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ORIGINAL RESEARCH

LncRNA DANCR Promotes Sorafenib Resistance via Activation of IL-6/STAT3 Signaling in Hepatocellular Carcinoma Cells

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Background: Hepatocellular carcinoma (HCC) is one of the major malignancies and the second most common cause of cancer-related death worldwide. Sorafenib, an approved first-line systematic treatment agent for HCC, is capable to effectively improve the survival of patients with advanced HCC. The long-noncoding RNA (lncRNA) differentiation antagonizing non-protein coding RNA (DANCR) has been reported to exert oncogenic functions in several kinds of human cancers. However, the role of lncRNA DANCR in sorafenib resistance in HCC remains unknown.

Methods: The expression levels of DANCR in HCC tissues were detected by qRT-PCR. DANCR overexpression and knockdown models were established and utilized to investigate the functional role of DANCR on sorafenib resistance in HCC cells. The MS2-binding sequences-MS2-binding protein–based RNA immunoprecipitation assay, RNA pull-down and luciferase reporter assay was used to detect the association between DANCR and PSMD10 mRNA. The activation of DANCR transcription mediated by STAT3 was assessed by luciferase reporter and chromatin immunoprecipitation assays.

Results: We found that DANCR was significantly overexpressed in HCC tissues and associated with prognosis of HCC patients. Overexpression and knockdown experiments demonstrated that DANCR promoted sorafenib resistance in HCC cells in vitro and in vivo. Mechanistically, the role of DANCR relied largely on the association with PSMD10. DANCR stabilized PSMD10 mRNA through blocking the repressing effect of several microRNAs on PSMD10. Besides, DANCR activated IL-6/STAT3 signaling via PSMD10. Furthermore, we revealed that DANCR transcription was enhanced by the activation of IL-6/STAT3 signaling, indicating a positive feedback loop of DANCR and IL-6/STAT3 signaling.

Conclusion: Collectively, our study is the first to elucidate the mechanism of DANCR-mediated sorafenib resistance via PSMD10-IL-6/STAT3 signaling axis, which provides a promising target for developing new therapeutic strategy for sorafenib tolerance of HCC.

Keywords: sorafenib, IL-6-STAT3 signaling, microRNA, feedback loop

Introduction

Hepatocellular carcinoma (HCC) is one of the major malignancies and the second most frequent cause of cancer-associated death around the world.[1] Even though the progress in clinical diagnosis and treatment of HCC has been achieved, the clinical outcome of HCC patients remains unsatisfactory. Most HCC patients are first diagnosed at the advanced stage which is unsuitable for surgical resection, and they are also insensitive to cytotoxic chemotherapies. Sorafenib is a multikinase inhibitor and one of the clinically approved drug for the advanced HCC patients.[2] Although the

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response rate of sorafenib was only 2–3.3%, sorafenib treatment was able to elongate the survival time of advanced HCC patients.[3] The activity of several tyrosine kinases contributing to tumor progression, including VEGFR, PDGFR, and Raf kinases, could be markedly suppressed by sorafenib treatment.[4] Nevertheless, only a few patients were sensitive to sorafenib, and some patients showed increasing sorafenib resistance gradually.[5] Hence, revealing the underlying mechanism is critical for improving the efficiency of sorafenib for HCC patients.

PSMD10 (also named Gankyrin) expression is commonly elevated in several types of cancers, including HCC, gliomas, lung cancer, breast cancer, colon cancer and esophageal cancer.[6] Increasing evidence demonstrated that upregulation of PSMD10 enhances HCC progression. PSMD10 expression was correlated with portal vein tumor thrombus and vascular invasion.[7] PSMD10 could induce epithelial–mesenchymal transition (EMT) and promote angiogenesis via activating PI3K-AKT-HIF-1α signaling pathway to promote TWIST1, VEGF, and MMP2 expression.[8] Recently, it was reported that PSMD10 could regulate sorafenib resistance in HCC cells. PSMD10 promoted autophagy to induce sorafenib resistance by association with ATG7 and activating its transcription.[9] In addition, the STAT3 activity and IL-6 expression were inhibited by PSMD10 knockout in nonparenchymal cells, leading to the suppression of sorafenib resistance.[10] These studies suggested the importance of PSMD10 in affecting the sorafenib tolerance of HCC patients.

Long non-coding RNAs (lncRNAs) function in regulating gene expression involving several biological processes in human diseases.[11] Mechanistically, lncRNAs form regulatory networks with miRNAs and mRNAs or associate with RNA bind proteins to modulate their function.[12,13] Recent studies demonstrated that some lncRNAs participate in sorafenib resistance. For example, depletion of endogenous lncRNA TUC338 can target RASAL1 3′-UTR and activate the RASAL1 pathway, which sensitizes HCC cells to the treatment of sorafenib.[14] NEAT1 suppresses sorafenib sensitivity by inhibiting drug-induced apoptosis via activating c-Met-Akt pathway.[15] SNHG1 contributes to sorafenib resistance by regulating SLC3A2-mediated activation of the Akt pathway.[16] FOXM1 forms a feedback loop with LINC-ROR to induce sorafenib tolerance in HCC cells.[17] However, little is known about the mechanism of lncRNAs in affecting sorafenib tolerance in HCC.

The differentiation antagonizing non-protein coding RNA (DANCR) was first found to repress epidermal cell differentiation.[18] DANCR also acts as an oncogene in tumor progression. For instance, DANCR is overexpressed in stem-like HCC cells, and predicts shorter overall survival time for HCC patients. DANCR upregulates CTNNB1 expression to enhance stemness features and tumorigenesis of HCC cells.[19] In bladder cancer, DANCR activates IL-11/STAT3 signaling pathway, which enhances tumor lymph node metastasis and growth.[20] Moreover, DANCR promotes nasopharyngeal carcinoma (NPC) metastasis. DANCR interacts with the NF90/NF45 complex and stabilizes HIF-1α mRNA in NPC cells.[21] Nevertheless, the functional significance of DANCR in sorafenib tolerance of HCC cells has not been investigated.

Here, we reported DANCR as an important regulator of sorafenib resistance in HCC cells by increasing PSMD10 expression and activating the IL-6/STAT3 signaling pathway. Our findings strongly suggested that DANCR may be a novel target for sorafenib resistance in HCC.

Materials and Methods

Tissue Samples

Fresh clinical HCC and paired normal liver tissues were collected from 66 HCC patients between January 2013 and January 2019 at the The First People's Hospital of Tianmen City. No patients had undergone chemotherapy before surgery. An ethical approval was obtained from Research Ethics Committee of The First People's Hospital of Tianmen City Hospital. Written informed consent was obtained from these patients. The study was performed in accordance with the World Medical Association Declaration of Helsinki.

Cell Culture

HEK-293T, Huh7 and Hep3B cells were obtained from the American Type Culture Collection (ATCC) and cultured at 37°C in an atmosphere containing 5% CO_2 and in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). Huh7/sorafenib-resistant (SR) and Hep3B/SR were constructed by long-term exposure to 5 μM sorafenib (Selleck), which was increased to 20 μM over 3 months. 5 μM sorafenib was added to the medium to maintain sorafenib resistance in the Huh7/SR and Hep3B/SR cells.

Cell Viability Analysis

2000 cells per well were seeded in triplicate in 96-well plates. 24 hrs later, the cells were treated with different

concentrations of sorafenib. After 48 hrs, the Cell Counting Kit-8 (CCK-8) was used to detect the cell viability according to the manufacturer's instructions.

Cell Apoptosis Detection

Cells were pretreated with sorafenib. After 48 hrs, cellular apoptosis was tested by Annexin V (ANXA5) and PI staining (Invitrogen), and then analyzed by flow cytometry.

Construction of Stable Cell Lines with Overexpression or Knockdown of DANCR

To construct stable cells overexpressing DANCR, pcDNA3.1-DANCR was transfected into cells. After 48 hrs, cells were treated with neomycin (800 μg/mL) for 4 weeks. To construct stable cells with DANCR knockdown, HEK-293T cells were cotransfected with a plasmid expressing DANCR shRNA (shRNA), pMD.2G, and psPAX2. After 24 hrs, the supernatant containing lentiviral particles was collected. Cells were infected with these lentiviral particles for 24 hrs. 1 μg/mL puromycin was used to screen out stable cells.

Western Blot

The cells were lysed in RIPA buffer (Beyotime). An equal quantity of protein was separated via SDS-PAGE and then transferred to a PVDF membrane. After being blocked with 5% nonfat milk, the membrane was incubated with primary antibodies overnight at 4°C. After three-times wash, the membrane was incubated with HRP-conjugated secondary antibody. The protein signal was detected by using Immobilon™ Western Chemiluminescent HRP Substrate (ECL) (Millipore).

qRT-PCR

Total RNA from tissues or cells was isolated using Trizol (Invitrogen). RNA was reverse transcribed, and obtained cDNA was amplified by using PrimeScript™ RT-PCR Kit (Takara). The relative expression was analyzed by $2^{-\triangle\triangle CT}$ method. The primer sequences were shown as follow: DANCR-forward: CTCGGAGGTGGATTCTGTTAG, DANCR-reverse: CTGCAGAGTATTCAGGGTAAGG; PSMD10-forward: GGGTGTGTGTCTAACCTAATGG, PSMD10-reverse: AGGGATTTATCGGCCAGAATAC; IL-6-forward: GGAGACTTGCCTGGTGAAA, IL-6-reverse: CTGGCTTGTTCCTCACTACTC.

The MS2-Binding Sequences-MS2-Binding Protein–Based RNA Immunoprecipitation Assay (MS2-RIP)

MS2-RIP assay was utilized to test the association between DANCR and PSMD10 mRNA and performed as previously described.[22] pcDNA-DANCR-MS2, or pcDNA-MS2 and pMS2-GFP (Addgene) was transfected into cell. After 48 hrs, RIP assay was carried out using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore) and GFP antibody (Abcam).

RNA Pull-Down Assay

RNA pull-down was performed as previously described.[22] In brief, DANCR were in vitro transcribed and biotin-labeled, and then incubated with whole-cell lysates. Complexes were isolated by using streptavidin agarose beads (Invitrogen). The pull-down RNA was then detected by qRT-PCR.

Chromatin Immunoprecipitation Assay (ChIP)

ChIP assays were performed by using EZ-ChIP-Chromatin Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. 4 μg of anti-STAT3 (Abcam) was used.

Mouse Xenograft Models

Approximately 5×10^6 control and DANCR-overexpressing Hep3B cells were subcutaneously injected into the right flank of 4-weeks-old BALB/c nude mice (8 mice per group). When the tumors grew to 50 mm^3, the mice were randomly subdivided into four groups and received oral administration of 60 mg/kg sorafenib or equal quantity of PBS per day. Xenograft size was measured every 5 days. Animal experiments were approved by the Ethical Committee of The First People's Hospital of Tianmen City Hospital according to the Guide for the Care and Use of Laboratory Animals by NIH.

siRNA Transfection

The siRNAs against PSMD10 were synthesized by GenePharma Company (Shanghai, China). Cells were transfected with siRNAs using the Lipofectamine RNAiMAX Reagent (Thermo) according to the manufacturers' instructions. Cells were subjected for further detection after 48 hrs.

Luciferase Reporter Assay

The 3′-untranslated region (3′-UTR) of PSMD10 mRNA was subcloned into the pmirGLO vector (pmirGLO-PSMD10). pmirGLO or pmirGLO-PSMD10 was cotransfected with miR-214, miR-1254, miR-199a, miR-605 mimics or miR-NC into stable cells with DANCR alteration by using Lipofectamine 2000. To detect the luciferase activity, Dual-Luciferase® Reporter Assay Kit (Promega) was used. 48 hrs later, the luciferase activity was measured.

Statistics

All statistical analyses were performed with SPSS software. For statistical comparisons, ANOVA, the chi-square test, or Student's *t*-test were performed. Pearson's correlation coefficient was used for statistical correlation. Survival curves were calculated using Kaplan-Meier's method and log-rank test. A p value <0.05 was considered to be statistically significant.

Results

Upregulation of DANCR Predicts Poor Prognosis in HCC

We first evaluated DANCR levels in 66 pairs of HCC and normal liver tissues and confirmed the higher levels of DANCR in HCC tissues (Figure 1A). Kaplan-Meier methods demonstrated a significant overall survival differences between the patients with high-DANCR and those with low-DANCR (Figure 1B). For further confirmation, HCC database of The Cancer Genome Atlas (TCGA) was analyzed. Consistent with our findings, analysis of TCGA datasets exhibited significantly higher DANCR expression in HCC tissues than normal liver tissues (Figure 1C). Additionally, the survival analysis via GEPIA online analysis tool (http://gepia.cancer-pku.cn/) showed that DANCR overexpression was correlated with shorter survival time (Figure 1D). These results indicate that DANCR may function in HCC progression.

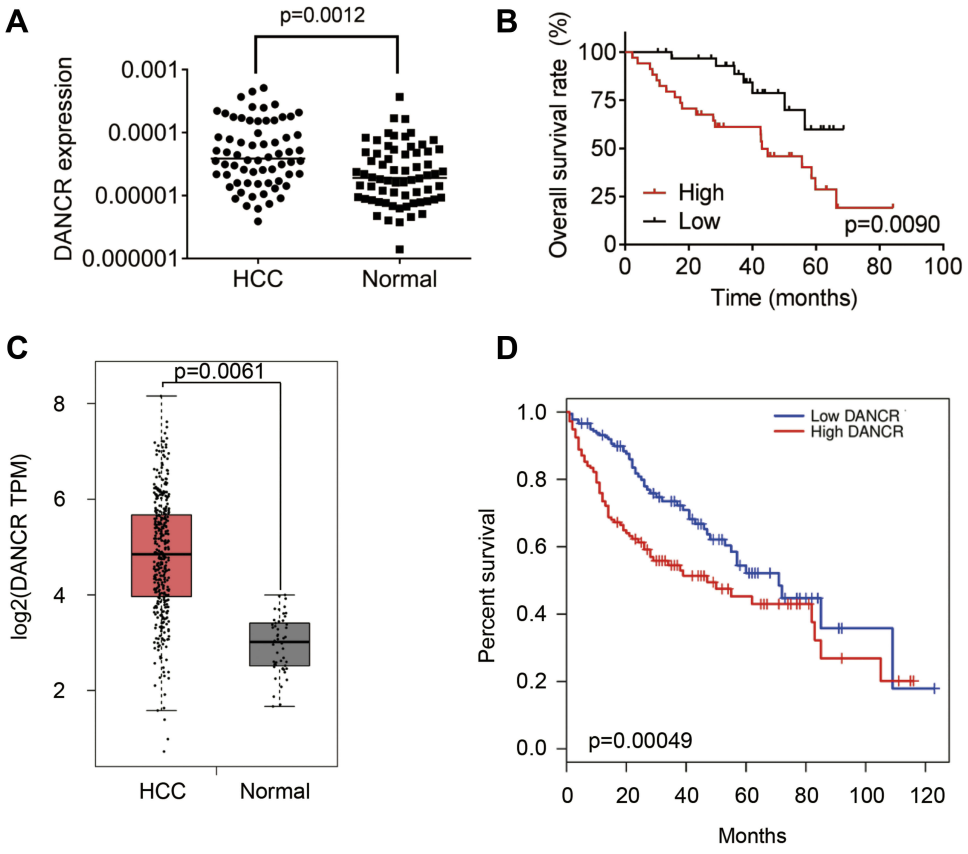

Figure 1 Upregulation of DANCR is correlated with poor prognosis in HCC. (**A**) Expression levels of DANCR in 66 pairs of tumor tissues and adjacent normal liver tissues from patients with HCC were measured by qRT-PCR and normalized to β-actin expression. (**B**) Kaplan-Meier analysis of overall survival time in HCC patients with low-DANCR expression (n=33) and high-DANCR expression (n=33). The median of DANCR expression in HCC tissues was taken as cutoff. (**C**) The differential expression of DANCR RNA expression between normal and HCC tissues from TCGA datasets was analyzed. (**D**) Kaplan-Meier survival analysis of the HCC patients according to DANCR RNA expression level was performed by GEPIA online analysis tool.

DANCR Enhance Sorafenib Tolerance in HCC Cells

The functional role of DANCR in sorafenib tolerance of HCC cells was then investigated. Sorafenib resistant (SR) Huh7 and Hep3B cell lines were constructed via repeated long-term exposure to increasing dose concentrations of sorafenib. To validate the sorafenib resistance in the HCC cell lines, the cells were treated with different concentrations of sorafenib. After 48 hrs, and cell viability was measured by CCK-8 assay (Figure 2A and B). As shown in Figure 2C, Huh7/SR and Hep3B/SR cells showed much higher half inhibitory concentrations (IC_{50}) than their parental cells, respectively, indicating that the HCC cells showed resistance to sorafenib. Interestingly, the DANCR was markedly increased in SR HCC cells (Figure 2D), suggesting that the upregulation of DANCR may enhance sorafenib resistance in HCC cells.

To further assess the role of DANCR in sorafenib tolerance in HCC cells, DANCR knockdown Huh7/SR and Hep3B/SR cells were constructed (Figure 2E) and treated with sorafenib at different concentrations. The results of CCK-8 assay demonstrated that DANCR knockdown significantly increased the sensitivity of sorafenib treatment in Huh7/SR and Hep3B/SR cells (Figure 2F and G). Consistently, sorafenib treatment led to an increase of apoptotic rate after DANCR knockdown as proved by flow cytometry assay (Figure 2H and Supplemental Figure 1A). Conversely, we constructed parental Huh7 and Hep3B cells with stably overexpressing DANCR (Figure 2I), and found that ectopic expression of DANCR significantly elevated sorafenib resistance in both Huh7 and Hep3B cells (Figure 2J and K). Consistently, flow cytometry assay also exhibited that the sorafenib-induced cell apoptosis was suppressed by DANCR overexpression (Figure 2L and Supplemental Figure 1B). Furthermore, tumors formed from overexpressing DANCR Hep3B cells showed poor responses to sorafenib in vivo (Figure 2M, Supplemental Figure 1C). Together, our results suggest that DANCR promotes the tolerance of HCC cells to sorafenib.

DANCR Interacts with and Stabilizes PSMD10 mRNA

We then investigated the underlying mechanism of DANCR-mediated sorafenib resistance. RNA interactome analysis from a previous study demonstrated that some cancer-related mRNA could be associated with DANCR.[19] It was found that PSMD10 mRNA may associate with DANCR. To validate this result, an MS2-binding sequences-MS2-binding protein–based RNA immunoprecipitation assay (MS2-RIP) was performed. Compared to the empty vector, IgG or GAPDH mRNA which did not have a complementary region with DANCR, the DANCR RIP in both Huh7 and Hep3B cells is significantly enriched for PSMD10 mRNA (Figure 3A). The RNA pull down assay also confirmed the specific association between DANCR and PSMD10 mRNA (Figure 3B). These data demonstrate a direct interaction between DANCR and PSMD10 mRNA.

Then, the biological consequence of DANCR-PSMD10 association was determined. Previous studies demonstrated that lncRNA could stabilize its interacting mRNA.[23–25] Overexpression of DANCR increased the PSMD10 mRNA in Huh7 and Hep3B cells (Figure 3C), while depletion of endogenous DANCR expression significantly downregulated PSMD10 mRNA level compared to control group in Huh7/SR and Hep3B/SR cells (Figure 3D). To test whether DANCR modulates the stability of PSMD10 mRNA, we used α-amanitin to block new RNA synthesis and then examined the degradation of PSMD10 and GAPDH mRNA over a 24 hr period. The overexpression of DANCR elongated the half-life of PSDM10 mRNA in Huh7 and Hep3B cells (Figure 3E). In contrast, downregulation of DANCR shortened the half-life of PSMD10 mRNA in Huh7/SR and Hep3B/SR cells (Figure 3F). Additionally, the PSMD10 mRNA level and its stability in Huh7/SR cells was much higher than that in Huh7 cells (Figure 3G and H). These data demonstrate that DANCR associates with and specially elevates the stability of PSMD10 mRNA.

DANCR Block miRNA-Induced PSMD10 Suppression via Binding to PSMD10 3′UTR

The mechanism by which DANCR promoted the stability of PSMD10 mRNA was then investigated. LncRNA was able to bind to 3′UTR of target mRNA to suppress miRNA-induced mRNA suppression.[19] We suspected that DANCR may stabilize PSMD10 mRNA in this manner. miR-214, miR-1254, miR-199a and miR-605 have been demonstrated to target PSMD10 3′UTR.[26–28] Overexpression of miR-214, miR-1254, miR-199a and miR-605 could dramatically downregulate PSMD10 level in control cells. This downregulation was retarded in Huh7 and Hep3B cells with DANCR overexpression (Figure 4A), while enhanced in Huh7/SR and Hep3B/SR cells with DANCR knockdown (Figure 4B). Moreover,

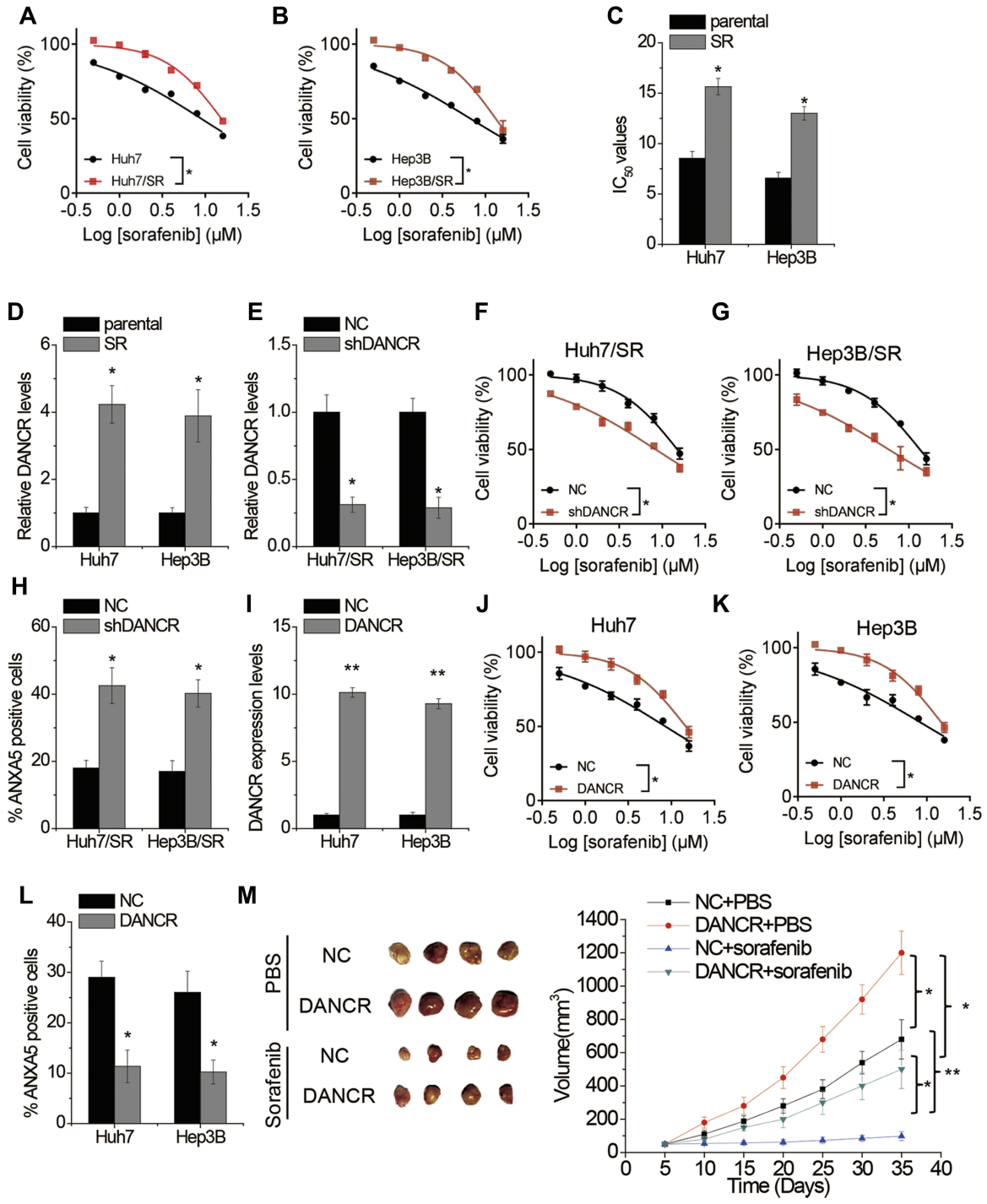

Figure 2 DANCR enhances sorafenib resistance in HCC cells. (**A**) Huh7 and Huh7/SR were treated with the indicated concentrations of sorafenib for 48 hrs. Cell viability was measured using the CCK-8 assay. (**B**) Hep3B and Hep3B/SR were treated with the indicated concentrations of sorafenib for 48 hrs. Cell viability was measured using the CCK-8 assay. (**C**) Based on the results of (**A**) and (**B**), the inhibitory concentration (IC_{50}) of sorafenib in parental and SR HCC cells was calculated. (**D**) The DANCR expression in parental and SR HCC cells was detected by qRT-PCR. (**E**) The DANCR expression was silenced by DANCR shRNA (shDANCR) in Huh7/SR and Hep3B/SR cells, and the knockdown efficiency was determined by qRT-PCR. (**F**) Control and DANCR knockdown Huh7/SR were treated with the indicated concentrations of sorafenib for 48 hrs. Cell viability was measured using the CCK-8 assay. (**G**) Control and DANCR knockdown Hep3B/SR were treated with the indicated concentrations of sorafenib for 48 hrs. Cell viability was measured using the CCK-8 assay. (**H**) Huh7/SR and Hep3B/SR cells with DANCR knockdown were subjected to sorafenib (20 μM) for 48 hrs and then analyzed for apoptosis. The percentage of cells was determined using the ANXA5 and propidium iodide staining assay. (**I**) The DANCR expression was overexpressed in Huh7 and Hep3B cells, and the overexpression efficiency was determined by qRT-PCR. (**J**) Control and DANCR overexpressing Huh7 were treated with the indicated concentrations of sorafenib for 48 hrs. Cell viability was measured using the CCK-8 assay. (**K**) Control and DANCR overexpressing Hep3B were treated with the indicated concentrations of sorafenib for 48 hrs. Cell viability was measured using the CCK-8 assay. (**L**) Huh7 and Hep3B cells with DANCR overexpression were subjected to sorafenib (20 μM) for 48 hrs and then analyzed for apoptosis. The percentage of cells was determined using the ANXA5 and propidium iodide staining assay. (**M**) Control and DANCR overexpressing Hep3B were injected into nude mice. When the average tumor volume reached 50 mm^3, the two groups of mice were subdivided into four groups randomly and were given 60 mg/kg sorafenib or PBS. Tumor sizes were calculated every 5 days. *p<0.05, **p<0.01.

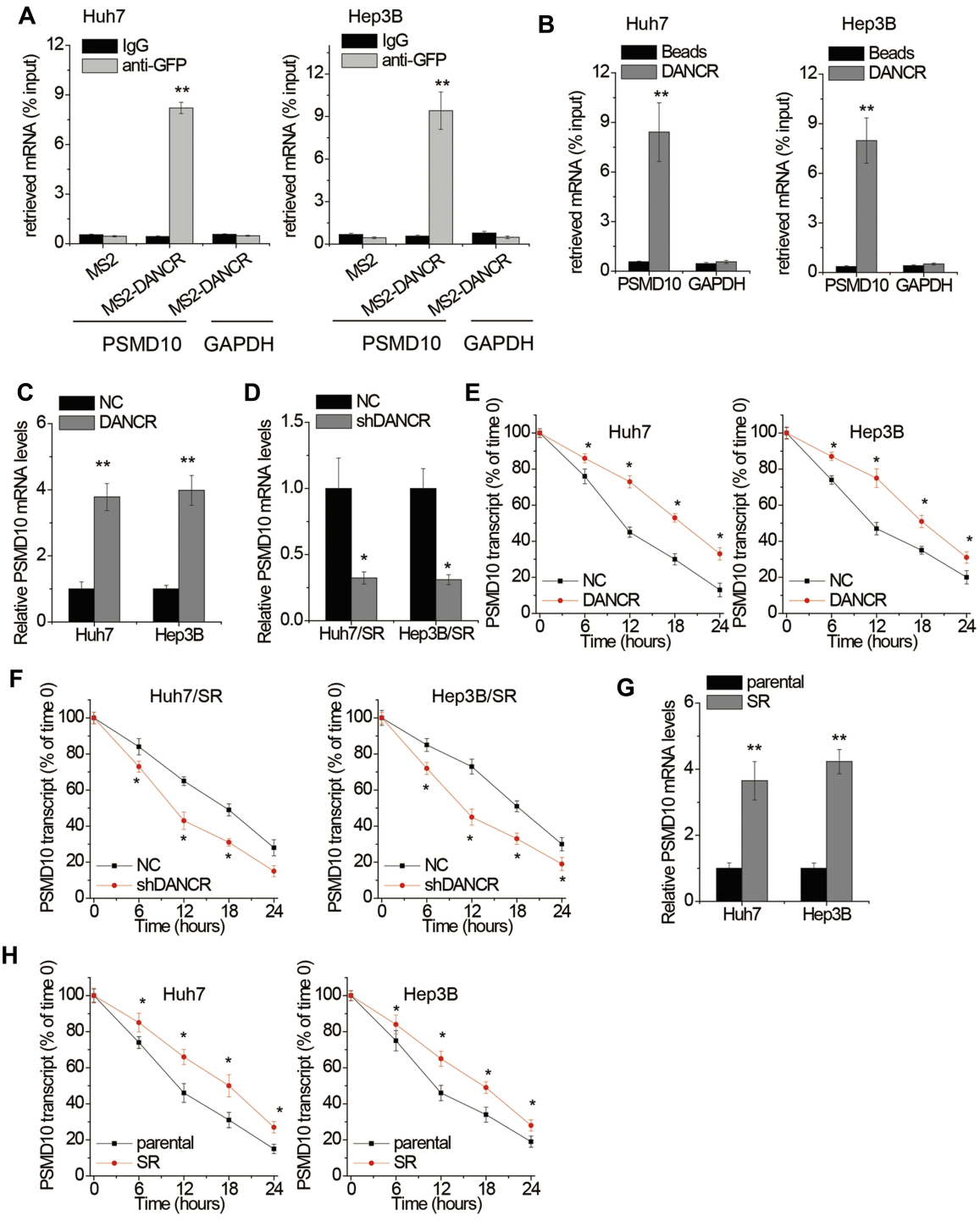

Figure 3 DANCR interacts with and stabilizes PSMD10 mRNA. (**A**) The MS2-RIP was performed to validate the interaction between DANCR and PSMD10 mRNA. RIP-derived RNA was measured by qRT-PCR. The levels of qRT-PCR products were expressed as a percentage of input RNA.GAPDH was taken as a negative control. (**B**) Huh7 and Hep3B cell lysates were incubated with biotin-labeled DANCR; after pull-down, mRNA was extracted and assessed by qRT-PCR. GAPDH was taken as a negative control. (**C**) The PSMD10 mRNA levels in Huh7 and Hep3B cells with or without DANCR overexpression were detected by qRT-PCR. (**D**) The PSMD10 mRNA levels in Huh7/SR and Hep3B/SR cells with or without DANCR knockdown were detected by qRT-PCR. (**E**) The stability of PSMD10 mRNA over time was measured by qRT-PCR relative to time 0 after blocking new RNA synthesis with α-amanitin (50 μM) in Huh7 and Hep3B cells with DANCR overexpression and normalized to 18S rRNA (a product of RNA polymerase I that is unchanged by α-amanitin). (**F**) The stability of PSMD10 mRNA over time was measured by qRT-PCR relative to time 0 after blocking new RNA synthesis with α-amanitin (50 μM) in Huh7/SR and Hep3B/SR cells with DANCR knockdown. (**G**) The relative expression level of PSMD10 mRNA in parental and SR HCC cells was detected by qRT-PCR. (**H**) The The stability of PSMD10 mRNA over time was measured by qRT-PCR relative to time 0 after blocking new RNA synthesis with α-amanitin (50 μM) in parental and SR HCC cells. *p<0.05, **p<0.01.

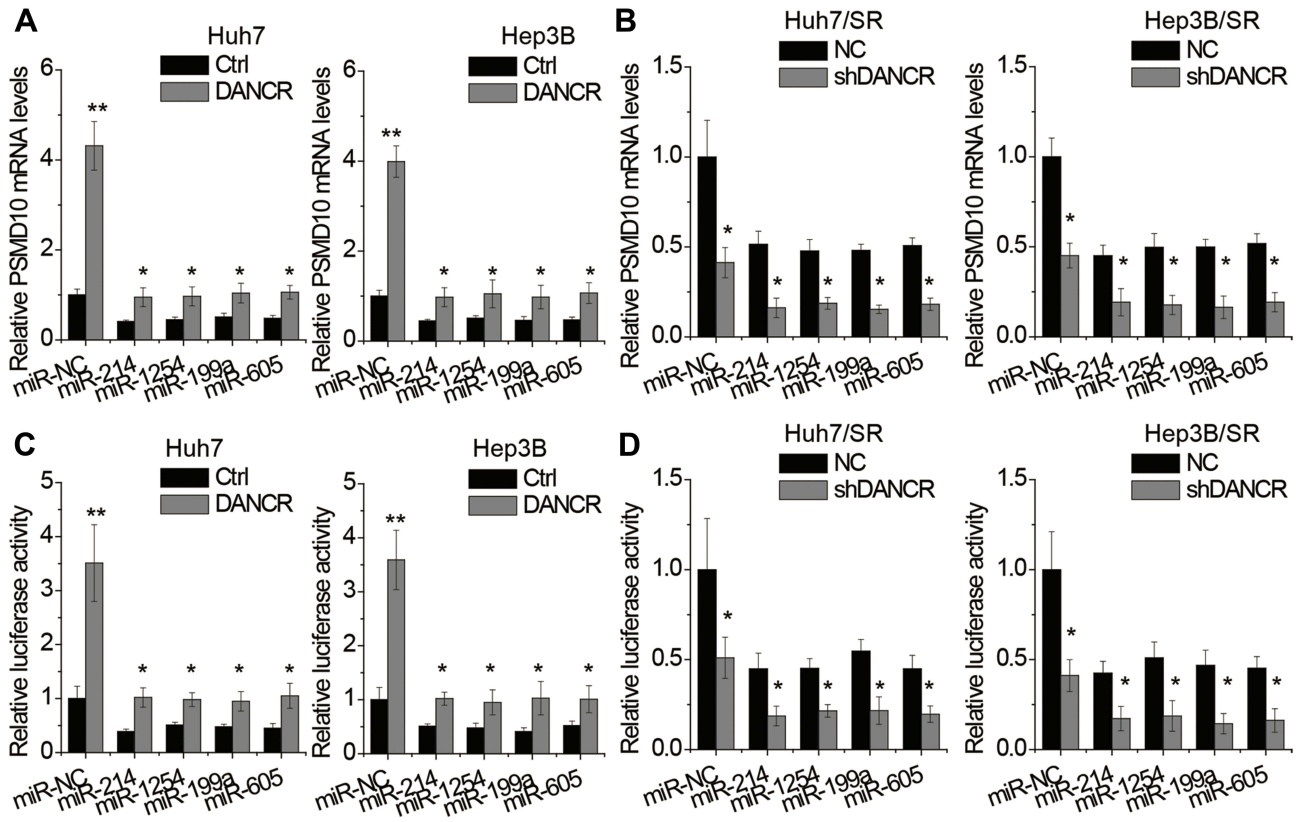

Figure 4 DANCR competitively bind to 3′UTR of PSMD10 to block miRNA-mediated PSMD10 suppression (**A**). Overexpression of DANCR attenuated suppression of PSMD10 mRNA by indicated microRNAs in Huh7 and Hep3B cells. (**B**) Knockdown of DANCR amplifies suppression of PSMD10 mRNA by indicated microRNAs in Huh7/SR and Hep3B/SR cells. (**C**) Luciferase activity of pmirGLO-PSMD10 in control and DANCR overexpressing Huh7 and Hep3B cells transfected with indicated microRNAs mimics. (**D**) Luciferase activity of pmirGLO-PSMD10 in control and DANCR knockdown Huh7/SR and Hep3B/SR cells transfected with indicated microRNAs mimics. *p<0.05, **p<0.01.

luciferase reporters containing PSMD10 3′UTR (pmirGLO-PSMD10) was constructed. The control reporter or pmirGLO-PSMD10 was transfected into the stable cells with DANCR alteration. Transfection of miR-214, miR-1254, miR-199a or miR-605 mimics repressed the luciferase activity of pmirGLO-PSMD10. DANCR overexpression rescued this downregulation in Huh7 and Hep3B cells (Figure 4C). Additionally, the microRNAs-mediated suppression of luciferase activity of pmirGLO-PSMD10 was enhanced in Huh7/SR and Hep3B/SR cells with DANCR knockdown (Figure 4D). Together, these findings reveal a novel regulatory mechanism of DANCR in modulating PSMD10 expression.

DANCR Transcript Level Is Positively Correlates with PSMD10 mRNA Level in HCC Tissues

To detect the pathological correlation between DANCR and PSMD10, PSMD10 mRNA level in 66 paired HCC and normal liver tissues was detected. As shown in Figure 5A and B, PSMD10 expression was markedly increased in HCC tissues and predicted shorter overall survival time of HCC patients. Moreover, DANCR expression positively correlated with PSMD10 mRNA levels in HCC tissues (R^2= 0.59, p<0.0001, Figure 5C). For further confirmation, analysis of the HCC database of TCGA showed that the DANCR expression was much higher in HCC tissues than normal liver tissues (Figure 5D). Upregulation of DANCR in HCC patients was associated with a poorer prognosis (Figure 5E). GEPIA online analysis also demonstrated a positive correlation between DANCR and PSMD10 mRNA levels in HCC tissues (Figure 5F).

DANCR Activates IL-6/STAT3 Signaling

Sustaining activation of IL-6/STAT3 signaling is crucial for sorafenib resistance.[29–31] Recent studies reported that PSMD10 could active IL-6/STAT3 signaling by facilitating the phosphorylation of Rb and IL-6 transcription, thereby promoting sorafenib resistance.[10,32] Based on these studies, we speculated that DANCR may have an effect on IL-6/STAT3 signaling via

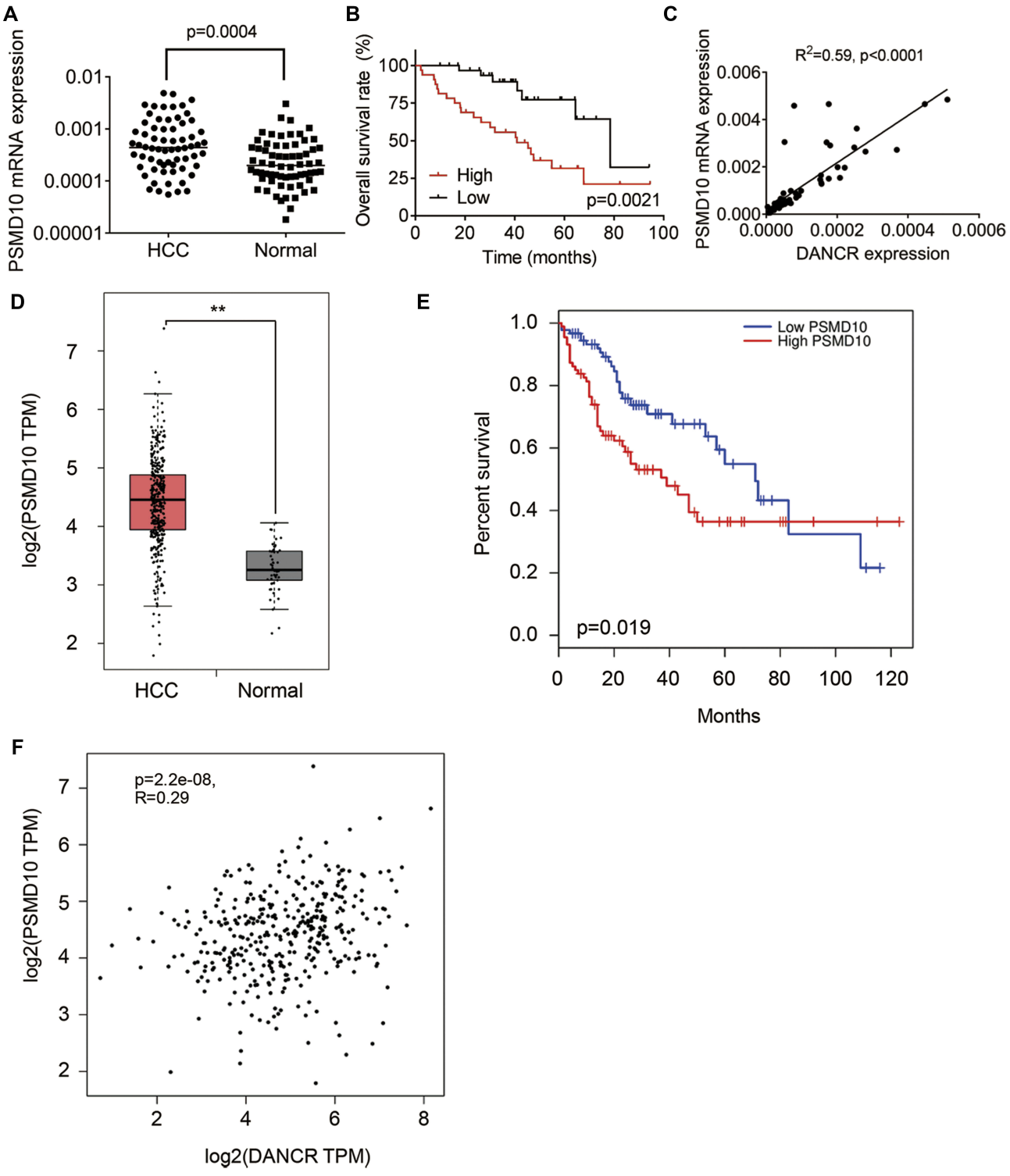

Figure 5 DANCR transcript level is positively correlates with PSMD10 mRNA level in HCC tissues. (**A**) Expression levels of PSMD10 mRNA in 66 pairs of tumor tissues and adjacent normal liver tissues from patients with HCC were measured by qRT-PCR and normalized to β-actin expression. (**B**) Kaplan-Meier analysis of overall survival time in HCC patients with low PSMD10 expression (n=33) and high PSMD10 expression (n=33). The median of DANCR expression in HCC tissues was taken as cutoff. (**C**) Pearson correlation analysis was performed to analyze the correlation between DANCR and PSMD10 mRNA level was measured in the same set of HCC tissues. (**D**) The differential expression of PSMD10 mRNA expression between normal and HCC tissues from TCGA datasets was analyzed. (**E**) Kaplan-Meier survival analysis of the HCC patients from TCGA datasets according to PSMD10 mRNA expression level was performed by GEPIA online analysis tool. (**F**) The correlation between DANCR and PSMD10 mRNA level from TCGA datasets was measured by GEPIA online analysis tool. **p<0.01.

PSMD10. The phosphorylation of STAT3, PSMD10 and IL-6 levels were much higher in Huh7/SR and Hep3B/SR cells compared to their parental cells (Supplemental Figure 2A and 2B). Overexpression of DANCR increased the phosphorylation of STAT3, which was abolished by PSMD10 knockdown in Huh7 and Hep3B cells (Figure 6A). Conversely, the phosphorylation of STAT3 was decreased by DANCR silencing in Huh7/SR and Hep3B/SR cells. Overexpression of PSMD10 reversed this downregulation (Figure 6B). Next, qRT-PCR and ELISA assays were utilized to test the IL-6 mRNA and protein expression, respectively. The IL-6 mRNA and protein levels were increased in the DANCR-overexpressed Huh7 and Hep3B cells compared with that in the control cells, whereas silence of PSMD10 attenuated this upregulation (Figure 6C and D). On the contrary, depletion of DANCR decreased IL-6 expression, which was reversed by restoration of PSMD10 expression in Huh7/SR and Hep3B/SR cells (Figure 6E and F). Together, our findings demonstrated that DANCR activated IL-6/STAT3 signaling in a PSMD10-dependent manner.

IL6-/STAT3 Signaling Can Activate DANCR Transcription to Form Feed-Back Regulatory Loop

Since IL-6/STAT3 signaling was important for HCC development and progressions, we investigated whether IL-6/STAT3 could have an effect on DANCR expression. Recombinant IL-6 (rIL-6) treatment elevated both DANCR and PSMD10 expression in Huh7 and Hep3B cells (Figure 7A). Conversely, DANCR and PSMD10 transcription was repressed by STAT3 inhibitor NSC 74859 treatment (Figure 7B). Of note, JASPAR online tool identified two potential STAT3-binding elements within DANCR promoter region (Figure 7C). The luciferase assay showed that IL-6 treatment activated DANCR promoter in a dose-dependent manner (Figure 7D), whereas STAT3 inhibitor treatment repressed DANCR transcription (Figure 7E). We individually deleted two predicted STAT3-binding sites and found either E1 or E2

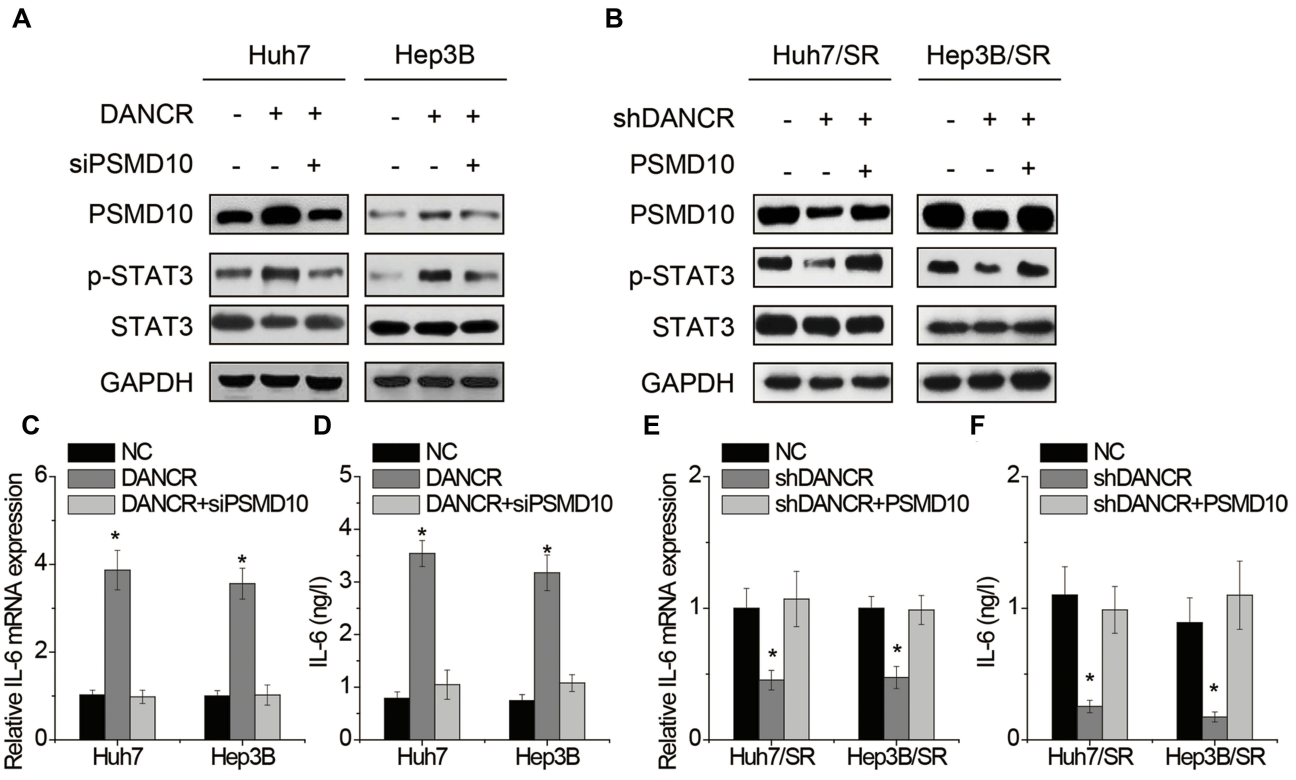

Figure 6 DANCR activates IL-6/STAT3 signaling (**A**). The siRNA targeting PSMD10 (siPSMD10) was transfected into DANCR overexpressing Huh7 and Hep3B cells. The expression of phosphorylation of STAT3 was then detected by Western blot. (**B**) The PSMD10 was transfected into DANCR knockdown Huh7/SR and Hep3B/SR cells. The expression of phosphorylation of STAT3 was then detected by Western blot. (**C** and **D**) The siRNA targeting PSMD10 (siPSMD10) was transfected into DANCR overexpressing Huh7 and Hep3B cells. The mRNA (**C**) and protein (**D**) levels of IL-6 were then detected by qRT-PCR and ELISA, respectively. (**E** and **F**) The PSMD10 was transfected into DANCR knockdown Huh7/SR and Hep3B/SR cells. The mRNA (**E**) and protein (**F**) level of IL-6 was then detected by qRT-PCR and ELISA, respectively. *p<0.05.

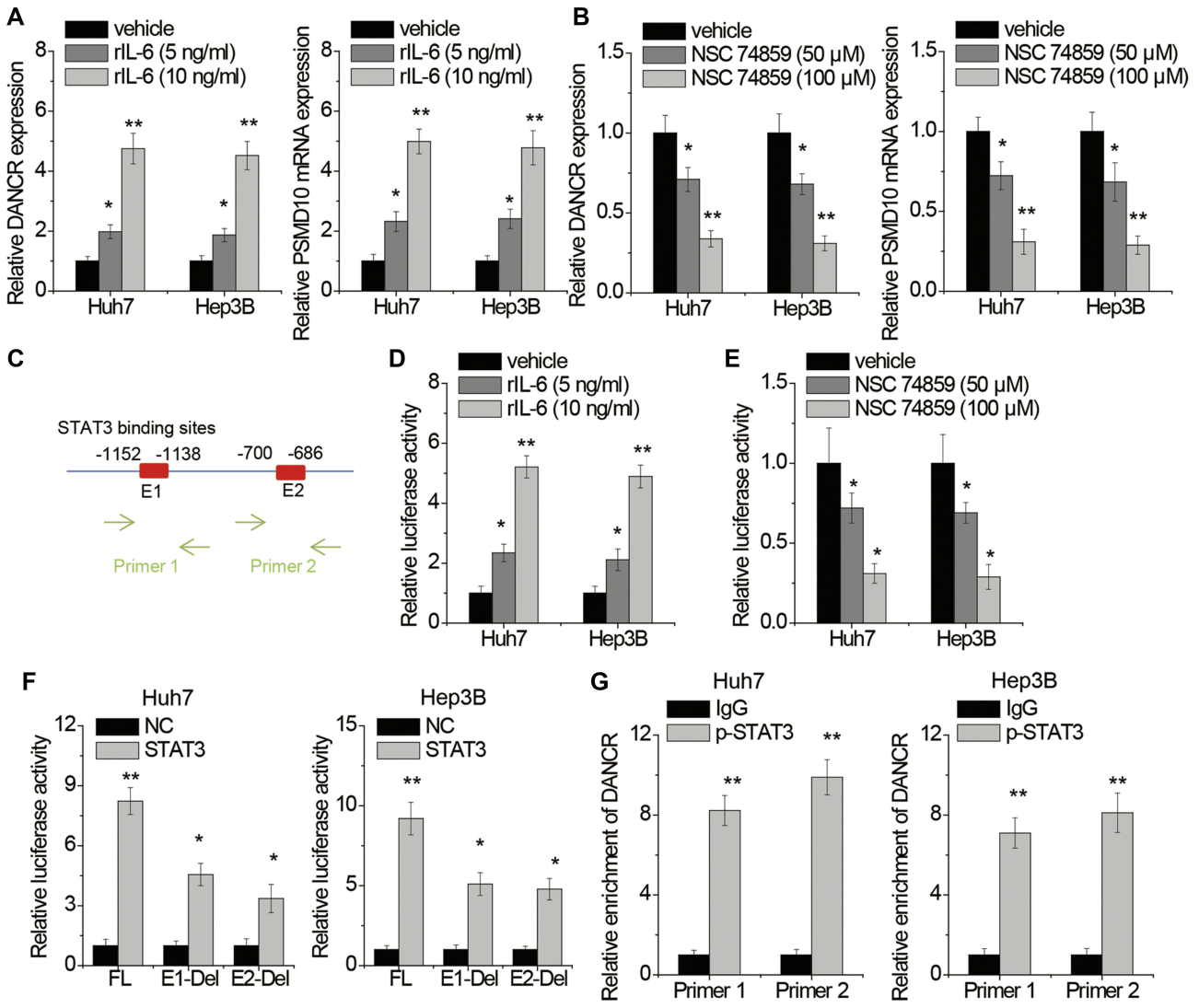

Figure 7 IL6-/STAT3 signaling can activate DANCR transcription. (**A**) The Huh7 and Hep3B cells were treated with indicated concentration of rIL-6. After 24 hrs, the DANCR and PSMD10 mRNA levels were detected by qRT-PCR. (**B**) The Huh7 and Hep3B cells were treated with indicated concentration of STAT3 inhibitor NSC 74859. After 24 hrs, the DANCR and PSMD10 mRNA levels were detected by qRT-PCR. (**C**) Schematic diagram of predicting STAT3-binding motif and two potential STAT3 responsive elements (E1 and E2) in the DANCR promoter region. (**D**) DANCR promoter activity when cells were treated with indicated concentration of rIL-6. (**E**) DANCR promoter activity when cells were treated with indicated concentration of NSC 74859. (**F**) Two predicted STAT3-binding sites of the DANCR promoter was individually deleted (E1-Del and E2-Del). STAT3 was transfected into Huh7 and Hep3B cells, transcriptional activities of the two DANCR promoter deletion mutants was determined by luciferase assay. (**G**) ChIP assays showed that p-STAT3 bound to the E1 and E2 element of DANCR promoter. IgG served as a negative control. *p<0.05, **p<0.01.

element absence suppressed DANCR promoter activity in part (Figure 7F), indicating that both E1 and E2 element was critical for STAT3-induced DANCR transcription. Moreover, ChIP assay showed that p-STAT3 could bind to E1 or E2 element in DANCR promoter (Figure 7G). In summary, our data indicate a regulatory feedback loop between DANCR and IL-6/STAT3 signaling pathway.

Discussion

The therapeutic options for these patients who are inherently nonresponsive to sorafenib are very limited. Therefore, it is important to reveal the underlying mechanisms of sorafenib tolerance in HCC patients. Here, we identified that lncRNA DANCR was highly expressed in HCC tissues and negatively associated with prognosis of HCC patients. We identified DANCR as being highly expressed in sorafenib resistant HCC cells. Knockdown and overexpression experiments demonstrated that DANCR facilitated sorafenib resistance in HCC cells in vitro and in vivo. Moreover, our mechanistic investigation revealed that DANCR was capable to interact with PSMD10 mRNA and increase its stability,

which induced the activation of IL-6/STAT3 signaling pathway. Meanwhile, the activated IL-6/STAT3 signaling triggered DANCR transcription reciprocally, suggesting a positive feedback loop regulation between DANCR and IL-6/STAT3 signaling in enhancing sorafenib resistance in HCC cells.

PSMD10 plays crucial roles in cancer initiation and progression by facilitating cell proliferation, migration, invasion, drug resistance and autophagy.[6,33] However, only a few studies revealed the regulatory mechanism of PSMD10 upregulation in human cancers. microRNAs, including miR-214, miR-1254, miR-199a and miR-605, have been reported to suppress PSMD10 expression.[26–28] A recent study revealed that lncRNA NBAT1 interacted with PSMD10 and promoted its degradation, thus promoting cell autophagy in non-small cell lung cancer cells.[34] Here, for the first time, our findings showed that DANCR posttranscriptionally regulated PSMD10. DANCR interacted with PSMD10 mRNA 3′UTR region to block the suppression of PSMD10 mediated by some microRNAs, such as miR-214, miR-1254, miR-199a and miR-605. Moreover, a positive correlation between DANCR and PSMD10 expression in HCC tissues was observed, indicating that PSMD10 was a bona fide downstream of DANCR.

Dysregulation of many endogenous signaling pathways participate in HCC progression. Among them, IL-6/STAT3 signaling pathway is capable to modulate a series of genes expressions to enhance tumor growth, metastasis, inflammation, angiogenesis as well as sorafenib resistance.[31,35,36] Recently, several lncRNAs have been found to have an impact on the STAT3 activation. For instance, IL-6 expression is regulated by lncRNA-DILC and lncRNA-SRLR.[31,37] LncRNA lncSox4 recruits STAT3 to form complex to activate Sox4 transcription and facilitate the self-renewal of liver tumor-initiating cells.[38] Moreover, lncRNA PVT1 increases the phosphorylation level of STAT3 protein to induce angiogenesis.[39] Recent studies demonstrated a close relationship between DANCR and STAT3. DANCR could activate IL-11/STAT3 signaling in bladder cancer cells.[20] DANCR interacts with STAT3 to activate STAT3 in NPC cells.[40] Here, we also showed that DANCR could activate IL-6/STAT3 signaling through the upregulation of PSMD10. Interestingly, activation of IL-6/STAT3 signaling promoted DANCR transcription reciprocally, indicating a positive regulatory feedback loop between DANCR and IL-6/STAT3 signaling pathway. Meanwhile, whether DANCR/IL-6/STAT3 loop is involved in growth, metastasis or self-renewal capacity in HCC needs to be investigated as well.

Conclusion

In summary, our findings demonstrated that DANCR contributes to sorafenib resistance by upregulating PSMD10 expression and activating the IL-6/STAT3 signaling pathway. Targeting DANCR-PSMD10-IL-6/STAT3 axis may be a promising therapeutic approach for overcoming sorafenib resistance in HCC cells.

Data Sharing Statement

The datasets used during this research are available.

Author Contributions

Yuan Liu, Lamei Chen, Huabing Yuan, Shenghong Guo and Gang Wu contributed to conception and design, and revised the manuscript for important intellectual content. Lamei Chen, Huabing Yuan and Shenghong Guo contributed to acquisition of data, or analysis and interpretation of data. All of the authors made the final approval of the version to be published and agreed to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin*. 2015;65(2):87–108. doi:10.3322/caac.21262
2. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med*. 2008;359(4):378–390. doi:10.1056/NEJMoa0708857
3. Cheng AL, Kang YK, Chen Z, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a Phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol*. 2009;10(1):25–34. doi:10.1016/S1470-2045(08)70285-7
4. Cervello M, Bachvarov D, Lampiasi N, et al. Molecular mechanisms of sorafenib action in liver cancer cells. *Cell Cycle*. 2012;11(15):2843–2855. doi:10.4161/cc.21193
5. Asghar U, Meyer T. Are there opportunities for chemotherapy in the treatment of hepatocellular cancer? *J Hepatol*. 2012;56(3):686–695. doi:10.1016/j.jhep.2011.07.031
6. Li H, Zhang J, Zhen C, Yang B, Feng L. Gankyrin as a potential target for tumor therapy: evidence and perspectives. *Am J Transl Res*. 2018;10(7):1949–1960.
7. Fu XY, Wang HY, Tan L, Liu SQ, Cao HF, Wu MC. Overexpression of p28/gankyrin in human hepatocellular carcinoma and its clinical significance. *World J Gastroenterol*. 2002;8(4):638–643. doi:10.3748/wjg.v8.i4.638
8. Fu J, Chen Y, Cao J, et al. p28GANK overexpression accelerates hepatocellular carcinoma invasiveness and metastasis via phosphoinositol 3-kinase/AKT/hypoxia-inducible factor-1alpha pathways. *Hepatology*. 2011;53(1):181–192. doi:10.1002/hep.24015
9. Luo T, Fu J, Xu A, et al. PSMD10/gankyrin induces autophagy to promote tumor progression through cytoplasmic interaction with ATG7 and nuclear transactivation of ATG7 expression. *Autophagy*. 2016;12(8):1355–1371. doi:10.1080/15548627.2015.1034405

10. Sakurai T, Yada N, Hagiwara S, et al. Gankyrin induces STAT3 activation in tumor microenvironment and sorafenib resistance in hepatocellular carcinoma. *Cancer Sci*. 2017;108(10):1996–2003. doi:10.1111/cas.2017.108.issue-10
11. Saha P, Verma S, Pathak RU, Mishra RK. Long noncoding RNAs in mammalian development and diseases. *Adv Exp Med Biol*. 2017; 1008:155–198.
12. Xuan W, Yu H, Zhang X, Song D. Crosstalk between the lncRNA UCA1 and microRNAs in Cancer. *FEBS Lett*. 2019. doi:10.1002/1873-3468.13470
13. Li M, Duan L, Li Y, Liu B. Long noncoding RNA/circular noncoding RNA-miRNA-mRNA axes in cardiovascular diseases. *Life Sci*. 2019;233:116440.
14. Jin W, Chen L, Cai X, et al. Long non-coding RNA TUC338 is functionally involved in sorafenib-sensitized hepatocarcinoma cells by targeting RASAL1. *Oncol Rep*. 2017;37(1):273–280. doi:10.3892/or.2016.5248
15. Chen S, Xia X. Long noncoding RNA NEAT1 suppresses sorafenib sensitivity of hepatocellular carcinoma cells via regulating miR-335-c-Met. *J Cell Physiol*. 2019. doi:10.1002/jcp.27567
16. Li W, Dong X, He C, et al. LncRNA SNHG1 contributes to sorafenib resistance by activating the Akt pathway and is positively regulated by miR-21 in hepatocellular carcinoma cells. *J Exp Clin Cancer Res*. 2019;38(1):183. doi:10.1186/s13046-019-1177-0
17. Zhi Y, Abudoureyimu M, Zhou H, et al. FOXM1-mediated LINC-ROR regulates the proliferation and sensitivity to sorafenib in hepatocellular carcinoma. *Mol Ther Nucleic Acids*. 2019;16: 576–588. doi:10.1016/j.omtn.2019.04.008
18. Kretz M, Webster DE, Flockhart RJ, et al. Suppression of progenitor differentiation requires the long noncoding RNA ANCR. *Genes Dev*. 2012;26(4):338–343. doi:10.1101/gad.182121.111
19. Yuan SX, Wang J, Yang F, et al. Long noncoding RNA DANCR increases stemness features of hepatocellular carcinoma by derepression of CTNNB1. *Hepatology*. 2016;63(2):499–511. doi:10.1002/hep.27893
20. Chen Z, Chen X, Xie R, et al. DANCR promotes metastasis and proliferation in bladder cancer cells by enhancing IL-11-STAT3 signaling and CCND1 expression. *Mol Ther*. 2019;27(2):326–341. doi:10.1016/j.ymthe.2018.12.015
21. Wen X, Liu X, Mao YP, et al. Long non-coding RNA DANCR stabilizes HIF-1alpha and promotes metastasis by interacting with NF90/NF45 complex in nasopharyngeal carcinoma. *Theranostics*. 2018;8(20):5676–5689. doi:10.7150/thno.28538
22. Liu F, Yuan JH, Huang JF, et al. Long noncoding RNA FTX inhibits hepatocellular carcinoma proliferation and metastasis by binding MCM2 and miR-374a. *Oncogene*. 2016;35(41):5422–5434. doi:10.1038/onc.2016.80
23. Yuan JH, Yang F, Wang F, et al. A long noncoding RNA activated by TGF-beta promotes the invasion-metastasis cascade in hepatocellular carcinoma. *Cancer Cell*. 2014;25(5):666–681. doi:10.1016/j.ccr.2014.03.010
24. Xiao Y, Pan J, Geng Q, Wang G. LncRNA MALAT1 increases the stemness of gastric cancer cells via enhancing SOX2 mRNA stability. *FEBS Open Bio*. 2019. doi:10.1002/2211-5463.12649
25. Diao P, Ge H, Song Y, et al. Overexpression of ZEB2-AS1 promotes epithelial-to-mesenchymal transition and metastasis by stabilizing ZEB2 mRNA in head neck squamous cell carcinoma. *J Cell Mol Med*. 2019;23(6):4269–4280. doi:10.1111/jcmm.2019.23.issue-6
26. Liu F, Lou K, Zhao X, et al. miR-214 regulates papillary thyroid carcinoma cell proliferation and metastasis by targeting PSMD10. *Int J Mol Med*. 2018;42(6):3027–3036. doi:10.3892/ijmm.2018.3902
27. Chen BF, Suen YK, Gu S, Li L, Chan WY. A miR-199a/miR-214 self-regulatory network via PSMD10, TP53 and DNMT1 in testicular germ cell tumor. *Sci Rep*. 2014;4:6413. doi:10.1038/srep06413
28. Li J, Tian F, Li D, et al. MiR-605 represses PSMD10/Gankyrin and inhibits intrahepatic cholangiocarcinoma cell progression. *FEBS Lett*. 2014;588(18):3491–3500. doi:10.1016/j.febslet.2014.08.008
29. Li R, Yanjiao G, Wubin H, et al. Secreted GRP78 activates EGFR-SRC-STAT3 signaling and confers the resistance to sorafeinib in HCC cells. *Oncotarget*. 2017;8(12):19354–19364. doi:10.18632/oncotarget.15223
30. Xie L, Zeng Y, Dai Z, et al. Chemical and genetic inhibition of STAT3 sensitizes hepatocellular carcinoma cells to sorafenib induced cell death. *Int J Biol Sci*. 2018;14(5):577–585. doi:10.7150/ijbs.22220
31. Xu Z, Yang F, Wei D, et al. Long noncoding RNA-SRLR elicits intrinsic sorafenib resistance via evoking IL-6/STAT3 axis in renal cell carcinoma. *Oncogene*. 2017;36(14):1965–1977. doi:10.1038/onc.2016.356
32. Zheng T, Hong X, Wang J, et al. Gankyrin promotes tumor growth and metastasis through activation of IL-6/STAT3 signaling in human cholangiocarcinoma. *Hepatology*. 2014;59(3):935–946. doi:10.1002/hep.v59.3
33. Wang X, Jiang B, Zhang Y. Gankyrin regulates cell signaling network. *Tumour Biol*. 2016;37(5):5675–5682. doi:10.1007/s13277-016-4854-z
34. Zheng T, Li D, He Z, Feng S, Zhao S. Long noncoding RNA NBAT1 inhibits autophagy via suppression of ATG7 in non-small cell lung cancer. *Am J Cancer Res*. 2018;8(9):1801–1811.
35. Yu H, Lee H, Herrmann A, Buettner R, Jove R. Revisiting STAT3 signalling in cancer: new and unexpected biological functions. *Nat Rev Cancer*. 2014;14(11):736–746. doi:10.1038/nrc3818
36. Yang C, Cai WC, Dong ZT, et al. lncARSR promotes liver cancer stem cells expansion via STAT3 pathway. *Gene*. 2019;687:73–81. doi:10.1016/j.gene.2018.10.087
37. Wang X, Sun W, Shen W, et al. Long non-coding RNA DILC regulates liver cancer stem cells via IL-6/STAT3 axis. *J Hepatol*. 2016;64(6):1283–1294. doi:10.1016/j.jhep.2016.01.019
38. Chen ZZ, Huang L, Wu YH, Zhai WJ, Zhu PP, Gao YF. LncSox4 promotes the self-renewal of liver tumour-initiating cells through Stat3-mediated Sox4 expression. *Nat Commun*. 2016;7:12598. doi:10.1038/ncomms12598
39. Zhao J, Du P, Cui P, et al. LncRNA PVT1 promotes angiogenesis via activating the STAT3/VEGFA axis in gastric cancer. *Oncogene*. 2018;37(30):4094–4109. doi:10.1038/s41388-018-0250-z
40. Zhang X, Yang J, Bian Z, Shi D, Cao Z. Long noncoding RNA DANCR promotes nasopharyngeal carcinoma progression by interacting with STAT3, enhancing IL-6/JAK1/STAT3 signaling. *Biomed Pharmacother*. 2019;113:108713. doi:10.1016/j.biopha.2019.108713