Knockdown of hsa_circ_0074298 suppressed the progression of pancreatic cancer by targeting the miR-519/SMOC2 axis

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Abstract

BACKGROUND: Pancreatic cancer (PC) is among the most malignant tumors in digestive system because of its fast progression, metastasis as well as resistance to chemotherapy. Former studies found that circRNAs that differentially expressed were correlated to progression and gemcitabine (GEM)-resistance in PC, and hsa_circ_0074298 aberrant expression played a role in PC progression, but the regulatory mechanism was unclear. **METHODS:** Rt-qPCR was used to detect hsa_circ_0074298 expression in PC cell lines. The CCK8, colony formation, and Transwell assays were used to evaluate the effect of hsa_circ_0074298 on PC cell migration and proliferation. Bioinformatics and luciferase reporter experiments were used to study the regulatory mechanism. Nude mouse xenografts were generated to examine the effect of hsa_circ_0074298 on tumor growth. **RESULTS:** The present study showed that hsa_circ_0074298 expression increased significantly in both PC cell lines and PC tissues. Downregulation of hsa_circ_0074298 caused a significant decrease in PC cell proliferation and in nude mouse xenograft growth. Hsa_circ_0074298 silencing also increased chemotherapy sensitivity to GEM. Bioinformatics analysis indicated that hsa_circ_0074298 was a miR-519 sponge and that the SMOC2 gene was a miR-519 target. MiR-519 downregulation or SMOC2 overexpression restored cell proliferation, migration, and chemoresistance after hsa_circ_0074298 silencing. The dual luciferase reporter assay showed that hsa_circ_0074298 interacted with miR-519, and miR-519 binding to the SMOC2 3'-UTR, then suppressed posttranscriptional SMOC2 expression. **CONCLUSIONS:** Taken together, hsa_circ_0074298 functioned as a tumor promoter through novel miR-519/SMOC2 axis, highlighting its possibility as a potential therapy for PC.

Background

Pancreatic cancer (PC) is among the major causes of cancer-relevant mortality in the world. Mortality caused by PCs of various classes accounted for 411,600 deaths in 2015 [1]. Chemotherapy, many times using gemcitabine (GEM), is now a common adjunct treatment for PC. However, resistance to gemcitabine is a main gap for effective chemotherapy of PC [2–4], although the molecular mechanism of PC resistance to GEM is still unclear.

Non-coding RNAs (ncRNAs) are RNAs not encoding proteins. It is already known that ncRNA contributes to a large amount of cellular RNAs, making at least 90% of human RNAs [5]. Former studies have illustrated that ncRNAs, like proteins, act as underlying factors in various cellular processes, including cell migration, proliferation, apoptosis, chemoresistance, angiogenesis, and the immune response [6, 7]. CircRNAs are considered as essential human disease regulators as well as biomarkers in many kinds of malignancies such like PC. The competing endogenous RNA (ceRNA) mechanism is known as an indispensable mechanism through which circRNAs regulate gene expression. Theoretically, circRNAs are molecular sponges for miRNA with its binding site, indirectly regulation gene expression [8–11].

Previous studies have found that circular RNA circ_0007534 that upregulated is associated with an unfavorable prognosis in pancreatic ductal adenocarcinoma patients, which regulates cell apoptosis,

invasion, as well as proliferation by sponging miR-892b and miR-625 [12]. CircRNA_100782 regulates pancreatic carcinoma proliferation via the IL6-STAT3 pathway [13]. Previous studies with microarray analysis involving circRNA expression profiles associated with GEM resistance in PC cells also revealed a family of circRNAs that were differentially expressed, including hsa_circ_0074298. Although hsa_circ_0074298 functions in PC progress, its mechanism of GEM resistance is not clear, so current investigation would identify hsa_circ_0074298 regulatory mechanism.

Materials & Methods

Ethics statement

We collected 30 fresh PC tissues and paired adjacent tissues after obtaining informed consent from patients at the Affiliated Zhongda Hospital, Southeast University, Nanjing. We snap-froze samples in liquid nitrogen and stored them at -80°C before RNA extraction and fluorescence *in situ* hybridization (FISH). The Ethics Committee of the Affiliated Zhongda Hospital of Southeast University approved this research, and the study was carried out in accordance with [The Code of Ethics of the World Medical Association](#).

FISH

We prepared particular probes to hsa_circ_0074298 (Dig-5'-GCCTCAACCACGGAGTTCCTTTTGCTCGG-3'-Dig) by Genesee Biotech (Guangzhou, China). We detected signals through fluorescein isothiocyanate (FITC)-conjugated anti-biotin antibodies and Cy3-conjugated anti-digoxin antibody (Jackson ImmunoResearch, West Grove, PA, US). We counterstained nuclei with 4,6-diamidino-2-phenylindole, and obtained images using Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Cell culture

We obtained PC cell lines (PANC-1, SW1990, AsPC-1, and BxPC-3) as well as normal human pancreatic duct epithelial cells, HPDE from the American Type Culture Collection. The culture medium consisted of fetal bovine serum (FBS; Invitrogen Life Technologies, Carlsbad, USA) of 10% in Dulbecco's Modified Eagle's Medium, as well as penicillin. We cultured cells in an incubator at 37 °C with CO₂ of 5%.

Bioinformatics analyses

We predicted circRNA/miRNA target genes using [Circular RNA Interactome](#), and predicted the interactive relations between miR-519 and SMOC2 through [TargetScanHuman](#).

Cell transfection

cDNA oligonucleotides specifically targeting hsa_circ_0074298 (sh-circ0074298), miR-519 inhibitor, and SMOC2 overexpression vector were synthesized by GenePharma (Shanghai, China). Both sh-circ0074298 and SMOC2 overexpression vectors were inserted into pGPH1/Neo. Expression of hsa_circ_0074298, miR-

519, and SMOC2 were then monitored using RT-qPCR and western blotting after transfection of PC cells for 48 h.

Transwell migration assay

We analyzed cell migration via Transwell chambers (Corning, Corning, NY, USA) following standard procedures. After incubation for 1 day, we removed cells on the chamber upper surfaces by cotton swabs. We fixed cells locating on the lower surfaces with methanol for ten minutes, followed by Crystal Violet staining. We imaged stained cells and counted them in five fields that randomly selected. In invasion experiments, we coated chamber inserts with 200 mg/mL Matrigel and dried them overnight under sterile conditions.

RNA extraction and RT-qPCR

We utilized TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) to separate total RNA from cultured PC cells and tissues. Using the Reverse Transcription Kit (Takara Biotechnology Co., Ltd., Dalian, China), we reverse-transcribed total RNA to cDNA. The primers used for RT-qPCR are: hsa_circ_0074298, forward: 5'-TTATTGATTATTACTGGCAAAAACG-3', reverse: 5'-CTATGTGGTAGCGTTTAATGTTGGT-3'; miR-519, RT primer, 5'-GTCGTATCCAGTGGTGTCTGTGAGTCGGCAATTGCACTGGATACGACCACTCT-3'; miR-519d, forward: 5'-CAAAGTGCCTCCCTTT-3' and reverse: 5'-CAGTGGTGTCTGTGAGT-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5'-CCAAAATCAGATGGGGCAATGCTGG-3' and reverse: 5'-TGATGGCATGGACTGTGGTCATTCA-3'; and U6, forward: 5'-CTCGCTTCGGCAGCACATA-3' and reverse: 5'-AACGATTCACGAATTTGCTG-3'. The thermal cycle was: 30 s at 95°C, 5 s for 40 cycles at 95°C, and 35 s at 60°C.

Cell proliferation assay

Following the CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan), we assessed cell growth of transfected cells in plates with 96 wells at 24, 48, and 72 h. We used a spectrophotometer (Thermo Scientific, Rockford, IL, USA) to detect the absorbance at 450 nm.

Colony formation assay

We transferred BxPC-3 and PANC-1 cells into plates with 6 wells for ten days. Then, we treated the colonies with 10% formaldehyde for half of an hour, followed by staining for 5 min with 0.5% Crystal Violet. Image-Pro Plus 6.0 (<https://www.mediacy.com/imageproplus>) was used for data analysis.

Cell cycle assay

A total of 2×10^5 cells/mL were diluted with RNase A in 75% ice-cold ethanol overnight, then we stained cells with propidium iodide (PI; 50 mg/mL; MultiSciences Biotech, Hangzhou, China) in the dark for 30 min at 4°C, followed by measuring with a flow cytometer (FACScan, BD Bioscience, San Jose, CA, USA).

Cell apoptosis assay

Flow cytometry binding buffer (100 μ L) was added after harvested cells were washed twice using ice-cold buffer. The mixture containing 5 μ L Annexin V/FICC and PI (BD, Franklin Lakes, NJ, USA) of 5 μ L was used for staining of cells for 15 min in the dark, followed by another 400 μ L binding buffer. We used FACSCalibur flow cytometer (BD Biosciences) to analyze cell apoptosis.

Western blot analysis

We washed cells with precooled phosphate-buffered saline and then lysed them with cell lysis solution (RIPA). We detected the protein concentration via BCA (Thermo Fisher Scientific, Waltham, MA, USA). We then transferred proteins to a polyvinylidene difluoride membrane, and blocked them in TBST (25 mM Tris, 140 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 5% skimmed milk and then incubated them for two hours. We then incubated membranes in primary antibodies against SMOC2 and GAPDH (Abcam, Cambridge, MA, U.S.A.) at 4°C overnight. After washing (3 $\times$ for 10 min) with TBST, we added secondary antibody and incubated them under room temperature for one hour. We analyzed results using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Dual-luciferase reporter assay

We cloned putative miR-519 binding site in the target gene, *SMOC2*, and wild-type (WT) or mutant (MUT) hsa_circ_0074298 3'-UTR into the psi-CHECK (Promega, Madison, WI, USA) vector downstream of the firefly luciferase 3'UTR, or hsa_circ_0074298 as a primary luciferase signal with Renilla luciferase for the normalization signal, and named them as SMOC2-Wt/circ-0074298-Wt and SMOC2-Mut//circ-0074298-Mut. The psi-CHECK vector provided the Renilla luciferase signal for normalization to compensate for distinctions between harvested efficiencies and transfection. We performed transfection of HEK293 cells through Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). We measured Renilla and firefly luciferase activities 1 day after transfection with the Dual-Luciferase Reporter Assay System (Promega, Mannheim, Germany) via a luminometer (Molecular Devices, San Jose, CA, USA), and analyzed relative Renilla luciferase activities following standard procedures.

Animal studies

For xenograft assays, we injected 1×10^6 modified (sh-circRNA, sh-circRNA + miR-519 inhibitor, or sh-circRNA + SMOC2) or control PANC-1 cells subcutaneously into male nude mice (Chinese Science Academy) right side. We measured tumor volumes (length $\times$ width² $\times$ 0.5) at the indicated time points, and excised tumors 4 weeks after injection.

We maintained all mice and handled them according to the instructions of the Animal Ethics Committee of Affiliated XXXX Hospital, XXXX University Medical School.

Statistical analysis

We conducted statistical analysis via SPSS statistical software (SPSS, Chicago, IL, USA). We utilized Student's *t*-test to analyze the data, which are denoted by the mean $\pm$ SD. $p < 0.05$ was regarded as statistical significance.

Results

hsa_circ_0074298 knockdown suppressed PC proliferation in *in vitro* and *in vivo* experiments.

Bioinformatics analysis suggested that hsa_circ_0074298 was derived and cyclized from a portion of the *HARS* gene exon and was located at chr5:140053489-140058712 (Figure 1A). Fluorescence in situ hybridization (FISH) detection showed that hsa_circ_0074298 expression increased in PC tissues comparing with their paired non-tumor tissues. Subcellular localization analysis showed that hsa_circ_0074298 was located predominantly in the cytoplasm (Figure 1B). We then performed qRT-PCR to determine hsa_circ_0074298 expression in 30 paired tissue specimens and four PC cells such as SW1990, BxPC-3, AsPC-1, and PANC1. We used HPDE cells as a control. Results demonstrated that hsa_circ_0074298 expression increased in PC tissues (Figure 1C) and cell lines (Figure 1D). BxPC-3 and PANC-1 cells had the highest hsa_circ_0074298 expressions, which were used in subsequent assays. We conducted Lentivirus-mediated hsa_circ_0074298 silencing of BxPC-3 and PANC-1 cells, and results verified that hsa_circ_0074298 expression decreased significantly (Figure 1E). Cell cycle distribution analysis showed that the percentage of S-phase cells significantly decreased and the percentage of G2/M-phase cells increased after hsa_circ_0074298 depletion, indicating a cell cycle arrest at the G2/M phase (Figure 1F). Both the CCK8 assay (Figure 1G and 1H) and colony formation assay (Figure 1I and 1J) showed that hsa_circ_0074298 depletion decreased cell proliferation in BxPC-3 and PANC-1 cells. PANC-1 cells transfected with or without hsa_circ_0074298 lentiviral interference vectors were used to assay tumor formation in nude mice xenografts. We determined results by measuring tumor volumes 5 days after grafting, with a vernier caliper, and indicated that hsa_circ_0074298 knockdown resulted in a reduced xenograft volume (Figure 1K).

hsa_circ_0074298 knockdown decreased migration capabilities and GEM resistance in PC.

Results validated that hsa_circ_0074298 depletion decreased the migration ability in BxPC-3 and PANC-1 cells (Figure 2A and 2B). To define the degree of the PANC-1-derived GEM-resistant subclone PANC-1-GEM resistance, we treated the derived and parental cells with different GEM concentrations. Cell viability was determined 2 days later using the CCK8 assay. Results illustrated that gemcitabine inhibited the parental PANC-1 cell viability, but PANC-1-GEM cells were totally resistant, when using the highest GEM concentration of 200 nM (Figure 2C). CCK8 detection also showed that hsa_circ_0074298 depletion decreased the proliferative ability of PANC-1-GEM cells after 100 nM GEM treatment for different times (Figure. 2D). Flow cytometry analysis with FITC-conjugated Annexin V/PI staining demonstrated that hsa_circ_0074298 depletion promoted cell apoptosis after treatment with 100 nM GEM for 48 h (Figure 2E and 2F). Overall, the results validated that hsa_circ_0074298 silencing inhibited PC cell migration and chemotherapy resistance, but the mechanism remains to be determined.

MiR-519/SMOC2 was the target of hsa_circ_0074298.

Bioinformatics analysis tool *Circular RNA Interactome* was utilized to select candidate hsa_circ_0074298 targets. Results validated that hsa_circ_0074298 had 12 more conservative miRNAs (Figure 3A). We then designed a hsa_circ_0074298 luciferase reporter screening protocol for the miRNAs. The results showed that miR-519 decreased the hsa_circ_0074298 luciferase reporter luciferase activity most by at least 80% (Figure. 3B). These results indicated that miR-519 had a more conserved binding site for hsa_circ_0074298. The RT-qPCR detection showed that miR-519 expression decreased in PC tissues when comparing with adjacent non-tumor tissues (Figure 3C). We subsequently conducted a dual-luciferase reporter assay in HEK293T cells. We cloned mutant and wild-type hsa_circ_0074298 sequences to construct the mutant vectors and reporter plasmids, respectively (Figure. 3D). Although it was found that miR-519 mimics co-transfected with the reporter plasmids decreased luciferase activity, in contrast, miR-519 mimics and mutated vector transfection showed no significant changes in luciferase activity. Hence, these data proved that miR-519 was a direct hsa_circ_0074298 target (Figure 3E). Next, bioinformatics analysis (<http://www.targetscan.org/>) showed that SMOC2 was a potential miR-519 target. To confirm that SMOC2 was a miR-519 target, we cloned mutant and wild-type SMOC2 sequences to construct mutant vectors and reporter plasmids, respectively (Figure. 3F). The results showed that the reporter plasmid and miR-519 mimic co-transfections visibly suppressed luciferase activity and mutated SMOC2 vectors, but miR-519 mimic co-transfection had no significant effect on luciferase activity. These results proved that miR-519 directly targeted SMOC2 (Figure. 3G), advising that miR-519/SMOC2 was the hsa_circ_0074298 target.

The miR-519 overexpression or SMOC2 downregulation reversed the hsa_circ_0074298 silencing suppressive effect on PC cell migration and proliferation.

RT-qPCR analysis validated that hsa_circ_0074298 expression were decreased after hsa_circ_0074298 silence, but treatment with the overexpressing SMOC2 or the miR-519 inhibitor did not affect hsa_circ_0074298 expression (Figure. 4A and 4B), showing that miR-519 and SMOC2 were hsa_circ_0074298 downstream. RT-qPCR detection also verified that hsa_circ_0074298 silencing promoted miR-519 expression, but SMOC2 overexpression had no effect on hsa_circ_0074298 depletion-induced SMOC2 expression (Figure 4C and 4D), showing that SMOC2 was miR-519 downstream. Western blot analysis demonstrated that hsa_circ_0074298 silencing decreased SMOC2 expression, but miR-519 inhibitor treatment reversed the inhibiting effect of hsa_circ_0074298 depletion on SMOC2 expression. After transfection with the SMOC2 overexpression vector, SMOC2 expression increased significantly (Figure 4E and 4F), suggesting that hsa_circ_0074298 promoted SMOC2 expression via sponging miR-519.

CCK8 assays (Figure 4G and 4H) and colony formation assays (Figure 4-K) showed that downregulation of miR-519 restored the proliferative ability in PANC-1 and BxPC-3 cells after hsa_circ_0074298 depletion. SMOC2 overexpression significantly promoted cells proliferation in BxPC-3 and PANC-1 cells even after hsa_circ_0074298 expression downregulation. The Transwell assay for migration analysis showed that

downregulation of miR-519 restored the migration ability in PANC-1 and BxPC-3 cells after hsa_circ_0074298 depletion. Furthermore, SMOC2 overexpression significantly promoted cell migration in PANC-1 and BxPC-3 cells, even after downregulation of hsa_circ_0074298 expression (Figure 4L-N).

The *in vivo* xenograft mouse models using PANC-1 cells also showed that downregulation of miR-519 or upregulation of SMOC2 recovered the tumor growth ability of PANC-1 after hsa_circ_0074298 knockdown (Figure. 4O), suggesting that miR-519/SMOC2 was a hsa_circ_0074298 downstream target. Overall, the results illustrated that Hsa_circ_0074298 promoted PC progression through sponging miR-519 and enhancing SMOC2 expression.

The miR-519 overexpression or SMOC2 downregulation reversed the hsa_circ_0074298 depletion suppressive effect on PC chemotherapy resistance.

The *in vivo* xenograft mouse models using PANC-1 cells also showed that downregulation of miR-519 or upregulation SMOC2 recovered the tumor growth ability of PANC-1 after hsa_circ_0074298 knockdown (Figure 4O), suggesting that miR-519/SMOC2 was a downstream target of the hsa_circ_0074298. Results verified that Hsa_circ_0074298 promoted PC progression by sponging miR-519 and enhancing SMOC2 expression.

CCK8 assay results also showed that downregulation of miR-519 or upregulation of SMOC2 recovered the hsa_circ_0074298 depletion-induced and proliferation-decreased ability using PANC-1-GEM cells after treatment with 100 nM GEM for 2 days (Figure 5A). Flow cytometry analysis with FITC-conjugated Annexin V/PI staining illustrated that hsa_circ_0074298 depletion promoted cell apoptosis after treatment with 100 nM GEM for 48 h, but downregulated miR-519 or upregulated SMOC2 recovered the survival ability of PANC-1-GEM cells (Figure 5B and 5C). Overall, the results validated that hsa_circ_0074298 promoted PC chemoresistance through sponging miR-519 and enhancing SMOC2 expression.

Discussion

Current investigation suggested that hsa_circ_0074298 expression increased in both PC tissues and PC cell lines. Hsa_circ_0074298 was 1,486 bp long and constructed with part of the HARS gene exon. Downregulation of hsa_circ_0074298 led to cell cycle arrest at the G2/M phase, and hsa_circ_0074298 silencing suppressed cell proliferation, migration, and GEM resistance. The hsa_circ_0074298 therefore functioned in PC progression and chemoresistance. Increasing studies have found that circRNA sponges have been characterized with high expression levels and a good amount of miRNA binding sites. They are potentially effective sponges comparing with those linear [14, 15]. Currently, sponging activity is the main function of some circRNAs, so in tumor development, circRNA-miRNA-mRNA interaction networks might be crucially important [16, 17].

To further identify the downstream miRNA, bioinformatics analysis was used to show that 12 different miRNAs were the targets of hsa_circ_0074298. Luciferase reporter studies confirmed that hsa_circ_0074298 could interface with miR-519. hsa_circ_0074298 downregulation promoted miR-519 expression, and miR-519 silencing restored the proliferation, migration, and GEM resistance after hsa_circ_0074298 depletion, suggesting that miR-519 had anti-tumor activity. Previous studies also found that upregulation of miR-519 inhibited cancer cell activity, including that of nasopharyngeal carcinoma and gastric cancer [18–20]. Together, these results strongly suggested that hsa_circ_0074298 expression promoted the pancreatic cancer progression by sponging miR-519.

Our further bioinformatics analyses showed that miR-519 bound to the SMOC2 3'UTR, and luciferase reporter studies confirmed that miR-519 interacted with the 3'UTR of SMOC2. Overexpression of SMOC2 restored the proliferation, migration, and GEM resistance after hsa_circ_0074298 depletion. An SMOC-2 named the SPARC-related modular calcium binding 2, which is a member of SPARC family, is highly expressed during wound healing and embryogenesis [21–23]. Its gene product is a matricellular protein that stimulates endothelial cell migration and proliferation, and angiogenic activity [24, 25]. This study also found that targeting the cancer stem cell signature gene, SMOC-2, inhibited endometrial carcinoma cell proliferation and overcame the chemoresistance [26]. Smoc2 potentiates carcinoma cell proliferation through cell cycle progression promotion [27, 28]. Present investigation showed that hsa_circ_0074298 promoted PC cell proliferation, migration, and chemoresistance by sponging miR-519 and enhancing SMOC2 expression.

Conclusions

In conclusion, results verified that hsa_circ_0074298 promoted proliferation in PC cells by possibly activating miR-519/SMOC2 signaling. All our results indicated that hsa_circ_0074298 could be candidate biomarker for PC prognosis and diagnosis, and may extend the use of drugs that target hsa_circ_0074298, which indicates the promising role regarding hsa_circ_0074298 in PC treatment.

Abbreviations

circRNAs, Circular RNAs; PC, pancreatic cancer; GEM, gemcitabine; ncRNAs, Non-coding RNAs; ceRNA, competing endogenous RNA; FITC, fluorescein isothiocyanate; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; CCK-8, Cell Counting Kit-8; RT-qPCR, quantitative reverse transcription-polymerase chain reaction.

Declarations

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Author Contributions

HC and DX were performed all the experiments and analyzed the data; HC and JZ carried out in all experiments and revised the manuscript; LW and JZ were designed the studies and prepared the manuscript with comments from all authors. All authors read and approved the final manuscript.

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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.

Ethics approval and consent to participate

The Ethics Committee of the Affiliated Zhongda Hospital of Southeast University approved this research, and the study was carried out in accordance with [The Code of Ethics of the World Medical Association](#). All patients consented to use of resected tissues for research purposes.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no conflicts of interests.

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References

1. **Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015.** *Lancet* 2016, **388**(10053):1459-1544.
2. Yoshida Y, Yamada T, Matsuoka H, Sonoda H, Fukazawa A, Yoshida H, Ishida H, Hirata K, Hasegawa S, Sakamoto K *et al*: **A Trial Protocol of Biweekly TAS-102 and Bevacizumab as Third-Line Chemotherapy for Advanced/Recurrent Colorectal Cancer: A Phase II Multicenter Clinical Trial (The TAS-CC4 Study).** *J Anus Rectum Colon* 2019, **3**(3):136-141.
3. Scheithauer W, Putora PM, Grunberger B, Eisterer W, Woll E, Prager G, Schabert-Moser R, Greil R, Glatzer M: **Patterns of care in metastatic pancreatic cancer: patient selection in clinical routine.** *Therap Adv Gastroenterol* 2019, **12**:1756284819877635.
4. Sugisawa N, Miyake K, Higuchi T, Oshiro H, Zhang Z, Park JH, Kawaguchi K, Chawla SP, Bouvet M, Singh SR *et al*: **Induction of Metastasis by Low-dose Gemcitabine in a Pancreatic Cancer Orthotopic Mouse Model: An Opposite Effect of Chemotherapy.** *Anticancer Res* 2019, **39**(10):5339-5344.
5. Birney E, Stamatoyannopoulos JA, Dutta A, Guigo R, Gingeras TR, Margulies EH, Weng Z, Snyder M, Dermitzakis ET, Thurman RE *et al*: **Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project.** *Nature* 2007, **447**(7146):799-816.
6. Xie N, Liu G: **ncRNA-regulated immune response and its role in inflammatory lung diseases.** *Am J Physiol Lung Cell Mol Physiol* 2015, **309**(10):L1076-1087.
7. Ding B, Lou W, Xu L, Fan W: **Non-coding RNA in drug resistance of hepatocellular carcinoma.** *Biosci Rep* 2018, **38**(5).
8. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP: **A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language?** *Cell* 2011, **146**(3):353-358.
9. Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J: **Natural RNA circles function as efficient microRNA sponges.** *Nature* 2013, **495**(7441):384-388.
10. Memczak S, Jens M, Elefsinioti A, Torti F, Krueger J, Rybak A, Maier L, Mackowiak SD, Gregersen LH, Munschauer M *et al*: **Circular RNAs are a large class of animal RNAs with regulatory potency.** *Nature* 2013, **495**(7441):333-338.
11. Guo JU, Agarwal V, Guo H, Bartel DP: **Expanded identification and characterization of mammalian circular RNAs.** *Genome Biol* 2014, **15**(7):409.
12. Hao L, Rong W, Bai L, Cui H, Zhang S, Li Y, Chen D, Meng X: **Upregulated circular RNA circ_0007534 indicates an unfavorable prognosis in pancreatic ductal adenocarcinoma and regulates cell proliferation, apoptosis, and invasion by sponging miR-625 and miR-892b.** *J Cell Biochem* 2019, **120**(3):3780-3789.

13. Chen G, Shi Y, Zhang Y, Sun J: **CircRNA_100782 regulates pancreatic carcinoma proliferation through the IL6-STAT3 pathway.** *Onco Targets Ther* 2017, **10**:5783-5794.
14. Wilusz JE, Sharp PA: **Molecular biology. A circuitous route to noncoding RNA.** *Science* 2013, **340**(6131):440-441.
15. Li J, Yang J, Zhou P, Le Y, Zhou C, Wang S, Xu D, Lin HK, Gong Z: **Circular RNAs in cancer: novel insights into origins, properties, functions and implications.** *Am J Cancer Res* 2015, **5**(2):472-480.
16. Liu J, Song S, Lin S, Zhang M, Du Y, Zhang D, Xu W, Wang H: **Circ-SERPINE2 promotes the development of gastric carcinoma by sponging miR-375 and modulating YWHAZ.** *Cell Prolif* 2019, **52**(4):e12648.
17. Liu G, Shi H, Deng L, Zheng H, Kong W, Wen X, Bi H: **Circular RNA circ-FOXO1 facilitates cell progression as ceRNA to target PPDPF and MACC1 by sponging miR-1304-5p in non-small cell lung cancer.** *Biochem Biophys Res Commun* 2019, **513**(1):207-212.
18. Yu G, Zhang T, Jing Y, Bao Q, Tang Q, Zhang Y: **miR-519 suppresses nasopharyngeal carcinoma cell proliferation by targeting oncogene URG4/URGCP.** *Life Sci* 2017, **175**:47-51.
19. Abdelmohsen K, Kim MM, Srikantan S, Mercken EM, Brennan SE, Wilson GM, Cabo R, Gorospe M: **miR-519 suppresses tumor growth by reducing HuR levels.** *Cell Cycle* 2010, **9**(7):1354-1359.
20. Zhou N, Zhou Y, Tang Y, Yu W: **[MiR-519 inhibits gastric cancer cell activity through regulation of HuR expression].** *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 2016, **41**(1):19-23.
21. Pazin DE, Albrecht KH: **Developmental expression of Smoc1 and Smoc2 suggests potential roles in fetal gonad and reproductive tract differentiation.** *Dev Dyn* 2009, **238**(11):2877-2890.
22. Rocnik EF, Liu P, Sato K, Walsh K, Vaziri C: **The novel SPARC family member SMOC-2 potentiates angiogenic growth factor activity.** *J Biol Chem* 2006, **281**(32):22855-22864.
23. Vannahme C, Gosling S, Paulsson M, Maurer P, Hartmann U: **Characterization of SMOC-2, a modular extracellular calcium-binding protein.** *Biochem J* 2003, **373**(Pt 3):805-814.
24. Nishimoto S, Hamajima Y, Toda Y, Toyoda H, Kitamura K, Komurasaki T: **Identification of a novel smooth muscle associated protein, smap2, upregulated during neointima formation in a rat carotid endarterectomy model.** *Biochim Biophys Acta* 2002, **1576**(1-2):225-230.
25. Maier S, Paulsson M, Hartmann U: **The widely expressed extracellular matrix protein SMOC-2 promotes keratinocyte attachment and migration.** *Exp Cell Res* 2008, **314**(13):2477-2487.
26. Lu H, Ju DD, Yang GD, Zhu LY, Yang XM, Li J, Song WW, Wang JH, Zhang CC, Zhang ZG *et al*: **Targeting cancer stem cell signature gene SMOC-2 Overcomes chemoresistance and inhibits cell proliferation of endometrial carcinoma.** *EBioMedicine* 2019, **40**:276-289.
27. Su JR, Kuai JH, Li YQ: **Smoc2 potentiates proliferation of hepatocellular carcinoma cells via promotion of cell cycle progression.** *World J Gastroenterol* 2016, **22**(45):10053-10063.
28. Shvab A, Haase G, Ben-Shmuel A, Gavert N, Brabletz T, Dedhar S, Ben-Ze'ev A: **Induction of the intestinal stem cell signature gene SMOC-2 is required for L1-mediated colon cancer progression.** *Oncogene* 2016, **35**(5):549-557.

Figures

Figure 1

Knockdown of hsa_circ_0074298 suppressed pancreatic cancer (PC) proliferation both in vivo and in vitro. (A) The genomic loci of the HARS gene and hsa_circ_0074298. (B) The expression and subcellular localization of hsa_circ_0074298 in PC tissue was analyzed using in situ hybridization. (C) RT-qPCR

showed the expression of hsa_circ_0074298 in PC tissue. Data are presented as the mean $\pm$ SD. $^{**}P < 0.01$ vs. normal. (D) RT-qPCR showed the expression of hsa_circ_0074298 in PC cell lines (PANC-1, SW1990, BxPC-3, and AsPC-1) and in HPDE normal human pancreatic duct epithelial cells. Data are presented as the mean $\pm$ SD. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. normal cells. (E) RT-qPCR detection of hsa_circ_0074298 expression in both PANC-1 and BxPC-3 cells following transfection with siRNA against hsa_circ_0074298 (sh-circ0074298) or the negative control (NC). Data are presented as the mean $\pm$ SD. $^{***}P < 0.001$ vs. NC. (F) Flow cytometry detection showing the percentages of cells in G1, S, or G2 phase in both PANC-1 and BxPC-3 cells. (G and H) The CCK8 assay shows the cell proliferation of both PANC-1 (G) and BxPC-3 (H) cells. Data are presented as the mean $\pm$ SD. $^{*}P < 0.05$; $^{***}P < 0.001$ vs. NC. (I and J) The colony formation assay showing proliferation in both PANC-1 and BxPC-3 cells. Data are presented as the mean $\pm$ SD; $^{***}P < 0.001$ vs. NC. (K) Knockdown of hsa_circ_0074298 attenuated PANC-1 tumor growth in xenografts of nude mice. Data are presented as the mean $\pm$ SD. $^{*}P < 0.05$; $^{***}P < 0.001$ vs. NC.

Figure 2

Knockdown of hsa_circ_0074298 decreased the migration capabilities and gemcitabine (GEM) resistance in pancreatic cancer (PC). (A and B) The Transwell assay showed the migration of both PANC-1 and BxPC-3 cells. Data are presented as the mean $\pm$ SD; $^{***}P < 0.001$ vs. the normal control (NC). (C) PANC-1 cells and the derived long-time GEM-treated subclone PANC-1-GEM were untreated (0 nM) or treated with GEM with concentrations from 10 to 200 nM as indicated. The viability was measured by the CCK8 assay, 48 h after treatment. Data are presented as the mean $\pm$ SD; $^{***}P < 0.001$ vs. the untreated group. (D) The CCK8 assays were used to evaluate the cellular proliferation of PANC-1-GEM cells with or without hsa_circ_0074298 silencing after treatment with 100 nM GEM for different times (0–72 h). Data are presented as the mean $\pm$ SD; $^{***}P < 0.001$ vs. the untreated group. (E and F) PANC-1-GEM cells with or without hsa_circ_0074298 silencing were treated with 100 nM GEM followed by staining with Annexin V-FITC and propidium iodide 48 h later, then evaluated by FACS-analysis. The percentage of FITC-positive

cells is shown as “Apoptosis rate (%)” Data are presented as the mean ± SD; ***P < 0.001 vs. the untreated group.

Figure 3

The miR-519/SMOC2 was the target of hsa_circ_0074298. (A) A schematic model showing the putative binding sites of 12 predicted miRNAs on hsa_circ_0074298. (B) The luciferase activity of hsa_circ_0074298 in HEK293T cells transfected with miRNA mimics, which are putative binding sites for the hsa_circ_0074298 sequence. Luciferase activity was normalized by Renilla luciferase activity. (C) RT-qPCR showing the relative expression of the miR-519 from pancreatic cancer tumor tissues and adjacent non-tumor tissues. Data are presented as the mean ± SD. ***P < 0.001. (D) The predicted binding sites of miR-519 in the hsa_circ_0074298. The mutated (Mut) version of hsa_circ_0074298 is also shown. (E) Relative luciferase activity was determined 48 h after transfection with miR-519 mimic/normal control

(NC) or with the hsa_circ_0074298 wild-type/Mut in HEK293T cells. Data are presented as the mean $\pm$ SD; $**P < 0.01$. (F) The predicted binding sites of miR-519 within the 3'-UTR of SMOC2. The mutated version of the 3'-UTR of SMOC2 is also shown. (G) Relative luciferase activity was determined 48 h after transfection with the miR-519 mimic/normal control or with the 3'-UTR-SMOC2 wild-type/Mut in HEK293T cells. Data are presented as the mean $\pm$ SD; $**P < 0.01$.

Figure 4

Overexpression of miR-519 or downregulation of SMOC2 reversed the suppressive effect of hsa_circ_0074298 silencing on pancreatic cancer (PC) cell proliferation and migration. (A-D) RT-qPCR detection showing the expression of hsa_circ_0074298 and miR-519 in PANC-1 and BxPC-3 cells. Data are presented as the mean $\pm$ SD; ***P < 0.001 vs. NC; ###P < 0.001 vs. sh-circ0074298. (E and F) Western blot analysis showing the expressions of SMOC2 in PANC-1 and BxPC-3 cells. Data are presented as the mean $\pm$ SD; ***P < 0.001 vs. NC; ###P < 0.001 vs. sh-circ0074298. (G and H) CCK8 assays were used to evaluate cell proliferation after 72 h of culture. Data are presented as the mean $\pm$ SD; ***P < 0.001 vs. NC; ###P < 0.001 vs. sh-circ0074298. (I-K) Colony formation assays showing the proliferation of PANC-1 and BxPC-3 cells. Data are presented as the mean $\pm$ SD; *P < 0.05; ***P < 0.001 vs. NC; ###P < 0.001 vs. sh-circ0074298. (L-N) Cell migration was assessed in PANC-1 and BxPC-3 cells using Transwell assays. Data are presented as the mean $\pm$ SD; ***P < 0.001 vs. NC; ###P < 0.001 vs. sh-circ0074298. (O) Xenograft tumors in nude mice from the four treatment groups (NC, sh-circRNA, sh-circRNA + miR-519 inhibitor, and sh-circRNA + SMOC2) after subcutaneous injection of PANC-1 cells. Xenograft volumes from the four treatment groups were measured at the indicated time points. Data are presented as the mean $\pm$ SD. ***P < 0.001 vs. the NC; ###P < 0.001 vs. sh-circ0074298.

Figure 5

Overexpression of miR-519 or downregulation of SMOC2 reversed the suppressive effect of hsa_circ_0074298 silencing on pancreatic cancer chemotherapy resistance. (A) CCK8 assays were used

to evaluate the cellular proliferation of PANC-1-GEM cells after treatment with 100 nM gemcitabine (GEM) for 48 h. Data are presented as the mean $\pm$ SD; ***P < 0.001 vs. NC; ###P < 0.001 vs. sh-circ0074298. (B and C) PANC-1-GEM cells in different group were treated with 100 nM GEM followed by staining with Annexin V-FITC and PI, 48 h later, then evaluated by FACS-analysis. The percentage of FITC-positive cells is shown as "Apoptosis rate (%)." Data are presented as the mean $\pm$ SD; ***P < 0.001 vs. NC; ###P < 0.001 vs. sh-circ0074298.