Downregulation of RRS1 Inhibits Proliferation, Migration, And Epithelial-Mesenchymal Transition In Hepatocellular Carcinoma

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Abstract

Background: Regulator of ribosome synthesis 1 (RRS1) is an important nuclear protein involved in the biogenesis of ribosomes, and its relationship with a variety of cancers is confirmed by several studies. However, the underlying mechanism of RRS1-related carcinogenesis remains largely elusive.

Methods: In this study, we aimed to investigate the role of RRS1 in hepatocellular carcinoma (HCC) by detecting the expression level of RRS1 in HCC tissues and para-carcinoma tissues and its effect on the proliferation, migration and epithelial-mesenchymal transition of HCC.

Results: Compared with para-carcinoma tissues, the expression of RRS1 was significantly higher in HCC tissues, which is associated with the poor prognosis for HCC patients. In addition, knockdown RRS1 in HCC cell lines inhibited proliferation, migration and epithelial-mesenchymal transition (EMT).

Conclusions: Our results indicated that RRS1 played an important role in proliferation, migration and EMT process of HCC. Hence, RRS1 might emerge as a promising therapeutic target for HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancer and life-threatening malignancy in the world(1). Surgical resection is still the cornerstone in the treatment of HCC(2). However, the recurrence rate and remote metastasis after radical resection for HCC has been reported to reach 60%~70% in 5 years(1). Therefore, it is necessary to find out the molecular mechanism of HCC invasion and migration, which is expected to develop more effective therapeutic strategies against this deadly cancer.

Epithelial-mesenchymal transition (EMT) is a biological process that epithelial cells lose their polarity and acquire the capacities of mesenchymal-like cells(3). It implies enhanced cellular invasion and epithelial plasticity. Previous studies have shown EMT is associated with HCC recurrence and metastasis(4-6). Therefore, it is important to clarify the molecular mechanism underlying EMT in HCC and to develop innovative strategies against HCC recurrence and metastasis.

Regulator of ribosome synthesis 1 (RRS1), first identified in yeasts, is a eukaryotic conserved nuclear protein composed of 203 amino acids, which plays an important role in the biogenesis of ribosome(7-10). It participates in the assembly of the 60s ribosomal subunit and escorts it out of the nucleus(11). In recent years, a growing number of reports have confirmed that RRS1 participates in the occurrence and development of many kinds of malignant tumors(12-16), but its mechanism in promoting tumors is not clarified yet. A previous study on 18 cases of hepatitis B virus (HBV)-related HCC shows that the mRNA expression abundance of RRS1 in HCC tissues is significantly higher than that in para-carcinoma tissues, and the expression level in HCC tissues is present at 3- to 4- fold increase than that in para-carcinoma tissues. The cellular functional experiment results were displayed in SMMC-7721 cells. The high expression of RRS1 might play a role in inhibiting apoptosis and promoting growth, and then maintain

and support the malignant transformation and proliferation of cancer cells, but the function and carcinogenic mechanism of RRS1 in HCC are still uncertain.

In this study, we first analyzed whether RRS1 affects cell proliferation, migration, and invasion in HCC. Besides, we investigated the prognostic value of RRS1 in HCC patients. Finally, we revealed the molecular mechanism of RRS1 promoting HCC occurrence and development.

Materials And Methods

Patients and specimens

All of the clinical specimens of HCC patients were retrieved from the paraffin specimens of the department of Pathology and Hepatobiliary surgery resection specimens of the Xingtai People's Hospital from 2015 to 2020, and we conducted retrospective analysis on its clinical pathological data and survival prognosis. This study was approved by ethics committee of Xingtai People's Hospital. Informed consent was obtained from every single patient in accordance with *Declaration of Helsinki*.

Cell lines and cell culture

Human hepatocellular carcinoma cell lines HepG2, HuH7, SMMC-7721, Bel-7402, Hep3B and human normal liver cell line L02 were all purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Among them, L02, HepG2, HuH7, SMMC-7721 were cultured with DMEM (Dulbecco's modified Eagle medium, Invitrogen, Carlsbad, CA) medium, Bel-7402 and Hep3B were cultured with RPMI-1640 medium (Invitrogen, Carlsbad, CA).

All media contained 1% Penicillin/Streptomycin (Invitrogen) and 10% fetal bovine serum (FBS). Cells were cultured in an incubator under the condition of 37°C, 5% CO₂.

Lentivirus-mediated short hairpin (sh)RNA delivery.

A pgCSiL-gFP lentivirus RNAi expression system (GeneChem Co., Ltd., Shanghai, China) were used for RRS1 knockdown. The effective targeting sequence for RRS1 (GCT GCC TTC ATT GAG TTT A) was selected by western blot analysis. A non-silencing shRNA sequence (TTC TCC GAA CGT GTT CAC GT) was used as a negative control, which targets no genes in humans, mice or rats as in NCBI RefSeq. The shRNA vectors and the lentiviral packaging plasmids pHelper1.0 and pHelper2.0 were cotransfected into the 293T cells using Lipofectamine 2000 (Invitrogen, Shanghai, China) to generate the respective lentiviruses. 3 days later, the viral stocks were collected from the 293T cells and were used to infect Huh7 cells. The Huh7 cells were infected with the RRS1-siRNA-lentivirus (Lv-shRRS1 group) or negative control lentivirus (Lv-shCon group) according to the recommended multiplicity of infection (MOI). The stem-loop-stem oligos (shRNAs) were synthesized, annealed, and ligated into the pgCSiL-gFP lentivirus RNAi expression system. The lentiviral-based shRNA-expressing vectors were confirmed by DNA sequencing.

After lentiviral infection, the Huh7 cells were observed under a fluorescence microscope (MicroPublisher 3.3RTV; Olympus Corp., Tokyo, Japan) 3 days after infection.

Cell proliferation

We used cell counting Kit-8 (CCK8) to detect cell viability. Untransfected group and cells transfected with shRRS1 were separately seeded into 96-well plates. The number of cells in each well is about $5 \times 10^3/100 \mu\text{l}$. Three parallel holes were set for each group. Cell viability was tested at 24, 48, 72 and 96 h. Add $10 \mu\text{l}$ CCK8 solution into each well and incubated at 37°C 5% CO_2 for 1 h, The optical density (OD) of each well was measured at 450 nm. Then calculate the average of every group and the proliferation curve was plotted based on the OD value.

Transwell analysis

Transwell chamber (Aperture: $8 \mu\text{m}$ Costar Cambridge, Massachusetts, U.S.) was used to test cell migration. In the upper chamber coated with matrigel, and DMEM medium ($500 \mu\text{l}$) containing 10% FBS was added to the lower chamber. The cells were suspended in DMEM medium and seeded into the upper chamber coated with matrigel. Then, DMEM medium ($500 \mu\text{l}$) were added with 10% FBS into the lower chamber. After incubating under 37°C and 5% CO_2 for 24h, cells on the lower surface of the membrane were fixed with methanol and stained with 0.1% crystal violet. The number of the migrated cells were counted under the microscope.

Immunohistochemistry

After being dewaxed with xylene, the paraffin sections were hydrated with gradient ethyl alcohol. Then, the sections were immersed in 3% H_2O_2 to inhibit endogenous peroxidase activity. The primary antibody was incubated for 1h at 37°C and washed with PBS three times for 3 min each. The secondary antibody was incubated at room temperature for 30 min and washed with PBS three times for 3 min each. Then, stained by Diaminobenzidine (DAB) for 5 min, counterstained by hematoxylin and differentiate by 0.1% HCl alcohol for 1s. Finally, the section was dehydrated with gradient alcohol, transparent by xylene, and sealed with neutral gum. The staining intensity scoring standards are as follows: 0, no staining; 1+, light staining; 2+, moderate staining; 3+, heavy staining. The staining area scoring criteria are as follows: 0, no staining under the microscope; 1+, <30% tissue staining is positive; 2+, 30% ~ 60% tissue staining is positive; 3+, >60% tissue staining is positive. The expression of RRS1 was evaluated by the comprehensive score of staining intensity and area. The highest score is 6 points, and the lowest score is 0 points. The criterion for positive RRS1 staining is brown staining in the nucleus.

RNA extraction and real-time PCR.

Total RNA of HCC tissues, para-carcinoma tissues and cells were obtained by Trizol reagent. RNA ($1 \mu\text{g}$) was reverse transcribed with PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan, RR047A).

Followed by real-time PCR detection using TaKaRa TB Green™ Premix Ex Taq™ (TaKaRa, Japan, RR820A). The primer sequences are as follows:

RRS1 forward primer: 5'-CCGAAAAGGGGTTGAACTTCC-3',

reverse primer: 5'-CCCTACCGGACACCAGAGTAA-3';

and GAPDH forward primer : 5'-TGACTTCAACAGCGACACCCA-3',

reverse primer: 5'-CACCTGTTGCTGTAGCCAAA-3'.

Western blot analysis

Protein of HCC tissues, para-carcinoma tissues and cells were extracted by RIPA lysis buffer with 1 mM PMSF. Then total protein content were quantified by using the BCA method. Equal amounts of proteins from tissues and cells were used for Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), and subsequently transferred onto the PVDF membrane. Block (proteins) at 37 °C by using 5% skim milk for 1h. Wash the membrane three times with TBST, and incubated with the primary antibody at 4 °C overnight. The next day, wash the membrane three times with TBST and incubate with the secondary antibody at 37 °C for 1 h. Blots were developed by using ECL plus. RRS1 (ab188161), E-Cadherin (ab231303), N-Cadherin (ab76011), Vimentin (ab20346), Snail (ab216347) primary antibodies were purchased from Abcam (Cambridge, MA, USA).

Statistical analysis

Statistical analysis were performed by SPSS 22 software (Chicago, IL, USA). Chi square test were used to analyze the correlation between RRS1 expression and clinical-pathological parameters. Continuous variables were analyzed by t-test, and survival probability was detected by Kaplan-Meier method. Differences between groups were statistically significant if $P < 0.05$. Each group experiment was repeated three times.

Results

1. RRS1 is highly expressed in HCC cancer tissues

We performed immunohistochemical staining on paraffin sections from 75 HCC tissues and para-carcinoma tissues to detect RRS1 expression. The results showed that the positive RRS1 protein staining was mainly located in the nucleus and was highly expressed in HCC tissues, while the paracancerous tissues were low ($P < 0.001$) (Figure 1A and 1B). Real-time PCR was performed to detect the RRS1 mRNA expression level of HCC and para-carcinoma tissues in 42 cases and it found that the expression level of RRS1 in HCC tissues was higher than that in para-cancerous tissues ($P < 0.05$) (Figure 1C and 1D).

Furthermore, western blot were used to detected protein expression of RRS1 in 16 cases. The results showed that the protein expression level of RRS1 in HCC tissues was significantly higher than that in

para-cancerous tissues. ($P < 0.01$) (Figure 1E and 1F).

2. RRS1 is associated with a poor clinical prognosis in HCC patients

We divided the expression level of RRS1 in HCC tissues into a high expression group ($n = 45$) and a low expression group ($n = 30$) according to the "immunohistochemistry" method in Materials and Methods. The results showed that the expression of RRS1 was associated with the tumor size ($P < 0.01$), clinical stage ($P < 0.01$), and lung metastasis ($P < 0.05$) of HCC (Table1).

We further analyzed the correlation between RRS1 expression and the clinical prognosis of HCC patients through the Kaplan-Meier survival curve. The results showed that the overall survival time of patients in the RRS1 high expression group was lower than that in the RRS1 low expression group ($P < 0.01$) (Figure 1G). Meanwhile, The recurrence-free survival time was also lower than that in the RRS1 low expression group ($P < 0.01$) (Figure 1H).

Therefore, our results showed that the high expression of RRS1 in HCC is closely associated with the tumor malignancy and poor clinical prognosis.

3. RRS1 is highly expressed in HCC cells, and shRRS1 can effectively silence the expression of RRS1

We selected human normal liver cell line L02 and five human hepatocellular carcinoma cell lines (SMMC-7721, HepG2, Hep3B, Bel-7402, Huh7) to detect the protein expression of RRS1 levels by western blot. The results showed that the expression of RRS1 in hepatocellular carcinoma cells (SMMC-7721, HepG2, Hep3B, Bel-7402, Huh7) is higher than that in normal liver cells (L02), and Huh7 cells showed the highest expression level of RRS1 in hepatocellular carcinoma cell lines (Figure 2A and 2B).

We further constructed shRRS1-RNA and transtected it into silenced the expression of RRS1 by transfect shRRS1-RNA into Huh7 cells to knockdown the expression of RRS1. To confirm transfection efficiency we observed the cells infected by shRRS1 and Lv-shCon vectors by fluorescence microscope. We found that more than 80% of Huh7 cells express GFP, which indicates a high infection efficiency of shRRS1 (Figure 2C). We verified the silencing effect of shRRS1 by real-time PCR and western blot. The results showed that shRRS1 can effectively knockdown the mRNA expression level of RRS1 ($P < 0.01$) (Figure 2D) and knockdown the protein expression level of RRS1 ($P < 0.01$) (Figure 2E and 2F).

4. Down-regulation of RRS1 can inhibit the growth and metastasis of Huh-7 cells

We silenced the expression level of RRS1 in Huh-7 cells and tested the proliferation ability of Huh-7 cells by CCK-8 assay. The results showed that compared with the negative control group, the proliferation ability of Huh-7 cells in the RRS1 knockdown group was suppressed ($P < 0.01$) (Figure 3A). Subsequently, we conducted cell migration experiments and the results showed that the migration of Huh-7 cells was inhibited after knockdown RRS1 expression (Figure 3B). In addition, the migratory cells per field and migration fold change of Huh-7 cells decreased after knockdown RRS1 expression (Figure 3C and 3D). To identify whether EMT participate in the biological process in HCC migration and invasion, we further

detected the expression of EMT-related proteins. The results showed that the expression of E-Cadherin in Huh-7 cells increased after knockdown the expression of RRS1. In contrast, the expression of N-Cadherin, Vimentin, and Snail decreased after knockdown the expression of RRS1 (Figure 3E and 3F).

Discussion

In this study, we found that RRS1 acted as a new tumor-promoting gene in HCC. It was associated with enhanced proliferation and invasion abilities. In addition, it could induce EMT in HCC cells.

RRS1 is a eukaryotic conservative nuclear protein made up of 203 amino acids(8). In secretory-deficient yeasts that nonsense mutations of an amino acid codon at the C-terminus of RRS1, the mutation of RRS1 significantly reduces the transcriptional inhibition of rRNA and ribosomal protein genes(17). It can be seen that RRS1 is an important protein in the signal transduction pathway of protein secretion and ribosome synthesis. In addition, RRS1 also acts as a messenger in the process of signal transmission and plays an important role in the occurrence of Huntington's disease(18, 19). In normal human cells, RRS1 is located in the nucleus. In addition, it is found that RRS1 is located both in the nucleus and the periphery of the nucleus in the HeLa cell line(20). After further inhibiting the expression of RRS1 by RNAi technology, the cell cycle of HeLa cells was significantly delayed. Therefore, it is speculated that RRS1 may be related to the occurrence and development of cervical cancer and other tumors(20).

Song et al(15). confirmed that RRS1 is a new gene related to breast cancer and may play an important role in breast cancer proliferation and apoptosis. Mechanism studies have shown that RRS1 may activate the p53 pathway to promote breast cancer proliferation through RPL11/MDM2. Another study confirmed that RRS1 is involved in the progression of papillary thyroid carcinoma(14). Knockout of RRS1 can up-regulate the expression of genes associated with cell apoptosis and metabolism, while down-regulating genes associated with cell proliferation and vascular development. This study confirmed the diagnostic value of RRS1 in thyroid cancer in children and adults, and the expression of RRS1 is positively correlated with age(14). However, the specific mechanism is still unclear.

The effect of RRS1 on the clinical prognosis and invasion in HCC has not been reported yet. Our results showed that RRS1 is highly expressed in HCC and significantly negatively correlated with the poor prognosis. Moreover, RRS1 might participate in the EMT process of HCC cells. EMT is an important biological process involved in tumor invasion and metastasis, which induces the epithelial cells to lose their polarity and adhesive properties and enhances the ability of migration and invasion(3, 4, 21). EMT has been confirmed to be involved in the progression of HCC and is associated with a poor prognosis(4, 16). To reveal the possible mechanism of RRS1 in the process of EMT in HCC, we transfected RRS1 shRNA to knockdown the RRS1 expression. We found that after knockdown RRS1, the protein levels of mesenchymal markers (N-cadherin, Vimentin, Snail) in HCC cell lines decreased significantly. In contrast, E-Cadherin increased significantly as an epithelial marker. Our data indicated that RRS1 induced the invasion of HCC cell lines, and further verified that RRS1 triggers EMT in cell lines, which plays a crucial role in HCC invasion and metastasis.

As far as we known, this is the first study that clarified the importance of RRS1 in HCC. However, this study still has some limitations. First, we need to further explore the regulation network of RRS1 and EMT-related signals. Second, it is important to determine the upstream signal responsible for the overexpression of RRS1 in HCC. Finally, the clinical significance of RRS1 in HCC requires further verification in large cohorts.

Conclusions

All in all, this study revealed that RRS promotes HCC cell proliferation, migration and EMT. RRS1 was a promising prognostic indicator and therapeutic targets for HCC.

Declarations

Ethics approval and consent to participate

Clinical data have been approved by the Ethics Committee of Xingtai People's Hospital and approved by the patients.

Consent for publication

All contributing authors agree to the publication of this article.

Availability of data and materials

All data are fully available without restriction.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YL, DXL conceived and designed the experiments. ZL and JTW performed the experiments. CYL, JLL and XCZ analyzed the data. XLZ, KPZ and ZGZ supervised the whole experimental work and revised the manuscript. YL and DXL wrote the paper. All authors read and approved the manuscript.

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Table

Table 1 Correlations between RRS1 Staining and Clinicopathologic Characteristics of 75 HCC Patients

Variables	RRS1 Staining		Total	P value
	low	high		
Age(years)				
<50	10	21	31	0.3392
≥50	20	24	44	
Sex				
male	23	35	58	0.8659
female	7	10	17	
AFP(ng/ml)				
<200	16	17	33	0.2370
≥200	14	28	42	
Tumor size(cm)				
<5	20	15	35	0.0088*
≥5	10	30	40	
Lung metastasis				
Negative	25	26	51	0.0243*
Positive	5	19	24	
Clinical stage				
I+II	18	13	31	0.0093*
III+IV	12	32	44	
Tumor number				
solitary	19	24	43	0.4773
mutiple	11	21	32	

Figures

Figure 1

RRS1 is highly expressed in HCC cancer tissues and is related to the poor clinical prognosis of HCC patients. **A.** The RRS1 protein expression in the 75 pairs of HCC cancer tissues and the corresponding normal tissues by immunohistochemistry assay. The representative results were shown as above and the scale was 50μm. **B.** Semi-quantitative statistical comparison of the RRS1 protein expression between HCC cancer tissues and the corresponding normal tissues. **C, D.** mRNA expression of RRS1 in the 75 pairs

of HCC cancer tissues and the corresponding normal tissues by qPCR assay. **E, F.** protein expression of RRS1 in the 42 pairs of OSCC cancer tissues and the corresponding normal tissues by WB. **G, H.** Kaplan-Meier survival curve analysis of the correlation between RRS1 protein expression in HCC and overall survival and recurrence-free survival. *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$, calculated by T test, error bar calculated by the standardized error.

Figure 2

RRS1 is highly expressed in HCC cells, and shRRS1 can effectively silence the expression of RRS1. **A, B.** RRS1 protein expressions in 5 HCC cell lines (SMMC-7721, HepG2, Hep3B, Bel-7402, Huh7) and in normal L02 cells by Western Blot. **C.** Observe the transfection efficiency of shRRS1 and Lv-shCon vector infection by fluorescence microscope. **D.** Down-regulation of RRS1 protein expressions in shRRS1 silencing plasmids by qPCR. **E, F.** Down-regulation of RRS1 protein expressions in shRRS1 silencing plasmids by Western Blot. *, $P<0.05$, **, $P<0.01$, calculated by T test, error bar calculated by the standardized error, the experiments were repeated three times.

Figure 3

Down-regulation of RRS1 can inhibit the growth and metastasis of Huh-7 cells. **A.** Proliferative levels in Huh-7 cells and the cells in the treatment groups detected by CCK-8 reagent kit. **B,C and D.** Knock down the expression of RRS1 to detect the migration ability of Huh-7 cells. **E and F,** Knocking down the expression of RRS1, the expression of EMT-related proteins was detected. *, $P < 0.05$, **, $P < 0.01$, calculated by T test, error bar calculated by the standardized error, the experiments were repeated three times.