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lncRNA VASH1-AS1/miR-199a-5p/PDCD4 Axis Regulate Proliferation, Apoptosis, and Cell Cycle in PDAC Progress

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Research Article

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Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) is a highly malignant tumor with clinical characterized by short course, rapid progression and rapid deterioration. At present, the studies on the regulatory factors of PDAC are not enough.

Methods: In this study, we applied transcriptome sequencing with PDAC tumor and normal tissues (5cases). Expression of miR-199a-5p was detected by qPCR in another 10 pairs of cancer and normal tissues. The miR-199a-5p mimic, miR-199a-5p mimic NC, miR-199a-5p inhibitor, and miR-199a-5p inhibitor NC were used to detect the function of miR-199a-5p in cell lines. Construction of double luciferase reporter vector of VASH1-AS1 and PDCD4 were used to confirm the binding of miR-199a-5p and 3'- UTR of human PDCD4 mRNA and VASH1-AS1, respectively. PDCD4 protein expression was detected by Western Blot. The cell apoptosis assay was performed using the Annexin V-FITC Kit. Cell proliferation was detected using CCK-8assay and EdU fluorescence analysis. Cell cycle and apoptosis analyzed by flow cytometry at excitation and emission wavelengths of 488 and 530 nm, respectively. SPSS software (version 22.0; SPSS, Chicago, USA) and GraphPad Prism 5.0 (GraphPad software, San Diego, California, USA) were used for data analysis.

Results: After data preprocessing, differentially expressed lncRNAs (606 up-regulated, 875 down-regulated), miRNAs (171 up-regulated, 188 down-regulated) and genes (4129 up-regulated, 3417 down-regulated) were identified. With dada analysis, a new VASH1-AS1/miR-199a-5p/PDCD4 regulation mode was discovered. In PDAC, the expression level of miR-199a-5p is negatively correlated with VASH1-AS1, and the further binding was performed between miR-199a-5p and PDCD4. The overexpression of miR-199a-5p can promote the proliferation and cycle of tumor cells, inhibit cell apoptosis. If the expression of miR-199a-5p is inhibited, the opposite result was obtained. By using overexpression vectors and siRNA of PDCD4, we also found that the low expression of PDCD4 can promote tumor cell proliferation and cycle, and inhibit cell apoptosis. If PDCD4 is highly expressed, the opposite result is obtained..

Conclusion: These findings suggest that lncRNA VASH1-AS1/miR-199a-5p/PDCD4 axis may regulate the formation of PDAC through synergy, and show potential application as an early diagnostic and prognostic marker for PDAC.

Background

Pancreatic cancer (PC), a highly malignant tumor of the digestive system, was prone to recurrence after surgery. It had a poor prognosis, with a 5-year survival rate of less than 8% [1–5]. The incidence of pancreatic cancer had been on the rise. Research on the pathogenesis of PC is ongoing, but no breakthrough so far, which had caused difficulties in diagnosis and treatment [6]. Approximately 70% of PDAC infiltrates to the matrix of normal pancreatic tissue. Although surgical resection was still the main way for PDAC treatment, it ass effective in only 10–15% of newly diagnosed patients. The number of

surgical resections carried out is low due to difficulty in early diagnosis, and lacked of other treatment options means that most patients with PDAC eventually die of metastasis [7].

In decades of miRNA research, more and more evidences showed that deregulation of miRNA expression levels were closely related to the development of tumors. In the study of PDAC, more than 80 types of miRNAs had been shown to regulate the proliferation and apoptosis through a variety of ways. Histone acetylation in the promoter zone can induce miR-21 up-regulation and associate with chemoresistance to gemcitabine and enhanced malignant potential in pancreatic cancer cells [8], miR-200a through regulating the DEK to suppress the metastasis of PDAC [9], miR-148a can target CCKBR and Bcl-2, then regulate growth and apoptosis of pancreatic cancer [10]. The expression pattern and mechanism of miRNA in various tumors are very different in previous reports. In liver cancer, ovarian cancer, prostate cancer, breast cancer, esophageal cancer, testicular cancer, and bladder cancer, miR-199a-5p is down-regulated and inhibited tumor invasion and metastasis by regulating SMAD1, Wnt/β-catenin, HIF-1α and other pathways [11–14]. However, studies such as Li et al. have found that miR-199a-5p is highly expressed in gastric cancer tissues and target the mTOR signaling pathway to promote the cancer processing [15]. High expression of miR-199a-5p has also been found in pancreatic cancer, which can target and regulate the FOXA2 tumor suppressor gene, thereby promoting tumor cell proliferation and invasion [16].

PDCD4 is a new tumor suppressor gene discovered in recent years. The higher expression of PDCD4 can increase the transcription of TIMP2 gene and inhibit the metastasis of breast cancer cells [17]. It can also interact with the transcription factor Twist1 to reduce the expression level of the target gene YB-1 of Twist1, thereby inhibiting cell proliferation [18].

In this study, we investigate whole transcriptome sequencing of the PDAC tumor and normal tissue samples (5cases). We discovered a new VASH1-AS1/miR-199a-5p/PDCD4 ceRNA network. Furthermore, we prove the relationships and functions in vitro between them using dual-luciferase report assay, CCK-8 assay, Flow Cytometry and EdU Fluorescence analysis.

Methods

Reagents and Chemicals

Fetal bovine serum (FBS) and Iscove's modified Dulbecco's medium (IMDM) were obtained from Gibco (USA). The CCK-8 assay kit, Annexin V-FITC assay kit, and Cell cycle assay kit were obtained from Thermo Fisher Scientific (USA). Primary antibodies against PDCD4 (1:500 dilution) and GAPDH (1:500 dilution) were purchased from Thermo Fisher Scientific (USA). HRP-conjugated goat anti-rabbit IgG (1:2000 dilution) and anti-mouse IgG (1:2000 dilution) were purchased from Invitrogen (USA). The EdU cell proliferation detection kit and antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin) were purchased from Solarbio (Beijing, China). All other chemicals were of reagent grade.

Ethics Statement

The tissues of fifteen patients with PDAC (cases) and normal tissues (controls) were used. PDAC was diagnosed by histopathologic evaluation of biopsied pancreatic tissue or by endoscopy. The cancerous and para-cancerous tissues were collected before surgery or radiotherapy. The study was approved by the Biomedical Ethics Committee of the West China Hospital of Sichuan University and was conducted in accordance with the Declaration of Helsinki and its later amendments. All participants provided written informed consent prior to being enrolled in this study.

RNA Extraction and Library Construction

The PDAC tissue samples (15 tumor and 15 normal) were collected and extracted according to the QIAamp Viral RNA Mini Kit. Small RNA sequencing libraries were constructed using the Multiplex Small RNA Library Kit for Illumina® (NEB, USA). LncRNA sequencing libraries were constructed using Stranded RNA Library Kit for Illumina® (NEB, USA). The constructed libraries were sequenced by Illumina Novaseq 6000 (Whbioacme, Co., Ltd, Wuhan).

Data Analysis

Raw data in fastq format were processed with Perl scripts. Quality filtering of clean data through removing reads containing adapters, poly-N, too short reads and low-quality reads from raw data. At the same time, the number of clean reads, clean Q20, clean Q30, and GC contents of the clean data were calculated. All data analysis about miRNA, mRNA, lncRNA were based on clean data. Reference genome and gene annotation files were downloaded directly from the genome website (GRCh38.p13). The known miRNA sequences were obtained in miRBase. miRNA expression levels were obtained using miRDeep2. Differential expression analysis of cancer compared with normal tissues was performed using DESeq2. miRNA target prediction was performed by TargetScan, picTar, microT, miRmap, RNA22, PITA and miRanda. Differential expressed mRNA and lncRNA analysis of cancer compared with normal tissues was performed using DESeq2. The screening criteria for significantly different genes are corrected P-values of 0.05, and log2 (fold change) $\geq$1. The target mRNA and lncRNA prediction of different expression miRNA uses Starbase database (version 3.0; starbase.sysu.edu.cn/index.php), which provides the prediction results of seven miRNA databases (TargetScan, picTar, microT, miRmap, RNA22, PITA and miRanda). The target genes were compared with different expression genes, and selected the overlap genes. The candidate DEL (different expression lncRNA)-DEM (different expression miRNA)-DEG (different expression mRNA) ceRNA network using Cytoscape software (version 3.8.2).

KEGG/GO pathway enrichment analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis uses cluster-profiler of R package. According to the KEGG annotations of genes, selecting all genes of human as background, and using $P<0.05$ as the significance threshold to obtain statistically significant high-frequency annotations relative to the background. In addition, Gene Ontology (GO) can be divided into three parts: Molecular Function, Biological Process and Cellular Component. We selected the all known

cancer-associated pathways according the previously study to construct the scatter diagram of KEGG and GO pathways.

Cell Culture

Human pancreatic cancer cells CFPAC-1 was purchased from the American Type Culture Collection (ATCC, USA). Cells were cultured in IMDM (Gibco, USA) supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin).

Inhibition and overexpression treatment

The miR-199a-5p sequence was retrieved from the miRBase database. The miR-199a-5p mimic, miR-199a-5p mimic NC, miR-199a-5p inhibitor, and miR-199a-5p inhibitor NC sequences were designed and synthesized by Ribobio (Guangzhou). To express PDCD4 and VASH1-AS1, a DNA fragment encoding the full-length PDCD4 and VASH1-AS1 were first amplified from the CFPAC-1 cell line (Table 1), and then cloned into pcDNA3.1 (Ribobio, Guangzhou) at the EcoR1 and Not1 sites, respectively.

Table 1 Mir-199a-5p mimics, inhibitor sequences, VASH1-AS1 and PDCD4 primer sequences

gene name	sequence
miR-199a-5p	5'-UAGCUUAUCAGACUGAUGUUGA-3'
miR-199a-5p reverse	5'-TCAACATCAGTCTGATAAGCTA-3'
miR-199a-5p mimic	5'-UAGCUUAUCAGACUGAUGUUGA-3'
	3'-AUCGAAUAGUCUGACUACAACU-5'
miR-199a-5p mimic NC	5'-UUUGUACUACACAAAAGUACUG-3'
	3'-AAACAUGAUGUGUUUUCAUGAC-5'
miR-199a-5p inhibitor	5'-UCAACAUCAGUCUGAUAAGCUA-3'
miR-199a-5p inhibitor NC	5'-CAGUACUUUUGUGUAGUACAAA-3'
PDCD4-Forward	5'-GAATTCTGGATGTAGAAAATGAGCAGA-3'
PDCD4-Reverse	5'-GCGGCCGCTCAGTAGCTCTCTGGTTTAAG-3'
VASH1-AS1-Forward	5'-GTCTCTTCCTTCCTAGGCC-3'
VASH1-AS1-Reverse	5'-TAGTGGTGCTTTTTAAATTTATTTTTAACTGTTT-3'

Vector constructions and Double Luciferase assay

The 3’-UTR of human PDCD4 and VASH1-AS1 containing the miR-199a-5p binding sites were cloned into pmiR-RB-REPORT vector (primers were listed in Table 2) (Ribobio, Guangzhou), which were named h-PDCD4-WT and h-VASH1-AS1-WT. Mutation in miR-199a-5p binding site was performed through

QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), with pmiR-RB-REPORT-WT as a template and named h-PDCD4-MUT and h-VASH1-AS1-MUT.

PDAC cells were seeded in a 96-well plate and incubated for 24 h before miRNA mimics or mimics NC were added. PDCD4 and VASH1-AS1 3'UTR Dual Luciferase Reporter vector or mutant vector were added to 15μL Opti-Mem medium and 25μL Lipofectamine 2000 (Invitrogen) for dilution. At 36 h after transfection, the fluorescence value was measured using the Dual Luciferase Reporter Assay System (Promega).

Table 2 PDCD4 and VASH1-AS1 3'-UTR amplification primers

primer name	sequence
PDCD4 3'-UTR-Forward	5'-TGTCTGACTGCCACTCCTTTC-3'
PDCD4 3'-UTR-Reverse	5'-AGCTGAGGTAATATGGGCTTG-3'
VASH1-AS1 3'-UTR-Forward	5'-TATGGATTCCTGCGGGTCAC-3'
VASH1-AS1 3'-UTR-Reverse	5'-CTGGCCTTGGGATGTGATGA-3'

Cell Transfection

CFPAC-1 cells (1×10^6 cells/mL) were seeded in a 6-well plate, and 2 mL of IMDM containing serum was added. The cells were placed in an incubator with a CO_2 concentration of 5 % and cultured at 37 °C until the cell confluence reached 70-80%. Separately, miR-199a-5p mimics, mimics NC, miR-199a-5p inhibitors, inhibitor NC, PDCD4-sirna, PDCD4-overexpression vector, VASH1-AS1-sirna and VASH1-AS1-overexpression vector were added to 100 μL of Opti-MEM serum-free medium. After incubation for 48 h, the cells were collected for subsequent experiments.

Real-time PCR

Total RNA was reverse transcribed to cDNA using a Reverse Transcription Kit (Takara Co., Ltd., Dalian, China). The designed primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). cDNA was amplified using SYBR® Premix Ex Taq™ (TaKaRa, Dalian). Gene expression levels were calculated by the ΔΔCt method with GAPDH and U6 as internal controls for mRNA, lncRNA and miRNA respectively. The primer sequences used in this manuscript were listed in Table3.

Table3 QPCR primer sequences for miR-199a-5p, PDCD4, VASH1-AS1 and internal controls

gene name	sequence
miR-199a-5p	Forward 5'-TCCCCCAGTGTTCAGACTAC-3'
	Reverse 5'-GCAGGGTCCGAGGTATTC-3'
U6	Forward5'-CGCTTCGGCAGCACATATAC-3'
	Reverse5'-AAATATGGAACGCTTCACGA-3'
PDCD4	Forward5'-TTGGAGGGGAAGGCTAGTCA-3'
	Reverse5'-AGCAATAAACTGGCCCACCA-3'
VASH1-AS1	Forward5'-TGGTCTAAGGAAGCTGGCAGGAG-3'
	Reverse5'-ATGATGGTGTGAAGGGCAGGAAAC-3'
GAPDH	Forward5'-TCAAGAAGGTGGTGAAGCAGG-3'
	Reverse5'-TCAAAGGTGGAGGAGTGGGT-3'

Western Blot Assay

Western blot assay in CFPAC-1 cells referred to the previous protocol [19], rabit-PDCD4 (1:500 dilution), rabbit GAPDH (1:200 dilution) and secondary antibody anti-rabbit IgG-HRP (1:1000 dilution) were used in our experiments. All results were expressed as the ratio of target protein to GAPDH.

CCK-8 Analysis

CCK-8 analysis in CFPAC-1 cells referred to the previous manuscript [20].

Flow Cytometry Analysis

Flow Cytometry Analysis in CFPAC-1 cells referred to the previous manuscript [19].

EdU Fluorescence Analysis

EdU Fluorescence Analysis in CFPAC-1 cells referred to the previous manuscript [21].

Statistical Analysis

SPSS software (version 22.0; SPSS, Chicago, USA) and GraphPad Prism 5.0 (GraphPad software, San Diego, California, USA) were used for data analysis. The data screening standard was three times the mean ± standard deviation. T-test was used to evaluate differences between groups, and a P value of <0.05 was regarded as to be statistically significant.

Results

Identification of DELs, DEMs and DEGs

The sequencing results showed that compared with the normal group, many genes showed significant differential expression. We identified the DELs, DEMs, and DEGs in cancer and normal tissues, with $P < 0.01$ and $|logFC| \geq 1$ as the thresholds. A total of DELs (606 up-regulated, 875 down-regulated), DEMs (171 up-regulated, 188 down-regulated) and DEGs (4129 up-regulated, 3417 down-regulated) were identified (Table4) (Fig.1A). The heat-map result showed clear separation and consistency expression profiles between cancer and normal samples. With analysis of the significantly DEMs, they are divided into three expression patterns. Type I is highly expressed in tumors and normal tissues like miR-199 family. They may play important roles in promoting the tumor cells' development. Type II show common expression in tumors and normal tissues like miR-450a-5p, and the expression in tumor tissues is up-regulated dozens to hundreds of times compared with normal tissues. These miRNAs may belong to tumor tissue-specific miRNA, which are activated during the development of tumor cells, thereby regulating the related target genes to promote tumor development. Type III is lowly expressed in tumors, but highly expressed in normal tissues like miR-148a-5p. These miRNAs may belong to tumor suppressor genes, which can inhibit tumor development through target gene regulation (Fig.1B).

Table 4 The number of significant different expression of miRNA/mRNA/lncRNA

gene type	total	up-regulate	down-regulate
miRNA	385	171	188
mRNA	7545	4129	3417
lncRNA	1481	875	606

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway analysis

To better understand the role of miRNA in PDAC, 35 significantly different miRNAs were selected to perform GO terms enrichment. The results show that many differentially expressed genes are enriched in intracellular receptor signaling pathway, positive regulation of catabolic process and other GO pathways related to cell growth and signal transfer (Fig.1C). Particularly, the candidate target genes of miR-199 family can be enriched into Wnt, TNF, Pancreatic cancer, FoxO, HIF1 and other pathways, which are close related to tumors formation. All these results indicated that miR-199 family and target genes may play critical roles in the development of PDAC (Fig.1D).

Predicted miRNA-target analysis and ceRNA network construction

35 miRNAs were selected with more significant differences contain three expression patterns for mRNA and lncRNA prediction. Separately, 3 to 5 predicted mRNA/lncRNA with the highest credibility were screened out for ceRNA network construction. Then, 45 lncRNAs, 65 mRNAs and 20 miRNAs were used to construct a ceRNA network. According to the DELs and DEGs, the negative correlation between miRNA and mRNA, and the negative correlation between miRNA and lncRNA were used as the screening basis.

Finally, we found an important ceRNA network of VASH1-AS1/miR-199a-5p/PDCD4 (Fig.1E). In this network, miR-199a-5p is negatively correlated with lncRNA in 178 samples using Starbase database V3.0 (Fig.1F). further confirmation was performed with 10 more samples and qPCR results indicated that the expression of miR-199a-5p in tumor tissues was generally higher than that in normal tissues (P-value=0.006) (Fig.1G)

Verification of VASH1-AS1/miR-199a-5p/PDCD4 Axis in CFPAC-1 cells

In order to verify the relationship between VASH1-AS1/miR-199a-5p/PDCD4 Axis, VASH1-AS1 mimic and inhibitor were used to modulate the expression of VASH1-AS1 in CFPAC-1 cells (Fig. 2A&2B). When the expression of VASH1-AS1 was overexpression, the expression of miR-199a-5p was down-regulated compared with the control group (Fig. 2C). If the expression of VASH1-AS1 was inhibited, the expression of miR-199a-5p was up-regulated compared with the control group (Fig. 2D). These results indicated that there may have a negative correlation between miR-199a-5p and VASH1-AS1. In order to further verify the relationship between miR-199a-5p and VASH1-AS1, we mutated the binding site of VASH1-AS1, and the expression of miR-199a-5p would not be affected. Conversely, wild type VASH1-AS1 can inhibit the expression of miR-199a-5p mimics (Fig. 2E).

Furthermore, miR-199a-5p mimic and inhibitor were applied to modulate the expression of miR-199a-5p in CFPAC-1 cells (Fig. 2F&2G). With qPCR analysis, the expression of PDCD4 were markedly decreased by miR-199a-5p mimics treatment, while miR-199a-5p inhibitor significantly increased the transcription of PDCD4 (Fig.2H&2I). As shown in Fig.2J, compared with mimic NC, the expression of PDCD4 protein was significantly down-regulated by miR-199a-5p mimic treatment, while miR-199a-5p inhibitor significantly up-regulated the expression of PDCD4 protein compared with inhibitor NC treatment. All these results indicated that miR-199a-5p could affect the expression of PDCD4. The regulatory effect of miR-199a-5p on PDCD4 was further analyzed by luciferase reporter assay. As shown in Fig.2K, compared with mimic NC, miR-199a-5p mimic could significantly reduce the activity of PDCD4 in WT cells. Conversely, the miR-199a-5p mimic could lead to the down-regulation of PDCD4 in PDCD4-MUT cells.

Effect of miR-199a-5p on Proliferation, Cell Cycle and Apoptosis in CFPAC-1 Cell

Fig. 3A&3B shown, compared with mimics NC, the cell proliferation rate was significantly increased with miR-199a-5p mimic. however, the cell proliferation rate was significantly decreased by miR-199a-5p inhibitor. As Fig.3C shown, the cell ratio of the G0/G1 phase and promoted cell cycle division were increased after miR-199a-5p mimic treatment compared with mimic NC, whereas miR-199a-5p inhibitor treatment can reduce the G0/G1 phase cell ratio and arrest the cell cycle effectively compared with inhibitor NC. Flow cytometric analysis suggested that the rate of apoptosis was markedly decreased after the expression of miR-199a-5p increased, while the rate of apoptosis was significantly increased associated with the inhibition of miR-199a-5p (Fig.3D). TO investigate the mechanism of cell proliferation, EdU fluorescence analysis was performed and similar results indicated that compared with mimic NC, miR-199a-5p mimic activated the proliferation activity in CFPAC-1 cells significantly. In

contrast, the miR-199a-5p inhibitor reduced the proliferation activity of CFPAC-1 cells compared to the inhibitor NC group effectively (Fig.3E).

Effect of PDCD4 on Cell Proliferation, Cell Cycle and Apoptosis in CFPAC-1 cells

To further prove whether PDCD4, the potential down-stream target of miR-199a-5p, can affect the biological function in CFPAC-1 cells, the si-PDCD4 and PDCD4 overexpression lines were generated in CFPAC-1 cells. Western blot analysis (Fig.4A) showed that the expression of PDCD4 was significantly increased by PDCD4 overexpression plasmid, whereas the expression of PDCD4 protein was inhibited by PDCD4 silencing. As Fig.4B-4C shown, the cell proliferation rate was significantly increased after si-PDCD4 transfection compared with si-NC. However, the cell proliferation rate was obviously decreased by PDCD4 overexpression compared with PDCD4 negative control. As Fig.4D shown, overexpression of PDCD4 can increase the G0/G1 phase cell ratio and affect the cell cycle effectively, and inhibiting the expression of PDCD4 can reduce the G0/G1 phase cell ratio and arrest the cell cycle effectively. Flow cytometric analysis suggested that the rate of apoptosis markedly decreased by PDCD4 silencing, while the rate of apoptosis was significantly increased after PDCD4 overexpressed (Fig.4E). As shown in Fig.4F, compared with PDCD4 NC, PDCD4 overexpression significantly inhibited the proliferation activity of CFPAC-1 cells. si-PDCD4 effectively enhanced the proliferation activity of CFPAC-1 cells, suggesting a negative regulatory relationship between PDCD4 and tumor proliferation in CFPAC-1 cells. Therefore, all above results indicated that PDCD4 could negatively promote the proliferation and cell cycle division of CFPAC-1 cells, and positively regulate the apoptosis in CFPAC-1 cells, which may lead to the negative regulation between miR-199a-5p on PDCD4.

Discussion

The role of ceRNA in the formation of tumors was becoming more and more important. LncRNA can competitively bind to miRNA, thereby affect the expression of mRNA which may affect the tumor formation. Cui et al. had found that LINC01133 inhibits Gastric cancer formation and metastasis by acting as a ceRNA for miR-106a-3p to regulate APC expression and the Wnt/β-catenin pathway, suggesting that LINC01133 may serve as a potential prognostic biomarker and anti-metastatic therapeutic target for Gastric cancer [22].

Through whole-transcriptome sequencing and bioinformatics analysis, we conducted a ceRNA network with VASH1-AS1/miR-199a-5p/PDCD4 axis. The relationships and functions of this axis were further verified by experiments. Interestingly, lncRNA VASH1-AS1 is a new lncRNA, and the role of VASH1-AS1 in tumor formation is rarely reported. In another hand, miR-199a-5p is a special kind of miRNA, and its expression pattern is completely opposite in different tumors. It plays a role as tumor suppressor in many tumors, but it plays a role as tumor-promoting gene in PDAC. PDCD4 has been reported to be a tumor suppressor that can inhibit tumor transformation, formation, and translation [23–24]. Therefore, if miR-199a-5p/PDCD4 and LncRNA VASH1-AS1 may apply as joint diagnostic markers, the abnormal

expression level of these markers can be detected in the blood in the early stage, which may help the early diagnosis of PDAC.

In summary, we found a novel mechanism can influence the progression of PDAC associate with VASH1-AS1/miR-199a-5p/PDCD4 axis. The functional and clinical application of VASH1-AS1/miR-199a-5p/PDCD4 axis needed further research, and it may contribute to early PDAC diagnosis and help with PDAC treatments.

Abbreviations

PDCD4: Programmed cell death 4, VASH1-AS1: Vasohibin 1 antisense RNA 1

PC: Pancreatic cancer; PDAC: Pancreatic ductal adenocarcinoma

FBS: Fetal bovine serum; IMDM: Iscove's Modified Dulbecco's Medium

PAGE: Polyacrylamide gels; PVDF: Polyvinylidene fluoride

3'UTR: 3'-untranslated regions (UTR); PI: Propidium Iodide

Declarations

Ethics approval and consent to participate

The study was approved by the Biomedical Ethics Committee of the West China Hospital of Sichuan University and was conducted in accordance with the Declaration of Helsinki and its later amendments. All participants provided written informed consent prior to being enrolled in this study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that there are no conflicts of interest.

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Authors' contributions

All authors participated in the study. Wei Liu performed the literature search and the experiments. Xinglei Li and Xing Huang performed the sequencing data analysis and collected the clinical samples. Bole Tian participated in the interpretation of data and revised the article for important intellectual content. All authors approved the final version of the article. The authors thank Editage Company for the help in editing the manuscript.

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Figures

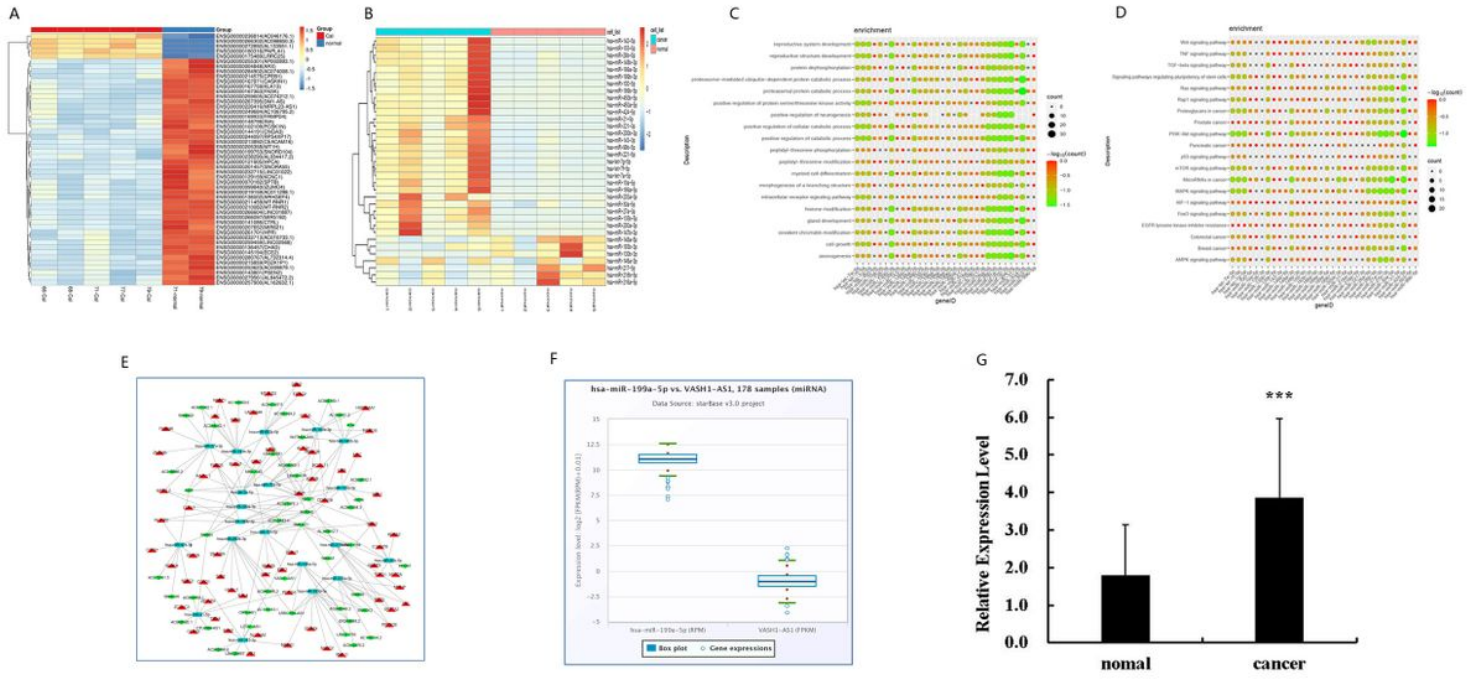

Figure 1

Whole transcriptome sequencing analysis and KEGG/GO enrichment. A: The differential analysis of expression of top 50 differential mRNA/lncRNA B: The differential analysis of candidate miRNA expression. C: GO terms enrichment of target genes of 35 significantly different miRNA. D: KEGG pathway enrichment of target genes of 35 significantly different miRNA. E: ceRNA network. F: expression correlated of miR-199a-5p and VASH1-AS1 from 178 samples. G: The expression level of miR-199a-5p was detected using qPCR in tumor and normal tissues, and the P-value=0.006

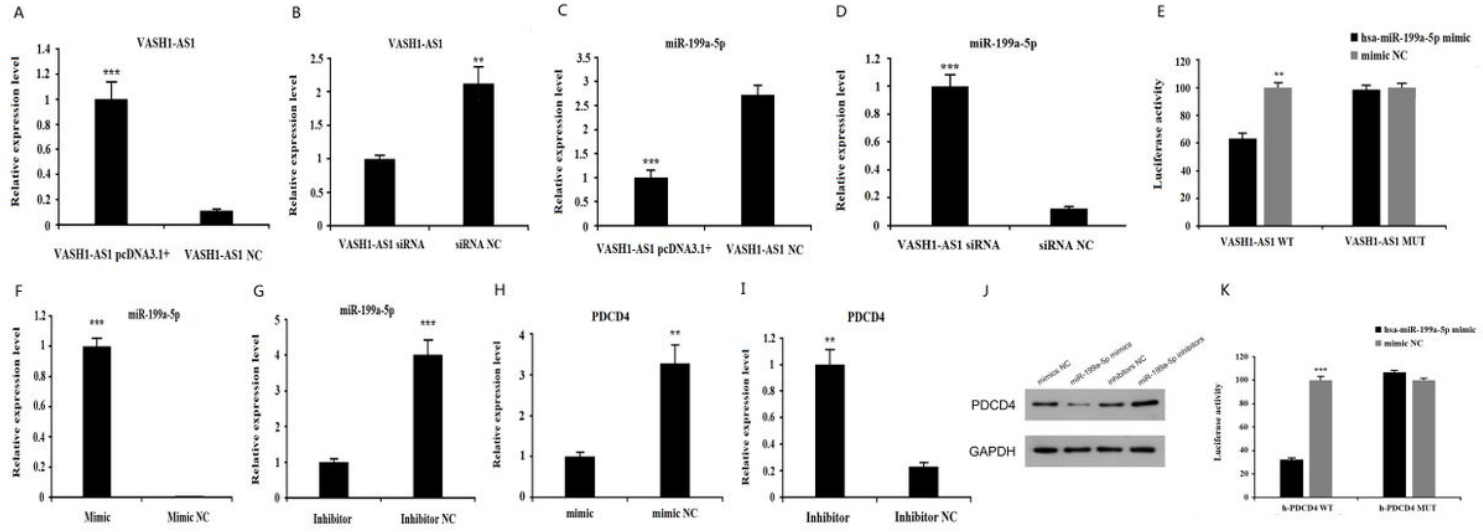

Figure 2

Effect of VASH1-AS1on miR-199a-5p targeting regulation in CFPAC-1 cells. A-B: The expression of VASH1-AS1 in CFPAC-1 cell after transfection with si-VASH1-AS1 and OE- VASH1-AS1. C-D: To verify the targeted regulation of VASH1-AS1 on miR-199a-5p, the expression of miR-199a-5p in CFPAC-1 cell after transfection with VASH1-AS1 for 48 h. E: The fluorescence intensity of VASH1-AS1 was analyzed by luciferase report in VASH1-AS1 WT and VASH1-AS1 MUT infection CFPAC-1 cell under miR-199a-5p mimic transfection. Values are expressed as means±SD. *P<0.05 vs mimic NC group. F-G: The expression of miR-199a-5p in CFPAC-1 cell after transfection with miR-199a-5p mimic or miR-199a-5p inhibitor. H-I: To verify the targeted regulation of miR-199a-5p on PDCD4, the mRNA expression of PDCD4 in CFPAC-1 cell after transfection with miR-199a-5p mimic or miR-199a-5p inhibitor for 48 h compared with NC group. J: The protein expression of PDCD4 in CFPAC-1 cell by western blot analysis under miR-199a-5p mimic or miR-199a-5p inhibitor transfection compared with NC group. K: The fluorescence intensity of h-PDCD4 was analyzed by luciferase report in h-PDCD4 WT and h-PDCD4 MUT infection CFPAC-1 cell under miR-199a-5p mimic or miR-199a-5p inhibitor transfection. Values are expressed as means±SD. *P<0.05 vs mimic NC or inhibitor NC group.

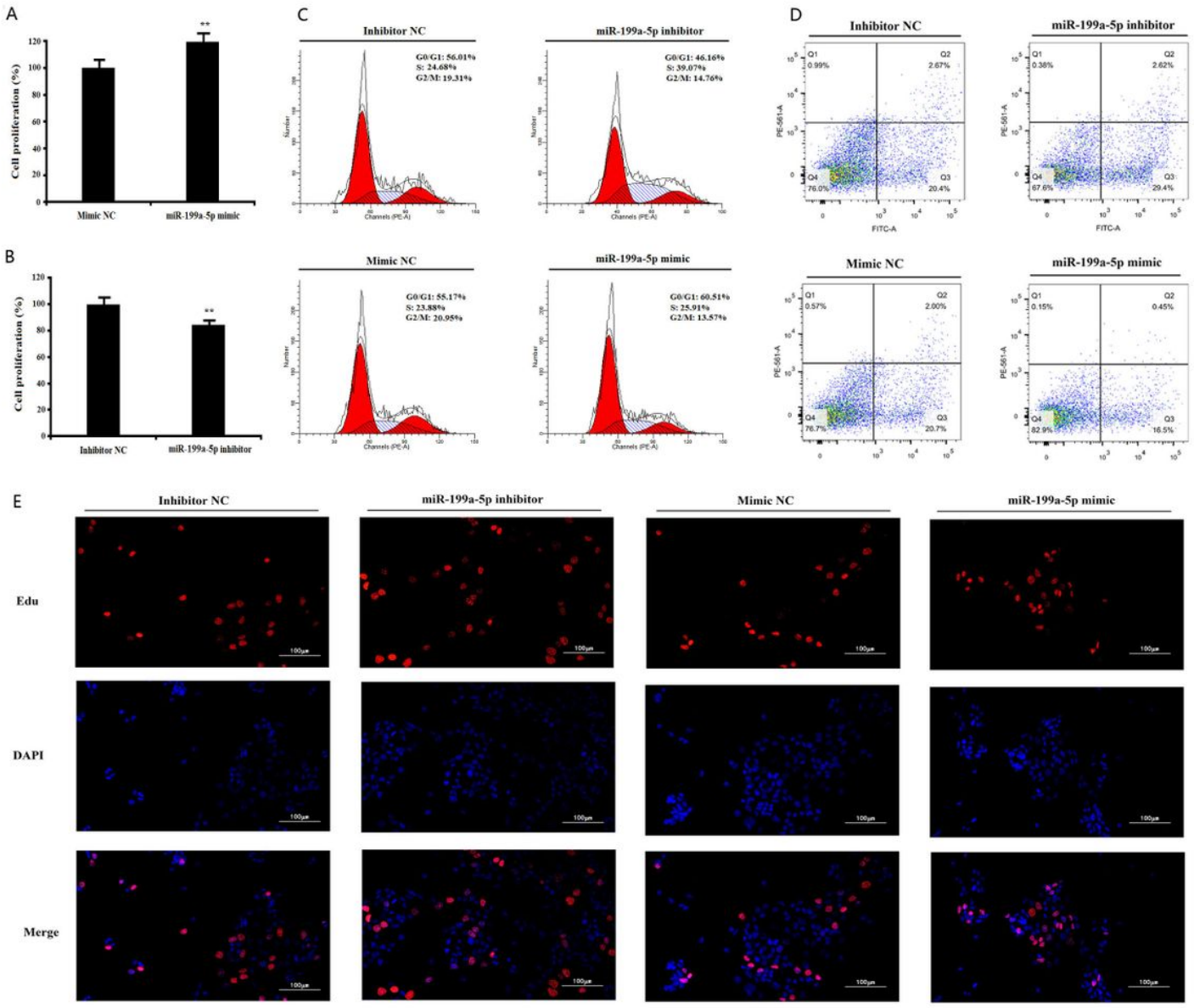

Figure 3

Effect of miR-199a-5p on CFPAC-1 cell proliferation, cycle and apopotosis. To investigate the effect of miR-199a-5p on the biological function of CFPAC-1 cells. miR-199a-5p mimic and miR-199a-5p inhibitor were transfected into CFPAC-1 cells for 48 h. A-B: The proliferation rate of CFPAC-1 cells was determined by CCK-8 assay. C: The cell cycle was measured by the assay kit after transfection with miR-199a-5p mimic and miR-199a-5p inhibitor. D: The apoptosis rate of CFPAC-1 cells was detected using the flow cytometry. E: The proliferation activity of CFPAC-1 cells was detected by EdU fluorescence analysis. Values are expressed as means±SD. *P<0.05 vs mimic NC or inhibitor NC group.

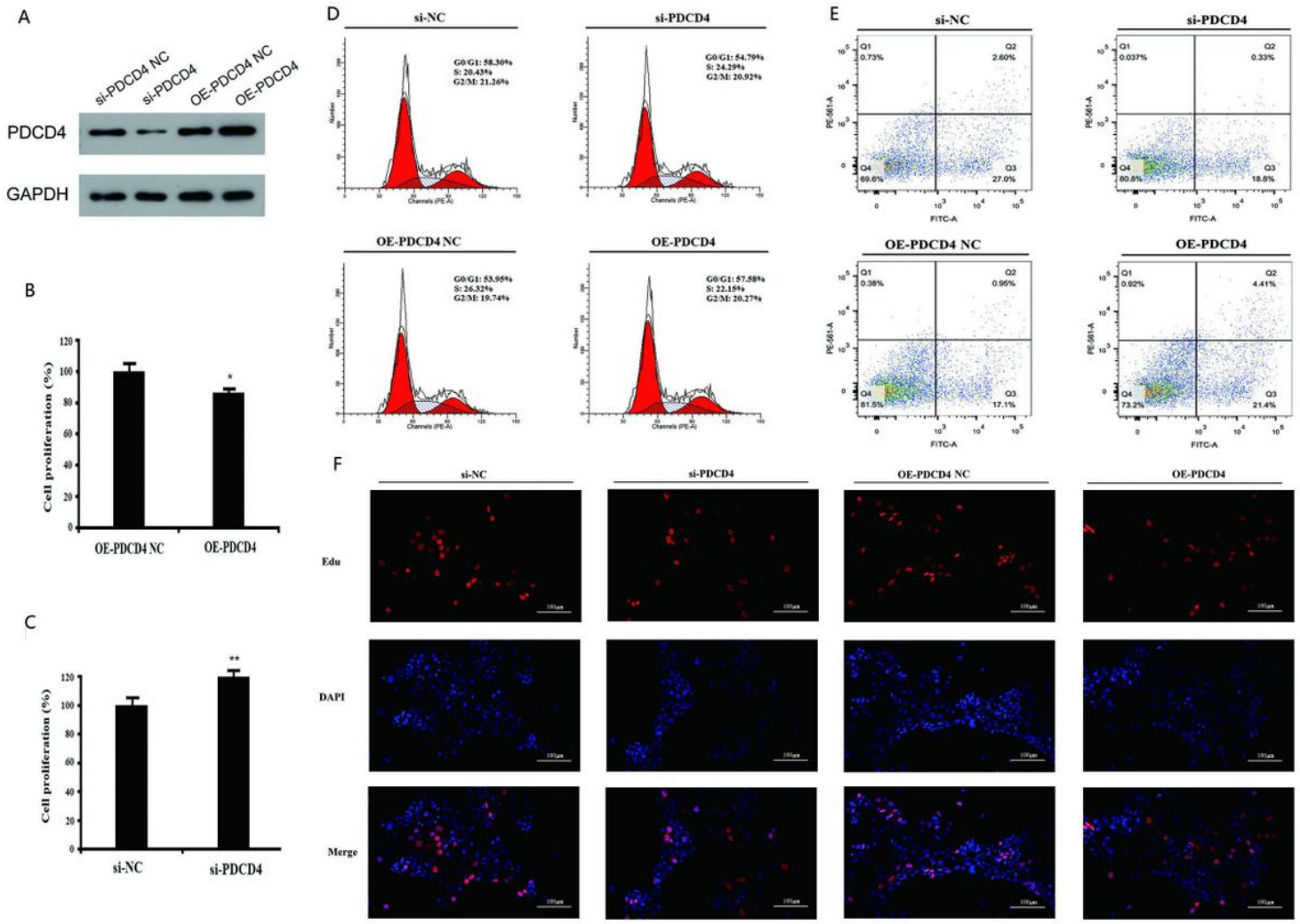

Figure 4

Effect of PDCD4 on CFPAC-1 cell proliferation, cycle division and apopotosis. To investigate the effect of PDCD4 on the biological function of CFPAC-1 cells. si-PDCD4 and PDCD4 overexpression plasmid were transfected into CFPAC-1 cells for 48 h. A: Protein expression of PDCD4 in CFPAC-1 cell by western blot analysis under PDCD4 silence or overexpression. B-C: The proliferation rate of CFPAC-1 cells was determined by CCK-8 assay. D: The cell cycle division was measured by the assay kit after transfection with si-PDCD4 and PDCD4 overexpression plasmid. E: The apoptosis rate of CFPAC-1 cells was detected using the flow cytometry. F: The proliferation activity of CFPAC-1 cells was detected by EdU fluorescence analysis. Values are expressed as means±SD. #P<0.05 vs PDCD4 NC or si-PDCD4 group.

Supplementary Files

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- SupplementalMaterial.docx