PVT1 promotes resistance to 5-FU in colon cancer via the miR-486-5p/CDK4 axis

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Abstract. Drug resistance in tumors is a major issue, limiting the curative efficacy of currently available cancer chemotherapeutics. 5-Fluorouracil (5-FU) is a commonly applied therapeutic drug in colon cancer patient regimens; however, the majority of patients develop resistance to 5-FU in the later stages of the disease, rendering this chemotherapy ineffective. Drug resistance is the main factor underlying the poor prognosis of patients with colon cancer. In recent years, a number of studies have confirmed that long non-coding (lnc)RNAs may play vital roles in tumor resistance. In the present study, the Gene Expression Omnibus (GEO) and lncRNADisease2 databases were screened for colon cancer-associated expression patterns of lncRNA plasmacytoma variant translocation 1 (PVT1). Subsequently, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect changes in PVT1 expression in resistant cell lines, and a Cell Counting Kit-8 (CCK-8) assay kit was used to assess the effects of PVT1 knockdown on the half maximal inhibitory concentrations of parental and 5-FU-resistant HCT116 cells. Subsequently, CCK-8, clone formation, and flow cytometric assays were performed to investigate the effects of PVT1 knockdown on the sensitivity of HCT116-5FU-resistant cells to 5-FU. Dual-luciferase assay, RNA pull-down and RNA immunoprecipitation assays verified the interactive regulation of PVT1, miR-486-5p and cyclin dependent kinase 4 (CDK4). PVT1 was highly expressed in HCT116-5FU-resistant cells, as compared to its expression in HCT116 parental cells. PVT1 knockdown significantly reduced the resistance of HCT116-5FU-resistant cells to 5-FU. In addition, PVT1 upregulated CDK4 expression by adsorbing miR-486-5p; however, CDK4 overexpression restored the effects of miR-486-5p inhibition on HCT116-5-FU-resistant cells. Additionally, PVT1 knockdown partially rescued CDK4 overexpression in HCT116-5-FU-resistant cells. On the whole, the findings of the present study suggest that PVT1 promotes the resistance of colon cancer cells to 5-FU by regulating the miR-486-5p/CDK4 axis. Therefore, PVT1 may prove to be a potential target for counteracting resistance to 5-FU in colon cancer therapy.

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Abbreviations: CRC, colorectal cancer; PVT1, plasmacytoma variant translocation 1; 5-FU, 5-fluorouracil; CDK4, cyclin dependent kinase 4; GEO, Gene Expression Omnibus; siRNA, small interfering RNA; ov, overexpression; NC, negative control; OD, optical density

Key words: PVT1, colon cancer, miR-486-5p, CDK4, 5-FU resistance

Introduction

Colorectal cancer (CRC) is the fourth most lethal cancer worldwide, following lung, liver and gastric cancer mortality, with ~700,000 individuals succumbing to CRC annually (1). Chemotherapy is the most common treatment for recurrent and metastatic colon cancer, and chemotherapeutic resistance is the largest limitation to cancer treatment efficacy. 5-FU is one of the oldest chemotherapeutics and it acts by suppressing tumor cell proliferation via the inhibition of DNA replication and RNA synthesis (2). Amongst patients receiving 5-FU, ~50% develop drug resistance, which is the main cause of the poor prognosis of patients with colon cancer.

Long non-coding (lnc)RNAs are an RNA subclass of >200 bp in length, lacking a protein-coding sequence. The lncRNA plasmacytoma variant translocation 1 (PVT1) gene has been reported to be highly expressed in esophageal, gastric, liver, colorectal, pancreatic, and other gastrointestinal cancers (3). PVT1 was first identified as a MYC agonist in mice (4). Subsequent analyses revealed that PVT1 could potentially upregulate epithelial-mesenchymal transition in esophageal cancer (5). PVT1 has also been reported to promote cell proliferation, activate the cell cycle and support a tumor stem cell-like population in hepatocellular carcinoma cells (6). This gene has been also reported to exert anti-apoptotic effects, and promote CRC tumor cell proliferation and CRC metastasis (7). In addition, PVT1 has been suggested to promote resistance to chemotherapeutics in a variety of gastrointestinal cancers and to enhance multidrug resistance in gastric

cancers (8). Furthermore, PVT1 has been reported to promote resistance to gemcitabine by regulating the Wnt/β-catenin and autophagy signaling pathways in pancreatic cancer (9), and also to regulate 5-FU resistance in gastric cancer (10) which could be reversed by the knockdown of PVT1 in 5-FU-resistant CRC cells (11). However, the mechanisms through which PVT1 confers resistance to 5-FU remain unclear.

The aim of the present study was to reveal the mechanisms underlying PVT1-mediated 5-FU resistance in colon cancer, and to determine whether PVT1 may be a promising possible therapeutic target in 5-FU-resistant cells, with the potential to improve the survival rate of patients who are resistant to 5-FU treatment.

Materials and methods

Cells, cell culture and transfection. The colon cancer cell line, HCT116 (CCL-247), and 293T (CRL-3216) cells were obtained from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS; HyClone; Cytiva) at 37˚C in a 5% CO_2 incubator. RNA/DNA was transfected into parental and 5-FU-resistant HCT116 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were harvested after 48 h of incubation at 37˚C. For PVT1 knockdown, 20 nM siRNA targeting PVT1 or 20 nM non-targeting control siRNA were transfected into cells. The siRNA sequences are presented in Table I. The siRNA sequences were synthesized at ribobio Technologies Cells were transfected with 50 ng CDK4 overexpression plasmid (Shanghai GenePharma Co., Ltd.) or 50 ng pcDNA3.1 empty vector (Shanghai GenePharma Co., Ltd.), in order to upregulate CDK4 expression, The overexpression and inhibition of microRNA (miRNA/miR)-486-5p was achieved by transfecting 20 nM miR-486-5p mimic or an miR-486-5p inhibitor (Guangzhou RiboBio Co., Ltd.) into parental and 5-FU-resistant HCT116 cells with Lipofectamine® 2000. A corresponding scrambled oligonucleotide sequence was used as a negative control. The miRNA mimics/inhibitor sequences are presented in Table I.

Construction of resistant strains. HCT116 cells (CCL-247, ATCC) were grown to 60-80% confluency, followed by the addition of 0.2 mM 5-FU (Beijing Solarbio Science & Technology Co., Ltd.) and incubation at 37˚C for 24 h. The culture medium was then discarded, and the cells were washed twice with phosphate-buffered saline (PBS) (Gibco; Thermo Fisher Scientific, Inc.). The conditioned medium was replaced with 5-FU-free medium. The cells were passaged after they resumed proliferation, and the 5-FU shock procedure was repeated 6-8 times. When a cell population had grown stably at this concentration, the cells were exposed to a higher concentration of 5-FU and passaging was continued, which resulted in the gradual increase of the drug concentration toleration threshold. The induction of 5-FU resistance lasted for 6 months until the cells could grow stably in a significantly increased drug concentration.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from parental and 5-FU-resistant HCT116 cells using TRIzol reagent (MilliporeSigma). RNA purity and integrity were assessed using RNA electrophoresis. Using random primers, total RNA was reverse transcribed into cDNA at 42˚C for 1 h (RevertAid First Strand cDNA Synthesis kit; cat. no. K1622; Thermo Fisher Scientific, Inc.). Target genes were amplified in a 20 μl reaction volume using SYBR-Green qPCR Mix (cat. no. AB1323A; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The following conditions were applied: 50˚C for 2 min; 95˚C for 2 min; followed by 40 cycles of 95˚C for 15 sec and 60˚C for 32 sec. The primer sequences used for the RT-qPCR analyses are listed in Table II. The expression levels of mRNA/lncRNA and miRNA were assessed using the $2^{-\Delta\Delta Cq}$ method (12), and experiments were performed in triplicate.

Table I. Sequences of siRNAs targeting PVT1 and miR-486-5p mimics/inhibitor.

Name	Sequence (5'-3')
siRNA-1	GGCACAUUUCAGGAUACUAAA
siRNA-2	GCUUAUUAUAGACUUAUAUGU
siRNA-3	GGGAUUUAGGCACUUUCAAUC
si-con	AACCGCUGAGUUGUCUGAUAU
miR-486-5p mimics	UCCUGUACUGAGCUGCCCCGAG
Mimics nc	GUCACUUGAUCCGAUGCGCCCG
miR-486-5p inhibitor	CUCGGGGCAGCUCAGUACAGGA
Inhibitor nc	GGCCAAGUAGGUCCUAGCGCAG

PVT1, plasmacytoma variant translocation 1.

Western blot analysis. HCT116 parental and 5-FU-resistant HCT116 cells were lysed in lysis buffer (Beyotime Institute of Biotechnology) containing a protease inhibitor cocktail (Bimake). Protein concentration was determined using the bicinchoninic acid method. Denatured proteins (20 μg) were then separated on a 1% gel via SDS-PAGE, transferred to PVDF membrane (Millipore), and incubated with a primary antibody at 4˚C overnight, prior to washing and incubation with an HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H+L) secondary antibody (1:5,000; SA00001-2; ProteinTech Group, Inc.) at room temperature for 1 h. Finally, proteins were visualized using an enhanced chemiluminescence reagent (ECL; Thermo Fisher Scientific, Inc.). Gray value analysis of strips was performed with ImageJ software (v.1.8.0; National Institutes of Health). Anti-GAPDH antibody (1:2,000; cat. no. 10494-1-AP; Proteintech Group, Inc.) was used as an internal reference. CDK4 was probed using anti-CDK4 antibody (1:1,000; cat. no. ab108357; Abcam). All the experiments were performed in triplicate.

Cell Counting Kit-8 (CCK-8) assay. HCT116 parental and 5-FU-resistant HCT116 cells ($3x10^3$ cells/well) were plated in 96-well plates. Following a 72-h time period, 10 μl CCK-8 reagent (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well containing cells, and the plates were incubated at 37˚C for 60 min. Proliferation was quantified at 450 nm using a Varioskan LUX Multifunctional microplate

Table II. Primer sequences used to analyze the expression of various RNAs and miRNAs.

Gene symbol	Forward (5'-3')	Reverse (5'-3')
PVT1	GCCTTCCCTCCTTCTGGAAG	GGTCCAGGTGGAGTCATG
CDK4	CCATCAGCACAGTTCGTGAGGT	TCAGTTCGGGATGTGGCACAGA
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
U6	CTCGCTTCGGCAGCACAT	TTTGCGTGTCATCCTTGCG
miR-486-5p	ACACTCCAGCTGGGTCCTGTAC TGAGCTGCCCCGAG	CTCAACTGGTGTCGTGGA

PVT1, plasmacytoma variant translocation 1.

reader (Thermo Fisher Scientific, Inc.). All the experiments were performed in triplicate.

Clone formation assay. Cultured HCT116 parental and 5-FU-resistant HCT116 cells were digested with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) and pipetted to break up cell clusters. A total number of 200 cells per dish were seeded in cell culture dishes containing 10 ml of pre-warmed RPMI-1640 culture medium (Gibco; Thermo Fisher Scientific, Inc.) and incubated at 4˚C in 5% CO_2 for 2 weeks. The cells were then fixed for 15 min in 5 ml of 4% paraformaldehyde and an appropriate amount of 1X Giemsa dye solution (G1015; Beijing Solarbio Science & Technology Co., Ltd.) was applied to the cells for 10-30 min at room temperature. Dishes were air-dried, and the clones were counted directly with the naked eye. Clone formation rate=(number of clones/number of inoculated cells) x100. Microscope photographs were acquired, and cells were observed by using an optical microscope (MF52; Guangzhou Micro-shot Technology Co., Ltd.). All the experiments were performed in triplicate.

Cell cycle assays. Cells ($1x10^6$) were harvested in a flow tube and washed with PBS. Pre-chilled 70-80% ethanol (5 ml) was added in a dropwise manner to the cells and incubated in the dark at 4˚C overnight. The cells were washed twice to completely remove the ethanol. Cells were incubated for 30 min in the dark at 37˚C with 0.5 ml PI/RNase staining solution (cat. no. C1052; Beyotime Institute of Biotechnology). Samples were stored at 4˚C in the dark, detected using FACSAria (BD Biosciences) and analyzed with FlowJo software (version 10.6.2; BD Biosciences). All experiments were performed in triplicate.

Dual-luciferase assay. CDK4-WT (wild type), CDK4-MUT (mutant), PVT1-WT, and PVT1-MUT sequences were synthesized and subcloned into the psiCHECK2 vector (Synbio Technologies). Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used to co-transfect psiCHECK2 subcloning plasmid with miR-486-5p mimic or mimic negative control into 293T cells. Following a 48-h incubation at 37˚C, Firefly and *Renilla* fluorescence values were obtained using a dual-luciferase reporter assay (Promega Corporation). Relative fluorescence values were Firefly fluorescence value/*Renilla* fluorescence value ratios. The miRNA mimic/mimic nc sequences used were as follows: miR-486-5p mimics: 5'UCC UGUACUGAGCUGCCCCGAG3', mimics nc: 5'GUCACU UGAUCCGAUGCGCCCG3'. Independent experiments were performed in triplicate.

RNA immunoprecipitation (RIP) assay. A total number of $1x10^7$ 5-FU-resistant HCT116 cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology) and supplemented with a protease inhibitor cocktail (Bimake); 10% of each 1 ml lysate was used as input. Protein A/G magnetic beads (Millipore) were coupled with anti-AGO2 antibody (1:50; cat. no. ab186733; Abcam) or normal rabbit IgG (negative control) (1:50; cat. no. 2729; Cell Signaling Technology, Inc.) at 4˚C for 4 h. Subsequently, these antibody-coupled magnetic beads were incubated with the lysis mixtures for 6 h at 4˚C, followed by the elution of captured RNA (RNeasy Mini Kit; Qiagen) and RT-qPCR, according to the instructions of the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (17-700; MilliporeSigma). All experiments were performed in triplicate.

RNA pull-down assay. Sense strand probes and probes for mutated PVT1 were synthesized *in vitro* and incubated with cell lysis complex solution at 4˚C for 4 h. Streptavidin magnetic beads were added and incubated at 4˚C for 1 h, RNA was extracted, and miR-486-5p was detected by RT-qPCR. The RNA probe sequences are demonstrated in Table III. All the experiments were performed in triplicate.

Database analysis. IC_{50} values of all CRC cell lines against 5-FU were queried from the GDSC database (https://www.cancerrxgene.org/). For RNA screening, the following was performed: The GSE100179 dataset contained data from three CRC samples (GSM2674208, GSM2674209 and GSM2674210) and three normal human tissue samples (GSM2674188, GSM2674189 and GSM2674190). The online tool GEO2R (ncbi.nlm.nih.gov/geo/geo2r) was used to recognize anomalously expressed lncRNA/mRNA that yielded a $\log_2$ |fold change (FC)| >2 with a P-value <0.05. Volcano plots was drawn to represent differentially expressed lncRNA/mRNAs. miRNA data were derived from the GSE98406 dataset, including three CRC samples (GSM2593633, GSM2593634 and GSM2593635) and three normal human samples (GSM2593614, GSM2593615 and GSM2593616). lncRNA-disease2 (http://www.rnanut.net/lncrnadisease/) was used for the screening of lncRNAs related to colon cancer. BiBiServ2 (https://bibiserv.cebitec.uni-bielefeld.de/sa), TargetScan

Table III. RNA pull-down probe sequences.

Name	Sequence (5'-3')
PVT1-WT	CTCCGGGCAGAGCGCGTGTGGCGGCCGAGCACATGGGCCCGCGGGCCGGGCGGGCTCGGGG CGGCCGGGACGAGGAGGGGCGACGACGAGCTGCGAGCAAAGATGTGCCCCGGGACCCCCGG CACCTTCCAGTGGATTTCCTTGCGGAAAGGATGTTGGCGGTCCCTGTGACCTGTGGAGACACG GCCAGATCTGCCCTCCAGCCTGATCTTTTGGCCAGAAGGAGATTAAAAAGATGCCCCTCAAGA TGGCTGTGCCTGTCAGCTGCATGGAGCTTCGTTCAAGTATTTTCTGAGCCTGATGGATTTACAG TGATCTTCAGTGGTCTGGGGAATAACGCTGGTGGAACCATGCACTGGAATGACACACGCCCGG CACATTTCAGGATACTAAAAGTGGTTTTAAGGGAGGCTGTGGCTGAATGCCTCATGGATTCTTA CAGCTTGGATGTCCATGGGGGACGAAGGACTGCAGCTGGCTGAGAGGGTTGAGATCTCTGTT TACTTAGATCTCTGCCAACTTCCTTTGGGTCTCCCTATGGAATGTAAGACCCCGACTCTTCCTCT GGTGAAGCATCTGATGCACGTTCCATCCGGCGCTCAGCTGGGTGAGCTGACCATACTCCCTGG AGCCTTCTCCCGAGGTGCGCGGGTGACCTTGGCACATACAGCCATCATGATGGTACTTTAAGT GGAGGCTGAATCATCTCCCCTTTGAGCTGCTTGGCACGTGGCTCCCTTGGTGTTCCCCTTTTAC TGCCAGGACACTGAGATTTGGAGAGAGTCTCACTCTGTGGTCCAGGCTGAAGTACAGTGGCA TGATCCCAGGTCACTGCAACCCCCACCTCCCGGGTTCAAGTGATCCTCCTGCCTCAGCCTCCC GAGTAGCTGGTATTACAGGCGTGTGCCACAAAGCCTGGCTAAGTTTTGTATTTTTAGTAGAGAC GGGGTTTCACCATGTTGGCCAGGTTGGTCTCGAACTCCTGACCTCAAGTGATCCACTCACTTT GGCCTTTCAACGTGCTGGGATTACAGGCGAGAGTCACCGCACCCGGACGACTCTGACATTTTT GAAGAGTCCAGAATCCTGTTACACCTGGGATTTAGGCACTTTCAATCTGAAAAAATACATATCC TTTCAGCACTCTGGACGGACTTGAGAACTGTCCTTACGTGACCTAAAGCTGGAGTATTTTGAGA TTGGAGAATTAAGAGCCAGTCTTGGTGCTCTGTGTTCACCTGGTTCATCTGAGGAGCTGCATCT ACCCTGCCCATGCCATAGATCCTGCCCTGTTTGCTTCTCCTGTTGCTGCTAGTGGACATGAGAA GGACAGAATAACGGGCTCCCAGATTCACAAGCCCCACCAAGAGGATCACCCCAGGAACGCTT GGAGGCTGAGGAGTTCACTGAGGCTACTGCATCTTGAGACTCAGGATGAAGACCCAGCTTGGG GCTGTCAAAGAGGCCTGAAGAGGCAGAACACCCCAGAGGAGCCTGGGGCCACCACCCAGCA TCACTGTGGGAAAACGGCAGCAGGAAATGTCCTCTCGCCTGCGTGCTCCACCTCGGTCCACGC CTTCCCTCCTTCTGGAAGCCTTGCCTGACCACTGGCCTGCCCCTTCTATGGGAATCACTACTGA CCTTGCAGCTTATTATAGACTTATATGTTTTTTGCATGTCTGACACCCATGACTCCACCTGGACC TTATGGCTCCACCCAGAAGCAATTCAGCCCAACAGGAGGACAGCTTCAACCCATTACGATTTCA TCTCTGCCCCAACCACTCAGCAGCAAGCACCTGTTACCTGTCCACCCCCACCCCTTCCCCCAAA CTGCCTTTGAAAAATCCCTAACCTATGAGCTTTGAATAAGATGAGTACGAACTTCATCGCCCACG TGGCGTGGCCGGCCTCGTGTCTATTAAATTCTTTTTC TACTAAAAAAAAAAAAAAAAAA
PVT1-WT	CTCCGGGCAGAGCGCGTGTGGCGGCCGAGCACATGGGCCCGCGGGCCGGGCGGAGAGCCCCG TCGGTCCTGGTCCTGGGCGACGACGAGCTGCGAGCAAAGATGTGCCCCGGGACCCCCGGCAC CTTCCAGTGGATTTCCTTGCGGAAAGGATGTTGGCGGTCCCTGTGACCTGTGGAGACACGGCC AGATCTGCCCTCCAGCCTGATCTTTTGGCCAGAAGGAGATTAAAAAGATGCCCCTCAAGATGG CTGTGCCTGTCAGCTGCATGGAGCTTCGTTCAAGTATTTTCTGAGCCTGATGGATTTACAGTGA TCTTCAGTGGTCTGGGGAATAACGCTGGTGGAACCATGCACTGGAATGACACACGCCCGGCAC ATTTCAGGATACTAAAAGTGGTTTTAAGGGAGGCTGTGGCTGAATGCCTCATGGATTCTTACAGC TTGGATGTCCATGGGGGACGAAGGACTGCAGCTGGCTGAGAGGGTTGAGATCTCTGTTTACTT AGATCTCTGCCAACTTCCTTTGGGTCTCCCTATGGAATGTAAGACCCCGACTCTTCCTGGTGAA GCATCTGATGCACGTTCCATCCGGCGCTCAGCTGGGCTTGAGCTGACCATACTCCCTGGAGCCT TCTCCCGAGGTGCGCGGGTGACCTTGGCACATACAGCCATCATGATGGTACTTTAAGTGGAGGC TGAATCATCTCCCCTTTGAGCTGCTTGGCACGTGGCTCCCTTGGTGTTCCCCTTTTACTGCCAGG ACACTGAGATTTGGAGAGAGTCTCACTCTGTGGTCCAGGCTGAAGTACAGTGGCATGATCCCA GGTCACTGCAACCCCCACCTCCCGGGTTCAAGTGATCCTCCTGCCTCAGCCTCCCGAGTAGCT GGTATTACAGGCGTGTGCCACAAAGCCTGGCTAAGTTTTGTATTTTTAGTAGAGACGGGGTTTC ACCATGTTGGCCAGGTTGGTCTCGAACTCCTGACCTCAAGTGATCCACTCACTTTGGCCTTTCA ACGTGCTGGGATTACAGGCGAGAGTCACCGCACCCGGACGACTCTGACATTTTTGAAGAGTCC AGAATCCTGTTACACCTGGGATTTAGGCACTTTCAATCTGAAAAAATACATATCCTTTCAGCACT CTGGACGGACTTGAGAACTGTCCTTACGTGACCTAAAGCTGGAGTATTTTGAGATTGGAGAATT AAGAGCCAGTCTTGGTGCTCTGTGTTCACCTGGTTCATCTGAGGAGCTGCATCTACCCTGCCCA TGCCATAGATCCTGCCCTGTTTGCTTCTCCTGTTGCTGCTAGTGGACATGAGAAGGACAGAATAA

Table III. Continued.

Name	Sequence (5'-3')
	CGGGCTCCCAGATTCACAAGCCCCACCAAGAGGATCACCCCAGGAACGCTTGGAGGCTGAGG
	AGTTCACTGAGGCTACTGCATCTTGAGACTCAGGATGAAGACCCAGCTTGGGGCTGTCAAAGA
	GGCCTGAAGAGGCAGAACACCCCAGAGGAGCCTGGGGCCACCACCCAGCATCACTGTGGGAA
	AACGGCAGCAGGAAATGTCCTCTCGCCTGCGTGCTCCACCTCGGTCCACGCCTTCCCTCCTTC
	TGGAAGCCTTGCCTGACCACTGGCCTGCCCCTTCTATGGGAATCACTACTGACCTTGCAGCTTA
	TTATAGACTTATATGTTTTTTGCATGTCTGACACCCATGACTCCACCTGGACCTTATGGCTCCAC
	CCAGAAGCAATTCAGCCCAACAGGAGGACAGCTTCAACCCATTACGATTTCATCTCTGCCCCAA
	CCACTCAGCAGCAAGCACCTGTTACCTGTCCACCCCCACCCCTTCCCCCAAACTGCCTTTGAA
	AAATCCCTAACCTATGAGCTTTGAATAAGATGAGTACGAACTTCATCGCCCACGTGGCGTGGCC
	GGCCTCGTGTCTATTAAATTCTTTTTCTACTAAAAAAAAAAAAAAAAAA

Underlining indicates the position of the mutation site. PVT1, plasmacytoma variant translocation 1.

(http://www.targetscan.org/) and miRDB (http://www.mirdb.org/) were used to analyze genes that potentially interact with miR-486-5p, PVT1, miR-486-5p and CDK4.

Statistical analyses. All data were analyzed using GraphPad Prism (version 8; GraphPad Software, Inc.). Data are expressed as the mean ± standard deviation. Data comparing two groups were analyzed using the unpaired Student's t-test, while data comparing multiple groups were analyzed by using one-way ANOVA, followed by the Bonferroni post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PVT1 is highly expressed in drug-resistant colon cancer cells. 5-FU is a chemotherapeutic drug commonly used for the treatment of colon cancer; however, drug resistance often poses a challenge in clinical settings. In the present study, to elucidate the mechanisms through which cells acquire resistance to 5-FU, the GEO and lncRNADisease2 databases were first used to screen for lncRNAs closely connected to the emergence and metastasis of colon cancer, including urothelial carcinoma-associated 1 (UCA1) and PVT1 (Fig. 1A and B). Among these, UCA1 has been revealed to be associated with colon cancer 5-FU resistance in previous studies (13,14). Thus, it was hypothesized that PVT1 may also be associated with 5-FU resistance in colon cancer. Firstly, according to the GDSC database query results, HCT116 colon cancer cells were highly sensitive to 5-FU (Fig. 1C). A 5-FU-resistant strain of HCT116 cells was then established and a CCK-8 assay was used to determine the IC_{50} values of parental and 5-FU-resistant HCT116 cells. The IC_{50} value of the 5-FU-resistant HCT116 cells was significantly higher than that of the parental cells (Fig. 1D). PVT1 expression was be elevated in the 5-FU-resistant HCT116 cells, as revealed using RT-qPCR (Fig. 1E). Thus, it was hypothesized that PVT1 may have contributed to the resistance of HCT116 cells to 5-FU; subsequently, nuclear and cytoplasmic separation was used in HCT116 and HCT116-5FU-resistant cells for the detection of the distribution of PVT1 in the nucleus and cytoplasm. The results demonstrated that PVT1 was evenly distributed in the nucleus and cytoplasm of HCT116 parental cells. However, it was more greatly distributed in the cytoplasm of HCT116-5FU-resistant cells (Fig. 1F and G). Thus, it was suspected that 5FU resistance may be attributed mainly due to the cytoplasmic role of PVT1.

PVT1 knockdown inhibits 5-FU resistance in colon cancer cells. To examine the effects of PVT1 expression on 5-FU resistance in colon cancer, PVT1 was knocked down in 5-FU-resistant HCT116 cells. Firstly, cells were transfected with three PVT1 siRNAs and the knockdown efficiency of each siRNA was detected using RT-qPCR. Since siRNA-2 produced the most intense knockdown effect (Fig. 2A), it was selected for use in follow-up experiments. Subsequently, the HCT116-5FU-resistant cells were treated with 1 mM 5-FU. CCK-8 assay demonstrated that PVT1 knockdown markedly reduced the IC_{50} value of 5-FU-resistant HCT116 cells (Fig. 2B) and reduced the proliferation of HCT116-FU-resistant cells treated with or without 5-FU (Fig. 2C). Cell cycle analyses revealed that PVT1 knockdown inhibited the cell cycle progression of 5-FU-resistant HCT116 cells treated with or without 5-FU (Fig. 2D). Clone formation assays demonstrated that PVT1 knockdown reduced the number of clones formed by 5-FU-resistant HCT116 cells treated with or without 5-FU (Fig. 2E). It was thus concluded that PVT1 knockdown notably reduced 5-FU resistance in colon cancer cells.

PVT1 absorbs miR-486-5p to regulate CDK4 expression. Subsequently, the mechanisms through which PVT1 confers drug resistance in cancer cells were investigated. An established lncRNA mechanism involves the regulation of gene expression by adsorbing miRNA (15). Therefore, miRNAs that were downregulated in colon cancer using the GEO database were analyzed first. The volcano plot shows the differential expression of the whole transcriptome, where green indicates downregulated miRNAs and red indicates upregulated miRNAs. Among these, as compared with normal tissues, the downregulated expression of miR-486-5p has the largest fold-change in colon cancer (Fig. 3A). Subsequently, BiBiServ2 software analysis indicated that PVT1 strongly interacted with miR-486-5p. Thus, dual-luciferase experiments

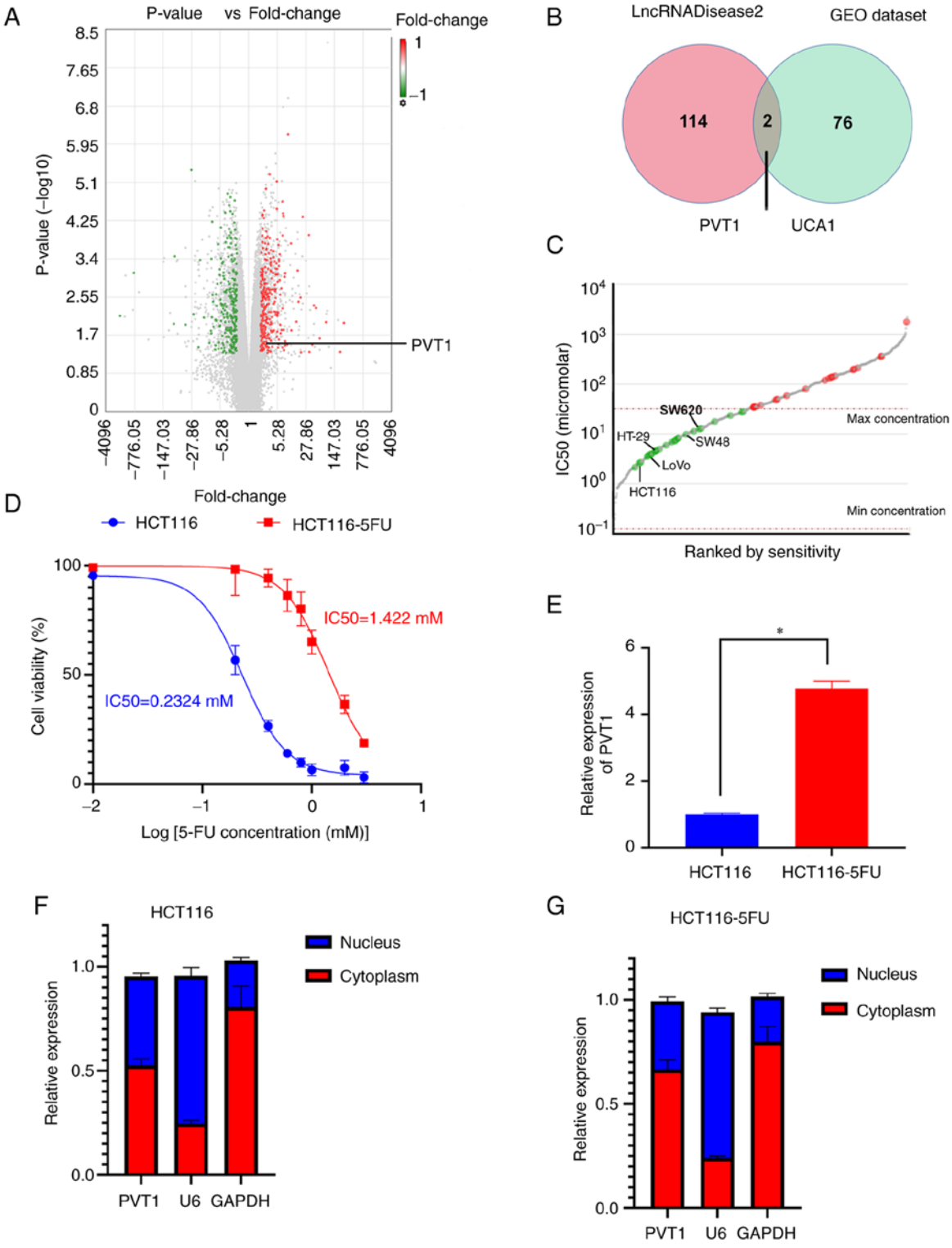

Figure 1. PVT1 is highly expressed in drug-resistant colorectal cancer cells. (A) Volcano plot demonstrating differentially expressed lncRNAs in colon cancer, identified in the GEO database. Green indicates downregulated lncRNAs, red indicates upregulated lncRNAs, and gray indicates non-differentially expressed lncRNAs and other types of RNAs (mRNAs and miRNAs). (B) Venn diagram illustrating the intersection of differentially expressed lncRNAs in colon cancer in the GEO database and lncRNAs related to colon cancer in the lncRNADisease2 database. (C) CCK-8 assay was used to determine 5-FU IC_{50} values for parental and 5-FU-resistant HCT116 cells. (D) RT-qPCR was used to quantify PVT1 expression in parental and 5-FU-resistant HCT116 cells. (E) PT-qPCR was used to detect the expression of PVT1 in the cytoplasm and nucleus of HCT116 cells. $^{*}P<0.05$ (F and G) RT-qPCR was used to detect the expression of PVT1 in the cytoplasm and nucleus of HCT116 and HCT116-5FU cells. PVT1, plasmacytoma variant translocation 1; lncRNAs, long non-coding RNAs; GEO, gene expression omnibus; 5-FU, 5-fluorouracil; RT-qPCR, reverse transcription-quantitative PCR.

were used to confirm miR-486-5p binding to PVT1 (Fig. 3B). Moreover, RNA pull-down and RIP assays were used to verify the binding of miR-486-5p to PVT1 in HCT116-5FU-resistant cells (Fig. 3C and D) and it was found that PVT1 could adsorb miR-486-5p. Additionally, the results of RT-qPCR demonstrated that PVT1 failed to affect the expression of miR-486-5p

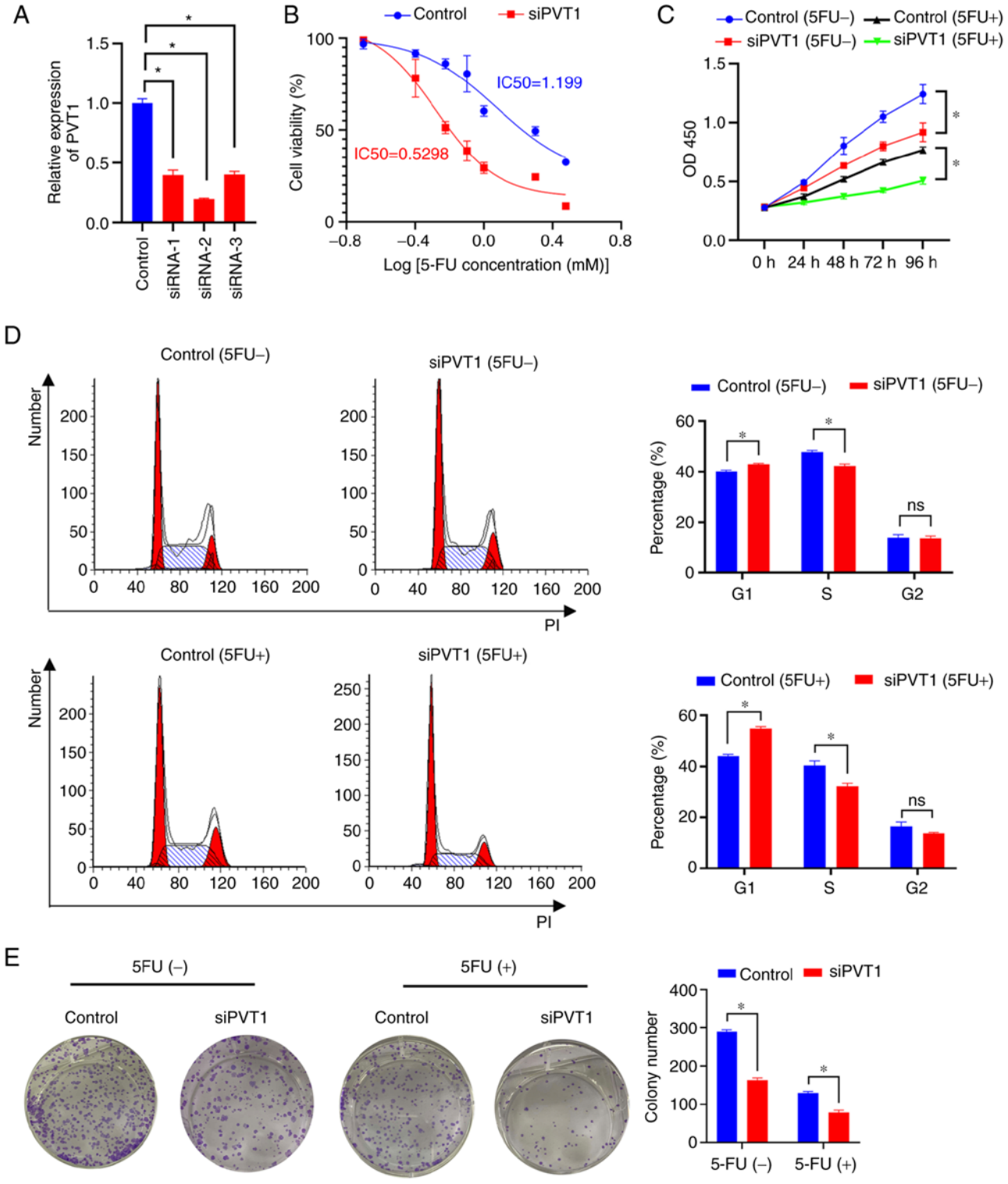

Figure 2. PVT1 knockdown reduces 5-FU resistance in HCT116-5FU-resistant cells (A) RT-qPCR was used for the detection of knockdown efficiency of PVT1 siRNAs in HCT116-5FU-resistant cells, (B) CCK-8 assay was used to detect the impact of PVT1 knockdown on the 5-FU IC_{50} values of HCT116-5FU-resistant cells. (C) CCK-8 assay was used to examine the effect of PVT1 knockdown on the proliferation of HCT116-5FU-resistant cells treated with or without 1 mM 5-FU. (D) Clone formation assay was used to evaluate the effects of PVT1 knockdown on stem like properties of HCT116-5FU-resistant cells treated with or without 1 mM 5-FU. (E) Flow cytometric analysis was used for the evaluation of PVT1 knockdown effect on cell cycle in HCT116-5FU-resistant cells treated with or without 1 mM 5-FU. *P<0.05. ns, not significant; PVT1, plasmacytoma variant translocation 1; 5-FU, 5-fluorouracil; RT-qPCR, reverse transcription-quantitative PCR.

(Fig. 3E). Since miRNAs mainly act on the 3'UTR of their target functional gene mRNA, nine possible miR-486-5p interacting mRNAs were obtained by using the combined analysis of TargetScan, miRDB and RNA-chip differential genes in the GEO database. Three of the upregulated miR-486-5p targeted gene candidates were CDK4, histone acetyltransferase 1 (HAT1) and twinfilin actin binding protein 1 (TWF1) (Fig. 4A). CDK4 was selected as an miR-486-5p target gene,

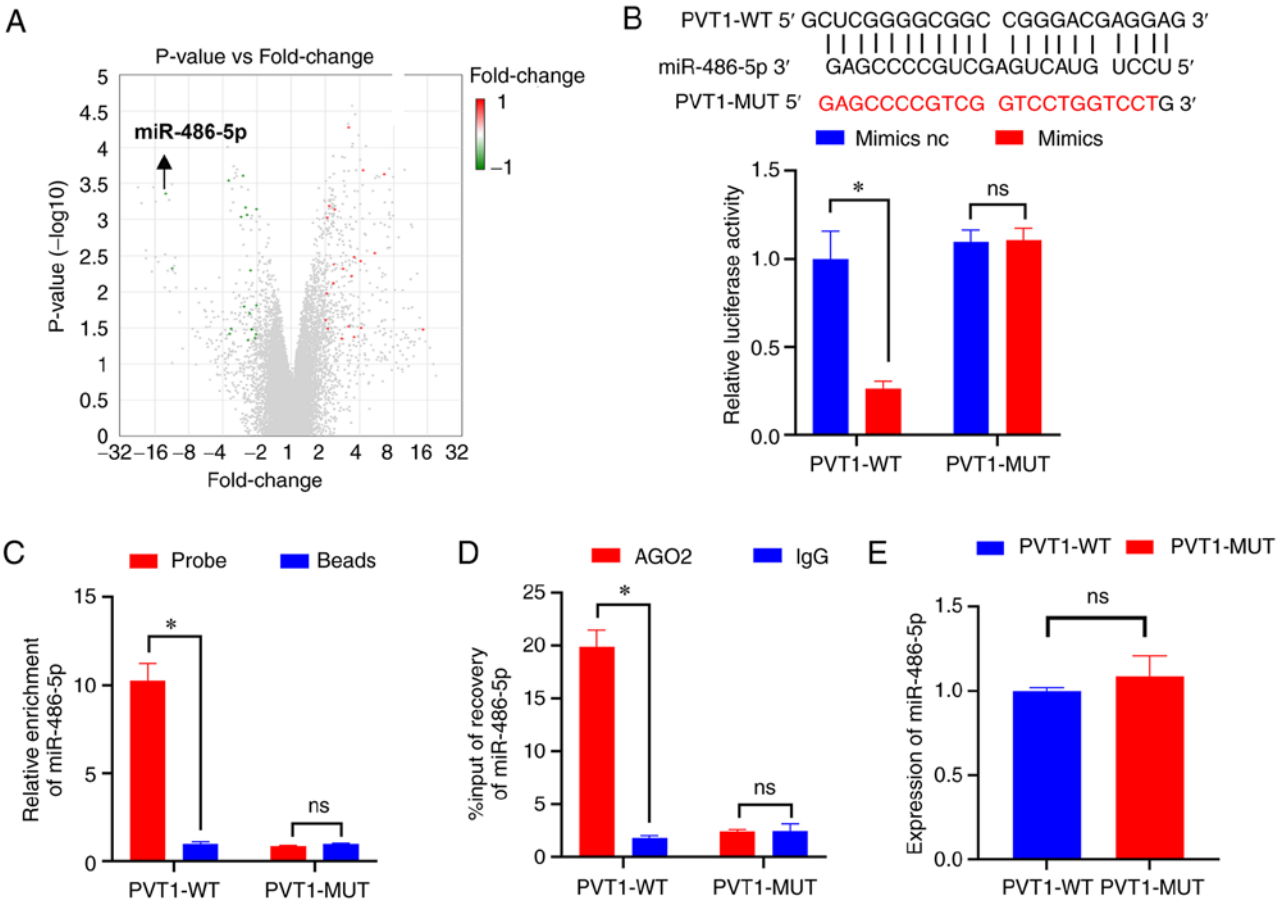

Figure 3. PVT1 adsorbs miR-486-5p for the upregulation of CDK4 expression. (A) Volcano map demonstrating differentially expressed miRNAs in colon cancer from the GEO database. (B) Dual luciferase experiments verified the interaction of PVT1 and miR-486-5p in 293T cells. (C) RNA pull-down experiment verified the interaction between PVT1 and miR-486-5p in HCT116-5FU-resistant cells with PVT1-WT and PVT1-MUT probe. (D) An AGO2-RIP experiment verified the binding of PVT1 to miR-486-5p in 5-HCT116-5FU-resistant cells transfected PVT1-WT and PVT1-MUT. (E) Reverse transcription-quantitative PCR was used for the evaluation of PVT1 effect on miR-486-5p. *P<0.05. ns, not significant; PVT1, plasmacytoma variant translocation 1; GEO, gene expression omnibus; 5-FU, 5-fluorouracil.

which is closely related to the proliferation cycle. Dual luciferase analysis verified that miR-486-5p binds to the 3'UTR region of CDK4 (Fig. 4B). The results of western blot analysis revealed that miR-486-5p suppressed CDK4 expression (Fig. 4C). RT-qPCR was also used for the detection of PVT1, miR-486-5p and CDK4 expression in drug-resistant and non-drug-resistant cells (Fig. 4D). The results demonstrated that the PVT1 and miR-486-5p expression levels were inversely associated, which also applied for CDK4 expression. Furthermore, western blot analysis revealed that 5-FU enhanced the expression of CDK4 in HCT116-5FU-resistant cells (Fig. 4E). In summary, PVT1 may promote colon cancer cell resistance to 5-FU via the miR-486-5p/CDK4 axis.

PVT1 regulates the resistance of colon cancer cells to 5-FU via the miR-486-5p/CDK4 axis. To further examine the effects of PVT1 on the resistance of colon cancer cells to 5-FU through the modulation of the miR-486-5p/CDK4 axis, and following the overexpression of miR-486-5p, CDK4 was also overexpressed in order to observe the regulatory effects of CDK4 and miR-286-5p on HCT116-5FU-resistant cells. Subsequently, PVT1 was downregulated and the effect of the PVT1, miR-286-5p and CDK4 interaction on the proliferation of HCT116-5FU drug-resistant cells was observed. Firstly, by using RT-qPCR and western blot analysis, PVT1, miR-486-5p and CDK4 expression was detected in each cell type. The results demonstrated that miR-486-5p overexpression (Fig. 5B) hardly affected PVT1 expression (Fig. 5A), although it inhibited CDK4 expression (Fig. 5C and D). Additionally, PVT1 knockdown decreased CDK4 expression (Fig. 5C and D). CCK-8 and clone formation assays demonstrated that CDK4 overexpression attenuated the inhibitory effects of miR-486-5p on the proliferation of HCT116-5FU-resistant cells, and PVT1 knockdown restored the suppressive effects of miR-486-5p on the proliferation of HCT116-5FU-resistant cells (Fig. 6A-C). Consistent with previous experimental results, flow cytometry revealed that CDK4 overexpression attenuated the inhibitory effects of miR-486-5p on the cell cycle progression of HCT116-5FU-resistant cells, and PVT1 knockdown restored the miR-486-5p-mediated sensitivity of HCT116-5FU cells (Fig. 6D). In summary, PVT1 may upregulate the resistance of colon cancer cells to 5-FU through the miR-486-5p/CDK4 axis.

Discussion

Due to an increase in the consumption of refined foods and limited exercise, CRC has become the third most common type

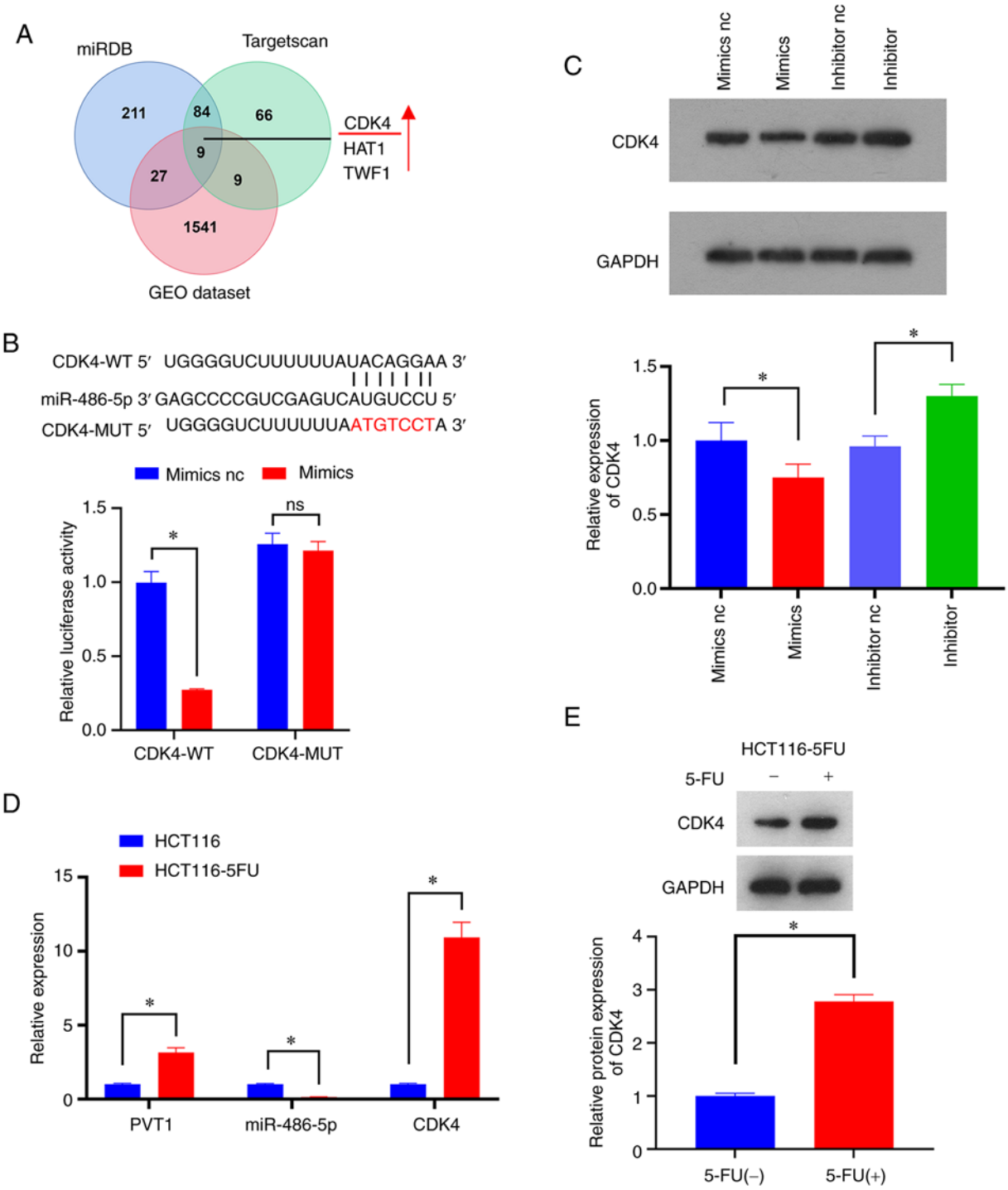

Figure 4. PVT1 adsorbs miR-486-5p to upregulate CDK4 expression. (A) A Venn map was created using GEO data along with TargetScan, miRDB to screen for miR-486-5p target genes (B) A dual luciferase experiment verified the interaction between miR-486-5p and the CDK4 3'UTR in 293T cells. (C) Western blot analysis was used for the detection of CDK4 expression in parental and 5FU-resistant HCT116 cells. (D) Reverse transcription-quantitative PCR was used to detect PVT1, miR-486-5p and CDK4 expression in parental and 5FU-resistant HCT116 cells. (E) Western blot analysis was performed to estimate the CDK4 protein levels in 5FU-resistant HCT116 cells treated with or without 1 mM 5-FU. *P<0.05. PVT1, plasmacytoma variant translocation 1; GEO, gene expression omnibus; 5-FU, 5-fluorouracil; HAT1, histone acetyltransferase 1; TWF1, twinfilin actin binding protein 1.

of cancer worldwide, with ~1.8 million new cases diagnosed each year (1). When a tumor produces local or remote metastases, 5-FU is often used as a chemotherapeutic agent (16). However, patients often develop drug resistance, leading to tumor recurrence (17). Therefore, the further understanding of the mechanisms responsible for the resistance of colon cancer to 5-FU is of utmost urgency.

In the present study, differentially expressed genes in colon cancer were first identified using the GEO database and the lncRNADisease2 database was then utilized to identify lncRNAs associated with colon cancer (UCA1 and PVT1). The mechanism by which UCA1 confers resistance op 5-FU in CRC has been previously investigated. UCA1 has been reported to increase the resistance of colon cancer cells to

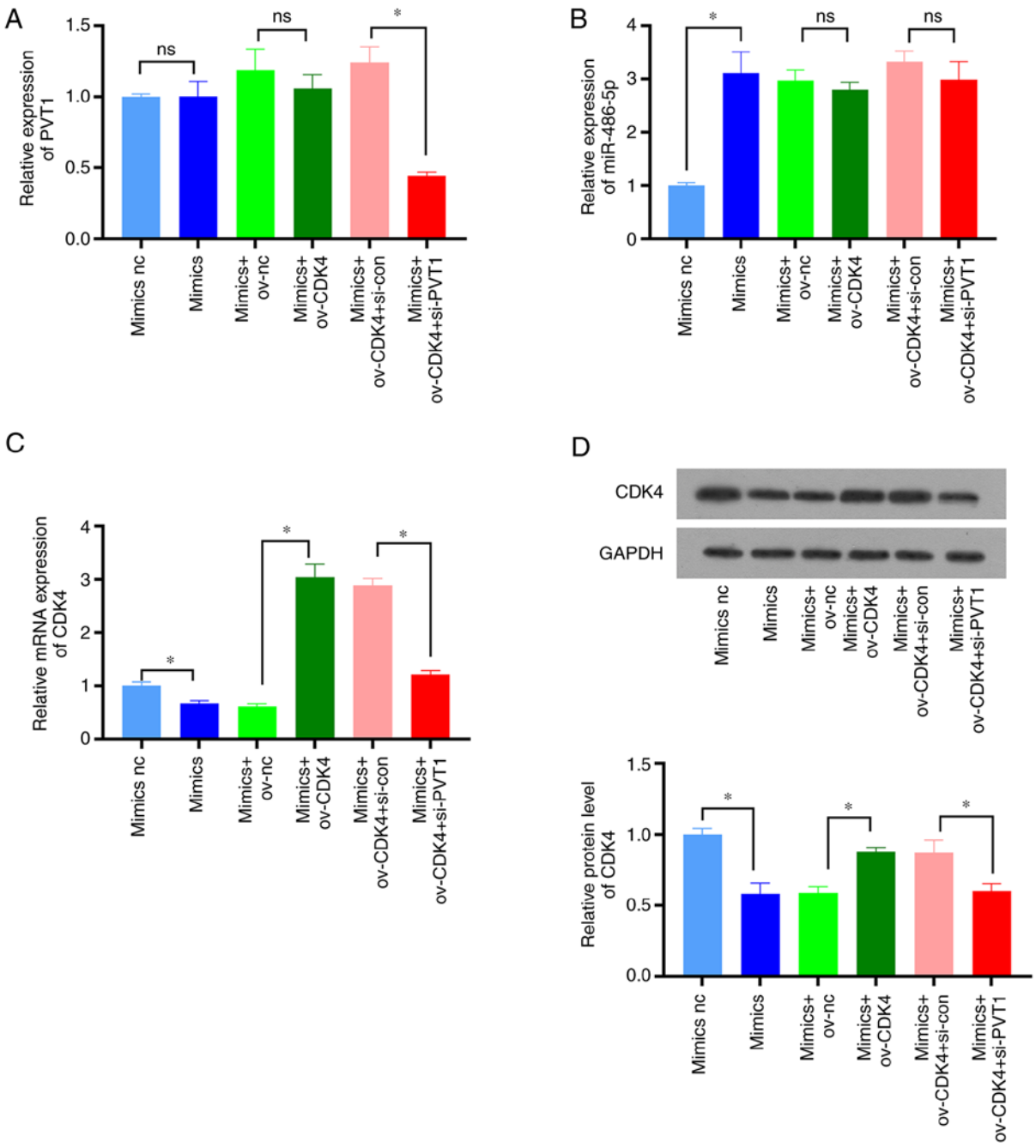

Figure 5. PVT1 regulates colon cancer cell 5-FU resistance via the miR-486-5p/CDK4 axis. Reverse transcription-quantitative PCR was used to detect (A) PVT1, (B) miR-486-5p and (C) CDK4 expression in HCT116-5-FU-resistant cells overexpressing miR-486-5p and CDK4, or in cells in which PVT1 expression was knocked down. (D) Western blot analysis was performed to detect CDK4 protein levels following the overexpression of miR-486-5p, and CDK4, or PVT1 knockdown in HCT116-5-FU-resistant cells. *P<0.05. PVT1, plasmacytoma variant translocation 1; 5-FU, 5-fluorouracil.

5-FU by suppressing miR-204-5p (14). UCA1 has also been suggested to promote the resistance of colon cancer to 5-FU via the miR-23b-3p/ZNF281 axis (13). Fan *et al* (11) demonstrated that knocking down PVT1 also inhibited the resistance of CRC to 5-FU and at the same time, the expression of PVT1 was significantly upregulated in 5-FU-resistant patients, compared to that in 5-FU-sensitive patients. However, the mechanisms by which PVT1 affects the resistance of colon cancer to 5-FU have not been investigated, at least to the best of our knowledge. Therefore, the present study examined the mechanisms through which PVT1 confers resistance to 5-FU in CRC.

lncRNAs often act as endogenous competitive RNAs in cells by sponging miRNAs to control target genes performing biological functions (18). Thus, differentially regulated miRNAs in colon cancer were analyzed using the GEO database and miRNA-miR-486-5p was selected, which had the largest fold-change among all downregulated miRNAs. Bibiserve2 software predicted and it was also experimentally verified that PVT1 bound to miR-486-5p that can inhibit the proliferation and cell cycle progression of HCT116-5FU-resistant cells. In line with the present study, Zhang *et al* (19) revealed that miR-486-5p inhibited colorectal cancer metastasis. Liu *et al* (20) demonstrated that lnc-NEAT1 may adsorb miR-486-5p, activating the

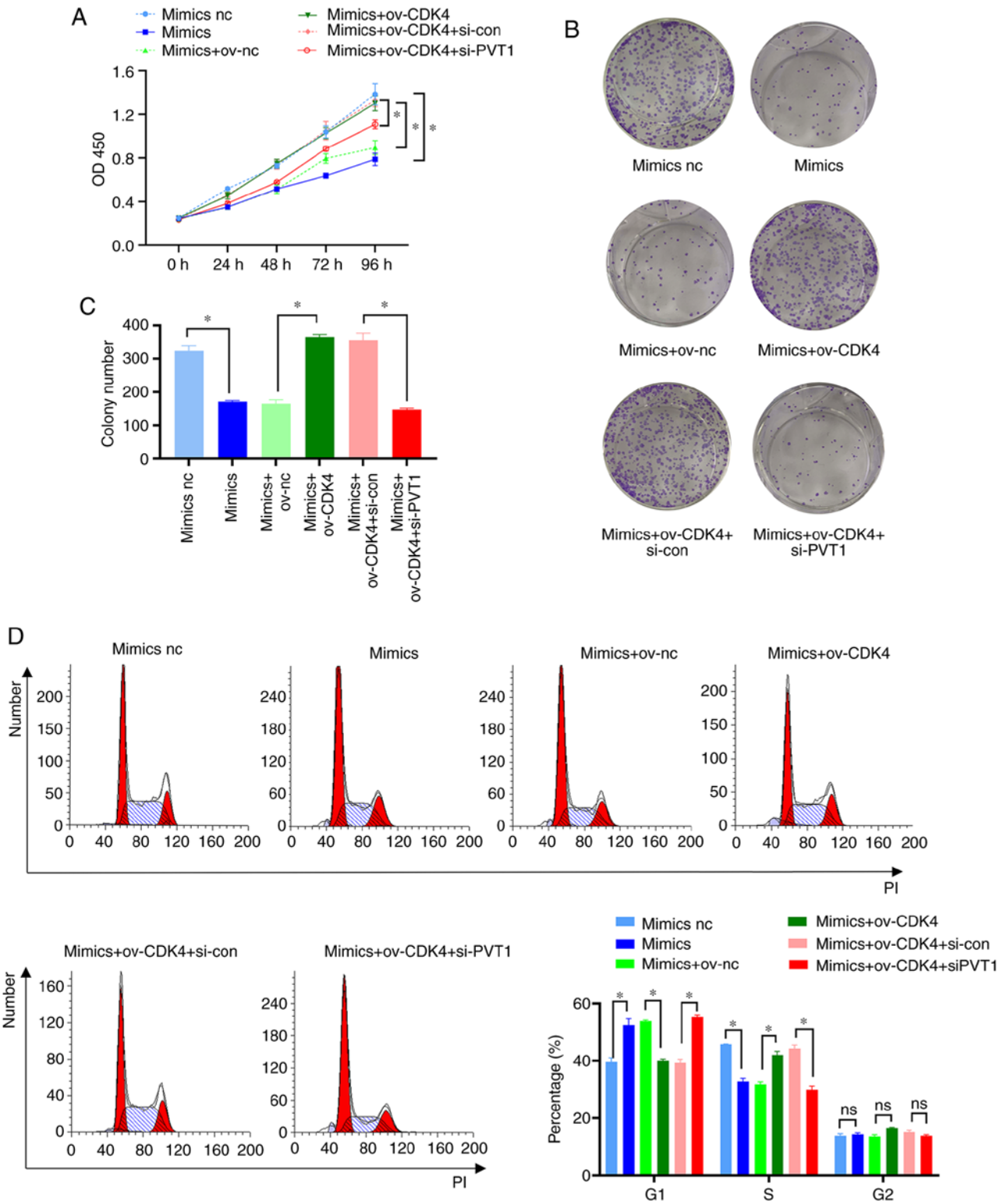

Figure 6. PVT1 regulates colon cancer cell 5-FU resistance via the miR-486-5p/CDK4 axis. (A) CCK-8 assay was used to detect the effects of the PVT1/miR-486-5p/CDK4 regulatory axis on the proliferation of HCT116-5FU-resistant cells. (B and C) A clone formation assay was used to assess the effects of the PVT1/miR-486-5p/CDK4 regulatory axis on clone formation by HCT116-5FU drug-resistant cells. (D) Flow cytometry was used to detect the effect of the PVT1/miR-486-5p/CDK4 regulatory axis on the cell cycle progression of HCT116-5FU drug-resistant cells. *P<0.05. PVT1, plasmacytoma variant translocation 1; 5-FU, 5-fluorouracil.

NR4A1/Wnt/β-catenin signaling pathway, thereby promoting colorectal cancer proliferation. Subsequently, the differential expression analysis of the GEO database for the identification of miR-486-5p target genes was performed and miRDB and TargetScan were screened. The results demonstrated that PVT1 may promote the drug resistance and cell cycle progression of colon cancer cells. Thus, a screening for cell cycle-related differentially expressed genes was performed and CDK4, a cyclin-dependent kinase that can combine with cyclin D to form a heterodimer that phosphorylates and inactivates retinoblastoma

protein to drive the transition from G1 to S phase was identified (21). It was experimentally verified that miR-486-5p may regulate CDK4 expression and that CDK4-knockdown restored the inhibitory effects of miR-486-5p on the proliferation and cell cycle progression of HCT116-5FU cells. PVT1 inhibited CDK4 expression and inhibited proliferation and cell cycle progression in HCT116-5FU cells. In summary, it was observed that PVT1 promoted drug resistance in colon cancer cells by upregulating the miR-486-5p target, CDK4.

A variety of CDK4/6 inhibitors have been used in clinical treatment, among which palbociclib and ribociclib have been approved for the clinical treatment of breast cancer. These CDK4/6 inhibitors are small molecule chemical therapeutics that mainly bind to CDK4/6 to inhibit cell cycle and proliferation. These inhibitors have been reported to easily induce drug resistance in cancer cells. However, PVT1 decreased the expression of CDK4/6 via miR-486-5p, thus indicating that PVT1 may regulate CDK4 mRNA expression. Therefore, PVT1 can still reduce the expression of CDK4 when CDK4 resistance to inhibitors develops, and PVT1 can be used as an adjuvant for CDK4 inhibitors.

The present study did not include *in vivo* validation experiments, in order to further verify the association between PVT1 and colon cancer drug resistance, and to clarify further the mechanisms by which PVT1 affects colon cancer drug resistance. Nevertheless, the present findings strongly support the potential application of targeting PVT1 to counteract 5-FU resistance in colon cancer in clinical practice.

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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.

Authors' contributions

ZH and ZL conceived and designed the study. ZL, RC, SH and XH performed the experiments. All authors have read and approved the final manuscript. ZH and ZL confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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