***RPS27A* is a Poor Prognostic Predictor for Hepatocellular Carcinoma via Interacting with METTL3-Mediated RNA Modifications**

Huiying Liu

Eastern Hepatobiliary Surgery Hospital

Jianjun Zhu

The Sixth Peoples' Hospital of Yancheng

Xiaoxia Kou

Eastern Hepatobiliary Surgery Hospital

Lingling Guo

Eastern Hepatobiliary Surgery Hospital

Hongjuan Zhang

Eastern Hepatobiliary Surgery Hospital

Xiaofeng Chen

Jiangsu Province Hospital

Jinrong Qiu (✉ [jqiu@njmu.edu.cn](mailto:jrqiu@njmu.edu.cn))

Shanghai Eastern Hepatobiliary Hospital <https://orcid.org/0000-0003-3684-6799>

Research Article

Keywords: RNA modification, hepatocellular carcinoma, RPS27A, prognosis, co-expression network

Posted Date: August 19th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-775176/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: Increasing evidence has pointed to the involvement of RNA modifications in the pathogenesis of human cancers. However, they are rarely studied in hepatocellular carcinoma (HCC).

Method: We summarized multiple types of RNA modification-related genes (RMRGs) from public references, and identified differentially expressed RMRGs (DEGs) between HCC tissues and matched normal samples, where their genetic variation were then investigated. The potential hub genes in the protein-protein interaction (PPI) network constructed by co-expression genes of RMRGs were recognized and verified in METTL3-knockdown HCC cell lines by quantitative PCR assay.

Results: Seventy-six RMRGs, including six writers, seven readers, and seven erasers, were collected, of which 34 were identified and validated as DEGs. *YTHDC2* exhibited the highest mutation rate, while *ADAT2* showed widespread deletions. High correlations were observed between the expressions of 34 RMRGs. The PPI network constructed by 1080 co-expression DEGs related to RNA regulations consisted of 513 nodes and 11557 edges, with *RPS27A* presented the most directed edges and maximum closeness centrality. Patients with high expression of *RPS27A* showed worse overall survival ($P < 0.01$) and disease-free survival ($P = 0.019$). Moreover, *RPS27A* was found upregulated on high-risk metastatic and recurrent HCC tissues. Quantitative PCR assay indicated that *RPS27A* was significantly decreased in cancer cell lines when *METTL3* was knocked down.

Conclusions: Remarkable differences were observed for RNA modifications between HCC and normal samples, and *RPS27A* could be a poor prognostic predictor for HCC via interacting with METTL3-mediated RNA modifications.

Introduction

Currently, more than 100 types of RNA modifications have been identified owing to the extensive usage of high-throughput sequencing technologies [1, 2]. It has been reported that these modifications are participated in RNA metabolisms at multiple steps, including the process of DNA transcription to mRNA translation and post-transcriptional modulations. After the initiation of mRNA transcription, the modification of m6A is carried out for un-matured mRNA by a multicomponent named writer complex [3]. The protein synthesis, known as mRNA translation is also fine-tuned widespread with three types of RNAs (mRNA, tRNA, and rRNA) been modified [4]. Many genes were identified that participated the process of RNA modifications and they were categorized into three groups which were called writers, erasers, and readers [5]. The deposition of RNA modification can be catalyzed by writers and recognized by readers, or removed by erasers. Accumulating evidence has demonstrated that the dysregulations of RNA modifications were associated with many disease progression including human cancers [6].

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors derived from digestive organ of the liver. The rising incidence makes it ranked the top 3 leading causes of cancer death worldwide [7]. Chronic liver diseases including virus infection, nonalcoholic liver, and hereditary

hemochromatosis, are the most important risk factors of HCC [8]. Many molecular variations have been reported that are associated with HCC pathogenesis by the investigation of multi-omics data [9, 10], however, the underlying molecular mechanisms are not completely elucidated.

RNA modifications have been documented that are comprehensively associated with tumorigenesis and cancer progressions, such as acute myeloid leukemia [11], breast cancer [12, 13], and liver cancer [14]. Methyltransferase-like 3 (*METTL3*), one of the writers of m6A modification, was reported that its overexpression promoted the progression of liver cancer, and silencing its expression suppressed tumorigenicity [14]. *WTAP* [15] and *KIAA1429* [16] were also found that they were up-regulated in HCC tissues, and both were associated with HCC prognosis. Nevertheless, different roles of RNA modifications on specific mRNAs were observed that downregulation of *METTL14* promotes the metastasis of HCC [17], which is opposite to that of *METTL3*. Additionally, the m5C methyltransferase of NSUN1 was discovered upregulating in lung and prostate cancer and served as a predictor of poor prognosis [18]. These findings suggest a very complex relationship between RNA modifications and HCC. Therefore, further exploring the expression of RNA modifications would facilitate better understanding of the potential roles of RNA modifications in HCC.

In this study, the landscape of RNA modifications in HCC was comprehensively investigated in seven independent datasets retrieved from public databases. A total of 76 RNA modification-related genes (RMRGs) were first collected from public references and their expressions were then assessed between HCC tissues and normal samples. Co-expression genes were then identified using the differentially expressed RMRGs and were used to construct a protein-protein interaction (PPI) network. Hub-genes were regarded according to the parameters of PPI network and verified on external independent datasets. This is the first time that we identified *RPS27A* as the hub gene which might facilitate the poor progression of HCC via RNA modifications.

Materials And Methods

Data preparation

Datasets used in this study were retrieved from Gene Expression Omnibus (GEO) database and The Cancer Genome Atlas (TCGA) (**Table 1**). For datasets from GEO, raw data were download and then normalized by the robust multi-array (RMA) method. For TCGA HCC (http://www.cbioportal.org/study?id=lihc_tcga), level 3 data, including RNA-Seq, copy number variation, somatic mutation, and the corresponding clinical features were obtained using TCGAbiolinks [19]. Differentially expressed genes (DEGs) between tumor and normal tissues were identified using the two datasets of GSE39791 [20] and GSE57957 [21], as both of them were generated by affymatrix platform and consisted of matched tumor and normal samples. The expression profile of TCGA HCC was used to verify the filtered DEGs and to identify co-expression genes. GSE130012 [22] and GSE146806 [23] are gene expression profiles of colorectal cancer and PAAD cell lines with *METTL3* knocked down. We selected these two datasets to validate the relationship between the identified hub gene and RNA modification-related genes. GSE14520

[24] is consisted of 488 tumor and paired non-tumor tissue of HCC samples, and we only selected 221 samples that were classified as high-risk and low-risk metastasis. GSE56545 contains 12 primary and 9 recurrent tumors.

Table 1 Datasets used in this study.

Data set	Normal	Tumor	Platform	Detail
GSE39791	72	72	GPL10558	Hepatocellular carcinoma
GSE57957	39	39	GPL10558	Hepatocellular carcinoma
TCGA	50	365	Illumina HiSeq	Hepatocellular carcinoma
GSE130012	3	3	Illumina HiSeq	colorectal cancer derived cell line
GSE146806	3	3	Illumina HiSeq	pancreas cancer derived cell line
GSE14520		221	GPL571	Hepatocellular carcinoma
GSE56545		21	GPL15433	Hepatocellular carcinoma

Collection of RNA modification-related genes

m6A modification is the most prominent type of RNA modification and many involved genes have been identified. However, other types of chemically modified DNA including m5C, m1a, etc., which also belong to RNA modifications are rarely investigated. Therefore, we first collected RNA modification-related genes (RMRGs) by searching public literature. Seventy-six genes that were reported involving in the modifications of mRNA, tRNA, miRNA, and rRNA by experimental data, were finally retrained (**Table 2**) from four references [5, 18, 25, 26].

Differential expression analysis

Low expression genes were defined as the expression values of more than 90% samples in each dataset equaled zero, and were then excluded before performing differential expression analysis. The normalized gene expression profiles of GSE39791 and GSE57957 were used to identify differentially expressed RMRGs (DEGs). Paired student t-test was used and the corresponding P-value was calculated to evaluate the differential expressions of RMRGs between tumor samples and matched normal samples. Adjusted P values were then calculated by Benjamini and Hochberg’s approach to controlling the false discovery rate (FDR). RMRGs with FDR less than 0.05 were recognized as DEGs.

Co-expression analysis

RNA-seq data of TCGA HCC was selected to perform co-expression analysis. The expression profiles of identified DEGs and other non-RMRGs were extracted and their correlation was assessed by Pearson correlation coefficient (Pearson's r). T-test P-value and the corresponding FDR was then calculated to evaluate the significance level of correlation. Non-RMRG was recognized as co-expression gene if its correlation coefficient was ≥ 0.6 and FDR was <0.05 .

Gene Ontology (GO) and KEGG pathway analyses

Functional enrichment analysis including GO and KEGG pathway was performed in the web-based tool of GeneCodis 3.0 [27]. A new algorithm that summarizes significantly enriched terms was implemented in GeneCodis 3.0 to conduct enrichment analysis. We used all the human genes as background and adjusted P-value of the hypergeometric test to determine significantly enriched biological process terms and KEGG pathways.

PPI network and Hub Gene identification

The protein-protein interaction (PPI) network of co-expression genes were constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins database (STRINGdb) [28]. We set 0.8 as the minimum cut-off of an interaction score. PPI network was then imported to Cytoscape v3.7.2 [29]. Network topological parameters were analyzed using the plugin of NetworkAnalyzer [30]. The node that had a maximum degree and closeness centrality was recognized as the hub gene of the PPI network.

Cell lines culture and transfection

Three HCC cell lines, SNU398, SNU449, and SNU475, were purchased from Shanghai Biochip Company Ltd. (Shanghai, China). Cells were propagated in Dulbecco's Modified Eagle's medium, then 100ul of the cell suspension was transferred to a six-well plate and incubated at 37°C with 5% CO₂ for one day. Next, the small interfering-METTL3 (si-METTL3) and si-NC (Empty vector) were added to 200ul of Opti-MEM (serum- and antibiotics-reduced) mixed with 3 ul lipofectamine 2000 to a final concentration of 20 nM. Then the above mixture was transferred to the six-well plate containing cell suspension, followed by adding 700 ul of pre-warmed DMEM (no serum and double antibodies). After incubation in a 37°C cell incubator for 4-6h, the medium was replaced by the complete medium with 10% fetal bovine serum and 1% double antibodies. Cells were collected for subsequent experiments after 48h.

Quantitative PCR

Total RNA was extracted from the transfected cells after 48h using the TRIzol™ Reagent (Thermo Fisher Scientific) under the instructions. The HiScript Q Select RT SuperMix for qPCR kit (vazyme, Nanjing, China) and SYBR Green Master Mix kit (vazyme, Nanjing, China) were utilized for cDNA synthesis and

quantitative PCR, respectively. Real-time qPCR was performed in the BioRad CFX96 PCR system. The three genes primers used in the study were as follows, GGACTCATGACCACAGTCCA (*GAPDH* forward), TCAGCTCAGGGATGACCTTG (*GAPDH* reverse), AACCAGGGTCTGGATTGTGA (*METTL3* forward), TCCAGTTGGGTTGCACATTG (*METTL3* reverse), ACTTCGTGGTGGTGCTAAGA (*RPS27A* forward), and CCCAGCACCACATTCATCAG (*RPS27A* reverse). *GAPDH* was selected as an internal reference, and $2^{-\Delta\Delta Ct}$ was calculated to quantify the relative expressions of *METTL3* and *RPS27A*.

Statistical analysis

Statistical analysis and figures plotting were conducted in R programming software (v3.6.1). Student t-test and Kruskal-Wallis rank test was used for the comparisons of paired groups and multiple groups respectively. For survival analysis, TCGA HCC patients were first divided into high group and low group according to the median expression value of hub gene. Then cox proportional hazards model was built to evaluate the association of gene expression with patient overall survival (OS) and disease-free survival (DFS) [31]. Difference between the survival curves of high group and low group was tested using the ‘survdif’ method in the package ‘survival’ [32]. In this study, we assigned ‘****’, ‘***’, ‘**’, and ‘*’ indicating P-value was < 1.0e-5, 0.001, 0.01, and 0.05 respectively.

Results

Deregulation of RMRGs in hepatocellular carcinoma

A total of 76 RMRGs belonging to 31 types of RNA modifications were chosen in this study, including 62 writers, 7 readers, and 7 erasers (**Table 2**). mRNA and tRNA were two major targets that accounted for almost 70% of the total targets (35.53% and 31.58%). Sixteen genes (21.05%) are related to m6A modification and 8 genes (10.53%) to m5C modification. Genes related to other types of modification were less than 5 except for m1G modification.

We attempted to assess the similarity between normal and HCC samples using a multidimensional scaling method based on the expression profiles of 76 RMRGs. The distances between samples can be interpreted by assigning observations to specific locations in a two-dimensional space. Results indicated that tumor and normal samples in both GSE39791 (72 vs 72) and GSE57957 (39 vs 39) were separated clearly and clustered together in each group (**Figure 1A&B**). Forty-six and 47 DEGs were identified in GSE39791 and GSE57957 respectively, with 35 genes shared by the two datasets (**Supplementary table 1**). We observed much more up-regulated genes (n=32) than down-regulated genes (n=3) in tumor samples (**Figure 1C**), of which 28 were writers, 5 were readers and 2 were erasers (**Table 2**). Among the 35 DEGs, 34 (97.14%) were then validated in TCGA HCC dataset (50 vs 50), including 33 up-regulated genes and 1 down-regulated gene (**Figure 1D, Supplementary table 2**).

Table 2 Summary of RNA modification-related genes.

Symbol	Type of RNA modification	Type Of Enzyme	Main Target	Role	Regulated
ADAR	Adenosine-to-inosine editing	Writer	ncRNA/mRNA		Ns
ADARB1	Adenosine-to-inosine editing	Writer	mRNA		Ns
ADAT2	m5C	Writer	tRNA		Up
ADAT3	m5C	Writer	tRNA		Ns
ALKBH1	m8A	Eraser	tRNA		Ns
ALKBH2	m10A	Eraser	tRNA		Ns
ALKBH3	m9A,m1A	Eraser	tRNA/mRNA		Ns
ALKBH5	m6A	Eraser	mRNA		Ns
ALKBH8	m5C	Writer	tRNA/mRNA		Up
APOBEC1	C-to-U	Writer	mRNA		Ns
APOBEC3G		Writer	mRNA		Ns
BCDIN3D	a-Phosphate methylation	Writer	miRNA		Ns
CBLL1	m6A	Writer	mRNA		Ns
CDK5RAP1	ms2i6A	Writer	tRNA		Up
CDKAL1	m1G	Writer	tRNA		Up
CTU1	m5C,mcm5s2U	Writer	tRNA/mRNA		Ns
CTU2	m5C,mcm5s2U	Writer	tRNA		Ns
DIS3L2	Uridylation	Reader	mRNA		down
DKC1	Pseudouridylase	Writer	rRNA/mRNA	Tumour suppressor	Up
DUS2	D	Writer	tRNA		Ns
ELP3	m5C,ncm5U,mcm5U	Writer	tRNA/mRNA		Ns
FBL	Nm	Writer	rRNA		Up
FTO	m6A	Eraser	mRNA	Oncogene	Up
HENMT1		Writer	pirna		Ns
IGF2BP1	m6A	Reader	mRNA		Ns
IKBKAP	cm5U	Writer	tRNA		Ns

JMJD6	m7A	Eraser	mRNA		Up
KIAA1429	m6A	Writer	mRNA		Ns
MEPCE	r-Phosphate methylation	Writer	rRNA		Ns
METTL1	m7G	Writer	tRNA	Tumor suppressor	Up
METTL14	m6A	Writer	mRNA		Ns
METTL3	m6A	Writer	tRNA/mRNA	Oncogene	Up
METTL6	m3C	Writer	tRNA/mRNA		Up
METTL8		Writer	mRNA		Ns
NAT10	ac4C	Writer	tRNA/mRNA		Up
NOP2	m5C	Writer	rRNA		Ns
NSUN2	m5C	Writer	tRNA/mRNA		Ns
NSUN3	m5C	Writer	tRNA		Up
NSUN4		Writer	rRNA		Ns
NSUN5		Writer	rRNA		Up
NSUN6	m5C	Writer	tRNA		down
NUDT16		Eraser	mRNA		Ns
PCIF1	m6Am	Writer	mRNA		Up
PUS1	Pseudouridylase	Writer	tRNA		Up
PUS10	Pseudouridylase	Writer	tRNA		Ns
PUS7	Pseudouridylase	Writer	tRNA		Up
QTRT1	Q	Writer	tRNA/mRNA		Ns
RBM15	m6A	Writer	mRNA		Ns
RBM15B	m6A	Writer	mRNA		up
RNMT	m7Gpp(pN)	Writer	mRNA		Up
TET1	hm5C	Writer	mRNA		Ns
TET2	hm5C	Writer	mRNA		Ns
TET3	hm5C	Writer	mRNA		Ns
TRDMT1	m5C	Writer	tRNA/mRNA		Ns

TRIT1	i6A	Writer	tRNA	Ns
TRMT1	m2,2G	Writer	tRNA	Up
TRMT10A	m1G	Writer	tRNA	Ns
TRMT10C	m1G	Writer	tRNA	Up
TRMT11	m2G	Writer	tRNA	Ns
TRMT12	m1G	Writer	tRNA	Up
TRMT2A	m5U	Writer	tRNA	Ns
TRMT5	m1G	Writer	tRNA	Up
TRMT6	m1A	Writer	tRNA/mRNA	Up
TRMT61A	m1A	Writer	tRNA/mRNA	Ns
TUT1	Uridylation	Writer	ncRNA/mRNA	Ns
WBSCR22		Writer	rRNA	Ns
WDR4	m7G	Writer	tRNA	Up
WTAP	m6A	Writer	mRNA	Up
YTHDC1	m6A	Reader	mRNA	Up
YTHDC2	m6A	Reader	mRNA	Oncogene Up
YTHDF1	m6A	Reader	mRNA	Up
YTHDF2	m6A	Reader	mRNA	Ns
YTHDF3	m6A	Reader	mRNA	Up
ZC3H13	m6A	Writer	mRNA	Ns
ZCCHC11	Uridylation	Writer	miRNA	Ns
ZCCHC6	Uridylation	Writer	miRNA	down

Ns: not significantly differential expression

Genomic features of RMRGs in hepatocellular carcinoma

Somatic mutations were found in 43 samples (11.81%) in 27 DEGs (7 genes showed no mutations), with *YTHDC2* exhibited the highest mutation frequency (**Figure 2A**). For copy number variation (CNV), the alterations were prevalent on amplification in copy number (**Figure 2B**). *TRMT12* and *YTHDF3* showed the highest proportion of amplification, while a widespread frequency of deletion were found in *ADAT2*, *TRMT5*, *ALKBH8*, and *YTHDC1*. Expressions of 33 DEGs were found up-regulated in tumor tissues except

for *NSUN6*, which presented decreased expression in tumor tissues (**Figure 2C**) and its down regulation was observed in early stage of HCC (**Supplementary figure 1**). Besides, prognostic analysis indicated that its high expression was associated with better patient outcome (**Supplementary figure 2**). Additionally, we found that *ADAT2*, which exhibited widespread deletions showed the lowest expression, and it was similar to *YTHDF3* and *JMJD6* (**Figure 2B&C**). Principle component analysis (PCA) based on the expression profiles of 34 DEGs indicated that HCC and normal samples could be clearly distinguished from each other (**Figure 2D**). Furthermore, expression profiles of these genes showed significantly positive correlations with each other (**Figure 2E**).

Co-expression analysis of RMRGs

Co-expression analysis identified 2511 genes, of which 1080 genes (43.01%) were differentially expressed between tumor and normal samples. We then performed GO and KEGG pathway enrichments for the 1080 co-expression DEGs to investigate their potential biological functions. A total of 788 GO terms were enriched, including 540 biological processes (BP), 152 cell components (CC), and 96 molecular functions (MF). While only 14 pathways were significantly enriched. The top 15 enriched GO terms and KEGG pathways were mainly related to cell division, ncRNA processing, RNA transport, and spliceosome (**Figure 3A&B**), which all involved in the process of RNA regulation.

Identification of hub genes

PPI network was constructed based on the 1080 co-expression DEGs by integrating the protein-protein connection information of STRINGdb [28] and then visualized in Cytoscape [29]. The network consisted of 513 nodes and 11557 edges, among which 7 nodes were RMRGs and they all presented up-regulated in tumor tissues (**Figure 4A**). The plugin NetworkAnalyzer [30] was used to assess the topological parameters of this undirected network. *RPS27A* was found obtained the most directed edges and maximum closeness centrality (**Figure 4B&C**). In a given undirected network, the number of edges represented the node degree and closeness centrality reflected information spreading speed from one node to other reachable nodes. Therefore, we regarded *RPS27A* as the hub gene of this network. Furthermore, no direct connections were found between *RPS27A* and *METTL3*, but three genes *POLR2K*, *POLR2J* and *NCBP2* showed direct interactions with both *RPS27A* and *METTL3* according to the PPI network (**Supplementary figure 3**). Besides, we investigated the methylation and copy number variation (CNV) data of *RPS27A* in TCGA HCC dataset. We found a weak positive correlation between the CNV and expression levels of *RPS27A* ($R = 0.28$, $P < 0.001$, **Supplementary figure 4A**). However, the methylation levels did not show strong correlation (**Supplementary figure 4B-C**).

Validation of hub genes in cell lines

To verify the relationship between *RPS27A* and RNA modifications, the expression profiles of two cancer cell lines, where *METTL3* were knocked-down were retrieved from GEO. Both *RPS27A* and *METTL3* were

significantly up-regulated on HCCs (**Figure 5A**). We then investigated their expressions in colorectal cancer cell lines (GSE130012) and pancreatic cancer cell lines (GSE146806). The expression of *METTL3* was significantly down-regulated in knocked-down (KD) cells than controls, and so as to *RPS27A* (**Supplementary figure 5A**). Furthermore, the expression of *RPS27A* showed high correlation with *METTL3* in both cancer cell lines (**Supplementary figure 5B**). Interestingly, on the other 14 cancer types, *RPS27A* showed significant upregulation on tumor tissues of ESCA and COAD, but downregulation on tissues of UCEC, CSC and BRCA (**Supplementary figure 5C**). We then constructed a small interfering RNA silencing system to knock out the expression of *METTL3* and meanwhile quantify the *RPS27A* expression in three HCC cell lines (**Supplementary figure 6A&B**). The result indicated that both *METTL3* and *RPS27A* expression was significantly decreased in the *METTL3*-KD cell lines compared to normal controls (**Figure 5B, Supplementary table 3**). Survival analysis was performed by using the TCGA HCC dataset to evaluate the association of *RPS27A* expression with prognosis. The result suggested that high group of HCC patients had worse OS ($P < 0.01$) and DFS ($P = 0.019$) (**Figure 5C**). A positive correlation was observed between the expression of *RPS27A* and pathological stages (**Supplementary figure 7A**). However, the expression levels did not show significant difference between virus infected HCC patients and non-virus infected HCC patients (**Supplementary figure 7B**).

Association of dysregulation RPS27A with poor prognosis of hepatocellular carcinoma

Survival analysis showed that HCCs with high-expression levels of *RPS27A* had worse DFS. We further investigated the relationship between *RPS27A* and the metastasis and recurrence of HCCs. According to the reference [33] which provided six HCC metastasis-related signatures, we evaluated the metastasis risk score of TCGA HCCs. It was found that high risk score of HCCs presented worse DFS (**Figure 6A**) and the expression levels of *RPS27A* showed positive correlation with metastasis risk scores (**Figure 6B**). *RPS27A* was up-regulated on the high-risk metastasis group (**Figure 6C**). In addition, the up-regulated *RPS27A* was also found in recurrent HCC tissues (**Figure 6D**).

Discussion

In this study, 76 genes belonging to 31 types of RNA modifications were collected from public references. The m6A modification was the most prominent and abundant form of RNA modifications, which accounted majority of RMRGs followed by m5C modification and m1A modification. Previous studies have focused on the relationship between m6A modification and HCC [5, 18], however, other types are rarely concerned. For the first time, we attempted to evaluate the association of over 30 types of RNA modifications, not merely concentrating on m6A modification, with HCC by integrating multiple datasets. The potential hub gene, *RPS27A*, identified by constructing a PPI network based on the co-expression genes, showed an unfavorable prognostic relationship with HCC patients. These findings revealed the extensive associations of RNA modifications with HCC from a landscape view. Further investigations will

provide important insights into the development of novel candidate therapeutic targets, diagnostic markers and prognostic predictors for HCC.

We observed extensive variations for RNA modifications between HCC and normal samples, as more than 60% of RMRGs were differentially expressed on both GSE39791 and GSE57957 datasets, and they were able to clearly separate HCC from normal samples. Almost all DEGs (97.06%) were up-regulated in tumor tissues, reflecting the activation of RNA modifications in HCC compared to normal tissues. The genomic variation analysis revealed that mutations of RMRGs were only found in about 10% of the samples, indicating a low overall mutation rate in HCC [34]. Interestingly, we also observed that copy number variations (CNV) commonly occurred in the regulators of RNA modifications and they were dominated by genomic amplification, which might interpret the extensive over-expression of RMRGs in HCC. Besides, most of the RMRGs showed strong correlations with each other, suggesting a synergistic regulation of these RMRGs which probably is related to the formed multi-functional complexes of many RMRGs in the process of RNA modifications [35–37].

Notably, we observed a down-regulated *NSUN6* in HCC tissues, which was contrary to all other DEGs. *NSUN6* belongs to the family of evolutionary conserved m5C RNA methylases [1] and has been reported its expression downregulated in tumor tissues compared to normal tissues [38]. Besides, patients with high expression of *NSUN6* showed a better survival rate. m5C was regarded as a prototypic DNA modification that participated in gene expression control and epigenetic regulation. It turned out that m5C regulators might be associated with cancerogenesis and progression of HCC via RNA decoration [39].

Ribosomal protein S27a (*RPS27A*) was recognized in this study as the hub gene of the PPI network constructed by co-expression genes of RMRGs. *RPS27A* is a member of the S27AE ribosomal protein family and encodes a fusion protein with ubiquitin at the N terminus and RPS27A at the C terminus. Several studies have reported the over-expression of *RPS27A* in HBV-induced HCC tissues [40–42]. This study revealed that the expression of *RPS27A* was significantly upregulated in HCC tissues. Furthermore, *RPS27A* expression was significantly downregulated in colorectal cancer cell lines (GSE130012) and pancreatic cancer cell lines (GSE146806) when *METTL3* was knockdown, and the observation was obtained in three *METTL3*-knockout HCC cell lines. These results suggested a possible regulation relationship between *RPS27A* and *METTL3*-mediated RNA modifications.

Currently, the association of *RPS27A* with RNA modifications is not well studied. Our findings provide a novel insight to unveil their potential relationship. According to the PPI network, no direct connections were found between *RPS27A* and *METTL3*, but three genes *POLR2K*, *POLR2J*, and *NCBP2* showed direct interactions with both *RPS27A* and *METTL3*. As expected, the silencing of *METTL3* in three HCC cell lines markedly decreased the expression of *RPS27A*, further revealed the possible regulation pattern between *METTL3* and *RPS27A* though no causal relationship was demonstrated. However, more thorough molecular evidence is needed to elucidate this direct or indirect regulation.

Meanwhile, we found expression of *RPS27A* negatively correlated with the HCC patients' survival rate, and *RPS27A* was also found upregulated on the high-risk metastasis group of HCC and recurrent HCC

tissues. These results suggested that *RPS27A* might interact with METTL3-mediated RNA modifications to contribute to poor outcomes of HCC patients. In addition, *RPS27A* was found as an important regulator in decreasing P53 expression in HPV-immortalized cervical epithelial cells [43]. In colorectal cancer, *RPS27A* not only interacts with apolipoprotein M to promote the growth and inhibit apoptosis of cancer cells [44] but also participates as a hub gene in regulating iNOS/NOS2-mediated poor prognosis [45]. Besides, this study also indicated that *RPS27A* was differentially expressed between normal and tumor tissues in other cancer types including ESCA, LUAD, LUSC, HNSC, UCEC, CESC, and BRCA. These findings suggest that *RPS27A* appears to be a common poor prognostic factor in multiple cancer types, representing a possible convergence of cancer prognostic regulatory pathways that may serve as a general therapeutic target.

Conclusions

In conclusion, we have comprehensively interrogated the possible associations of RNA modifications with HCC for the first time and then demonstrated that *RPS27A* may be a poor prognostic factor for HCC by interacting with METTL3-mediated RNA modification via constructing PPI network and silencing gene expression. Nevertheless, the prevalent alterations of involved molecules and pathways are still poorly understood. Therefore, more thorough work including in vivo and vitro experiments is needed to explore this complicated molecular mechanism.

Abbreviations

HCC: hepatocellular carcinoma; RMRG: RNA modification-related gene; PPI: protein-protein network; DEG: differentially expressed gene; GEO: Gene Expression Omnibus; TCGA: The Cancer Genome Atlas.

Declarations

Ethics approval and consent to participate

All data used in this study are publicly accessible to any researcher, our research did not require the approval of an ethics committee.

Consent for publication

Not applicable.

Data Availability Statement

The five datasets used in this study are publicly available.

Conflict of Interest

All authors declare no conflict of interest.

Acknowledgments

This study was not supported by any fund. We declare that submitted manuscript and other materials are not under consideration for publication elsewhere. All authors listed have read the complete manuscript and have approved submission of the paper.

Author Contributions

Study design: JR Q and XF C.

Data collection and analysis: HY L, JJ Z, and HJ Z.

Manuscript writing: HY L, JJ Z, XX K and LL G.

Manuscript and results revising: XF C and JR Q.

Supplementary Materials

Supplementary materials including 7 figures and 3 tables are listed in the file ‘Supplementary materials’.

References

1. Frye M, Harada BT, Behm M, He C: **RNA modifications modulate gene expression during development.** *SCIENCE* 2018, **361**(6409):1346-1349.
2. Boccaletto P, Machnicka MA, Purta E, Piatkowski P, Baginski B, Wirecki TK, de Crécy-Lagard V, Ross R, Limbach PA, Kotter A *et al*: **MODOMICS: a database of RNA modification pathways. 2017 update.** *NUCLEIC ACIDS RES* 2018, **46**(D1):D303-D307.
3. Ke S, Pandya-Jones A, Saito Y, Fak JJ, Vågbo CB, Geula S, Hanna JH, Black DL, Darnell JJ, Darnell RB: **m(6)A mRNA modifications are deposited in nascent pre-mRNA and are not required for splicing but do specify cytoplasmic turnover.** *Genes Dev* 2017, **31**(10):990-1006.
4. Roundtree IA, Evans ME, Pan T, He C: **Dynamic RNA Modifications in Gene Expression Regulation.** *CELL* 2017, **169**(7):1187-1200.
5. Esteve-Puig R, Bueno-Costa A, Esteller M: **Writers, readers and erasers of RNA modifications in cancer.** *CANCER LETT* 2020, **474**:127-137.

6. 6. Jonkhout N, Tran J, Smith MA, Schonrock N, Mattick JS, Novoa EM: **The RNA modification landscape in human disease.** *RNA* 2017, **23**(12):1754-1769.
7. 7. Wallace MC, Preen D, Jeffrey GP, Adams LA: **The evolving epidemiology of hepatocellular carcinoma: a global perspective.** *Expert Rev Gastroenterol Hepatol* 2015, **9**(6):765-779.
8. 8. Budny A, Kozłowski P, Kamińska M, Jankiewicz M, Kolak A, Budny B, Budny W, Niemunis-Sawicka J, Szczypiór G, Kurniawka B *et al*: **Epidemiology and risk factors of hepatocellular carcinoma.** *Pol Merkur Lekarski* 2017, **43**(255):133-139.
9. 9. Craig AJ, von Felden J, Garcia-Lezana T, Sarcognato S, Villanueva A: **Tumour evolution in hepatocellular carcinoma.** *Nat Rev Gastroenterol Hepatol* 2020, **17**(3):139-152.
10. Sia D, Villanueva A, Friedman SL, Llovet JM: **Liver Cancer Cell of Origin, Molecular Class, and Effects on Patient Prognosis.** *GASTROENTEROLOGY* 2017, **152**(4):745-761.
11. Li Z, Weng H, Su R, Weng X, Zuo Z, Li C, Huang H, Nachtergaele S, Dong L, Hu C *et al*: **FTO Plays an Oncogenic Role in Acute Myeloid Leukemia as a N(6)-Methyladenosine RNA Demethylase.** *CANCER CELL* 2017, **31**(1):127-141.
12. Cai X, Wang X, Cao C, Gao Y, Zhang S, Yang Z, Liu Y, Zhang X, Zhang W, Ye L: **HBXIP-elevated methyltransferase METTL3 promotes the progression of breast cancer via inhibiting tumor suppressor let-7g.** *CANCER LETT* 2018, **415**:11-19.
13. Zhang C, Samanta D, Lu H, Bullen JW, Zhang H, Chen I, He X, Semenza GL: **Hypoxia induces the breast cancer stem cell phenotype by HIF-dependent and ALKBH5-mediated m⁶A-demethylation of NANOG mRNA.** *Proc Natl Acad Sci U S A* 2016, **113**(14):E2047-E2056.
14. Chen M, Wei L, Law CT, Tsang FH, Shen J, Cheng CL, Tsang LH, Ho DW, Chiu DK, Lee JM *et al*: **RNA N6-methyladenosine methyltransferase-like 3 promotes liver cancer progression through YTHDF2-dependent posttranscriptional silencing of SOCS2.** *HEPATOLOGY* 2018, **67**(6):2254-2270.
15. Chen Y, Peng C, Chen J, Chen D, Yang B, He B, Hu W, Zhang Y, Liu H, Dai L *et al*: **WTAP facilitates progression of hepatocellular carcinoma via m⁶A-HuR-dependent epigenetic silencing of ETS1.** *MOL CANCER* 2019, **18**(1):127.
16. Lan T, Li H, Zhang D, Xu L, Liu H, Hao X, Yan X, Liao H, Chen X, Xie K *et al*: **KIAA1429 contributes to liver cancer progression through N6-methyladenosine-dependent post-transcriptional modification of GATA3.** *MOL CANCER* 2019, **18**(1):186.
17. Ma JZ, Yang F, Zhou CC, Liu F, Yuan JH, Wang F, Wang TT, Xu QG, Zhou WP, Sun SH: **METTL14 suppresses the metastatic potential of hepatocellular carcinoma by modulating N(6) - methyladenosine-dependent primary MicroRNA processing.** *HEPATOLOGY* 2017, **65**(2):529-543.
18. Barbieri I, Kouzarides T: **Role of RNA modifications in cancer.** *NAT REV CANCER* 2020, **20**(6):303-322.
19. Colaprico A, Silva TC, Olsen C, Garofano L, Cava C, Garolini D, Sabedot TS, Malta TM, Pagnotta SM, Castiglioni I *et al*: **TCGAbiolinks: an R/Bioconductor package for integrative analysis of TCGA data.** *NUCLEIC ACIDS RES* 2016, **44**(8):e71.
20. Kim JH, Sohn BH, Lee HS, Kim SB, Yoo JE, Park YY, Jeong W, Lee SS, Park ES, Kaseb A *et al*: **Genomic predictors for recurrence patterns of hepatocellular carcinoma: model derivation and validation.**

PLOS MED 2014, **11**(12):e1001770.

21. Mah WC, Thurnherr T, Chow PK, Chung AY, Ooi LL, Toh HC, Teh BT, Sauntharajah Y, Lee CG: **Methylation profiles reveal distinct subgroup of hepatocellular carcinoma patients with poor prognosis.** *PLOS ONE* 2014, **9**(8):e104158.
22. Shen C, Xuan B, Yan T, Ma Y, Xu P, Tian X, Zhang X, Cao Y, Ma D, Zhu X *et al*: **m(6)A-dependent glycolysis enhances colorectal cancer progression.** *MOL CANCER* 2020, **19**(1):72.
23. Müller S, Bley N, Busch B, Glaß M, Lederer M, Misiak C, Fuchs T, Wedler A, Haase J, Bertoldo JB *et al*: **The oncofetal RNA-binding protein IGF2BP1 is a druggable, post-transcriptional super-enhancer of E2F-driven gene expression in cancer.** *NUCLEIC ACIDS RES* 2020, **48**(15):8576-8590.
24. Roessler S, Jia HL, Budhu A, Forgues M, Ye QH, Lee JS, Thorgeirsson SS, Sun Z, Tang ZY, Qin LX *et al*: **A unique metastasis gene signature enables prediction of tumor relapse in early-stage hepatocellular carcinoma patients.** *CANCER RES* 2010, **70**(24):10202-10212.
25. Delaunay S, Frye M: **RNA modifications regulating cell fate in cancer.** *NAT CELL BIOL* 2019, **21**(5):552-559.
26. Begik O, Lucas MC, Liu H, Ramirez JM, Mattick JS, Novoa EM: **Integrative analyses of the RNA modification machinery reveal tissue- and cancer-specific signatures.** *GENOME BIOL* 2020, **21**(1):97.
27. Tabas-Madrid D, Nogales-Cadenas R, Pascual-Montano A: **GeneCodis3: a non-redundant and modular enrichment analysis tool for functional genomics.** *NUCLEIC ACIDS RES* 2012, **40**(Web Server issue):W478-W483.
28. Szklarczyk D, Franceschini A, Wyder S, Forslund K, Heller D, Huerta-Cepas J, Simonovic M, Roth A, Santos A, Tsafou KP *et al*: **STRING v10: protein-protein interaction networks, integrated over the tree of life.** *NUCLEIC ACIDS RES* 2015, **43**(Database issue):D447-D452.
29. Su G, Morris JH, Demchak B, Bader GD: **Biological network exploration with Cytoscape 3.** *Curr Protoc Bioinformatics* 2014, **47**:8-13.
30. Doncheva NT, Assenov Y, Domingues FS, Albrecht M: **Topological analysis and interactive visualization of biological networks and protein structures.** *NAT PROTOC* 2012, **7**(4):670-685.
31. Andersen PK, Gill RD: **Cox's Regression Model for Counting Processes: A Large Sample Study.** 1982, **10**(4):1100-1120.
32. Harrington DP, Fleming TR: **A Class of Rank Test Procedures for Censored Survival Data.** *BIOMETRIKA* 1982, **69**(3):553-566.
33. Yuan S, Wang J, Yang Y, Zhang J, Liu H, Xiao J, Xu Q, Huang X, Xiang B, Zhu S *et al*: **The Prediction of Clinical Outcome in Hepatocellular Carcinoma Based on a Six-Gene Metastasis Signature.** *CLIN CANCER RES* 2017, **23**(1):289-297.
34. Chen M, Wong C: **The emerging roles of N6-methyladenosine (m6A) deregulation in liver carcinogenesis.** *MOL CANCER* 2020, **19**(1):44.
35. Du H, Zhao Y, He J, Zhang Y, Xi H, Liu M, Ma J, Wu L: **YTHDF2 destabilizes m(6)A-containing RNA through direct recruitment of the CCR4-NOT deadenylase complex.** *NAT COMMUN* 2016, **7**:12626.

36. Alexandrov A, Martzen MR, Phizicky EM: **Two proteins that form a complex are required for 7-methylguanosine modification of yeast tRNA.** *RNA* 2002, **8**(10):1253-1266.
37. Liu J, Yue Y, Han D, Wang X, Fu Y, Zhang L, Jia G, Yu M, Lu Z, Deng X *et al.*: **A METTL3-METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation.** *NAT CHEM BIOL* 2014, **10**(2):93-95.
38. Selmi T, Hussain S, Dietmann S, Heiß M, Borland K, Flad S, Carter JM, Dennison R, Huang YL, Kellner S *et al.*: **Sequence- and structure-specific cytosine-5 mRNA methylation by NSUN6.** *NUCLEIC ACIDS RES* 2020.
39. Dominissini D, Rechavi G: **5-methylcytosine mediates nuclear export of mRNA.** *CELL RES* 2017, **27**(6):717-719.
40. Kim MY, Park E, Park JH, Park DH, Moon WS, Cho BH, Shin HS, Kim DG: **Expression profile of nine novel genes differentially expressed in hepatitis B virus-associated hepatocellular carcinomas.** *ONCOGENE* 2001, **20**(33):4568-4575.
41. Lim KH, Kim KH, Choi SI, Park ES, Park SH, Ryu K, Park YK, Kwon SY, Yang SI, Lee HC *et al.*: **RPS3a over-expressed in HBV-associated hepatocellular carcinoma enhances the HBx-induced NF-κB signaling via its novel chaperoning function.** *PLOS ONE* 2011, **6**(8):e22258.
42. Fatima G, Mathan G, Kumar V: **The HBx protein of hepatitis B virus regulates the expression, intracellular distribution and functions of ribosomal protein S27a.** *J GEN VIROL* 2012, **93**(Pt 4):706-715.
43. Chen L, Wang H: **Nicotine Promotes Human Papillomavirus (HPV)-Immortalized Cervical Epithelial Cells (H8) Proliferation by Activating RPS27a-Mdm2-P53 Pathway In Vitro.** *TOXICOL SCI* 2019, **167**(2):408-418.
44. Mu Q, Luo G, Wei J, Zheng L, Wang H, Yu M, Xu N: **Apolipoprotein M promotes growth and inhibits apoptosis of colorectal cancer cells through upregulation of ribosomal protein S27a.** *EXCLI J* 2021, **20**:145-159.
45. Ma B, Li J, Xing Y, Wang L, Wu W, Song C, Tian F: **Up-regulated iNOS/NOS2 is associated with the poor prognosis of colorectal cancer: an integrated bioinformatics analysis.** In:2019; 2019.

Figures

Figure 2

Landscape of genetic and expression variation of 34 differentially expressed RMRGs in HCC. A: Somatic mutation profiles of 34 DEGs in 364 HCC patients. Columns represented HCC patients. Total mutation burden of each patients was presented on upper barplot. The right-side numbers indicated the mutation frequency of each gene. The stacked barplot below showed fraction of conversions in each sample. B: CNV frequency of 24 differentially expressed RMRGs. Note: no CNV data was found in 10 genes. C: FPKM of 34 differentially expressed RMRGs in tumor and matched normal samples. D: PCA for the expression

profiles of 34 differentially expressed RMRGs to distinguish tumors from normal samples. E: Correlation matrix of 34 differentially expressed RMRGs. The circle size and color represented correlation coefficient. Red color, blue color, and white color indicated positive, negative and no correlation respectively.

Figure 3

Functional enrichment analysis of co-expression DEGs. Top 15 enriched GO terms (A) and KEGG pathways (B) were presented.

Figure 4

PPI network analysis of 1080 co-expression DEGs. A: PPI network visualized by Cytoscape. Red triangles represented RMRGs. Dashed circle indicated the hub gene of this network. B: Number of directed edges of all nodes. Directed edge also referred to degree. C: Closeness centrality of all nodes.

Figure 5

Validation of RPS27A in independent datasets and HCC cell lines. A: FPKM of RPS27A and METTL3 in paired tumor and normal samples. B: Relative expression of METTL3 and RPS27A in three normal and METTL3-knockdown HCC cell lines. Each treatment was repeated three times. Error bar indicate mean $\pm$ sd. Si-NC: vector control, si-METTL3: small interfering METTL3. FPKM: Fragments Per Kilobase Million, KD: knock down. C: Survival curve of OS and DSS for high group and low group of HCC patients. OS: overall survival. DFS: disease free survival.

Figure 6

Association of dysregulation deregulated RPS27A with poor prognosis of hepatocellular carcinoma. A: Survival curve of high-risk and low-risk metastasis group of HCCs. The division of high risk and low risk was based on the median risk score. Dashed lines indicated 95% confidence intervals. Hazard ratio and p value were estimated by log-rank test. B: Correlation of the metastasis risk score with expression levels of RPS27A. The correlation coefficient was calculated by Spearman method. C: Expression levels of RPS27A on high-risk and low-risk metastasis group of HCC on GSE14520 dataset. D: Expression levels of RPS27A on recurrent and non-recurrent HCC tissues on GSE56545 dataset. NRTumor: non-recurrent tumor, RTumor: recurrent tumor.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterials.docx](#)