis in CRC cells. **(A-D)** Cell apoptosis was calculated in SW480 and LOVO cells transfected with si-con and si-lncPCAT-1 using flow cytometry. **(E-F)** The Bcl-2 and Bax protein expression were measured in SW480 and LOVO cells transfected with si-con and si-lncPCAT-1 using Western blot. *p<0.05*p<0.05.

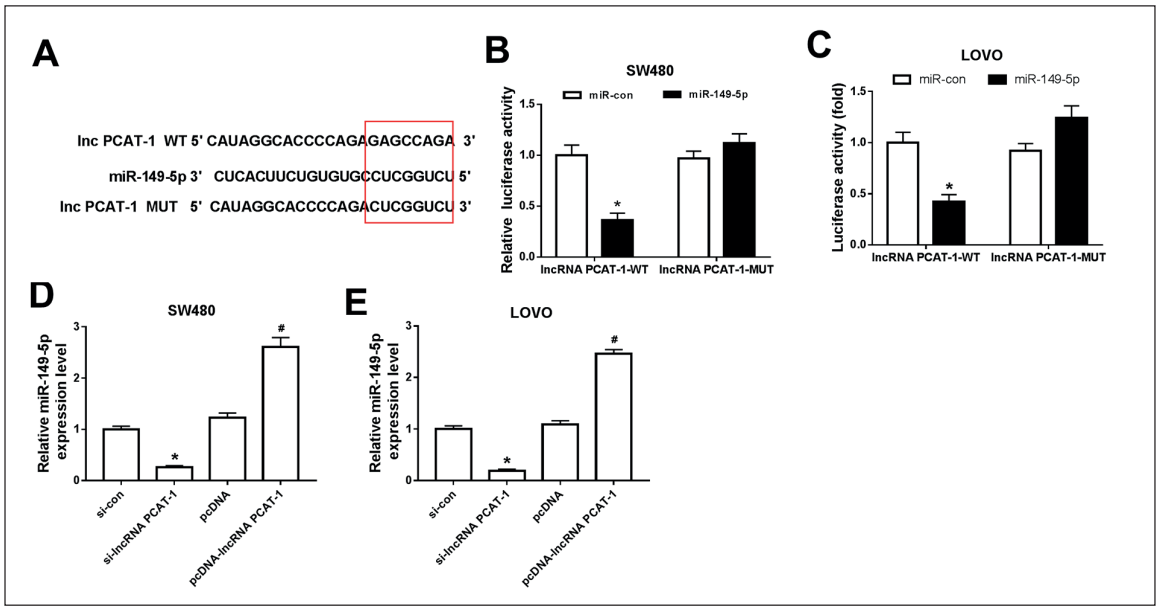

Figure 5. MiR-149-3p was a target miRNA of PCAT-1. **(A)** The predicted binding sites of miR-149-5p to PCAT-1 sequence were shown. **(B-C)** Luciferase activities of SW480 and LOVO cells cotransfected with miR-149-5p mimic and miR-con and luciferase reporters containing PCAT-1-WT or PCAT-1-MUT were analyzed. **(D-E)** The expression of miR-149-5p in SW480 and LOVO cells transfected with si-con, si-lncPCAT-1, pcDNA and PcDNA-lncRNA PCAT-1 were detected using qRT-PCR. *p<0.05.

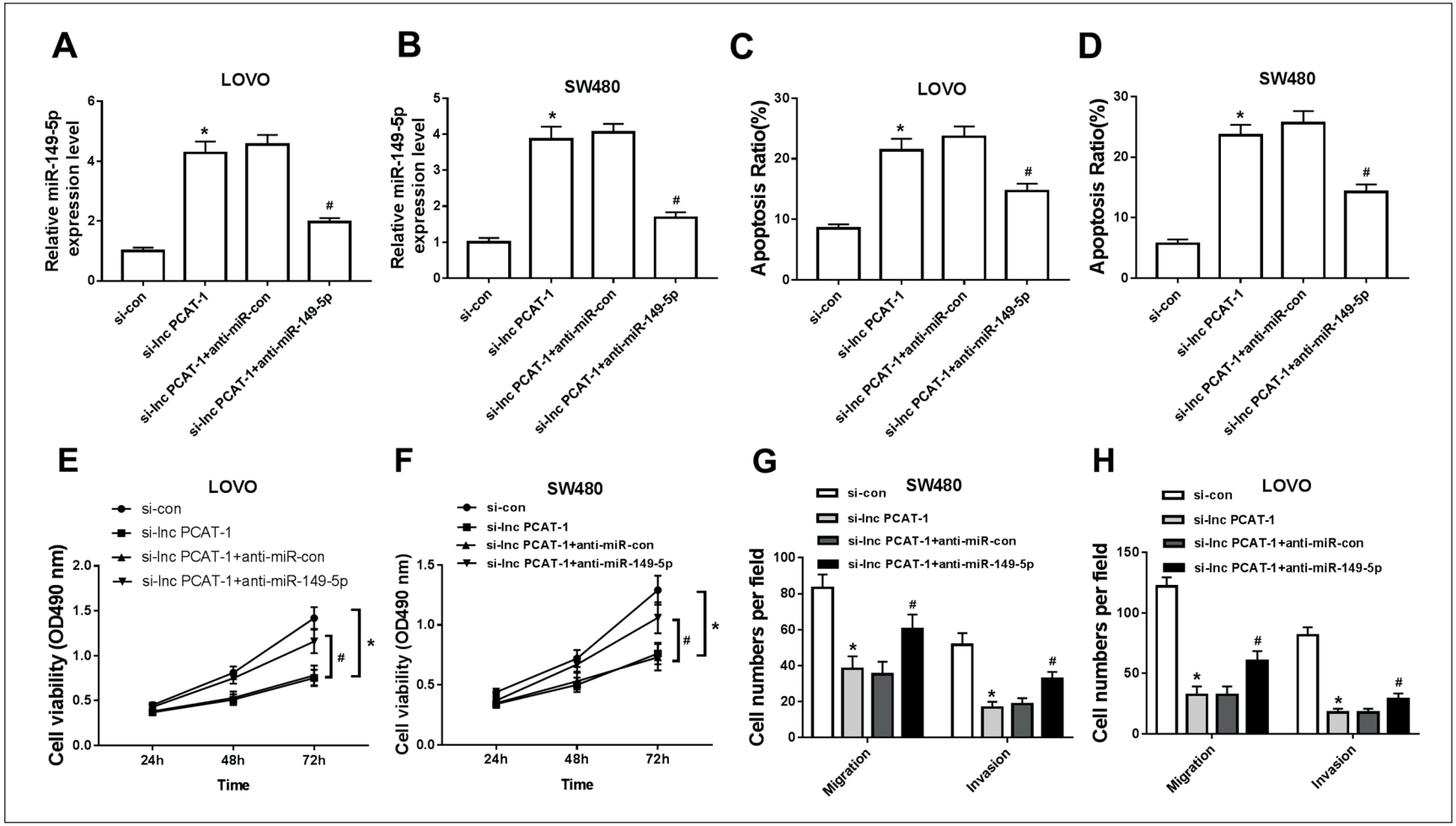

Figure 6. MiR-149-3p reversed the suppressive effects of PCAT-1 on the cell growth of CRC cells. **(A-B)** The expression of miR-149-5p in SW480 and LOVO cells transfected with si-con, si-lncPCAT-1, si-lncPCAT-1+anti-miR-con and si-lncPCAT-1+anti-miR-149-5p was detected using qRT-PCR. **(C-D)** Cell apoptosis in SW480 and LOVO cells transfected with si-con, si-lncPCAT-1, si-lncPCAT-1+anti-miR-con and si-lncPCAT-1+anti-miR-149-5p was detected using flow cytometry. **(E-F)** Cell proliferation in SW480 and LOVO cells transfected with si-con, si-lncPCAT-1, si-lncPCAT-1+anti-miR-con and si-lncPCAT-1+anti-miR-149-5p was detected using MTT assay. **(G-H)** Cell migration and invasion in SW480 and LOVO cells transfected with si-con, si-lncPCAT-1, si-lncPCAT-1+anti-miR-con and si-lncPCAT-1+anti-miR-149-5p were detected using qRT-PCR. *$p<0.05$.

miR-149-5p in SW480 and LOVO cells (Figure 6E to 6H). The results suggested that miR-149-3p reversed the suppressive effects of PCAT-1 on the cell growth of CRC cells.

Discussion

Accumulating evidence revealed that lncRNA was associated with cell progression in cancers, such as cell proliferation, migration and apoptosis[18-20]. Meanwhile, lncRNAs are also involved in prognosis and drug resistance of cancers[12,21,22]. Importantly, abnormal expressions of lncRNAs have been found to play an important role in CRC[23]. For example, lncRNA H19 functions as miRNA sponges could promote epithelial to mesenchymal transition[24]. Han et al[22] reported that lncRNA CRNDE expressed high in CRC and promoted cell proliferation and chemoresistance in CRC. In addition, lncRNA HOTTIP has been revealed to be associated with CRC progression and prognosis[25]. Furthermore, in several cancers, lncRNA PCAT-1 has been identified as a predictive biomarker and was correlated with poor prognosis. More than that, lncRNA PCAT-1 promoted cell proliferation, invasion and migration[26-28]. In this study, the expression of PCAT-1 was upregulated in CRC tissues and cells. Function experiments showed that knockdown of PCAT-1 inhibited cell proliferation, invasion and migration while promoted cell apoptosis in CRC using qRT-PCR, MTT assay, transwell assay and flow cytometry. Thus, we identified that PCAT-1 was an oncogene of CRC and regulated cell progression of CRC.

The regulatory mechanism of lncRNA PCAT-1 has been reported in cancers[15,29,30]. In the study of Gu et al[29] and Zhang et al[31], lncRNA PCAT-1 targeted miR-129-5p and promoted cell invasion and metastasis in hepatocellular carcinoma and human ovarian cancer. In this study, we found that miR-149-5p was a target miRNA of lncRNA PCAT-1 using luciferase reporter assay. Therefore, we predicted that lncRNA PCAT-1 affected cell progression and tumor formation through targeting miR-149-5p. Emerging studies have revealed that miR-149-5p expressed low in cancers, which has been identified to be a tumor suppressor[32-34]. For example, in renal cell carcinoma, miR-149-5p was associated with cellular migration, proliferation and apoptosis[35]. In this study, we found that the expression of miR-149-5p was downregulated in CRC tissues and cells. Moreover, PCAT-1 expression was negatively related to miR-149-5p expression in CRC tissues. Rescue experiments demonstrated that suppression of miR-149-5p reversed the effects of inhibition of PCAT-1 on cell proliferation, migration, invasion and apoptosis. Taken together, all data suggested that PCAT-1/miR-149-5p axis played an important role in CRC.

Conclusions

In summary, inhibited PCAT-1 expression suppressed cell proliferation, migration, and invasion, while promoted cell apoptosis, suggesting that PCAT-1 was an oncogene of CRC and regulated cell progression in CRC. LncRNA PCAT-1 affected CRC progression through modulating miR-149-5p, providing a new regulatory network of PCAT-1 in CRC. These findings enhanced the understanding of CRC pathogenesis and provided a novel therapeutic for CRC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the First People's Hospital of Xianyang City. The methods used in this study were performed in accordance with relevant guidelines and regulations. Written consent was obtained from the participants or guardians of participants under 16 years old.

Availability of Data and Materials

All original data and materials are available from the corresponding author upon request.

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