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ORIGINAL ARTICLE

Genomic gain/methylation modification/hsa-miR-132-3p increases *RRS1* overexpression in liver hepatocellular carcinoma

Xiaoxia Zhang[1] | Peilin Cong[2,3,4,5] | Li Tian[2,3,4,5] | Yinggang Zheng[2,3,4,5] | Hong Zhang[6] | Qiong Liu[2,3,4,5] | Tingmei Wu[2,3,4,5] | Qian Zhang[2,3,4,5] | Huanghui Wu[2,3,4,5] | Xinwei Huang[2,3,4,5] | Lize Xiong[2,3,4,5]

[1]Department of Hospital Infection Management, Shanghai Fourth People's Hospital, School of Medicine, Tongji University, Shanghai, China

[2]Clinical Research Center for Anesthesiology and Perioperative Medicine, Tongji University, Shanghai, China

[3]Shanghai Key Laboratory of Anesthesiology and Brain Functional Modulation, Shanghai, China

[4]Translational Research Institute of Brain and Brain-Like Intelligence, Shanghai Fourth People's Hospital, School of Medicine, Tongji University, Shanghai, China

[5]Department of Anesthesiology and Perioperative Medicine, Shanghai Fourth People's Hospital, School of Medicine, Tongji University, Shanghai, China

[6]Department of Rehabilitation Medicine, Shanghai Fourth People's Hospital, School of Medicine, Tongji University, Shanghai, China

Correspondence
Lize Xiong and Xinwei Huang, Clinical Research Center for Anesthesiology and Perioperative Medicine, Tongji University, Shanghai 200434, China.
Email: lizexiong@tongji.edu.cn and huanggenetics@tongji.edu.cn

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Abstract

This study aimed to determine the upstream regulatory factors affecting ribosome biogenesis regulator 1 homolog (*RRS1*) expression and the development and prognosis of liver hepatocellular carcinoma (LIHC). The expression profiles of *RRS1* were evaluated in pan-cancer tissues and liver tumor cell lines. The associations of *RRS1* with pan-cancer survival, immune infiltrations, immune checkpoints, and drug sensitivity were identified. We explored the potential upstream regulatory mechanisms of *RRS1* expression. Hsa-miR-132-3p knockdown, CCK-8 assays, transwell, and wound healing assays were performed to validate the regulatory effect of hsa-miR-132-3p on *RRS1* expression and the development of LIHC. Our findings demonstrated that *RRS1* was significantly elevated in 27 types of cancers. *RRS1* predicts a poor outcome of LIHC, lung adenocarcinoma, head and neck cancer, and kidney papillary cell carcinoma. *RRS1* expression showed a significant association with immune cell infiltrates and the expression of immune checkpoints-related genes in LIHC tissues. Increased *RRS1* expression may have a negative effect on these anticancer drugs of LIHC. Low methylation of the *RRS1* promoter and its genomic gain may elevate *RRS1* expression and predict poor prognosis for LIHC. Increased hsa-miR-132-3p expression may elevate

Abbreviations: BLCA, bladder cancer; BRCA, breast cancer; CHOL, bile duct cancer; CNV, copy number variation; COAD, colon cancer; CTRP, cancer therapeutics response portal; ESCA, esophageal cancer; GBM, glioblastoma; GDSC, Genomics of Drug Sensitivity in Cancer; GEO, Gene Expression Omnibus; GSCA, Gene Set Cancer Analysis; HAVCR2, hepatitis A virus cellular receptor 2; HNSC, head and neck cancer; HPA, Human Protein Atlas; IHC, immunohistochemistry; KCM, melanoma; KICH, kidney chromophobe; KIRC, kidney clear cell carcinoma; KIRP, kidney papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; PDCD1, programmed cell death 1; PDCD1LG2, programmed cell death 1 Ligand 2; PRAD, prostate cancer; READ, rectal cancer; *RRS1*, ribosome biogenesis regulator 1 homolog; SIGLEC15, sialic acid binding Ig like lectin 15; STAD, stomach cancer; TCGA, The Cancer Genome Atlas; THCA, Thyroid cancer; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TSS, transcriptional start site.

Xiaoxia Zhang, Peilin Cong, and Li Tian contributed equally to this work and share first authorship.

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RRS1 expression and result in poor prognosis for LIHC. Hsa-miR-132-3p inhibition can decrease *RRS1* expression and the development of liver tumor cell lines. Low methylation of the *RRS1* promoter, *RRS1* genomic gain, and hsa-miR-132-3p upregulation in LIHC may promote *RRS1* upregulation and thus lead to the development and poor prognosis for LIHC. *RRS1* is a promising therapeutic target for LIHC.

KEYWORDS
genomic gain, hepatocellular carcinoma, hsa-miR-132-3p, methylation modification, ribosome biogenesis regulator 1 homolog

1 | INTRODUCTION

Liver hepatocellular carcinoma, the most common form of primary liver cancer, is the fourth most common cause of tumor-related death worldwide.[1] Despite improved screening and discoveries and the combined use of multiple therapeutic strategies have improved the prognosis for LIHC patients, LIHC shows a rapid clinical course with an increased mortality rate, and the long-term outcome of this malignancy remains limited.[2] Therefore, it there is an urgent need to explore the molecular mechanisms of LIHC and determine predictive biomarkers for prognostic evaluation and targeted treatment.

RRS1, located in the nucleolus and endoplasmic reticulum, participates in 25S rRNA maturation, ribosome biogenesis, and the assembly of the 60S subunit.[3] The dysregulation of *RRS1* in tumor cells can significantly change their functions via disturbing ribosome biosynthesis and triggering the ribosomal stress response, which activates the oncogenic pathway and induces tumor onset.[4,5] Previous studies have reported that elevated *RRS1* expression could promote the development and progression of multiple cancers.[4,6–8] Although the downstream molecular mechanisms for *RRS1* mediating LIHC have been partly reported, including that *RRS1* promotes LIHC development by attenuating the RPL11-MDM2-p53 signaling,[2] the upstream or other regulatory mechanisms of RRS1-mediated LIHC, such as epigenetic modification, microRNA, and copy number regulation, which are closely related to cancer occurrence,[9–11] remain unclear. At present, little is known about the expression status of *RRS1* in a large sample of pan-carcinoma tissues.

In this study, we aimed to identify the expression profiles of *RRS1* pan-cancers and to determine their roles in the clinical prognosis for pan-cancers. Based on the known carcinogenic effect of *RRS1* in LIHC, we focused on the potential regulatory factors affecting *RRS1* expression, including copy number variation, DNA methylation, and microRNAs. In addition, we underlined *RRS1* association with immune infiltrations, immune checkpoints, and drug sensitivity.

2 | MATERIALS AND METHODS

2.1 | Data acquisition and preprocessing

The mRNA-seq data (count) of 730 tumor-adjacent and 10,363 pan-cancer tissues, microRNA-seq data (reads per million mapped reads, RPM) of 679 tumor-adjacent and 10,403 pan-cancer tissues, mRNA-seq data (transcript per million, TPM) of 7862 GTEx donor tissues, Illumina Human Methylation 450 data (Beta value) of 50 tumor-adjacent and 380 LIHC tissues, and corresponding clinical information were downloaded from TCGA database of the UCSC Xena public datasets (https://xenabrowser.net/datapages/). Among the above datasets, pan-cancer mRNA-seq data consisted of 50 normal tissues and 374 LIHC tissues. Pan-cancer microRNA-seq data included 50 normal tissues and 375 LIHC tissues. Four mRNA-array datasets of LIHC were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), of which GSE101685 included eight normal liver tissues and 24 LIHC tissues. GSE36376 included 193 normal adjacent tissues and 240 LIHC tissues. GSE60502 consisted of 18 normal adjacent tissues and 18 LIHC tissues. GSE10143 contained 307 liver tissues with hepatitis or cirrhosis and 80 LIHC tissues. In addition, the paired *RRS1* expression and promoter methylation matrix data including 375 TCGA LIHC tissues were obtained from the EWAS Data Hub.[12] All sample information for the datasets is shown in Table S1.

2.2 | Expression analysis for *RRS1* in pan-cancers and LIHC

First, we evaluated the expression status of *RRS1* between pan-cancers via the UALCAN database,[13] an interactive web portal to perform the in-depth analyses of TCGA gene expression and methylation data. The standardized RNA-sequencing count matrix of pan-cancer samples was converted to a TPM matrix referring to a previous report.[14] RPM and TPM matrixes were then converted to $\log_2$ (RPM or TPM + 1) matrixes. As some cancer types lacked control samples, we added the corresponding mRNA data ($\log_2$ (TPM + 1)) of GTEx normal tissues of each cancer type into TCGA mRNA data ($\log_2$ (TPM + 1)) of each cancer type and merged TCGA tumor-adjacent samples and GTEx normal samples into the control samples after data batch normalization using R packages (*sva* and *limma* 3.40.6). The Wilcoxon rank sum test was utilized to perform the differential expression analysis for genes between control/tumor-adjacent samples and pan-cancer samples.

In addition, four normalized mRNA-array datasets, including GSE101685, GSE36376, GSE60502, and GSE10143, were obtained

from the GEO database, and the Wilcoxon rank sum test was used to validate the expression status of *RRS1* between the control liver and LIHC tissues. Finally, we estimated the protein expression difference of RRS1 between adjacent non-LIHC and LIHC tissues by IHC in the HPA database (https://www.proteinatlas.org/). A p-value <0.05 was considered statistically significant. Based on TCGA and the GEO datasets, we used *pROC* and *ggplot2* packages to perform Receiver Operating Characteristic (ROC) curve and evaluate the prognostic capacity of *RRS1* in LIHC and distinguish LIHC from the normal groups.

2.3 | Survival analysis for *RRS1* in pan-cancers and LIHC

The association of *RRS1* with the overall survival of pan-cancers was evaluated using the Mantel–Cox test in the GEPIA 2 database.[15] A p-value <0.05 was considered as statistically significant.

2.4 | Correlation analysis of *RRS1* with immune infiltrations, immune checkpoints, and drug sensitivity

The proportion of six immune cell types in LIHC and normal liver tissues was calculated based on TCGA LIHC data via the TIMER algorithm.[16] The differential immune cell score between the two groups was assessed using the Wilcoxon rank sum test. Spearman correlation analysis was utilized to evaluate the association between *RRS1* expression and immune cell score. In addition, Wilcoxon rank sum test was used to analyze the expression difference of immune checkpoints-related genes lymphocyte-activation gene 3 (*LAG3*), *CD274*, *PDCD1*, cytotoxic T-lymphocyte associated protein 4 (*CTLA4*), programmed cell death 1 Ligand 2 (*PDCD1LG2*), *SIGLEC15*, *HAVCR2*, and *TIGIT* between normal liver and LIHC tissues based on TCGA dataset. The association between their expression with *RRS1* expression was determined in pan-cancers using Spearman correlation analysis. Moreover, we assessed the correlation of *RRS1* with drug sensitivity based on GDSC and CTRP databases using GSCA.[17] A p-value <0.05 was considered as statistically significant.

2.5 | Identification of potential regulatory factors affecting *RRS1* expression

The methylation status of the *RRS1* promoter between normal liver tissues and LIHC and subtype LIHC tissues was evaluated using UALCAN. We then estimated the correlation of the methylation of the *RRS1* promoter with the overall survival of LIHC and *RRS1* expression based on 371 LIHC samples and the corresponding information obtained from the EWAS Data Hub platform.[12] In addition, we used Spearman analysis to evaluate the correlation of each methylation site (−5000 bp to +5000 bp) of the *RRS1* TSS with *RRS1* expression based on TCGA methylation data for LIHC. We then analyzed the CNV distribution of *RRS1* between gain LIHC, loss LIHC, and normal LIHC groups and identified the correlation for CNV in *RRS1* with *RRS1* expression in LIHC using DriverDBv3, a multi-omics database for cancer driver genes.[18] Finally, upstream binding microRNAs of *RRS1* were predicted based on seven prediction programs PicTar, microT, PITA, RNA22, miRmap, miRanda, and TargetScan via The Encyclopedia of RNA Interactomes (ENCORI) database.[19] The predicted microRNAs were acquired according to their appearance in three or more programs. Based on the pan-cancer microRNA datasets, we then used Spearman analysis to identify the correlation of *RRS1* expression with its target microRNA expression. Wilcoxon rank sum test was utilized to estimate the expression difference of target microRNAs between normal and pan-cancer tissues. Furthermore, overall survival analysis for target microRNAs was performed using the R packages *survival* and *survminer*. A p-value <0.05 was considered statistically significant.

2.6 | Cell lines and cell culture

The normal liver cell line (LX2) and liver tumor cell lines (Hep3B and HepG2) were obtained from Hunan Fenghui Biotechnology Co. Ltd. (Changsha City, China). All cell lines were grown in DMEM (Gibco) and supplemented with 10% FBS (Gibco). Unless otherwise specified, all cell cultures were grown in 5% CO_2 at 37°C.

2.7 | Cell transfection

The miR-132-3p inhibitor and relevant negative controls were purchased from Genechem (Shanghai, China). In order to inhibit the expression of miR-132-3p, HepG2 and Hep3B cells were transfected with negative control lentivirus or knockdown lentivirus using Polyplus transfection (Genechem, China). The protein or mRNA was extracted after 48–72 h. Western blot (WB) and qRT-PCR were used to detect cell transfection efficiency, and follow-up experiments were performed.

2.8 | Cell viability

HepG2 and Hep3B cells treated with knockdown lentivirus were seeded at 5×10^3 per well in a 96-well plate. The viability of cells was measured using a Cell Counting Kit-8 (Dojindo, Japan) daily for 5 days. After incubating for 1 h the absorbance at 450 nm was measured on a microplate reader.

2.9 | Scratch assay

HepG2 and Hep3B cells treated with knockdown lentivirus were seeded into a six-well cell culture plate. After 24 h of culture, the cells were scratched and the floating cells and debris were washed

away with PBS. The scratch distance was photographed and recorded under a microscope. After 48h, the floating cells were washed off with PBS, and the scratch distance was recorded under a microscope.

2.10 | Transwell

At 1 h before the experiment, Matrigel (Corning) was mixed with medium according to the protocol, and then added to the upper well, followed by culture at 37°C, 5% CO_2 for 1h. After gel coagulation, HepG2 and Hep3B cells treated with knockdown lentivirus were diluted with serum-free DMEM to obtain 5×10^5 cells in each well. At the same time, DMEM with 20% serum was added to the lower well, and then wells were incubated at 37°C, 5% CO_2. After 24h, cells were washed with PBS and fixed with paraformaldehyde for 20min. Then we gently wiped the nontransferred cells on the membrane with a cotton swab. After crystal violet staining for 20min, this was washed with PBS until the background was colorless. Finally, photographs were taken and results counted under a microscope.

2.11 | Western blotting

Cells were collected and extracted using a total cell protein extraction kit (Thermo Fisher, USA), and the total protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Next, 10% SDS-PAGE was prepared and the samples were analyzed. *RRS1* antibodies (Affinity, USA) were used to detect the corresponding proteins and β-actin (Affinity, USA) was used as an internal reference for quantitative analysis.

2.12 | Real-time PCR

Total RNA of cells was extracted with TRIzol (Vazyme, China) and reverse transcribed into cDNA using the PrimeScript RT reagent kit (Vazyme, China). The miRNA of the treated cells was extracted with the MiPure Cell miRNA Kit (Vazyme, China) and reverse transcribed using an miRNA 1st Strand cDNA Synthesis Kit (Vazyme, China) in a reaction mixture containing an miR-specific stem–loop reverse transcription primer (5-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCGCGT-3). Then qRT-PCR was carried out using the SYBR Green Master Mix (TaKaRa, Japan) on a Quant Studio 1 Real-Time PCR system (Thermo Fisher, USA) to assess gene expression. GAPDH was used as a reference for mRNA levels, and U6 was used as a reference for miRNA levels. The primer details are summarized in Table S2.

2.13 | Statistical analysis

All statistical analyses were based on multiple database analysis platforms as well as R software 4.1.0 version and the attached packages. For cell experiments, comparisons of mRNA or protein levels between different groups were conducted using one way-ANOVA test or Student's *t* test. The significant differences were defined as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

3 | RESULTS

3.1 | Elevated *RRS1* expression in pan-cancers and LIHC

Using the UALCAN database, we evaluated the expression status of *RRS1* between pan-cancers. We found that *RRS1* was broadly expressed in all tumor tissue types with low cancer specificity (Figure 1A). We then performed a differential expression analysis for *RRS1* between 24 pan-cancer samples that had control samples. As shown in Figure 1B, the expression of *RRS1* was significantly increased in all tumor types compared with control samples, including LIHC, BRCA, COAD, LUAD, LUSC, PRAD, THCA, STAD, HNSC, KIRP, CHOL, READ, KIRC, KICH, ESCA, BLCA, and GBM ($p < 0.05$). We then conducted a differential expression for *RRS1* between normal TCGA and GTEx control samples and TCGA pan-cancer samples. Our results indicated that *RRS1* expression was significantly elevated in 27 types of cancers including LIHC compared with the corresponding control samples ($p < 0.05$; Figure 1C), which validated the above findings. Similarly, the differential analysis in four GEO datasets suggested that *RRS1* also showed a consistent upregulation in LIHC ($3.1E-40 < p < 0.022$; Figure 1D–G). Moreover, as displayed in Figure 1H, the IHC of the HPA database exhibited that RRS1 protein was not detected in normal adjacent tissues, but highly expressed in LIHC tissues. In addition, our WB experiment for RRS1 protein conducted on a normal liver cell line (LX2) and liver tumor cell lines (Hep3B and HepG2) showed that RRS1 was significantly elevated in liver tumor cell lines (Figure S1). ROC curve analysis indicated that the areas under the ROC curve (AUCs) for *RRS1* were 0.856 and 0.919 for the training set and the test set, respectively (Figure 1I), which indicated a good predictive ability of *RRS1* for LIHC.

3.2 | Elevated expression of *RRS1* predicts a poor prognosis in LIHC

In order to explore whether *RRS1* could serve as the prognostic factor of LIHC, we performed a survival analysis. Our findings indicated that HNSC, KIRP, and LUAD patients with high *RRS1* expression had poor overall survival (Figure 2A). LIHC patients with high *RRS1* expression showed poor overall survival, poor disease-specific survival, and poor progress-free interval ($1.52 < HR < 1.75$; $0.004 < p < 0.016$; Figure 2B–D). These findings revealed that *RRS1* influences the prognosis of LIHC, KIRP, LUAD, and HNSC.

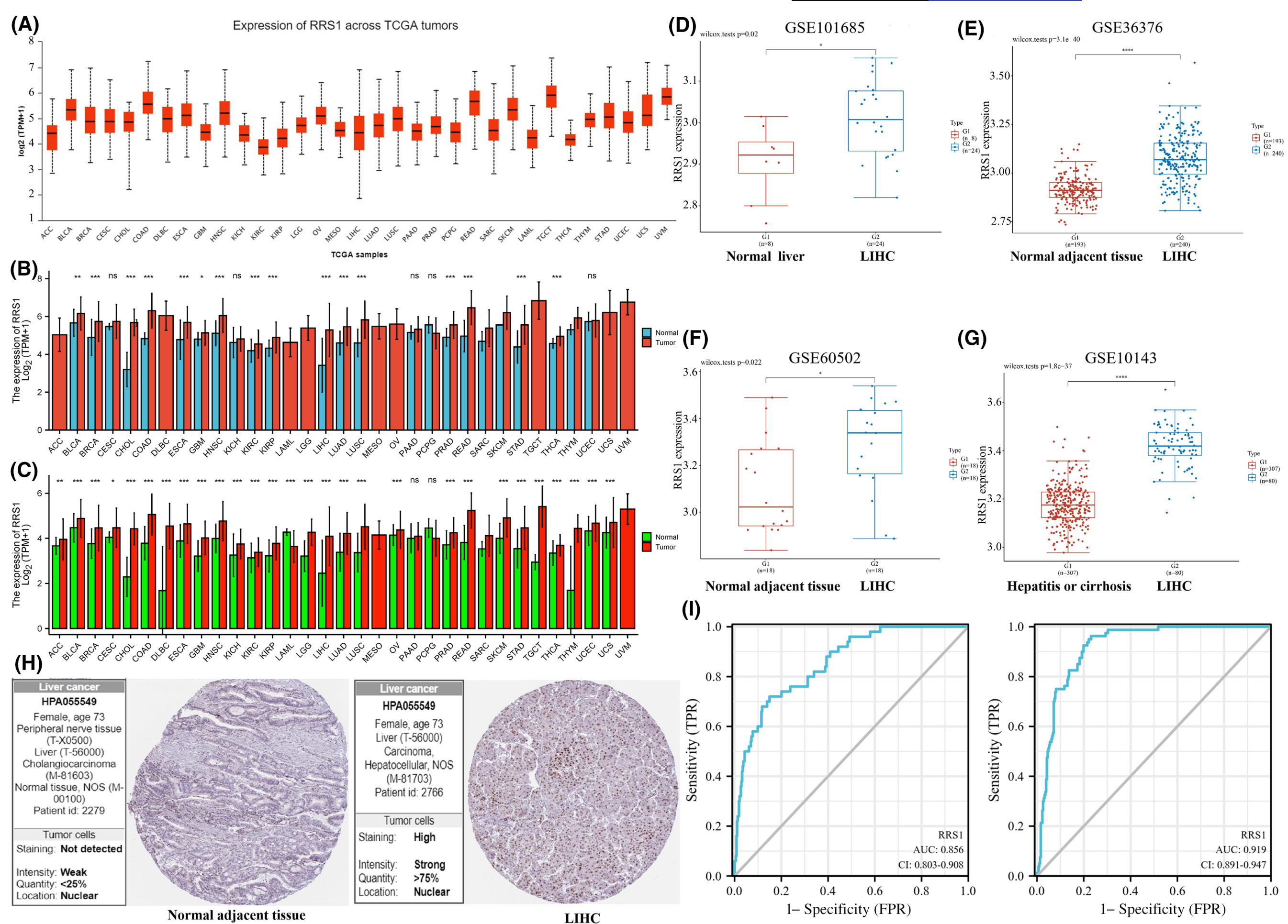


FIGURE 1 Expression profiles of ribosome biogenesis regulator 1 homolog (RRS1) in pan-cancers. (A) Expression of RRS1 across TCGA tumors. (B) Differential expression of RRS1 between TCGA control and tumors. (C) Differential expression of RRS1 between TCGA and GTEx normal samples and tumor samples. (D–G) RRS1 expression was increased in liver hepatocellular carcinoma compared with normal liver tissues, adjacent liver tissues, or liver tissues with hepatitis or cirrhosis. These datasets (GSE101685, GSE36376, GSE60502, and GSE10143) were obtained from Gene Expression Omnibus (GEO) databases. (H) The protein expression level of RRS1 via immunohistochemical staining from the HPA database. (I) Receiver Operating Characteristic curve for RRS1 based on TCGA and GEO datasets. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001;****p-value < 0.0001.

3.3 | Association of *RRS1* expression with immune checkpoints and immune infiltrations in LIHC

Immune checkpoints broadly expressed on the immune cell or tumor cell populations affect drug resistance and inhibit immune response through inactivating immune cells.[20,21] In recent years, tumor immunotherapy based on immune checkpoint blocking has developed into an emerging strategy for LIHC treatment.[22] For instance, by blocking checkpoints such as *CTLA-4*, programmed cell death 1 (*PD-1*), and *CD274* molecule (PD-L1), drugs targeting these immune checkpoints have been successful in LIHC with significantly favorable outcomes.[23] In this study, we evaluated the expression difference of eight checkpoints between normal liver tissues and LIHC tissues. As displayed in Figure 3A,B, *CD274*, *LAG3*, *PDCD1LG2* were downregulated in LIHC tissues compared with normal liver tissues (2.11E-08 < p < 1.0E-04), while *CTLA4*, *SIGLEC15*, and *PDCD1* were highly expressed in LIHC tissues (6.19E-06 < p < 0.017). We then identified the correlation of these checkpoints with *RRS1* expression in pan-cancers, and our findings suggested that *RRS1* expression showed a consistent negative association with eight checkpoint gene expression levels in both COAD and SKCM. In addition, *RRS1* expression was found to be positively correlated with *CD274*, *HAVCR2*, *PDCD1*, and *TIGIT* expression in LIHC (Figure 3C).

The dynamic cross-talk between immune cells and tumor cells governs LIHC development. Immune infiltration by immune cells such as T cells, dendritic cells, and natural killer (NK) cells is related to improved prognosis and favorable outcomes.[24] In the current study, we explored the association of immune cells with LIHC development and *RRS1* expression. We found that CD8^{+} cell and macrophage infiltrates were lower in LIHC tissues than in normal liver tissues (3.65E-04 < p < 1.23E-03), whereas CD4^{+} cell and dendritic cell infiltrates were higher in LIHC tissues (8.79E-13 < p < 1.35E-05;

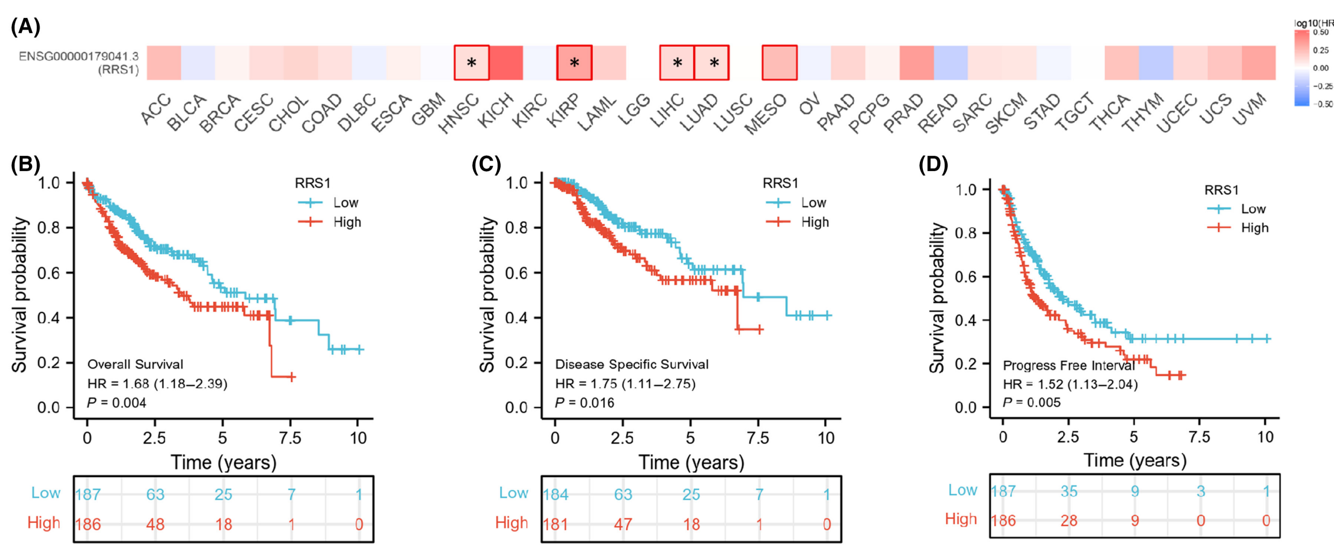


FIGURE 2 Elevated expression of ribosome biogenesis regulator 1 homolog (RRS1) predicts poor prognosis in liver hepatocellular carcinoma (LIHC). (A) Association of RRS1 expression with survival of pan-cancers. (B–D) Association of RRS1 expression with overall survival, disease-specific survival, and progress-free interval of hepatocellular carcinoma (LIHC). *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

Figure 4A,B). Furthermore, our results showed that *RRS1* expression was positively correlated with B-cell, $CD4^+$ cell, neutrophil, and dendritic cell infiltrates ($0.14 < Cor < 0.18$; $0.001 < p < 0.007$; Figure 4C).

3.4 | Correlation of *RRS1* expression with drug sensitivity

In order to explore whether *RRS1* had an effect on anticancer drug responses, we determined the association of *RRS1* expression with drug susceptibility on GDSC and CTRP databases. As summarized in Table S3, *RRS1* was negatively associated with 59 drug responses in GDSC and 148 drug responses in CTRP (false discovery rate p-value < 0.05). Among these, *RRS1* consistently presented a negative relationship with the sensitivity of seven drugs AZD8055, OSI-027, PHA-793887, PI-103, PIK-93, SNX-2112, and TPCA-1 in both GDSC and CTRP databases. Four anticancer drugs AZD8055, OSI-027, PI-103, and SNX-2112 have been reported to show inhibitory effects in LIHC cells, such as inhibiting LIHC cell proliferation and inducing apoptosis and death of LIHC cells.[25–28] These results indicated that elevated *RRS1* expression may have a negative effect on these anticancer drugs of LIHC and thus could affect the outcomes of LIHC patients.

3.5 | DNA methylation influences *RRS1* expression and prognosis of LIHC

DNA methylation modification has emerged as a driver in tumorigenesis, metastasis, and therapeutic responses of human malignancies via inducing the hypermethylation of tumor suppressor genes or hypomethylation of oncogenes in cancer.[29] In our study, we estimated the methylation status of the *RRS1* promoter between normal liver and LIHC and its subtypes, and identified the influence of *RRS1* methylation on its expression and overall survival of LIHC patients. We found that the *RRS1* promoter had a significantly lower methylation level in LIHC tissues than in normal liver tissues ($p = 1.04E\text{-}07$; Figure 5A). Compared with normal liver tissues, the methylation level of the *RRS1* promoter was lower in stage 1, stage 2, or stage 3 LIHC tissues ($7.28E\text{-}08 < p < 1.77E\text{-}03$; Figure 5B). Similarly, grade 1, grade 2, grade 3, or grade 4 LIHC tissues had a lower methylation level of *RRS1* promoter than normal liver tissues ($1.39E\text{-}06 < p < 7.63E\text{-}04$; Figure 5C). We found that LIHC tissues with N0 metastasis showed lower methylation of the *RRS1* promoter relative to normal samples ($p = 1.56E\text{-}07$; Figure 5D). In addition, the methylation level of the *RRS1* promoter was lower in both male and female LIHC tissues than in the control samples ($2.91E\text{-}09 < p < 7.06E\text{-}03$; Figure 5E). We also observed that the methylation level of this gene promoter in LIHC tissues from the young, middle-aged and elderly was lower than that in normal samples ($6.43E\text{-}07 < p < 1.60E\text{-}05$; Figure 5F). Moreover, the *RRS1* promoter was found to have a lower methylation in both TP53 mutant and non-TP53 mutant LIHC tissues compared to normal liver tissues ($4.59E\text{-}07 < p < 3.351E\text{-}06$; Figure 5G). Survival analysis suggested that LIHC patients with high methylation levels of the *RRS1* promoter could have a poor overall survival ($p = 0.09$; Figure 5H). Correlation analysis indicated that the high methylation level of *RRS1* promoter showed a negative association with *RRS1* expression ($r = -0.15$; $p = 0.002$; Figure 5I). These results revealed that low methylation of the *RRS1* promoter in LIHC may elevate *RRS1* expression and result in a poor prognosis for LIHC.

Furthermore, we evaluated the correlation of methylation sites (−5000 bp to +5000 bp of *RRS1*) with *RRS1* expression in LIHC tissues. We found that six *RRS1* promoter methylation sites cg14204843 [TSS-502bp], cg15537436 [TSS-288bp], cg24803876 [TSS-276bp], cg21768436 [TSS-142bp], cg25352650 [TSS-151bp], and cg23306542 [TSS-709bp] were significantly negatively correlated

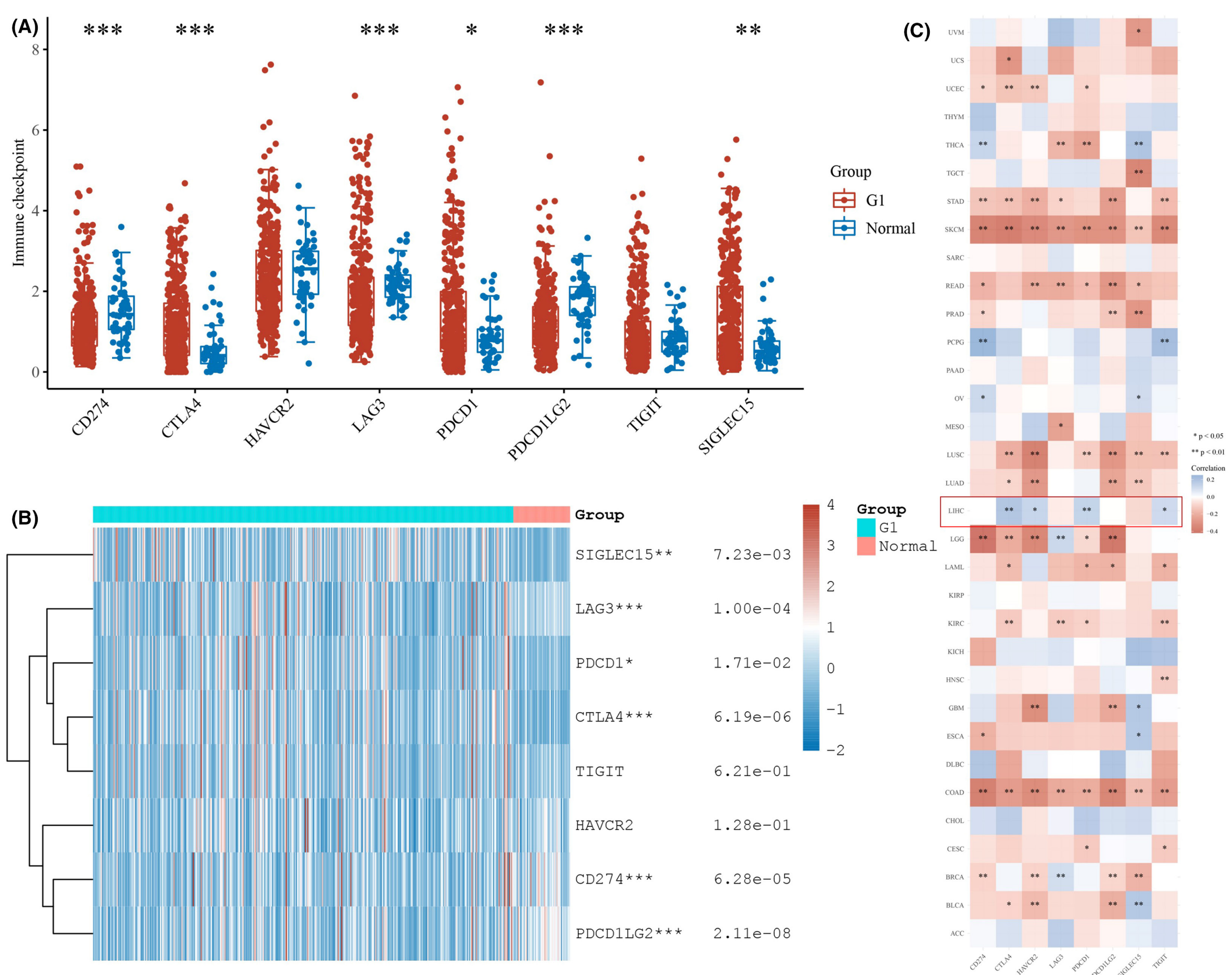


FIGURE 3 Correlation analysis of ribosome biogenesis regulator 1 homolog (RRS1) expression with immune checkpoint genes. (A, B) Differential expression of immune checkpoint genes between normal liver and liver hepatocellular carcinoma (LIHC) tissues. (C) Association of RRS1 expression with immune checkpoint gene expressions in LIHC. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

with *RRS1* expression ($-0.414 < R < -0.106$, $p < 0.042$; Figure 6A). Five methylation sites, cg08090772 (TSS +3390 bp), cg20171999 (TSS +1816 bp), cg20295442 (TSS +3415 bp), and cg00007076 (TSS +1350 bp), also showed a negative association with *RRS1* expression ($-0.265 < R < -0.233$, $p < 0.001$; Figure 6B). Moreover, four methylation sites, cg25046651 (TSS +3756 bp), cg19283840 (TSS +3392 bp), cg01988129 (TSS +3686 bp], and cg01586524 (TSS +569 bp), showed a significantly positive relationship with *RRS1* expression ($0.147 < R < 0.240$, $p < 0.005$; Figure 6C). These findings revealed that abnormal methylation of the *RRS1* promoter region and its downstream regions may affect *RRS1* expression.

3.6 | Genomic gain of *RRS1* may drive *RRS1* expression

Somatic changes in cancer genomes often result in transcript alterations.[30] In this study, we assessed the expression difference *RRS1* between gain LIHC, loss LIHC, and normal LIHC groups. We found that *RRS1* was overexpressed in LIHC tissues with *RRS1* gain (log2FC = 1.13; p = 3.46E-16) and downregulated in LIHC tissues with *RRS1* loss, compared with LIHC tissues with normal *RRS1* copy number alterations (CNVs; log2FC = −1.01; p = 3.25E-05). Furthermore, *RRS1* expression had a positive correlation with *RRS1* genomic gain (Cor = 0.66; p = 1.96E-35; Figure 7A). These findings indicated that *RRS1* genomic gain in LIHC may drive overexpression of *RRS1* and thus lead to a poor prognosis of LIHC.

3.7 | Increased hsa-miR-132-3p expression may elevate *RRS1* expression and result in a poor prognosis of LIHC

MicroRNAs (miRNAs) are endogenous noncoding RNAs regulating gene expression and can affect tumorigenesis by modulating the target mRNAs.[31] To determine whether *RRS1* overexpression

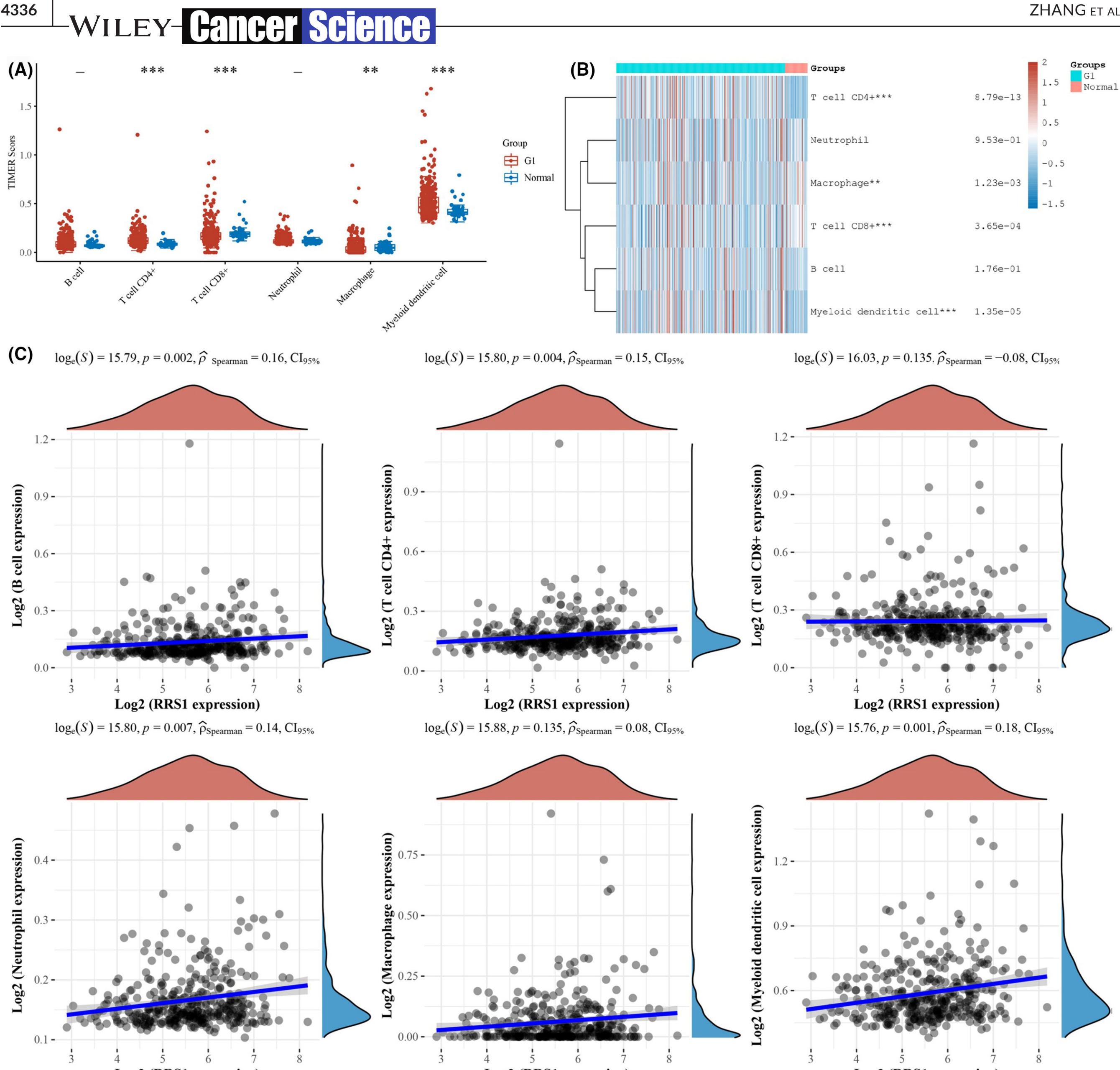


FIGURE 4 Correlation analysis of ribosome biogenesis regulator 1 homolog (RRS1) expression with immune infiltrations. (A, B) Differential levels of immune cell infiltrations between normal liver and liver hepatocellular carcinoma (LIHC) tissues. (C) Association of RRS1 expression with immune cell infiltrations in LIHC. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

is driven by miRNAs, we evaluated the upstream miRNAs of *RRS1* based on seven prediction programs, analyzed the expression status of these miRNAs between normal liver and LIHC tissues, and identified their influence on survival of LIHC patients. As displayed in Figure 7B, hsa-miR-132-3p, hsa-miR-212-3p, hsa-miR-216a-5p, hsa-miR-224-5p, hsa-miR-299-5p, and hsa-miR-455-3p were predicted serve as the upstream targets of *RRS1*, among which hsa-miR-132-3p and hsa-miR-212-3p expression had a significantly positive association with *RRS1*expression in LIHC, while hsa-miR-216a-5p expression showed a significantly negative relationship with *RRS1* expression. Overall survival analysis indicated that LIHC patients with high hsa-miR-132-3p and hsa-miR-212-3p expression presented a poor prognosis (HR = 1.64 and 2, respectively; $p < 0.006$; Figure 7C). Additionally, hsa-miR-132-3p and hsa-miR-224-5p were obviously elevated in LIHC tissues compared with normal tissues, while hsa-miR-299-5p and hsa-miR-455-3p were obviously decreased in LIHC tissues ($p < 0.001$; Figure 7D). These findings revealed that increased hsa-miR-132-3p expression in LIHC may elevate *RRS1* expression and result in poor prognosis for LIHC.

3.8 | Inhibition of hsa-miR-132-3p can decrease *RRS1* expression and the development of liver tumor cell lines

To validate the regulatory effect of hsa-miR-132-3p on *RRS1* expression and the role of hsa-miR-132-3p in LIHC, we performed a series of cell experiments in LX2, Hep3B, and HepG2 cell lines. As shown

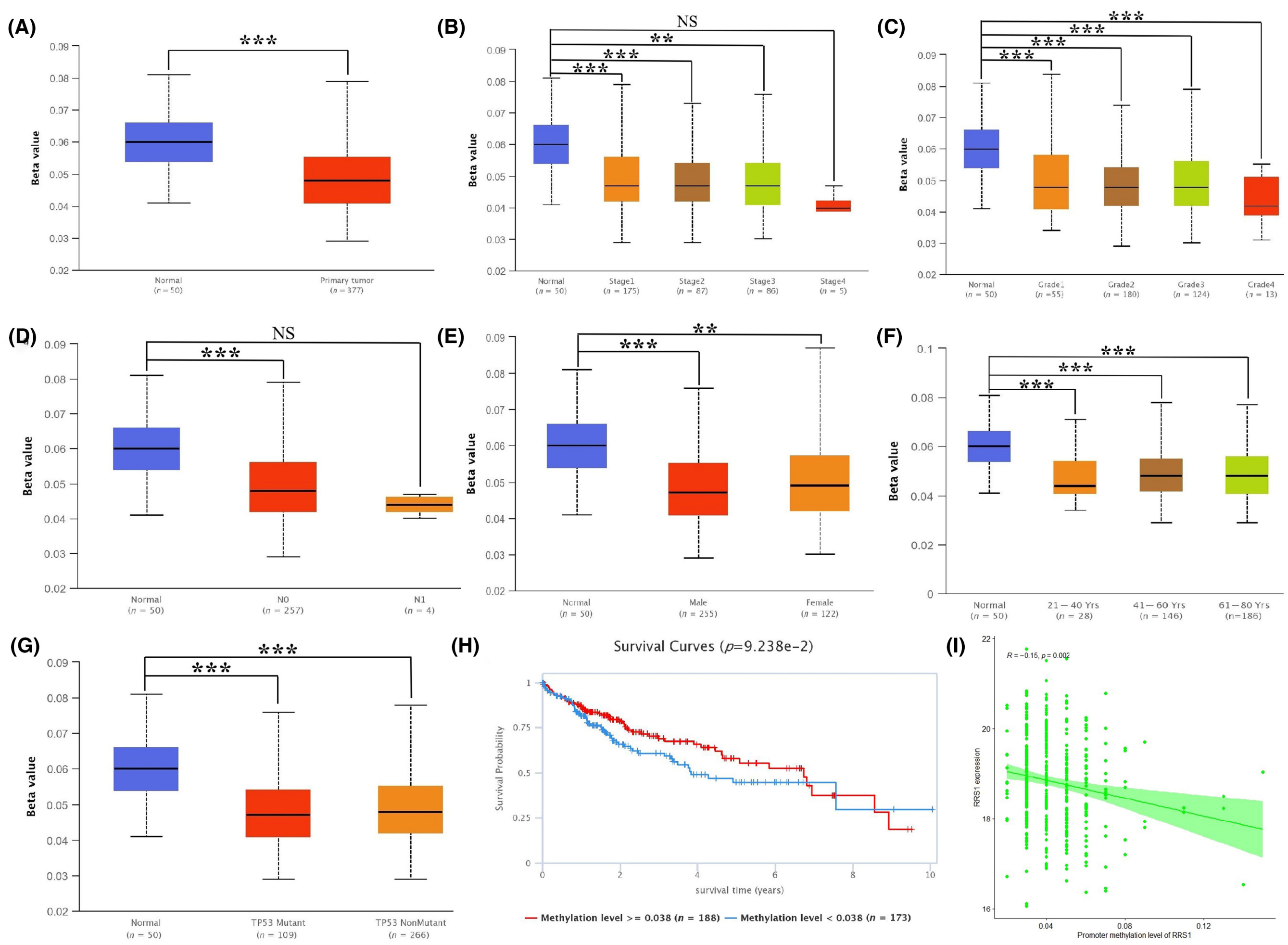


FIGURE 5 Methylation status of ribosome biogenesis regulator 1 homolog (RRS1) promoter in liver hepatocellular carcinoma (LIHC) and its influence on RRS1 expression and survival of LIHC patients. (A) Methylation difference of RRS1 promoter between LIHC tissues and normal liver tissues. (B) Methylation difference of RRS1 promoter between different stage LIHC tissues. (C) Methylation difference of RRS1 promoter between different grade LIHC tissues. (D) Methylation difference of RRS1 promoter between LIHC tissues with different metastasis types. (E) Methylation difference of RRS1 promoter between male and female LIHC tissues. (F) Methylation difference of RRS1 promoter between LIHC tissues with different age. (G) Methylation difference of RRS1 promoter between TP53 mutant and non-TP53 mutant LIHC tissues. (H) Association of high methylation levels of the RRS1 promoter with overall survival in LIHC patients. (I) Correlation of methylation level of RRS1 promoter with RRS1 expression. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

in Figure 8A,B, successful hsa-miR-132-3p knockdown can obviously reduce *RRS1* expression at mRNA and protein levels in Hep3B and HepG2 cells. CCK-8 assays were performed to determine the proliferation of Hep3B and HepG2 cells. We found that hsa-miR-132-3p knockdown significantly inhibited Hep3B and HepG2 cell growth (Figure 8C). In addition, transwell and wound healing assays revealed that hsa-miR-132-3p knockdown decreased migration and invasion (Figure 8D,E). Those results collectively suggest that the loss of hsa-miR-132-3p can reduce the proliferation, migration, and invasion of liver tumor cells in part by decreasing *RRS1* expression.

4 | DISCUSSION

RRS1 regulates ribosomal biosynthesis and stress response, cell cycle, and its expression promotes LIHC cell growth.[2,4] In this article, we performed a large-sample expression analysis for *RRS1* in pan-cancers and LIHC. Our results indicated that *RRS1* mRNA was overexpressed in 27 types of cancers. Seven datasets containing 1417 control and LIHC samples consistently revealed that *RRS1* expression was significantly elevated in LIHC at the mRNA level. Furthermore, *RRS1* was also validated elevated LIHC at the protein level. These findings reveal that *RRS1* was closely associated with carcinogenesis. Overall survival analyses revealed that RRS1 influenced the prognosis of LIHC, KIRP, LUAD, and HNSC.

We then estimated the association of *RRS1* with eight immune checkpoint genes and immune infiltrates, we found that *CD274*, *LAG3*, and *PDCD1LG2* were downregulated in LIHC tissues, while *CTLA4*, *SIGLEC15*, and *PDCD1* were overexpressed in LIHC tissues. *RRS1* expression was found to be positively correlated with *CD274*, *HAVCR2*, *PDCD1*, and *TIGIT* expression, and showed a positive correlation with B-cell, CD4$^+$ cell, neutrophil cell, and dendritic cell

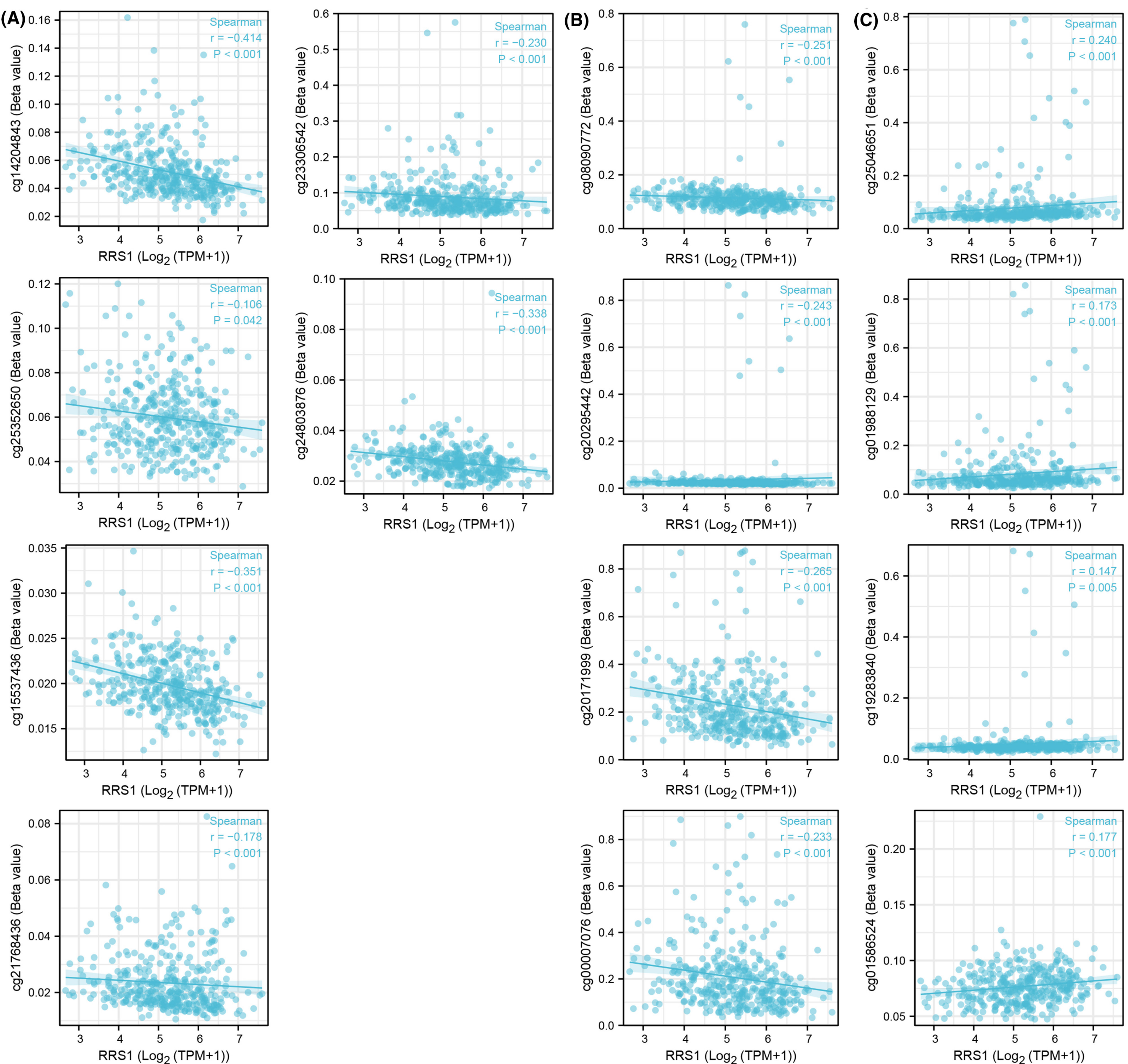
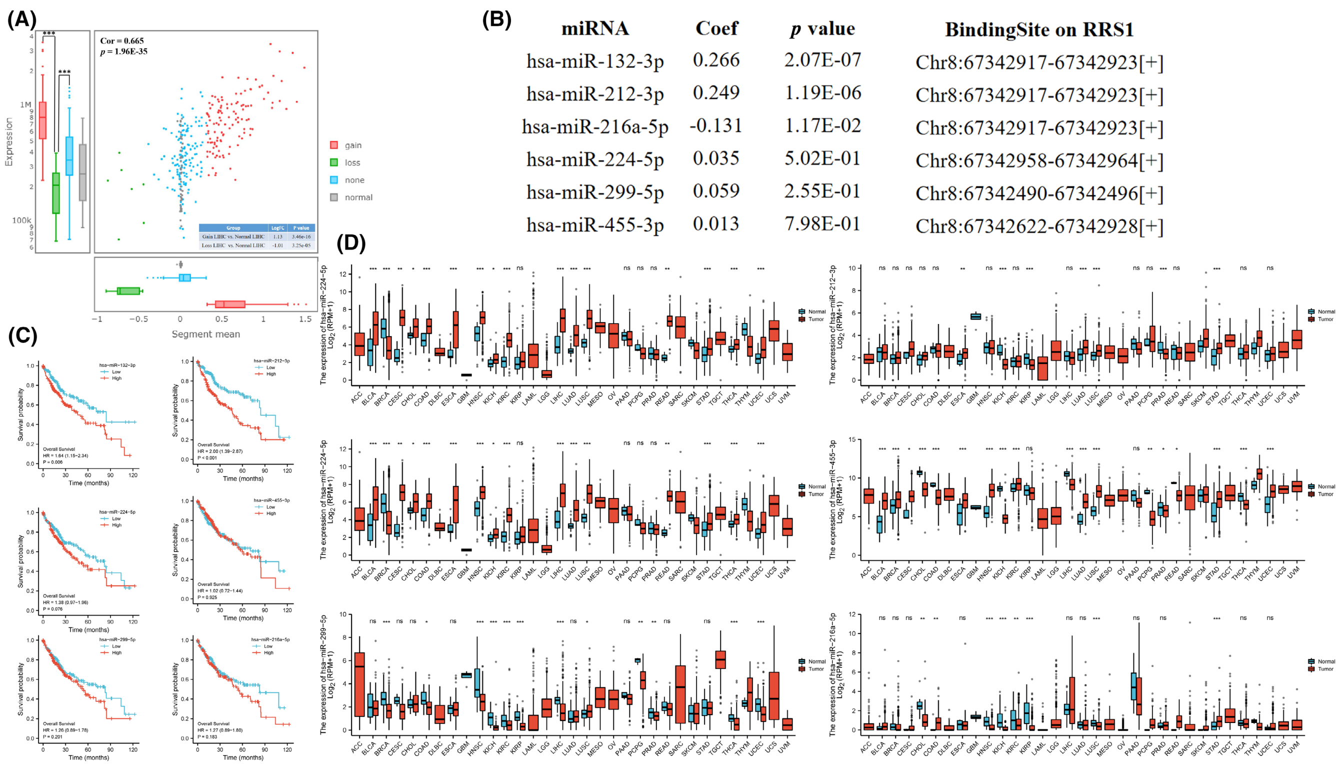


FIGURE 6 Abnormal methylation of ribosome biogenesis regulator 1 homolog (*RRS1*) promoter region and its downstream regions is correlated with RRS1 expression. (A) Increased methylation levels of RRS1 promoter region (TSS −709 bp to TSS −142 bp) were negatively related to RRS1 expression. (B) Increased methylation levels of RRS1 downstream methylation sites cg08090772, cg20171999, cg20295442, and cg00007076 were negatively related to RRS1 expression. (C) Increased methylation levels of RRS1 downstream of methylation sites cg25046651, cg19283840, cg01988129, and cg01586524 were positively correlated with RRS1 expression.

infiltrates. *CD274* (*PD-L1*), expressed mainly by tumor cells, dendritic cells, and macrophages, allows tumor cells to proliferate, grow and spread by impeding immune function.[32] Overexpression of *CD274* in LIHC cells inhibits the function of T cells in the liver tumor microenvironment. Blocking *CD274* has emerged as a promising treatment strategy for LIHC.[33] PDCD1 (PD-1), an immunosuppressive protein expressed in T cells, NK T cells, B cells, and dendritic cells, and overexpression of *PDCD1* can suppress anticancer immunity and affect the course of LIHC.[34] Moreover, *HAVCR2* can promote LIHC development by triggering autosecretion of IL-6 and then facilitating tumor growth via the STAT3 signaling pathway. Increased *HAVCR2* expression also promotes cell migration and invasion through accelerating epithelial–mesenchymal cell transition.[33] These findings indicate the possibility that elevated *RRS1* may result in a poor outcome for LIHC patients by regulating B-cell, T-cell, and dendritic cell infiltrates, and the expression of *PDCD1*, *CD274*, and *HAVCR2*. Future studies are needed to further explore the role of *RRS1* in immune checks and immune infiltrates.

We determined the association of *RRS1* expression with multiple drug susceptibility on GDSC and CTRP databases. Notably,



miRNA	Coef	p value	BindingSite on RRS1
hsa-miR-132-3p	0.266	2.07E-07	Chr8:67342917-67342923[+]
hsa-miR-212-3p	0.249	1.19E-06	Chr8:67342917-67342923[+]
hsa-miR-216a-5p	-0.131	1.17E-02	Chr8:67342917-67342923[+]
hsa-miR-224-5p	0.035	5.02E-01	Chr8:67342958-67342964[+]
hsa-miR-299-5p	0.059	2.55E-01	Chr8:67342490-67342496[+]
hsa-miR-455-3p	0.013	7.98E-01	Chr8:67342622-67342928[+]

FIGURE 7 Effect of ribosome biogenesis regulator 1 homolog (RRS1) copy number variation (CNV) distribution and microRNAs on RRS1 expression and survival of liver hepatocellular carcinoma (LIHC) patients. (A) The scatter plot and boxplots show a detailed view of the CNV distribution and correlation in LIHC. (B) Correlation between expressions of microRNAs with RRS1 expression. (C) Association between expression of microRNAs with overall survival in LIHC patients. (D) Differential expression of RRS1 and microRNAs between control and pan-cancer samples. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

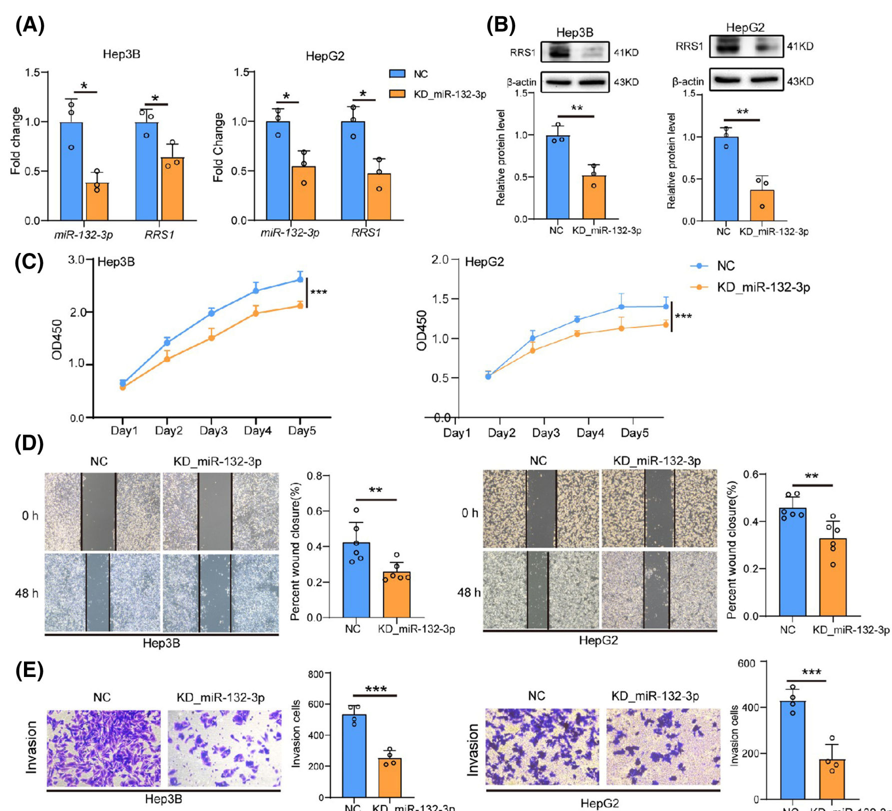


FIGURE 8 Inhibition of hsa-miR-132-3p can decrease ribosome biogenesis regulator 1 homolog (RRS1) expression and the development of liver tumor cell lines. (A) RT-PCR shows that hsa-miR-132-3p was successfully knocked down in Hep3B and HepG2 cells and hsa-miR-132-3p knockdown significantly decreased the RRS1 mRNA expression at mRNA in Hep3B and HepG2 cells. (B) hsa-miR-132-3p knockdown significantly decreased the RRS1 protein expression at mRNA in Hep3B and HepG2 cells. (C) CCK-8 assays exhibited that hsa-miR-132-3p knockdown significantly inhibited Hep3B and HepG2 cell growth. (D) Wound healing assays revealed that hsa-miR-132-3p knockdown decreased the migration of Hep3B and HepG2 cells. (E) Transwell assay suggests that the loss of hsa-miR-132-3p can reduce the invasion of Hep3B and HepG2 cells. *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

RRS1 expression showed a negative relationship with four drugs AZD8055, OSI-027, PI-103, and SNX-2112 that have been reported to show inhibitory effects in LIHC, such as inhibiting LIHC cell proliferation and inducing apoptosis and death of LIHC cells via blocking the MAPK, PI3K, AKT, or mTOR pathways.[25–28] These findings indicated that elevated *RRS1* expression may have a negative effect on these anticancer drugs for LIHC and thus could affect the outcomes of LIHC patients.

In order to determine the potential upstream regulatory mechanisms for *RRS1* expression in LIHC, we comprehensively evaluated the regulatory effects of DNA epigenetic modification and somatic genomic copy number alterations of *RRS1* and its upstream target miRNAs on *RRS1* expression. Our results identified that methylation of the *RRS1* promoter was significantly decreased in LIHC and subtype LIHC tissues, and had a negative relationship with *RRS1* expression and a positive association with outcome of LIHC patients. Further analysis showed that abnormal methylation of the *RRS1* promoter region (TSS −709 bp to TSS −142 bp) sites, cg14204843, cg15537436, cg24803876, cg21768436, cg25352650 and cg23306542, and its downstream regions (TSS +569 bp to TSS +3756 bp) may affect *RRS1* expression. Furthermore, we found that *RRS1* genomic gain was elevated in LIHC and positively correlated with overexpression of *RRS1*. In our study, we first determined that an upstream molecule hsa-miR-132-3p of *RRS1* showed a consistent overexpression with *RRS1* in LIHC tissues and had a positive correlation with *RRS1* expression. In addition, hsa-miR-132-3p expression and *RRS1* expression exhibited a consistently poor outcome in LIHC patients. To validate the regulatory effect of hsa-miR-132-3p on *RRS1* expression and the development of LIHC, we performed a series of cell experiments in liver normal and tumor cell lines, including hsa-miR-132-3p knockdown, CCK-8 assays, transwell and wound healing assays. We found that hsa-miR-132-3p knockdown could obviously reduce *RRS1* expression in Hep3B and HepG2 cells and inhibit their cell growth, migration, and invasion, suggesting that loss of hsa-miR-132-3p could reduce the proliferation, migration, and invasion of liver tumor cells in part by decreasing *RRS1* expression. Collectively, these findings revealed that low methylation of the *RRS1* promoter, *RRS1* genomic gain, and increased hsa-miR-132-3p expression in LIHC may elevate *RRS1* expression and affect the development and prognosis of LIHC (Figure 9).

The strengths of this article are: we identified the upstream regulatory mechanisms of *RRS1* expression, such as abnormal methylation modification and CNVs of *RRS1*. We also first validated the regulatory effect of hsa-miR-132-3p on *RRS1* in LIHC tissues and cell lines and confirmed the role of hsa-miR-132-3p in the proliferation, migration, and invasion of LIHC. There were several limitations as follows: (1) this study found that methylation modification and CNVs of *RRS1* and hsa-miR-132-3p could regulate *RRS1* expression and promote LIHC development, but more specific regulatory mechanisms need to be further explored. For example, what degree of methylation and CNVs of *RRS1* can drive LIHC development; (2) the above regulatory mechanisms of *RRS1*-mediated LIHC development need to be verified in an animal model of LIHC; (3) we focused on the bioinformatics analysis of RRS1, the detailed influence of *RRS1* on immune checkpoint genes, immune infiltrates, anticancer drug susceptibility needs to be further explored *in vivo* and *in vitro* experiments.

Our findings demonstrate that *RRS1* is broadly upregulated in pan-cancers and is closely associated with carcinogenesis. *RRS1* expression can predict poor outcome for LIHC, KIRP, LUAD, and

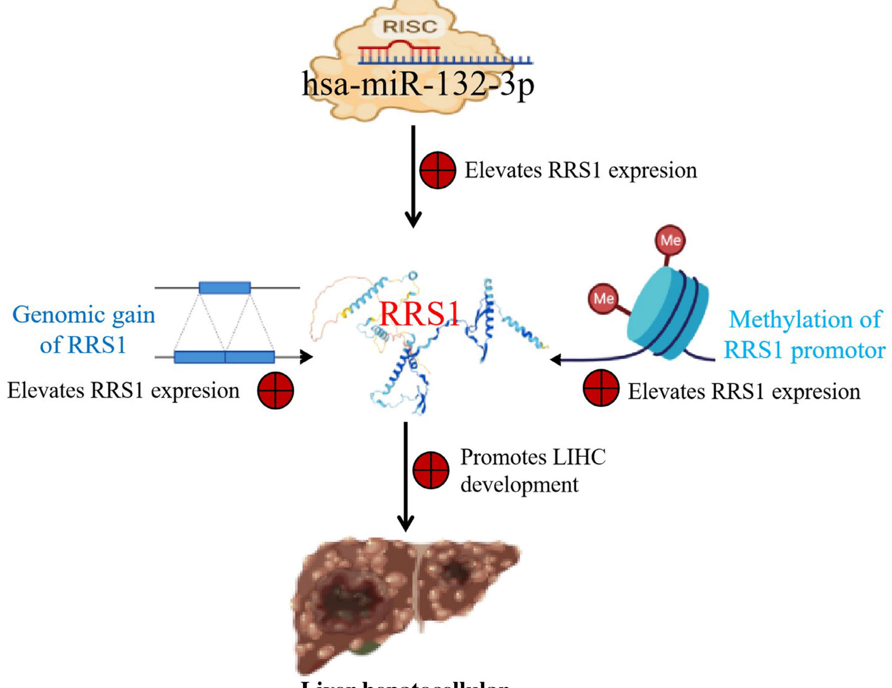


FIGURE 9 Genomic gain/methylation modification/hsa-miR-132-3p-mediated ribosome biogenesis regulator 1 homolog (RRS1) overexpression promotes liver hepatocellular carcinoma (photograph material is from https://www.biorender.com/).

HNSC. Elevated *RRS1* expression showed a close relationship with immune cell infiltrates and the expression of *PDCD1*, *CD274*, and *HAVCR2*. Increased *RRS1* expression may have a negative effect on these anticancer drugs of LIHC. Low methylation of the *RRS1* promoter, *RRS1* genomic gain, and hsa-miR-132-3p upregulation in LIHC may promote the expression of *RRS1* and thus lead to the development and poor prognosis for LIHC. *RRS1* is a promising therapeutic target for LIHC.

AUTHOR CONTRIBUTIONS

Xinwei Huang and Lize Xiong designed research; Xinwei Huang, Peilin Cong, Xiaoxia Zhang, and Li Tian drafted the manuscript and revised the paper; Xinwei Huang, Xiaoxia Zhang, and Li Tian performed analyses; Xinwei Huang, Peilin Cong, Tingmei Wu, Yinggang Zheng, Qian Zhang, Huanghui Wu, Hong Zhang, and Qiong Liu participated in data consolidation and plotting. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

No conflicts of interest, financial or otherwise, are declared by the authors.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. The datasets that support the findings of our study are openly available in the GEO database at https://www.ncbi.nlm.nih.gov/geo/, TCGA of UCSC Xena at https://xena.ucsc.edu/, DriverDBv3 database at http://driverdb.tms.cmu.edu.tw/, ENCORI database at https://starbase.sysu.edu.cn/, GEPIA 2 database (http://gepia2.cancer-pku.cn/), and the UALCAN database at http://ualcan.path.uab.edu/index.html, The Human Protein Atlas (HPA) at https://www.proteinatlas.org/, EWAS Data at https://ngdc.cncb.ac.cn/ewas/datahub/index, and GSCA (http://bioinfo.life.hust.edu.cn/web/GSCALite/).

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: N/A.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

ORCID

Peilin Cong https://orcid.org/0000-0002-4522-0272

Xinwei Huang https://orcid.org/0000-0002-8568-6261

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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