High expression of serine and arginine-rich splicing factor 9 (SRSF9) is associated with hepatocellular carcinoma progression and a poor prognosis

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Abstract

Background: Serine and arginine-rich splicing factor 9 (SRSF9) has been linked to the occurrence and progression of various cancers; however, its effects and mechanism of action hepatocellular carcinoma (HCC) have not been reported. In this study, we used a bioinformatics approach and in vitro assays to evaluate the expression of SRSF9 in HCC, its prognostic value, and its underlying regulatory mechanisms, including analyses of related pathways and the role of methylation.

Methods: Transcriptomic and DNA methylation data for 357 HCC cases and 50 paratumor tissues in The Cancer Genome Atlas database were obtained. Additionally, protein expression data for cell lines and tissue samples were obtained from the Human Protein Atlas. The CMap databased was used to predict candidate drugs targeting SRSF9.

Various cell lines were used for in vitro validation.

Results: SRSF9 expression was significantly elevated in HCC and was negatively regulated by its methylation site cg06116271. The low expression of SRSF9 and hypermethylation of cg06116271 were both associated with a longer overall survival time. A correlation analysis revealed ten genes that were co-expressed with SRSF9; levels of CDK4, RAN, DENR, RNF34, and ANAPCS were positively correlated and levels of RBP4, APOC1, MASP2, HP, and HPX were negatively correlated with SRSF9 expression. The knockdown of SRSF9 in vitro inhibited the proliferation and migration of HCC cells and significantly reduced the expression of proteins in the Wnt signaling pathway (DVL2 and β-catenin) and cell cycle pathway (Cyclin D and Cyclin E). A CMap analysis identified two drugs, camptothecin and apigenin, able to target and inhibit the expression of SRSF9.

Conclusions: This study expands our understanding of the molecular biological functions of SRSF9 and cg06116271 and provides candidate diagnostic and therapeutic targets for HCC.
Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor. Due to the high heterogeneity of tumor cells and high malignancy, the prognosis is ultimately poor [1]. To improve the prognosis of patients with HCC, comprehensive treatment strategies based on surgery have been adopted. With the application of novel treatment schemes, including immunotherapy [2], the survival time still has not increased significantly. The complex mechanisms and multiple risk factors for HCC drive research aimed at revealing the pathogenesis of HCC and the development of corresponding treatment strategies. At the molecular level, HCC is characterized by expression changes in a large number of genes, abnormal epigenetic regulation, and changes in the activity of cellular pathways, leading to abnormalities in the microenvironment of HCC as well as proliferation, differentiation, migration, and even metastasis [3]. Therefore, identifying genes related to prognosis may provide a basis for the development of targeted biotherapies to improve the prognosis of patients with HCC.

Serine and arginine-rich splicing factor 9 (SRSF9) is an important gene with regulatory effects in the pathological process of a variety of tumors. For example, an abnormal increase in the expression level of SRSF9 in oral squamous cell carcinoma leads to a significant reduction in survival time via the regulation of alternative splicing [4]. However, there are no comprehensive and systematic reports on the role of SRSF9 in HCC. Therefore, we collected transcriptomic and DNA methylation data for a large sample of patients with HCC along with detailed clinical information from The Cancer Genome Atlas (TCGA) database to explore associations between SRSF9 and prognosis, clinical characteristics, and genetic pathways in HCC. We verified the results of the bioinformatics analyses by knocking down the expression of SRSF9 in vitro. This is the first detailed study of the role of SRSF9 in the pathological process of HCC. This study not only expands our understanding of the roles of SRSF9 in tumor biology but also clarifies the pathogenesis of HCC at the molecular level. Our results provide sufficient evidence for the clinical value of SRSF9 as a biological target for the treatment of HCC.

Material and methods

Data collection

TCGA was jointly launched by the National Cancer Institute (NCI) and National Human Genome Research Institute (NHGRI) in 2006 [5]. It is an important data source for global cancer research, including clinical data, mRNA expression data, and methylation data for various cancers. Data for 374 HCC samples and 50 paratumor tissues were used for a comparative analysis of SRSF9 expression. Samples with missing information, such as survival time and tumor grade, were screened. Finally, 357 HCC samples with complete clinical and methylation data were retained for further data mining and analyses (Additional file 1: Table S1).

The Human Protein Atlas (HPA) (www.proteinatlas.org) is one of the most widely accessed protein databases in the world, including continuously updated tissue and cell distribution information for numerous human proteins [6]. The expression levels of proteins in 64 cell lines, 48 normal human tissues, and 20 tumor tissues detected by immunoassays are available in this database of the human proteome. We utilized the HPA database to query the expression levels of the protein encoded by SRSF9 in normal liver tissues and HCC tissues. To validate the results of the bioinformatics analysis, five pairs of matched HCC and normal liver samples from laboratory were used to verify the protein levels of SRSF9 at the tissue level.

Gene set enrichment analysis

A gene set enrichment analysis (GSEA) is a conventional tool for obtaining biological information from gene expression data [7], which is based on the KEGG pathway database [8]. The enrichment of genes was analyzed by an initial database of defined gene sets. Functional enrichment of genes showing correlated expression patterns with SRSF9 in HCC-related pathways were performed using GSEA 3.0.jar. $P < 0.05$ and false discovery rate (FDR) $< 0.25$ were thresholds for significance.
CMAP analysis
The Connectivity Map (CMAP) database contains transcriptome data for cultured cells treated with active small molecules and uses a pattern matching algorithm to predict gene expression changes caused by drugs. In this study, genes with positive co-expression were regarded as upregulated and vice versa. Then, a CMAP analysis was performed to obtain a collection of drug molecules. The top two drugs based on the strength of the negative correlation were selected as candidate drugs for the treatment of HCC.

Cell culture and treatment
The normal liver cell line L02, hepatoblastoma cell line HepG2, and hepatocellular carcinoma cell lines Huh-7 and Hep3B were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin, and placed in a sterile incubator with 5% CO2 at 37 °C. In the drug intervention experiments, cells were treated with S-adenosyl methionine (SAM) for 8 h, decitabine (DAC) for 72 h, and camptothecin for 12 h, and apigenin for 12 h. In the SRSF9 knockdown experiment, control shRNA (shNC) or shRNA targeting SRSF9 (shSRSF9) (5′-GAT CCG GAAGGACACATGCCGAGAATTCAGATTTCC GCATGTCATCCTTTTTTTA-3′) was transfected into Huh-7 and Hep3B cells using Lipo2000 (Invitrogen, Waltham, MA, USA). After 24 h of incubation, the cells were used in subsequent experiments.

Real-time RT-PCR
Total RNAs from cells and tissues were extracted using TRIzol reagent (Invitrogen) and then reverse transcribed into cDNA using the Titan One Tube RT-PCR Kit (Roche, Mannheim, Germany). The expression of selected genes was detected by RT-PCR via TaqMan Fast Advanced Master Mix (Thermo Scientific, Waltham, MA, USA). GAPDH was recognized as an internal reference, and the primer sequences were as follows: 5′-CTA CAAGTACGGCGCATCC-3′ (sense) and 5′-CCCCGA CCTCCAATAATCTCT-3′ (antisense) for SRSF9; 5′-CAC CCACTCCTCCACCTTTGA-3′ (sense) and 5′-ACC ACCCTGTGTGCTGTAGCCA-3′ (antisense) for GAPDH. All experiments were repeated three times, and P-values less than 0.05 were statistically significant.

Immunoblotting and immunofluorescence
Whole proteins of tissues and treated cells were extracted using radioimmunoprecipitation (RIPA) lysis buffer containing a protease inhibitor cocktail (ProteinTech, Wuhan, China). Centrifugation was performed at 4 °C and 13,000 rpm for 20 min. The protein samples were analyzed by 10% SDS-PAGE and transferred to a PVDF membrane (Thermo Scientific). The membrane was sealed with 5% skim milk at 24 °C for 1 h, incubated with primary antibodies (SFRS9, Cat 17926-1-AP, Proteintech; DVL2, Cat 12037-1-AP, Proteintech; β-catenin, Cat 17565-1-AP, Proteintech; Cyclin D, Cat 29639-1-AP, Proteintech; Cyclin E, Cat 101554-1-AP, Proteintech) at 4 °C overnight, and washed with TBST three times (10 min each). Furthermore, the membrane was incubated with HRP-conjugated secondary antibody (HRP, Cat SA00001-2; Proteintech) at room temperature for 1 h, and washed with TBST three times (10 min each). Finally, the HRP signal was detected using the chemiluminescence detection system (Applygen Technologies, Beijing, China).

For immunofluorescence experiments, adherent cells were fixed with 4% paraformaldehyde and then 0.3% Triton X-100 was applied to increase the permeability of the cell membrane. Cell samples were sealed in 10% serum at room temperature for 1 h and then incubated with the specific antibody (Ki67, Cat 27309-1-AP; Proteintech) overnight at 4 °C. Samples were washed with 0.5% TBST and incubated with the secondary antibody labeled with fluorescent (Alexa Fluor 594, Cat A11037; Invitrogen) for 1 h at room temperature in the dark. Then, samples were incubated with DAPI solution for 10 min. Finally, images were obtained using a fluorescent microscope and processed using ImageJ.

MTT assay
Huh-7 and Hep3B cells treated with shRNA for 24 h were seeded into 96-well plates at a density of 2000 cells per well. Then, 20 μL of MTT solution (5 mg/mL) was added to each well at 0 h, 24 h, 48 h, 72 h, and 96 h and then incubated at 37 °C for 4 h. Formazan crystals were dissolved in 150 μL of DMSO in 96-well plates and incubated in the dark for 15 min. The absorbance of Huh-7 and Hep3B cells at 490 nm was detected by a microplate reader.

Colony formation and Transwell assays
Huh-7 and Hep3B cells transfected with shRNA for 24 h were resuspended and cultured in a 6-well plate at a density of 500 cells per well. After 14 d of incubation within complete medium, cell colonies were fixed with 4% paraformaldehyde and then stained with crystal violet solution. After images of the staining results were obtained, cell colonies were counted and analyzed.

Huh-7 and Hep3B cells treated with shRNA were resuspended in the medium with 5% FBS. Cells were cultured in Transwell chambers at a density of 105 cells per well. In addition, 600 μL of medium containing 20% FBS was
added to the lower chamber of the 24-well plate. After 48 h of incubation, cells that migrated to the outer side of the Transwell membrane were fixed and stained. Stained cells were counted and analyzed.

**Statistical analysis**
All experiments were repeated three times independently and all results were analyzed using GraphPad 9.0. The unpaired Student’s t-test or one-way ANOVA was used for comparisons between groups. The data are described as means ± standard deviation (SD). P-value less than 0.05 was regarded as statistically significant.

**Results**

**mRNA and protein expression levels of SRSF9 were significantly increased in HCC**
In this study, expression changes in SRSF9 in HCC and its impact on prognosis were evaluated. The mRNA expression of SRSF9 was significantly higher in HCC tissue samples (n = 374) than in paratumor samples (n = 50 cases) in TCGA (Fig. 1A). To verify these results, we collected HCC and normal tissue samples (n = 5 each) and three HCC cell lines. The RT-qPCR results showed that the mRNA expression of SRSF9 was significantly higher in both HCC tissue samples and three HCC cell lines than in the control group (Fig. 1B, C). We further examined the protein expression level of SRSF9 in HCC by immunohistochemistry. SRSF9 expression was significantly higher in HCC tissues than in control tissues in both the HPA database and clinical samples (Fig. 1D; Additional file 1: Figure S1A). Finally, we found that the expression level of SRSF9 was correlated with the 1-year survival rate of patients with HCC by an ROC curve analysis based on TCGA data (area under the curve, AUC = 0.737) (Fig. 1E). Based on these results, we speculate that SRSF9 has an important regulatory role in the pathological process of HCC.

**High expression of SRSF9 is regulated by DNA methylation**
mRNA expression is frequently regulated by DNA methylation. To explain the high expression of SRSF9 in HCC, methylation data for HCC were collected from TCGA.
Seven methylation sites for *SRSF9* in HCC tissues were identified, and low levels of methylation were maintained at all sites (Fig. 2A). A correlation analysis identified only one methylation site that was significantly correlated with gene expression, i.e., the methylation level of cg06116271 had a negative correlation with *SRSF9* expression \((R = -0.15, P = 0.0047)\) (Fig. 2B). A high methylation status of cg06116271, indicating low *SRSF9* expression, was correlated with a better prognosis in HCC (Fig. 2C). These results suggested that methylation contributes to the regulation of the expression of *SRSF9* in HCC. To verify this, 200 μM SAM, which increases DNA methylation levels [9], was used to stimulate selected HCC cell lines. RT-PCR results showed that the mRNA levels of *SRSF9* were remarkably reduced by nearly 50% upon treatment with SAM (Fig. 2D). Conversely, there was a statistically significant upregulation of *SRSF9* expression after treatment with the demethylation drug DAC [10] (Fig. 2E). The results of drug treatment experiments clearly demonstrated that *SRSF9* expression is regulated by DNA methylation; therefore, the high expression of *SRSF9* in HCC can likely be attributed to its hypomethylation status.

**High expression of *SRSF9* is associated with malignant features of HCC**

Based on the impact of clinical characteristics on prognosis, we evaluated whether *SRSF9* is also related to the poor prognosis of HCC. We selected data for 357 patients with HCC and explored the relationship between *SRSF9* expression and the histopathological stages of HCC by the chi-squared test. As shown in Fig. 3A–C, higher *SRSF9* expression was significantly positively correlated with malignant characteristics of HCC, such as tumor stage III versus stage I \((P = 0.0075)\); pathologic T3 versus T1 \((P = 0.015)\); histologic grade G3 versus G1 \((P = 0.0022)\), and G3 versus G2 \((P = 0.019)\). Furthermore, a univariate analysis demonstrated that a variety of factors are unfavorable in HCC, such as *SRSF9*, Pathologic M, Pathologic T, and tumor stage, while BMI is a favorable factor for HCC (Fig. 3D). After excluding other influencing factors, the results of a multivariate analysis indicated that *SRSF9* is an independent prognostic factor for HCC as well as Pathologic T (Fig. 3E). Overall, these results suggested that high *SRSF9* expression acted as an adverse factor associated with HCC.
High SRSF9 expression reduces overall survival in HCC by promoting the malignant behavior of tumor cells

Pathogenic genes often reduce the survival rate of patients with malignant tumors; accordingly, data were divided into high and low expression groups according to the median expression value of SRSF9. Then, the Kaplan–Meier (KM) curve showed that overall survival of patients in the high expression group (n = 178) was significantly lower than that of the low expression group (n = 179) (Fig. 1F). In vitro experiments were performed to verify the effect of SRSF9 on cell behavior in HCC. shRNA was used to knock down the mRNA expression of SRSF9 in Hep3B and Huh-7 cells (Additional file 1: Figure S1B). Then, a CCK8 assay demonstrated that the rates of proliferation of Hep3B cells and Huh-7 cells with the knockdown of SRSF9 were significantly lower than those of the control group (Fig. 4B, C). Next, fluorescence staining results for Ki67 (a marker of the proliferative capacity of Hep3B cells and Huh-7 cells) suggested that as the expression level of SRSF9 decreased, the protein level of Ki67 also decreased (Fig. 4D, E), and the ability of Hep3B cells and Huh-7 cells to form colonies was also inhibited (Fig. 4F). In addition, the migratory ability of shRNA-treated cells was also verified by a Transwell assay, demonstrating that reduced SRSF9 expression inhibited the migratory ability of Hep3B and Huh-7 cells (Fig. 4G). These results suggest that SRSF9 plays an important pathogenic role in HCC; however, its mechanism of action needs to be further evaluated.

SRSF9 functions via the Wnt signaling pathway and cell cycle related pathways

A GSEA was performed to predict the signaling pathways mediating the effects of SRSF9. The enrichment of SRSF9 was screened by FDR less than 25%, and related pathways were chosen according to $P < 0.05$. The significant pathways were as follows: Wnt signaling pathway, cell cycle, spliceosome, and DNA replication (Fig. 5A–D). Detailed data are provided in Additional file 1: Table S2. These results suggested that the effects of SRSF9 are mediated by these signaling pathways, and thus these pathways contribute to the progression of HCC. To further verify the GSEA results, DVL2 and β-catenin, related to the Wnt signaling pathway, were significantly downregulated, as determined by western blotting, in Hep3B cells.
and Huh-7 cells with SRSF9 knockdown (Fig. 5E, F). The expression levels of DVL2 and β-catenin were also evaluated in HCC tissues and normal tissues, revealing higher SRSF9 expression levels in HCC tissues than in normal tissues (Additional file 1: Figure S2A). Furthermore, the cell cycle pathway identified as enriched in the GSEA of SRSF9 was also validated. The protein expression levels of Cyclin D and Cyclin E, key proteins related to the cell cycle pathway, were also reduced by the knockdown of SRSF9 in Hep3B and Huh-7 cells (Fig. 5G, H). These results were consistent with those obtained in HCC tissues (Additional file 1: Figure S2B). Collectively, the in vitro experiments confirmed that the function of SRSF9 may be mediated by various mechanisms, such as regulation of the Wnt signaling pathway and cell cycle pathway, in HCC.

**Co-expressed genes and potential therapeutic drugs targeting SRSF9**

A co-expression analysis was performed to explore the molecular mechanism underlying the effects of SRSF9 in HCC. Hundreds of co-expressed genes were identified by setting a co-expression coefficient of less than -0.4 or greater than 0.4 and \( P < 0.05 \) as thresholds. The top five genes with the most highly positive (CDK4, RAN, DNR, RNF34, and ANAPC5) and negative (RBP4, APOC1, MASP2, HB, and HPX) correlations with SRSF9 expression were selected as candidate co-expressed genes (Fig. 6A, B).
We regarded the genes with positive correlations as upregulated and those with negative correlations as downregulated. Using the CMap database, candidate small molecule drugs were obtained. We set the screening criteria as $P < 0.001$ and selected the first two small molecule drugs, camptothecin and apigenin, as likely to inhibit the expression of $SRSF9$, according to the maximum negative correlation coefficient. The chemical formula and 2D and 3D structures of candidate drugs were obtained from the PubChem database (Fig. 6C, D).

To verify whether the screened drugs have inhibitory effects on the expression of $SRSF9$, the hepatoblastoma cell line HepG2 and hepatocellular carcinoma cell lines Huh-7 and Hep3B were treated with camptothecin (10 μM) and apigenin (40 μM) for analyses of $SRSF9$ expression by RT-PCR. As shown in Fig. 6E, F, the expression of $SRSF9$ was significantly lower in cells treated with either drug than in the control group. These results suggest that camptothecin and apigenin could indeed reduce the expression level of $SRSF9$ in HCC.

**Discussion**

We detected a significant relationship between the occurrence of HCC and the dysregulation of $SRSF9$ expression at the transcriptome level. The origin of HCC is accompanied by the activation of oncogenes and the inactivation of tumor suppressor genes [11]. Oncogenes are usually highly expressed in cancers [12], consistent with our results demonstrating that $SRSF9$ expression is significantly increased in HCC at the mRNA level based on TCGA data and at the protein level based on HPA data and laboratory HCC samples. Moreover, the activation of oncogenes can lead to the malignant progression of cancer, as the high expression of $SRSF1$ in breast cancer is positively associated with a higher tumor grade [13]. Consistently, high $SRSF9$ expression in this study was positively correlated with malignant physiopathological features of HCC. In addition, increased malignancy eventually leads to a poor prognosis [14]. For example, the increased expression of $YKT6$ is correlated with the tumor size, Edmondson
Fig. 6 Co-expression analysis and CMap analysis of SRSF9. A, B Co-expressed genes with SRSF9. C, D Chemical formulae and 2D and 3D structures of candidate drugs, camptothecin and apigenin, from the PubChem database; E, F mRNA level of SRSF9 in cells treated with camptothecin and apigenin. ****P<0.0001 versus control group.
Grade, metastasis, and microvascular invasion and predicts a poorer prognosis in patients with HCC [15]. In this study, a Kaplan–Meier curve revealed that the increase in SRSF9 expression can significantly reduce the overall survival time of patients. Further univariate and multivariate analyses suggested that SRSF9 is an independent risk factor for prognosis in HCC. In general, these results suggest that SRSF9 contributes to the pathogenesis of HCC and may influence disease progression.

DNA methylation in epigenetics is a very important gene regulatory mechanism, and low methylation in the genome generally contributes to the activation of oncogenes [16]. The hypomethylation status of the promoter region of HJLIRP is related to its high expression, which promotes the malignant progression of HCC and is associated with a poor patient prognosis [17]. Our results revealed that all seven methylation sites of SRSF9 remained hypomethylated in HCC and showed identified that the methylation status of cg06116271 could negatively regulate the expression of SRSF9. Importantly, the hypermethylation of cg06116271 was associated with a better prognosis in patients with HCC. Therefore, the cg06116271 methylation site is a candidate biological target for the treatment of HCC.

Previous studies have suggested that DNA hypomethylation can activate the Wnt signaling pathway and promote tumor formation in HCC [18]. The activation of the Wnt pathway in HCC may be involved in the maintenance of tumor stem cells and cell proliferation, differentiation, infiltration, and migration [19]. In this study, we confirmed that SRSF9 influences key proteins in the Wnt pathway by specifically knocking down SRSF9 via in vitro experiments. As a key splicing factor, SRSF9 affects the Wnt pathway and cell cycle pathway and influences the malignant progression of HCC by regulating cell proliferation and migration. Our comprehensive study of the role of SRSF9 in the progression of HCC provides a precise and novel therapeutic target for diagnosis and treatment.

To promptly translate the study results for the benefit of patients with HCC, two drugs, camptothecin and apigenin, predicted to inhibit SRSF9 expression were identified using the CMap database and verified using HCC cell lines. Numerous studies have reported the importance of camptothecin and apigenin for HCC therapy. Camptothecin downregulates the expression of Nrf2 and affects invasion, metastasis, and angiogenesis in HCC [20]. Apigenin can inhibit the proliferation of HCC by affecting the expression of microRNAs [21]. The results of this study further support the clinical value of camptothecin and apigenin, targeting SRSF9, providing a basis for improving the rate of survival and prognosis of patients.

Although the combination of large-scale data analyses using public databases and in vitro experiments confirmed the impact of SRSF9 on the prognosis of HCC from multiple perspectives, this study still had some shortcomings. First, according to a GSEA, SRSF9 can regulate a variety of cancer-related cellular signaling pathways. However, it was not possible to verify the roles of all candidate cell signaling pathways. Second, as a retrospective analysis, all indexes with predictive value for prognosis should be included. Unfortunately, data for important clinical features, such as the Child–Pugh stage and aluminum and bilirubin levels, were lacking, which is an inherent disadvantage of public databases. These shortcomings will be addressed in future research.

**Conclusion**

Our comprehensive analysis of the mechanism of action of SRSF9 in HCC, high SRSF9 expression regulated by the cg06116271 site predicted a poor prognosis. Furthermore, SRSF9 can promote HCC proliferation and migration by regulating the Wnt pathway and cell cycle pathway. This study not only provides insight into the pathological mechanism underlying HCC but also provides a new target for diagnosis and treatment. Targeted therapy focusing on SRSF9 may provide a personalized treatment strategy for patients with HCC, with better clinical effectiveness.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12920-022-01316-7.

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**Author contributions**

YJX designed and managed the entire study; ZGS and LB performed the main experiments; analyzed the data and drafted the original manuscript; SH and WGK analyzed the data and wrote a part of manuscript text; WDY and WLQ analyzed the data; LSN and WZY revised the manuscript; WSY provided professional advice about the study. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets generated and/or analysed during the current study are not publicly available due the data is being used in an ongoing, unpublished article, but are available from the corresponding author on reasonable request.

**Declarations**

**Ethics approval and consent to participate**

The sample information stored in the public database complies with the declaration of Helsinki (as revised in 2013). All experiments involving cell lines and
human tissues are approved by the Ethics Committee of Affiliated Hospital of North China University of Technology (No. 20220223004).

Competing interests
The authors declare that they have no competing interests.

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