JBUON 2020; 25(5): 2205-2214
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

IRF-1 mediated long non-coding RNA PVT1-214 promotes oxaliplatin resistance of colorectal cancer via miR-128 inhibition

Deyong Tong[1]*, Erwen Zou[1]*, Li Bai[2], Jianjun Ma[1], Na Guo[1], Huhui Wang[1], Li Jiang[1]

[1]Department of Oncology, 970th Hospital of the PLA Joint Logistics Support Force, no. 7 Zhichu South Rd, Yantai 264000, China. [2]Department of Gastroenterology, 970th Hospital of the PLA Joint Logistics Support Force, no. 7 Zhichu South Rd, Yantai 264000, China.

*These authors contributed equally to this work.

Summary

Purpose: *Long non-coding RNA (lncRNA) plasmacytoma variant translocation 1-214 transcript (PVT1-214) is a notable lncRNA involved in gastric cancer and colorectal cancer (CRC) so far. Nowadays, the biological function of PVT1-214 on the response of CRC to chemotherapy is still unclear. We aimed to explore the molecular mechanism of PVT1-214 and its regulatory mechanism in advanced CRC.*

Methods: *The levels of PVT1-214, microRNA (miR)-128, and interferon regulatory factor-1 (IRF-1) in CRC tissues and cell lines were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). Log-rank test was applied to evaluate the role of high PVT1-214 levels in shortening the overall survival of CRC patients. Chi-square test was to assess the relation between PVT1-214 expression and clinicopathological features of CRC patients. CCK8 assays tested the cell proliferation of oxaliplatin-resistant CRC cells (HCT116/Oxa and SW480/Oxa) with PVT1-214 knockdown. The underlying regulatory mechanism between PVT1-214 and miR-128 was predicted by bioinformatics and verified by RNA transfection, qRT-PCR and western blotting. Chromatin immunoprecipitation (ChIP) assay was done to examine the relationship between or IRF-1 and the PVT1-214 gene.*

Results: *High levels of PVT1-214 expression were more likely to be present in patients with late-stage (IV), chemotherapy resistance, and inferior overall survival. PVT1-214 was aberrantly elevated in oxaliplatin-resistant CRC tissues and cell lines (HCT116/Oxa and SW480/Oxa). PVT1-214 knockdown reduced cell proliferation, migration and invasion of oxaliplatin-resistant CRC cells in vitro. Moreover, IRF-1 was found to be a negative transcription regulator of PVT1-214 and decreased PVT1-214 levels in oxaliplatin-resistant CRC cells. Besides, PVT1-214 repressed miR-128 function by binding to the complementary sites of miR-128.*

Conclusions: *IRF-1/PVT1-214 may markedly boost the oxaliplatin-resistance of CRC, resulting in the late TNM stage and poor survival. These findings suggest that the IRF-1/PVT1-214 axis may be a helpful target for intervention in CRC.*

Key words: *colorectal cancer, interferon regulatory factor-1, long non-coding RNA, plasmacytoma variant translocation 1-214 transcript, miR-128, oxaliplatin resistance*

Introduction

The annual incidence of colorectal cancer (CRC) and its associated deaths rank third among both men and women [1]. Various therapy options have extended the overall survival of metastatic CRC to 3 years; unfortunately, relapses remain a significant barrier to survival improvement [2]. The chemoresistance of residual tumor cells is an essential cause of CRC recurrence after regu-

Corresponding author: Li Jiang, MD. Department of Oncology, 970th Hospital of the PLA Joint Logistics Support Force, no. 7 Zhichu South Rd, Yantai 264000, China.
Tel and Fax: +86 0535 87146943, Email: hanxiaogx@126.com
Received: 17/02/2020; Accepted: 03/03/2020

lar treatments [3]. The loss of SMAD family member 4, a biomarker of resistance to 5-fluorouracil (5-FU), is associated with shorter recurrence-free survival [4]. Increasing in tumor tissues from recurrent patients, circular RNA homeodomain-interacting protein kinase three is involved in oxaliplatin resistance and autophagy inhibition by boosting Beclin1 and bcl2 signaling [5]. Currently, the first-line chemotherapy strategy of CRC is still based on oxaliplatin and 5-FU. Therefore, novel prevention for CRC chemoresistance, especially oxaliplatin-resistance, may be a promising strategy to intervene in the development and relapse of CRC.

Studies have reported that low survival rates and tumor stem cell characteristics are mediated by long non-coding RNAs (lncRNAs) [6]. LncRNAs are transcripts with more than 200 bases and cannot encode proteins [7] but inhibit miR function through interactions [8]. Generally, carcinogenic lncRNAs are abnormally expressed in malignant tumors, enhancing cell proliferation, metastasis and chemoresistance [9]. Inhibition of these carcinogenic lncRNAs can impair cellular malignant biological behavior, thereby controlling cancer growth and improving the therapeutic outcomes [10]. It has been reported that the dysregulation of lncRNA is linked to the resistance to chemotherapeutics of CRC cells. LncRNA nuclear paraspeckle assembly transcript 1 (NEAT1) is found to be related to the 5-FU-resistant features [11]. Cancer-associated fibroblast (CAF)-derived lncRNA CCAL (colorectal cancer-associated lncRNA) can not only be transmitted to CRC cells through exosomes but also enhance the oxaliplatin resistance of CRC cells by up-regulating β-catenin expression [12]. LncRNA Linc00467 plays an essential role in the maintenance of chemoresistance (5-FU) via ferritin light chain (FLC) [13]. Therefore, studying the critical role of lncRNA in tumorigenesis, especially chemoresistance, may have far-reaching value for identifying novel molecular targets in the development of CRC.

Although increasing lncRNAs are found to be involved in the resistance properties of CRC, the upstream regulators of these lncRNAs are rarely reported. In our investigation, we found that lncRNA plasmacytoma variant translocation 1-214 transcript (PVT1-214) was up-regulated in CRC tissues rather than in adjacent normal ones. The data of our study identified that PVT1-214 promoted the chemoresistance of CRC, being mediating by interferon regulatory factor-1 (IRF-1). All these indicated that the IRF-1/ PVT1-214 pathway could be a helpful molecular target for improving the prognosis of CRC patients.

Methods

Chemotherapy responses and follow-up in CRC patients

The Ethics Committee of the 970th Hospital of the PLA Joint Logistics Support Force approved the study. All patients provided written informed consent before the study entry. After an enteroscopic biopsy, a total of 75 CRC patients were collected from January 2014 to October 2014. All patients diagnosed by biopsy received chemotherapy. Any distant metastasis (M1 stage, the 7th AJCC TNM staging system) was confirmed by magnetic resonance imaging (MRI) or bone scanning. Tissue specimens were frozen in -80 °C for further experiments.

The enrollment criteria of qualified patients were as follows: CRC verified by biopsy, no neoadjuvant therapy, no surgery, arranged chemotherapy treatment finished, KPS (Karnofsky performance status) scores >70. Patients were defined as unqualified if they met the following criteria: Absence of measurable disease, second malignancies, incomplete chemotherapy, incomplete follow-up data, and other conditions that required medical treatment.

At least two cycles of the first-line platinum-based chemotherapy (Oxaliplatin 130 mg/m^2 iv on day 1, Capecitabine 1000 mg/m^2 po bid on days 1-14) were administered to all these patients. An enhanced computed tomography before chemotherapy, another one after 2-3 cycles of chemotherapy were performed to evaluate the response to treatment. According to the RECIST1.1 criteria about tumor response (complete response, CR; partial response, PR; stable disease, SD; progressive disease, PD), all those patients were divided to chemosensitive group (n=45, including CR and PR) and chemoresistant group (n=30, including SD and PD) [14]. The overall survival of patients was followed up, defined as the time from the first-time chemotherapy to death or lost to follow-up.

Total RNA extraction and quantitative PCR analysis

Total RNA of tissue and cell lines was extracted using RNAiso Plus (TAKARA, Beijing, China) according to the instructions. The extracted RNA was synthesized to cDNA by the PrimeScript™ R.T. reagent Kit (TAKARA, Beijing, China). Quantitative PCR was done using SYBR® Green Realtime PCR Master Mix (TOYOBO, Shanghai, China) on the Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. The quantitation of the target RNA expression was assessed using the endogenous control by the $2^{-\Delta\Delta Ct}$ method (Glyceraldehyde-phosphate dehydrogenase, GAPDH). Qubit Flex Fluorometer (Thermo Fisher Scientific, USA) was used to evaluate the quality of the prepared RNA, and cDNA was measured. All sequences for primers are shown in Supplementary Table I.

Cell lines and culture

The CRC cell lines SW620, SW480, HCT116, HCT8, HT29, and normal human colonic epithelial cell line HIEC-6 were purchased from the Chinese Academy of Sciences (Shanghai Institute of Cell Biology, Shang-

hai, China). A continuous exposure with an escalating dose of oxaliplatin was applied to establish oxaliplatin-resistant SW480 and HCT116 cell lines (SW480/Oxa and HCT116/Oxa). Briefly, oxaliplatin (Sigma-Aldrich, St Louis, MO, USA) with an initial concentration of 0.5 μM was administered to cells in the exponential growth phase. Oxaliplatin dosage was increased two-fold when SW480/HCT116 exhibited resistance to the current concentration until oxaliplatin was increased to 8 μM. The induced cell line was defined as oxaliplatin-resistant when it could maintain a normal morphology and activity in the highest concentration of oxaliplatin (8 μM) for eight weeks. The cell lines SW620, HCT116, HCT8, HT29, and HIEC-6 were cultured in 10 % fetal bovine serum (FBS) supplementing RPMI (Roswell Park Memorial Institute) 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA). SW480 was cultured in 10% FBS-supplementing Dulbecco Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Waltham, MA, USA). Moreover, SW480/Oxa and HCT116/Oxa were cultured in the medium mentioned above, plus two μM oxaliplatin. All of them were in a 37°C atmosphere with 5 % CO_2.

Determination of half maximal inhibitory concentration

The CRC cells (1×10^5 cells/ml) were seeded into a 96-well plate for overnight culture. Then, different wells were treated with various concentrations (0, 2, 4, 6,8 μM) of oxaliplatin for 48 h. Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Kyushu, Japan) was used to measure cell viability based on the protocol. The cell viability of each well was analyzed at optical density (OD) 450 nm to compute the toxicity curves of oxaliplatin.

Gene knockdown, overexpression and cells transfection

For gene overexpression, lentiviral vectors (pcDciR vector, Geenseed Biotech, Guangzhou, China) were used to construct IRF-1 expressing particles (oe-IRF-1). IRF-1-cDNA or Vector-cDNA (MOI=20) with polybrene was transfected into SW480/Oxa and HCT116/Oxa. For gene knockdown, the pLVX-puro expression vector

Supplementary Table I. The primers for genes detected by real-time PCR

Gene	*Forward primer (5'-3')*	*Reverse primer (5'-3')*
PVT1-214	GGACATGATACCTGGATGTG	CCTGAGTCTCAAGATGCAGTAG
miR-128	CCACCTCTACGCATCATTCA	CCAAGCTCGTCTGGTTCTC
PVT1-214 shRNA	GATCCGCAGCAGCCATCTGGTAATTATTCAAGACG TAATTACCAGATGGCTGCTGCTTTTTT GTCGACA	GCTTGTCGACAAAAAAGCAGCAGCCATCTGGTAA TTACGTCTTGAATAATTACCAGATGGCTGCTGCG
shRNA control	GATCCGCAGCAGCCATACAAGAATTATTCAAGACG TAATTCTTGTATGGCTGCTGCTTTTTT GTCGACA	AGCTTGTCGACAAAAAAGCAGCAGCCATACAAGA ATTACGTCTTGAATAATTCTTGTATGGCTGCTGCG
β-actin	TGACGTTGACATCCGTAAAGACC	CTCAGGAGGAGCAATGATCTTGA
GAPDH	CCCTTCATTGACCTCAACTACA	ATGACAAGCTTCCCGTTCTC

Supplementary Table II. Prediction of transcription factors (Match Score>10) in the JASPAR (https://jaspar.genereg.net/) databases

Matrix ID	*Name*	*Score*	*Relative score*	*Sequence ID*	*Start*	*End*	*Strand*	*Predicted sequence*
MA0516.1	SP2	17.5545	0.962264789	NC_000008.11:127792533-127794632	1783	1797	+	GCCCCGCCTCCGCGC
MA0516.2	SP2	14.1062	0.863634792	NC_000008.11:127792533-127794632	738	754	+	GTAACCCCCACCCCATT
MA0516.1	SP2	13.8286	0.911701506	NC_000008.11:127792533-127794632	2067	2081	-	GCCCCTCCTCGTCCC
MA0052.4	MEF2A	12.1748	0.872470895	NC_000008.11:127792533-127794632	926	940	+	TGCTAAAATTACAAA
MA0516.1	SP2	11.6615	0.882292332	NC_000008.11:127792533-127794632	1935	1949	+	CCCCCTCCCCCGAGG
MA0052.2	MEF2A	11.3723	0.880952153	NC_000008.11:127792533-127794632	926	940	+	TGCTAAAATTACAAA
MA0052.2	MEF2A	11.2637	0.879489495	NC_000008.11:127792533-127794632	1087	1101	+	ATCTCTAAATAAATA
MA0052.4	MEF2A	11.25	0.857360075	NC_000008.11:127792533-127794632	1087	1101	+	ATCTCTAAATAAATA
MA0516.1	SP2	11.1861	0.87584011	NC_000008.11:127792533-127794632	1778	1792	+	AGCCCGCCCCGCCTC
MA0516.1	SP2	10.8059	0.870681317	NC_000008.11:127792533-127794632	584	598	-	GCTCCTCCCTCTTTC
MA0050.1	IRF1	10.7851	0.855220334	NC_000008.11:127792533-127794632	1073	1084	+	CAGAGTGAAACT
MA0052.3	MEF2A	10.6265	0.875014873	NC_000008.11:127792533-127794632	1088	1099	+	TCTCTAAATAAA
MA0516.1	SP2	10.589	0.867737958	NC_000008.11:127792533-127794632	742	756	+	CCCCCACCCCATTGC
MA0052.4	MEF2A	10.5819	0.846442164	NC_000008.11:127792533-127794632	239	253	-	CTTTTAAAATATAAA
MA0516.1	SP2	10.5243	0.86685993	NC_000008.11:127792533-127794632	641	655	-	GCCCCTCTTCTCCCC
MA0516.1	SP2	10.4524	0.865884021	NC_000008.11:127792533-127794632	1856	1870	-	TCCCCGCCCGCCGCC
MA0516.2	SP2	10.4401	0.820463777	NC_000008.11:127792533-127794632	1775	1791	+	CCAAGCCCGCCCCGCCT
MA0893.2	GSX2	10.181	0.975374728	NC_000008.11:127792533-127794632	67	74	+	GCCATTAG

(Genechem, Shanghai, China) and a short hairpin RNA (Genechem, Shanghai, China) (shRNA sequence is shown in Supplementary Table I) were used to decrease the expression of PVT1-214 (sh-PVT1-214 and sh-Vec). Twenty-four h after transfection, a new medium replacement was done to the culture medium. Stably transfected cells were selected by Puromycin (1 μg/ml). The Puromycin (1 μg/ml, 2-3 times) selection was made until green fluorescence was shown in all cancer cells via fluorescence microscope (Olympus IX71, Japan).

Proliferation ability of tumor cells

CRC cells were cultured in 96-well plates (5×10^3 cells/well) for five days. Based on the protocol, CCK-8 (Dojindo, Kumamoto, Kyushu, Japan) was used to measure cell viability every 24 h. The analyzed OD at 450nm at each test was accumulated to compute the proliferation curves.

Migration and invasion ability of tumor cells

Transfected CRC cells were incubated for 24 h. According to the manufacturer's instruction of invasion (Matrigel) assay, 5×10^5 transfected cells in 200 μL of medium (fetal bovine serum, FBS free) were seeded in the upper chamber and 600 μL of medium with 10% FBS was added into the lower chamber using BioCoat Matrigel chambers (Corning, New York, NY, USA). After 24 h of incubation, the remaining cells were removed from the polycarbonate membrane of the upper chamber. Moreover, the cells in the lower chamber were fixed, stained, and photographed by a microscope (Olympus, Tokyo, Japan). For the migration assay, the chamber was not pre-coated with Matrigel.

Dual-luciferase reporter assay

The wildtype lncRNA PVT1-214 sequences containing miR128-binding sites or mutant sites were cloned to psiCHECK-2 plasmid (Promega, Madison, WI, USA). Then, following the manufacturer's instructions, the Renilla luciferase reporter vector was transfected into Oxa-resistant CRC cells along with the synthetic vectors, miR-128, anti-miR-128, or relative controls. The above transfected CRC cells were seeded for incubation 48 h. The luciferase activities of the target gene were tested by a Dual Luciferase-Reporter Assay System (Promega, Madison, WI, USA).

Western blotting assay

RIPA lysis buffer (Solarbio, Beijing, China) was applied to extract total proteins, supplemented with 1%

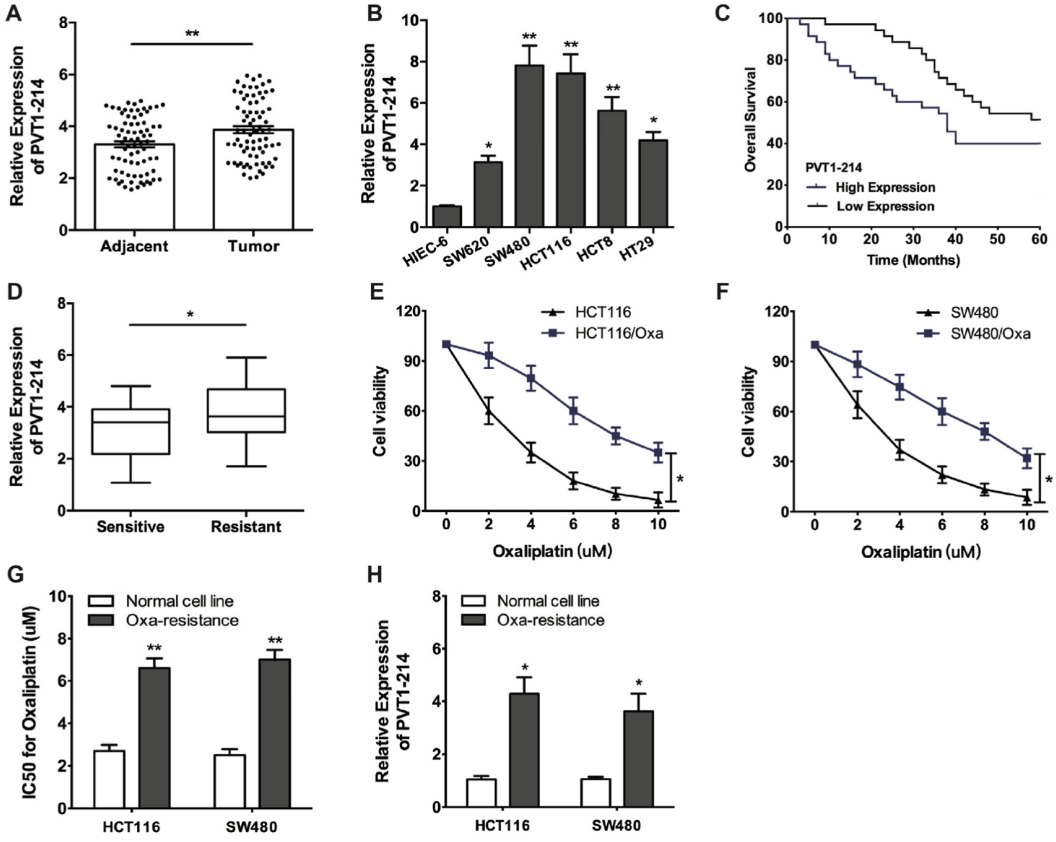

Figure 1. PVT1-214 expression is highly related to oxaliplatin-resistant and inferior survival in CRC. **A:** Expression of PVT1-214 in CRC tissues and adjacent normal tissues shown by qPCR. **B:** PVT1-214 levels in CRC cells (SW620, SW480, HCT116, HCT8, and HT29) and HIEC-6. **C:** Overall survival of CRC patients with high and low PVT1-214 expression. **D:** Expression of PVT1-214 in CRC tissues with different responses to oxaliplatin (Sensitive, n=45; Resistant n=30). **E:** Cell viability of HCT116 and HCT116/Oxa by CCK8 assay. **F:** Cell viability of SW480 and SW480/Oxa by CCK8 assay. **G:** IC50 of oxaliplatin -sensitive and -resistant CRC by CCK8 assays. **H:** Expression of PVT1-214 in CRC cells (Sensitive: HCT116 and SW480) with different responses to oxaliplatin (Resistant: HCT116/Oxa and SW480/Oxa). *p<0.05, **p<0.01.

phosphorylation and protease inhibitors (Solarbio, Beijing, China). According to the manufacturer's protocol, the concentration of the protein samples was tested by the bicinchoninic acid (BCA) protein assay kit (Tiangen, Beijing, China). After denatured at 96°C for 10 min, 9% SDS-PAGE (Solarbio, Beijing, China) was used to divide the target proteins. The PVDF membrane (Solarbio, Beijing, China) was used for transfer. After incubation with 5% non-fat milk for a blockade of non-specific signals, PVDF membranes were incubated with primary antibodies against MRP1 (1:1000), P-gp (1:2000), IRF-1 (1:3000), and GAPDH (1:4000) (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Then, the PVDF membrane was dealt with HRP conjugated secondary antibody (1:5000, Cell Signaling Technology, Danvers, MA, USA). The protein blots were photographed using a Western imaging system (General Electric Company, USA). The density of bands was quantified by Image J software (Bio-Rad, Hercules, CA, USA).

Chromatin immunoprecipitation (ChIP) assay

According to the instruction, the ChIP Assay Kit (Beyotime, Shanghai, China) was used to investigate the relation between IRF-1 and PVT1-214. Briefly, 1% formaldehyde was used to cross-link cells. After a sonication on ice for producing 200-500 bp DNA fragments, chromatin was stained and incubated with primary antibodies against IRF-1 or IgG (isotype control) (Abcam, San Francisco, CA, USA) overnight at 4°C. The antibody precipitated the target chromatin DNA. After the DNA was recovered, a qPCR assay was applied to evaluate it.

Statistics

All data were presented as mean ± standard deviation of 3 independent experiments. The Student's t-test made a comparison between the means from two groups. Survival analysis was performed using the Kaplan-Meier method, followed by comparison using the Log-rank test. All statistical analyses were carried out by SPSS 22.0 software. Statistical significance was set at $p<0.05$.

Results

PVT1-214 expression is highly related to oxaliplatin-resistant and inferior survival in CRC patients

By analyzing 75 pairs of tissues from patients, we found declined expression of PVT1-214 mostly in CRC tissues relative to adjacent normal tissues (Figure 1A). Then, we observed that the expression of PVT1-214 was higher in CRC cell lines (SW620,

Table 1. Relation between PVT1-214 expression and clinicopathological features in CRC (n = 75).

Variables	*Total N*	*PVT1-214 expression*		*p value*
		Low n=37	*High n=38*	
Gender				0.933
Male	49	24	25	
Female	26	13	13	
Age, years				0.535
≤60	29	13	16	
>60	46	24	22	
Tumor size, cm				0.925
≤5	45	22	23	
>5	30	15	15	
Tumor location				0.948
Right hemicolon	31	15	16	
Left hemicolon	44	20	22	
Lymphovascular invasion				0.073
Present	45	26	19	
Absent	30	11	19	
Distant metastasis				0.072
Present	25	16	9	
Absent	50	21	29	
TNM stage				0.028*
II+III	39	24	15	
IV	36	13	23	
Chemotherapy response				0.024*
Sensitive	45	27	18	
Resistant	30	10	20	

*p<0.05 represents significant difference

SW480, HCT116, HCT8, and HT29) than in normal human colonic epithelial cell line (HIEC-6) (Figure 1B). Based on Kaplan-Meier analysis, a higher expression of PVT1-214 was associated with inferior overall survival (Figure 1C, p<0.05). Moreover, CRC patients with advanced TNM stages (IV) or chemotherapy resistance exhibited notably decreased PVT1-214 expression than CRC tissues with less advanced TNM stages (II/III, Table 1, p=0.028) or chemotherapy sensitivity (Table 1, p=0.024; Figure 1 D, p<0.05), respectively. Thus, we adopted the oxaliplatin-resistant CRC cell lines HCT116/Oxa and SW480/Oxa to investigate the association between chemotherapeutics response and PVT1-214. Under increasing oxaliplatin pressure (0-10 μM), SW480/Oxa and HCT116/Oxa cells showed higher viability (Figure 1E & F, p<0.05) and IC_{50} (Figure 1G, p<0.05), compared to SW480 and HCT116 cells. Last but not least, quantitative analysis showed that PVT1-214 expression in SW480/Oxa and HCT116/Oxa cells were higher than their counterparts (Figure 1H, p<0.05). Altogether, our results indicated that PVT1-214 is up-regulated in CRC tissues, and it demonstrates an oncogenic role in CRC by inducing oxaliplatin- resistance.

PVT1-214 knockdown enhanced drug response of oxaliplatin-resistant CRC cells in vitro

A short hairpin expressing (sh-PVT1-214) vector was transfected into SW480/Oxa and HCT116/Oxa cells to repress PVT1-214 expression (Figure 2A). Then, we performed molecular experiments *in vitro* to verify the regulatory function of PVT1-214 on drug response in CRC cells tolerant to oxaliplatin. CCK8 assays revealed that the proliferation of HCT116/Oxa (Figure 2B, p<0.05) and SW480/Oxa (Figure 2C, p<0.05) cells decreased notably after PVT1-214 knockdown. Moreover, compared to HCT116 and SW480 cells, PVT1-214 knockdown strongly suppressed the cell migration (Figure 2D, p<0.05) and invasion (Figure 2E, p<0.05) of HCT116/Oxa and SW480/Oxa cells, as shown by transwell assay. Western blot assay suggested that several intracellular biomarkers for drug tolerance (Multidrug Resistance-associated Protein 1, MRP1 and P-glycoprotein, P-gp) were significantly decreased

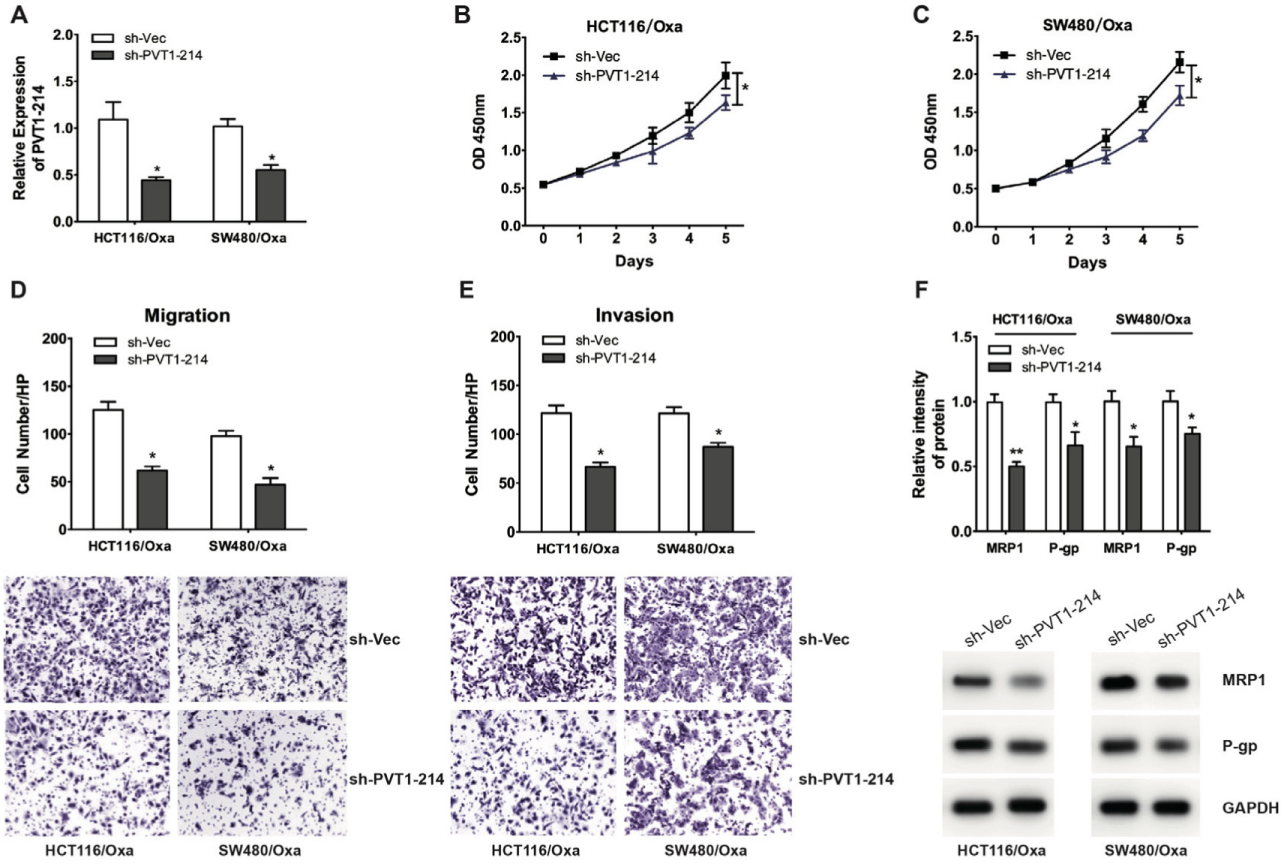

Figure 2. PVT1-214 knockdown enhanced drug response of oxaliplatin-resistant CRC cells *in vitro*. **A:** PVT1-214 expression was decreased by a short hairpin RNA in HCT116/Oxa and SW480/Oxa cells. **B,C:** Proliferative ability of HCT116/Oxa and SW480/Oxa after PVT1-214 knockdown. **D:** Migration ability of HCT116/Oxa and SW480/Oxa transfected with knockdown vector or control (×200). **E:** Invasion ability of HCT116/Oxa and SW480/Oxa after PVT1-214 knockdown (×200). **F:** Western blot assay showed the MRP1 and P-gp protein expression in HCT116/Oxa and SW480/Oxa after PVT1-214 knockdown. *p<0.05, **p<0.01.

in HCT116/Oxa and SW480/Oxa cells with PVT1-214 knockdown compared to vector control (Figure 2F, p<0.05). Therefore, these data suggest that PVT1-214 can mediate the destructive properties (proliferation, migration and invasion), especially the oxaliplatin response of CRC cells *in vitro*.

IRF-1 decreases PVT1-214 expression in CRC cells

To further investigate the upstream regulatory mechanism of PVT1-214 in CRC, we predicted the transcription factors which may target to the promoter of PVT1-214 using the JASPAR (https://jaspar.genereg.net/) databases. The transcription factor IRF-1 is recommended by JASPAR (Supplementary Table II). The binding sites in the PVT1-214 sequence are shown in Figure 3A. ChIP assays exhibited that the PVT1-214 promoter was significantly pulled down by the antibody against IRF-1 rather than the isotype antibody (Figure 3B). A synthesized lentiviral IRF-1-overexpressing (oe-IRF-1) vector was transfected into SW480/Oxa and HCT116/Oxa cells to augment the IRF-1 signal (Figure 3C), leading to a notable decrease of PVT1-214 levels (Figure 3D). Furthermore, the overexpression of IRF-1 strongly restrained mirgation (Figure 3E) and invasion (Figure 3F) in SW480/Oxa and HCT116/Oxa cells. Therefore, these results indicate that IRF-1 acts as a promotor for PVT1-214 in CRC.

PVT1-214 modulates oxaliplatin response in CRC by targeting miR-128

Bioinformatic analysis by miRTarBase predicted the complementary binding potential between PVT1-214 and miR-128 (Figure 4A). The assays validated that the luciferase activity of miR-128 was significantly repressed by the wildtype PVT1-214 rather than the mutant form in CRC cells (Figure 4B and C, p<0.05). Compared to the sh-Vec treated cells, the expression of miR-128 inclined both in CRC cells and oxaliplatin-resistant CRC cells transfected with sh-PVT1-214 (Figure 4D and E, p<0.05). Furthermore, verifying that PVT1-214 could target miR-128, the expression of miR-128 in oxaliplatin-resistant CRC tissues (Figure 4F, p<0.05) and cells (Figure 4G, p<0.05) was markedly lower than oxaliplatin-sensitive ones, shown by qRT-PCR. Collectively, our data suggested that PVT1-214 interacted with miR-128 as a competing endogenous RNA to modulate oxaliplatin response in CRC.

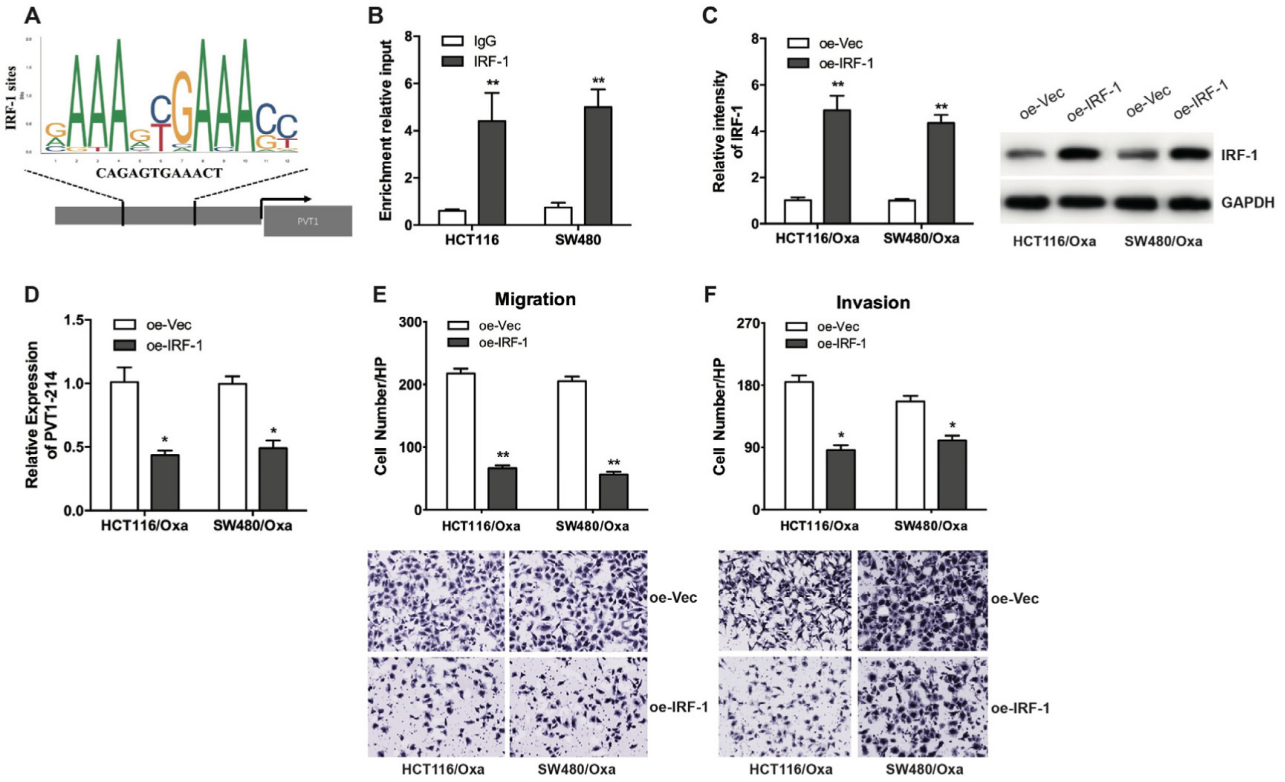

Figure 3. IRF-1 decreases PVT1-214 expression in CRC cells. **A:** JASPAR database reveals the complementary binding sites within the promoter region of PVT1-214. **B:** The targeting ability of IRF-1 to the PVT1-214 promoter validated by ChIP assay and qPCR. **C:** Western blot assay showed the IRF-1 protein expression after overexpression of IRF-1. **D:** PVT1-214 expression was decreased by lentiviral vectors of IRF-1 in HCT116/Oxa and SW480/Oxa cells. **E:** Migration ability of HCT116/Oxa and SW480/Oxa transfected with IRF-1 overexpressing vector or control (×200). **F:** Invasion ability of HCT116/Oxa and SW480/Oxa after IRF-1 overexpression (×200). *p<0.05, **p<0.01.

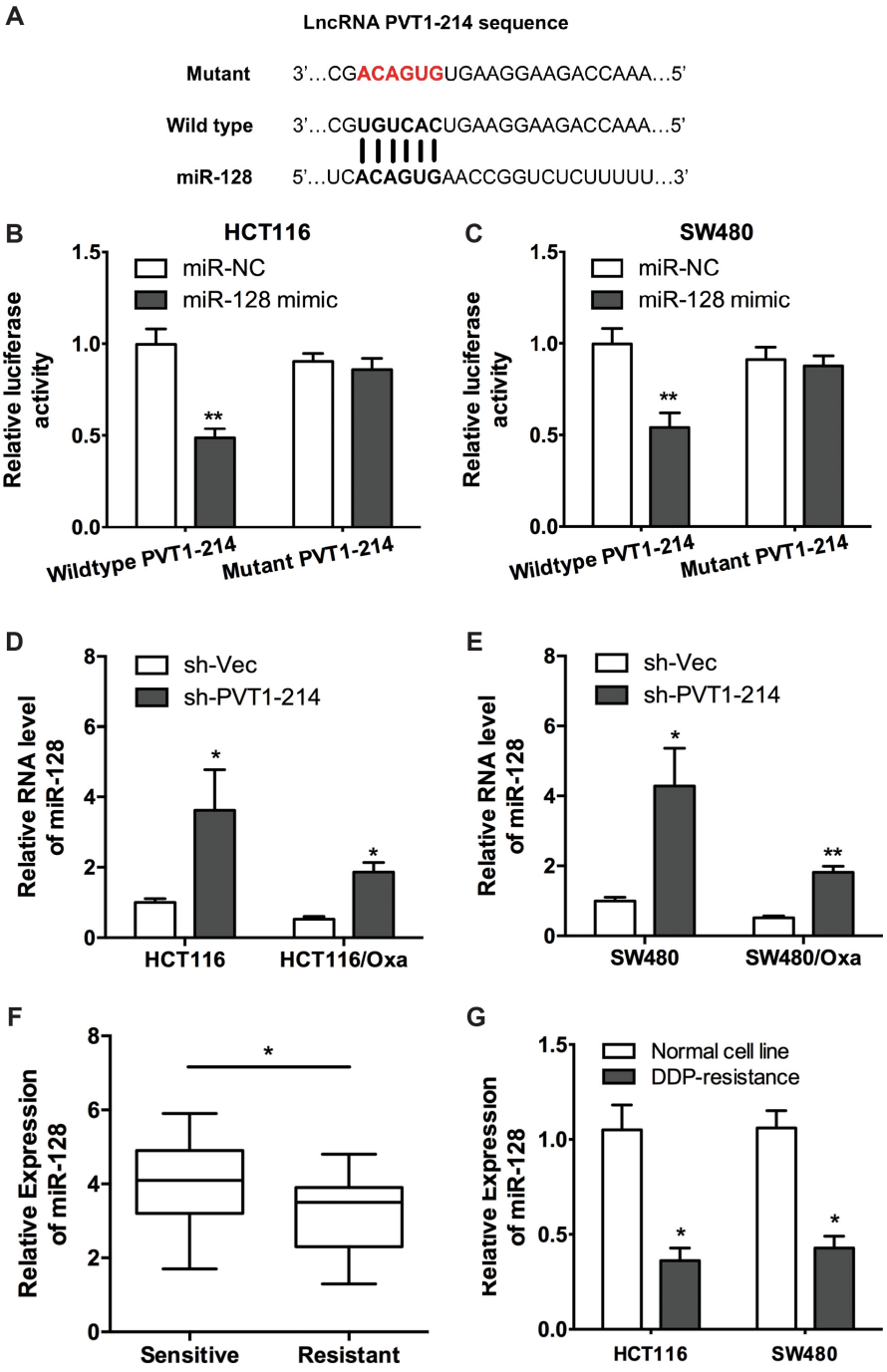

Figure 4. PVT1-214 modulates oxaliplatin response in CRC by targeting miR-128. **A:** Bioinformatics tools reveal the complementary binding sites within PVT1-214 and miR-128. **B,C:** Luciferase reporter assay confirmed the molecular binding between PVT1-214 and miR-128 in HCT116 (B) and SW480 **(C)**. **D,E:** qRT-PCR showed the miR-128 expression in CRC cells transfected with PVT1-214 knockdown vector or control in HCT116/Oxa **(D)**and SW480/Oxa **(E)** cells. **F:** Expression of miR-128 in CRC tissues with different responses to oxaliplatin (Sensitive, n=45; Resistant n=30). **G:** miR-128 expression in oxaliplatin -sensitive and -resistant CRC cells. *p<0.05, **p<0.01.

Discussion

Despite extensive research on the molecular mechanisms of drug resistance, there is still lack of an effective intervention strategy. Studying the genetic characteristics of the chemoresistant phenotype in cancer cells may lead to the discovery of more effective therapeutic targets. We investigated the molecular mechanism of PVT1-214/miR-128 regulatory axis in the regulation of chemoresistance in CRC cells and explored potential new interventions in tumor progression. Previously, investigators have confirmed that PVT1 acted as an oncogene in CRC, boosting the resistance to chemotherapeutics. Initially, researchers found up-regulated PVT1 appears to be associated with shorter

survival of CRC [15]. Moreover, PVT1 can be up-regulated by the transcription factor RUNX2, thereby promoting cell proliferation of CRC [16]. Elevated Circular RNA PVT1 in CRC also can promote tumor cell invasion and metastasis by inhibiting miR-145 [17]. PVT1 silencing can reverse the resistance to oxaliplatin of CRC cells [18]. Among all transcripts of PVT1, PVT1-214 was found to have the largest increase [19]. However, little is known about the chemotherapy resistance mediated by PVT1-214 and its upstream transcription factor in CRC. In this study, we found that the aberrant increase of PVT1-214 was strongly related to chemoresistance characteristics and maybe an independent predictor of inferior overall survival of CRC (Figure 1). Knockdown PVT1-214 in CRC cells inhibits the proliferation of *in vitro* oxaliplatin-resistant cell lines (Figure 2). PVT1-214 can alter the biological behavior of tumor cells via miR-128 regulation (Figure 4). Moreover, IRF-1 has been predicted and verified as the upstream regulatory factor of PVT1-214 (Figure 3). Altogether, these results indicate that IRF-1/PVT1-214 does involve in chemoresistance maintenance and inferior prognosis in CRC patients.

IRF-1, as a transcription factor, can be competitively repressed by IRF-2 function [20]. Activated IRF-1 often inhibits the malignant properties in various human cancers, especially CRC. IRF-1 reduces the risk of CRC by reducing the intestinal inflammation and suppressing intestinal dysplasia [21]. The activation of the Wnt/β-catenin axis can degrade IRF-1, leading to the occurrence of CRC [22]. IRF1, as a promoter for Ras association domain-containing protein 5 activation, can arrest cell cycle rather than induce apoptosis of CRC cells, to repress the development of CRC [23]. Activating crosstalk between IRF1 and miR-29b can sensitize the response of tumor cells to IFN-γ [24]. However, few reports have exhibited the role of IRF-1 in CRC chemoresistance and its underlying mechanism. Our data represent a pioneering research to show that IRF-1 functions as a tumor-suppressing gene targeting to the regulatory sites of PVT1, triggering the tumor response to oxaliplatin (Figure 3). Collectively, our results suggest that IRF-1 acts as a modulator of the malignant properties of CRC cells in the transcription factor/lncRNA interaction pathway.

As competing endogenous RNAs (ceRNAs), sponging target miRs for post-transcriptional regulation is a functional property of lncRNAs [25]. In our study, we applied bioinformatics analysis to predict the interaction between the lncRNA PVT1-214 and miR-128 (Figure 4). The luciferase activity of miR-128 was inhibited by wildtype lncRNA PVT1-214 but was not inhibited by the mutant form, thus demonstrating the direct-binding between them (Figure 4B,C). Furthermore, our results showed that lncRNA PVT1-214 knockdown released the expression of miR-128 in tumor cells (Figure 4D,E). Therefore, since lncRNA PVT1-214 displayed an oncogenic role and competitive repression of miR-128, it is clear that miR-128 acts as a significant tumor suppressor for tumor development. In a previous study, inhibition of miR-128 boosted resistance of ovarian cancer cells to oxaliplatin by decreasing the level of ATP-binding cassette transporter family class C 5 protein [26]. Increased ZEB1 can reverse the inhibitory function of miR-128 overexpression on the sensitive response of gastric cancer cells to oxaliplatin [27]. An investigation by Liu et al found that exosome-derived miR-128-3p could counteract Bmi1 protein activity, leading to enhanced oxaliplatin sensitivity of CRC [28]. Our results represent a novel investigation of the critical role of the lncRNA PVT1-214-miR-128-interaction in the chemoresistance of CRC.

Conclusion

We have demonstrated that a high level of lncRNA PVT1-214 expression was associated with advanced TMN stage, chemotherapy resistance, and inferior prognosis of CRC. LncRNA PVT1-214 can strongly induce the proliferation and metastasis of oxaliplatin-resistant CRC *in vitro*. Furthermore, PVT1-214 was mediated by IRF-1 and regulated miR-128 expression via complementary binding in CRC chemoresistant cells. These data suggest that the IRF-1/PVT1-214/miR-128 axis may be a promising therapeutic target for CRC.

Conflict of interests

The authors declare no conflict of interests.

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