Research Article

RPNs Levels Are Prognostic and Diagnostic Markers for Hepatocellular Carcinoma

Wangyang Zheng,1,2,3 Yuling Zheng,4 Xue Bai,5 Yongxu Zhou,1,2 Liang Yu,1,2 Daolin Ji,2,6 Kaiming Leng,7 Nanfeng Meng,1 Hang Wang,1 ZiYue Huang,1 Yi Xu,1,2,8 and Yunfu Cui1

1Department of Hepatopancreatobiliary Surgery, Second Affiliated Hospital of Harbin Medical University, Harbin 150086, China
2The Key Laboratory of Myocardial Ischemia, Harbin Medical University, Ministry of Education, Harbin 150086, China
3Department II of Gastroenterology, Third Affiliated Hospital of Harbin Medical University, Harbin 150086, China
4Second Affiliated Hospital of Harbin Medical University, Department of Pediatric, Harbin 150086, China
5Department of Clinic of Internal Medicine, Ulm University, Ulm, Germany
6Department of Hepatopancreatobiliary Surgery, Fourth Affiliated Hospital of Harbin Medical University, Harbin 150086, China
7Department of Hepatopancreatobiliary Surgery, Qingdao Municipal Hospital, Qingdao, China
8Department of Pathology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong, China

Correspondence should be addressed to Yi Xu; xuyihrb@pathology.hku.hk and Yunfu Cui; yfcui777@hotmail.com

Received 20 December 2021; Revised 4 May 2022; Accepted 23 May 2022; Published 29 August 2022

Academic Editor: Prasanna Kumar Santhekadur

Copyright © 2022 Wangyang Zheng et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The ribophorin family (RPN) is an essential regulatory subunit of the proteasome. By influencing the ubiquitin-proteasome system activity, ribophorins (RPNs) are responsible for almost all physiology and pathology processes of mammalian cells. Nevertheless, little is known about the role of RPNs in HCC. In this work, we first evaluated the transcriptional levels and the prognostic and diagnostic value of RPNs based on the public database. Firstly, we found all RPNs were surprisingly consistently upregulated in HCC tissues. Moreover, the RPNs’ expression pattern is correlated with HCC tumor grade. The TCGA HCC platforms’ data indicated that RPN2, RPN3, RPN6, RPN9, RPN10, RPN11, and RPN12 have robust diagnosis values. Then, survival analysis revealed that the high expression of RPN1, RPN2, RPN4, RPN5, RPN6, RPN9, and RPN11 was correlated with unfavourable HCC overall survival. Then, genetic alteration, immune infiltration feature, gene-genes network, and functional enrichment for RPNs indicated that RPNs have many potential biosynthesis activities expert for UPS functions. Moreover, western blot and qRT-PCR results confirmed these results. The silencing of RPN6 and RPN9 significantly reduced HCC cells’ proliferation, migration, and invasion ability in vitro. An in vivo tumor model further validated the oncogene effect of RPN6 on HCC cell growth. Moreover, RPN6 and RPN9 could promote cell migratory and invasive potential by affecting the epithelial-mesenchymal transition (EMT) process. In summary, this study suggests that the RPN family has the potential to be potential biomarkers and targets for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) ranks as the fifth most common cancer and the second cause of cancer-related death worldwide [1, 2]. In the last two decades, the incidence of HCC has been increasing globally while even doubling in the United States [3]. Although surgery has become a standard treatment for HCC, most patients have reached the late stage when being diagnosed and are not surgical candidates [4, 5]. The five-year survival rate for
patients with HCC is only 7%. Therefore, it is urged to find new biomarkers for the detection, diagnosis, and prognosis and new targets for molecule therapy [6].

The ubiquitin-proteasome system (UPS) is highly conserved in eukaryotic cells [7]. UPS is responsible for almost all cellular progress, functioning as degrading cellular proteins. Incorrect protein degradation may give rise to many diseases, including cancers, in versatile ways, reported in proliferation, autophagy, and drug resistance [8–10]. The Ribophorin family have 14 members: RPN1, RPN2, RPN3/PSMD3, RPN4/PSMD9, RPN5/PSMD12, RPN6/PSMD11, RPN7/PSMD6, RPN8/PSMD7, RPN9/PSMD13, RPN10/PSMD4, RPN11/PSMD14, RPN12/PSMD8, RPN13/ADRM1, and RPN14/PAAF1. They construct the 19 s (regulatory particles) of the 26 s proteasome. Some subunits (RPN1, RPN10, and RPN13), their specificity structure, and location even determine whether proteasomes work or not and which protein should be degraded [11]. Therefore, many RPNs have been deregulated and have robust oncogene functions in cancers [12–15]. Moreover, some studies reported that RPNs’ expression increased after virus infection [16]. As we all know, most HCC patients are initiated by chronic liver hepatitis virus B or C infections. Therefore, RPNs may do something special in HCC pathology [17].

Many cancer targets and new biomarkers have been identified and verified [18, 19]. This study extended the knowledge on RPNs and HCC to appraise distinct prognostic values, predict potential functions and putative targets, and then evaluate by experiments.

2. Materials and Methods

2.1. Specimens and Cell Lines. Between January 2016 and January 2018, 54 HCC specimens and corresponding non-cancerous tissues were harvested from patients at the Second Affiliated Hospital of Harbin Medical University. These fresh specimens were preserved in liquid nitrogen. None of the patients received radiotherapy or chemotherapy before surgery. This study was authorized by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. Hcclm3, Huh7, and Wrl-68 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Hcclm3 and Huh7 were maintained in DMEM of the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Hcclm3, Huh7, and Wrl-68 cellswereobtainedfromtheSecond

2.2. Cell Transfection and RNA Isolation. Small-hairpin RNA directed against RPN6 and RPN9 were designed and synthesized (Gene Chem, Shanghai, China). Their sequences are listed in Table S1. An empty Sh-NC vector was used as a control. The procedure of lentiviral infection was conducted by the instructions of the manufacturer. The selection of qualified cells was performed using puromycin for 3-4 weeks. TRIzol (Sigma, MO, USA) was used for total RNA isolation in HCC tissue specimens and cultured cells. Then, we checked the many features such as nucleic acid concentration, OD230, OD260, and OD280 of RNAs.

2.3. qRT-PCR and Western Blotting. RNAs were applied to synthesize the complementary DNA (cDNA) with a First Strand cDNA Synthesis Kit (Roche, Germany). RT-qPCR was carried out using the FastStart Universal SYBR Green Master Kit (Roche, Germany). The primer sequences are listed in Table S1. GAPDH was used for the internal control of RNA expression. Sequences of all of the gene primers are listed in Table S1. The mRNA fold change data were normalized and calculated using equation (2)−△△CT. Western blot was carried out following standard protocols as previously described [20].

2.4. Tumor Xenograft Study. Hcclm3 cells were transfected with sh-RPN6 or the sh-NC control. After collecting these cells, 3 × 10⁶ cells were injected subcutaneously into either side of female BALB/c nude mice (6–8 weeks of age, n = 3 per group). Tumor growth was measured, and tumor volumes were calculated every three days. The mice were euthanized 18 days after injection, and the tumors weights were measured.

2.5. CCK-8 and Wound Scratch Assay. CCK-8 (Cell counting kit-8) (Dojindo, Japan) was employed to determine cell viability. A density of 4 × 10⁴ cells per well was seeded in 96-well plates. Ten μl of reagent was added to each well and maintained for two h at 37°C. At 0, 24, 48, 72, and 96 h, the cells were measured by the reader (Tecan, Switzerland) at a wavelength of 450 nm. Cell motility was measured by wound scratch experiments. Using a 200 μl pipette tube, we created an acellular area. Then, cells were placed in a serum-free DMEM medium. The area was measured at 0, 24, and 36 h.

2.6. Migration and Invasion Assays. Coated with Matrigel (for invasion) or not (for migration), transwell chambers (Corning, New York, USA) were applied to further access cellular motility. 5 × 10⁴ cells were resuspended in 200 μL of FBS-free DMEM in the higher chambers while lower chambers were placed with 600 μL 10% FBS DMEM. After incubating for 48 h at 37°C, cells on the upper surface of the chambers were eliminated. Then, cells passed through the membranes were fixed and stained. The numbers of invasive or migrated cells were counted using a microscope.

2.7. ONCOMINE Analysis. To analyze the relative expression of RPNs in a variety of tumors, ONCOMINE, a free open-accessin-depth bioinformatic database (https:// ONCOMINE.org), was used, including 715 datasets and 86,733 samples. The mRNA expression profiles of RPNs in HCC were confirmed by this database, using a students’ t-test to generate a p-value.

2.8. UALCAN Database. UALCAN (https://ualcan.path. uab.edu) is an easy-to-use online tool to analyze online microarray data from the TCGA databases. Besides, they
also provide some clinical features and survival prognosis data based on gene expression differences in 31 cancer types. In our study, we analyzed the expression pattern of RPNs in HCC and the relationships between RPNs’ mRNA expression pattern and patients’ tumor grades. Moreover, we used Bonferroni correction to test the \( p \)-value significance.

2.9. UCSC Xena. UCSC Xena (https://xena.ucsc.edu/) is a free and open-access online tool for getting homogenized data from multiple databases, including TCGA, ICGC, TARGET, GTEx, and CCLE [21]. The database provides information on copy number, methylation, somatic mutation, gene expression, protein expression, and clinical data.

2.10. Kaplan–Meier Plotter Database. The Kaplan–Meier Plotter (https://kmplot.com/analysis/) is an online tool established by TCGAplot patient survival information and is used to evaluate the prognostic value of RPNs in HCC [22]. The prognostic value was evaluated by four indexes, OS (overall survival), PFS (progression-free survival), RFS (recurrence-free survival), and DSS (disease-specific survival).

2.11. Chiportal Analysis. The Chiportal database (https://www.chiportal.org/) is an open-access platform which provides interactive exploration of multidimensional Cancer Genomics data [23]. We analyzed the genetic alterations of the RPN family genes in HCC based on the 8 studies containing 1308 samples. Genomic profiles, mutations, survival data, and mRNA expressions were mentioned for analyzing the 14 RPN family genes.

2.12. GeneMANIA and Functional Enrichment Analysis. GeneMANIA (https://www.genemania.org) is an online platform for deriving hypothetical genes interaction networks based on those locations or functions. By querying a list of genes, GeneMANIA can generate multiply genes...
Table 1: Significant changes of RPNs’ expression in the transcriptional level between cancer and normal tissues (oncomine database).

| Gene ID | Types of HCC versus normal | Fold Change | p Value | t Test | References |
|---------|----------------------------|-------------|---------|--------|------------|
| RPN1    | Hepatocellular Carcinoma versus Normal | 1.249 | 1.09E-14 | 7.904 | Roessler [26] |
|         | Hepatocellular Carcinoma versus Normal | 1.019 | 0.024 | 2.009 | TCGA |
| RPN2    | Hepatocellular Carcinoma versus Normal | 1.713 | 1.54E-24 | 22.605 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.468 | 3.44E-7 | 5.927 | Roessler [27] |
| Hepatocellular Carcinoma versus Normal | 1.022 | 3.27E-4 | 3.790 | Guichard [28] |
| Hepatocellular Carcinoma versus Normal | 1.074 | 4.1E-8 | 6.045 | TCGA |
| Hepatocellular Carcinoma versus Normal | 1.033 | 2.13E-5 | 4.248 | Guichard [28] |
| Hepatocellular Carcinoma versus Normal | 1.465 | 0.012 | 2.546 | Wurmbach [27] |
| RPN3    | Hepatocellular Carcinoma versus Normal | 1.231 | 0.001 | 3.074 | Chen [29] |
| Hepatocellular Carcinoma versus Normal | 1.286 | 7.37E-17 | 8.617 | Roessler [27] |
| Hepatocellular Carcinoma versus Normal | 1.046 | 0.001 | 3.165 | TCGA |
| Hepatocellular Carcinoma versus Normal | 1.020 | 0.009 | 2.414 | Guichard [28] |
| RPN4    | Hepatocellular Carcinoma versus Normal | 1.112 | 0.010 | 2.337 | Chen [29] |
| Hepatocellular Carcinoma versus Normal | 1.239 | 7.50E-11 | 6.573 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.256 | 0.023 | 2.058 | Roessler [26] |
| RPN5    | Hepatocellular Carcinoma versus Normal | 1.692 | 5.83E-37 | 13.892 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.110 | 2.46E-9 | 6.430 | TCGA |
| Hepatocellular Carcinoma versus Normal | 1.042 | 9.3E-7 | 4.986 | Guichard [28] |
| Hepatocellular Carcinoma versus Normal | 1.034 | 0.005 | 2.778 | Guichard [28] |
| Hepatocellular Carcinoma versus Normal | 1.153 | 0.020 | 2.062 | Chen [29] |
| RPN6    | Hepatocellular Carcinoma versus Normal | 1.414 | 1.00E-5 | 5.881 | Chen [29] |
| Hepatocellular Carcinoma versus Normal | 1.654 | 3.73E-36 | 13.732 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.407 | 0.011 | 2.571 | Wurmbach [27] |
| Hepatocellular Carcinoma versus Normal | 1.041 | 0.006 | 2.590 | TCGA |
| Hepatocellular Carcinoma versus Normal | 1.011 | 0.024 | 1.997 | Guichard [28] |
| RPN7    | Hepatocellular Carcinoma versus Normal | 1.465 | 1.02E-13 | 10.595 | Roessler [26] |
| RPN8    | Hepatocellular Carcinoma versus Normal | 1.162 | 9.26E-16 | 4.329 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.373 | 0.014 | 2.375 | Wurmbach [27] |
| RPN9    | Hepatocellular Carcinoma versus Normal | 1.458 | 4.73E-29 | 12.000 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.400 | 0.016 | 2.422 | Wurmbach [27] |
| RPN10   | Hepatocellular Carcinoma versus Normal | 2.265 | 2.10E-45 | 25.343 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 2.533 | 1.20E-11 | 9.023 | Wurmbach [27] |
| Hepatocellular Carcinoma versus Normal | 2.078 | 1.66E-11 | 9.550 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.097 | 9.6E-7 | 6.126 | Guichard [28] |
| Hepatocellular Carcinoma versus Normal | 1.098 | 9.4E-18 | 10.186 | Guichard [28] |
| Hepatocellular Carcinoma versus Normal | 1.318 | 2.06E-18 | 10.720 | TCGA |
| Hepatocellular Carcinoma versus Normal | 1.340 | 8.84E-5 | 4.036 | [30] |
| RPN11   | Hepatocellular Carcinoma versus Normal | 2.243 | 4.71E-74 | 22.195 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 2.061 | 3.8E-7 | 5.876 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.266 | 5.88E-5 | 3.941 | Chen [29] |
| Hepatocellular Carcinoma versus Normal | 1.723 | 0.008 | 2.864 | Wurmbach [27] |
| RPN12   | Hepatocellular Carcinoma versus Normal | 1.543 | 7.28E-34 | 12.413 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.477 | 0.003 | 2.928 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.148 | 0.007 | 2.507 | Chen [29] |
| Hepatocellular Carcinoma versus Normal | 1.031 | 0.002 | 2.981 | TCGA |
| Hepatocellular Carcinoma versus Normal | 1.015 | 0.033 | 1.854 | Guichard [28] |
| RPN13   | Hepatocellular Carcinoma versus Normal | 1.044 | 3.63E-5 | 4.500 | Guichard [28] |
| Hepatocellular Carcinoma versus Normal | 1.091 | 5.17E-10 | 6.763 | TCGA |
| Hepatocellular Carcinoma versus Normal | 1.288 | 1.40E-19 | 9.407 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.371 | 0.002 | 3.094 | Roessler [26] |
| Hepatocellular Carcinoma versus Normal | 1.174 | 0.007 | 2.487 | Chen [29] |
| Hepatocellular Carcinoma versus Normal | 1.030 | 0.001 | 3.126 | Guichard [28] |
| RPN14   | Hepatocellular Carcinoma versus Normal | 1.343 | 0.007 | 2.657 | Wurmbach [27] |
| Hepatocellular Carcinoma versus Normal | 1.131 | 3.89E-5 | 3.994 | Roessler [26] |
with similar functions and illustrate the relationship between the queried gene set and the dataset by constructing an interactive network [24]. This study used GeneMANIA to build a gene-gene interaction network for RPNs. We used the STRING database to perform the functional enrichment analysis [25].

2.13. Statistical Analysis. We used GraphPad Prism 5.01 software and SPSS 22.0 to analyze the data in this study. The diagnosis value of RPNs was measured by ROC curves, which were plotted using the SPSS software. Quantitative data were expressed as mean ± S.D.

3. Results

3.1. All RPNs’ Transcriptional Levels Are Extremely High in Cancer Tissues. We first analyzed all of the RPN1-RPN14 mRNA expression levels in multiple human cancers using the Oncomine database (Figure 1). The analysis showed that the transcriptional levels of all RPNs were upregulated in...
human cancers. Some RPNs, such as RPN10 and RPN11, were upregulated in HCC patients.

3.2. Relationship between the Transcriptional Levels of RPNs and the Clinicopathological Parameters of HCC Patients.

Then, we compared the RPNs’ transcriptional levels in HCC with those in normal samples using the UALCAN database (Figure 2). All RPNs were surprisingly upregulated in HCC tissues than normal, with a low p-value. After Bonferroni correction, the p-value of all RPNs is still lower than the corrected (p < 0.003571). Moreover, we examined our findings in the ONCOMINE database (Table 1). In Roessler’s dataset, RPN1 was upregulated with a fold change of 1.249 ($p = 1.09E - 14$), RPN2 with a fold change of 1.713 ($p = 1.54E - 74$), RPN3 with a fold change of 1.286 ($p = 7.37E - 17$), RPN4 with a fold change of 1.239 ($p = 7.50E - 11$), RPN5 with a fold change of 1.692 ($p = 5.83E - 37$), RPN6 with a fold change of 1.654 ($p = 3.73E - 36$), RPN7 with a fold change of 1.465 ($p = 1.02E - 23$), RPN8 with a fold change of 1.162 ($p = 9.26E - 6$), RPN9 with a fold change of 1.458 ($p = 4.73E - 29$), RPN10 with a fold change of 2.265 ($p = 2.10E - 85$), RPN11 with a fold change of 2.243 ($p = 4.71E - 74$), RPN12 with a fold change of 1.543 ($p = 7.28E - 31$), RPN13 with a fold change of 1.288 ($p = 1.40E - 19$), and RPN14 with a fold change of 1.131 ($p = 3.89E - 5$), which is in accordance with our finding [26]. Next, using the UALCAN database, we analyzed whether the RPNs’ transcriptional levels were related to clinicopathological characteristics (Figure 3). As shown in Figure 3, when tumor grade increases, the RPNs’ expression pattern also increases. The mRNA levels of all RPNs were

Figure 5: Prognostic value of the mRNA expression for distinct RPNs in HCC (Kaplan–Meier Plotter).
positive for tumor differentiation with \( p < 0.05 \). That means the measurement of RPNs’ levels may help determine the patient’s tumor stage.

3.3. Diagnosis and Prognostic Significance of the RPNs in HCC.

To evaluate the value of RPNs in the diagnosis of HCC, the computing receiver operating characteristic (ROC) analysis was used. Using the data obtained from the HCC platform from the UCSC website, we draw the ROC curves to analyze the RPNs’ diagnostic significance. As the figure showed (Figure 4), RPN2 (Area = 0.818 and \( p < 0.0001 \)), RPN3 (Area = 0.728 and \( p < 0.0001 \)), RPN6 (Area = 0.704 and \( p < 0.0001 \)), RPN9 (Area = 0.650 and \( p < 0.0001 \)), RPN10 (Area = 0.880 and \( p < 0.0001 \)), and RPN11 (Area = 0.815 and \( p < 0.0001 \)) were all helpful for HCC diagnosis. Our finding suggested that RPN2, RPN3, RPN6, RPN9, RPN10, RPN11,
and RPN12 have the potential to be useful as biomarkers for HCC.

Then, we evaluated the prognostic role of RPNs in HCC using the Kaplan–Meier Plotter databases (Figure 5). In the OS (overall survival) group, lower RPN1, RPN2, RPN5, RPN6, RPN9, and RPN11 expressions indicated better survival. In the PFS (progression-free survival) group, reduced RPN4, RPN5, and RPN11 were correlated with better survival. In the RPS (recurrence-free survival) group, the downregulation of RPN10 was connected with better survival. In the DSS (disease-specific survival) group, the downregulation of RPN2, RPN5, RPN9, and RPN11 may indicate better survival. These findings were consistent with the UALCAN analysis and suggested that some RPNs such as RPN5, RPN9, and RPN11 have significant prognostic value for HCC.

3.4. Genetic Alteration and Immune Infiltration Analyses of RPNs in HCC Patients. We used the cbioPortal online tool to analyze the genetic changes of RPNs in HCC. According to the TCGA dataset, the highest genetic variation rate in RPNs is RPN10 (5%), the lowest mutation rate is RPN4 (0.1%), and the others are RPN8 (0.2%), RPN14 (0.3%), RPN1 (0.3%), RPN3 (0.3%), and RPN7 (0.3%) (Figure 6(a)). Although changes are little in RPNs, those changes were correlated with overall patient survival (Figure 6(b)).

The TIMER database is used to evaluate the relationship between the transcription level of RPNs and the level of immune infiltration in HCC. It was found that RPNs are involved in the inflammatory response and immune cell infiltration, which may affect the clinical outcome of HCC patients. The analysis results are shown in Figure 7. RPN2 expression was positively correlated with the infiltration of B cells, macrophages, and dendritic cells. RPN4 was negatively associated with the infiltration of CD4+ T cells. RPN5 was negatively correlated with the infiltration of CD4+ T cells while positively correlated with neutrophil infiltration. RPN6 expressions were positively associated with the infiltration of macrophages and neutrophils. RPN7 was negatively correlated with the infiltration of CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells. RPN8 was negatively associated with the infiltration of purity and CD4+ T cells. RPN9 was negatively correlated with the infiltration of CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells. RPN11 was positively associated with the infiltration of neutrophils while negatively correlated with purity cells. RPN12 was positively associated with the infiltration of CD4+ T cells. RPN13 was negatively correlated with B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells. RPN14 was positively associated with the infiltration of purity cells while negatively with the B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils, and dendritic cells. These results indicated that the level of RPNs’ expression is associated with the level of immune infiltration in HCC.

3.5. The GGI and Functional Enrichment of RPNs. Identifying more details about RPNs could boost the understanding of their potential functions in HCC. Therefore, we constructed a GGI using the GeneMANIA database. In
Figure 8, the 14 central nodes of RPNs are surrounded by 20 genes strongly associated with RPNs in physical interactions, co-localization, co-expression, prediction, genetic interactions, and pathways. The top five genes most associated are PSMC1 (proteasome 26S subunit, ATPase 1), PSMC6 (proteasome 26S subunit, ATPase 6), PSMD10 (proteasome 26S subunit, non-ATPase 10), PSMC4 (proteasome 26S subunit, ATPase 4), and PSMC3 (proteasome 26S subunit, ATPase 3). They all have genetic interactions and are correlated with functions such as proteasome accessory complex, proteasome complex, and regulation of the cellular amino acid metabolic process.

As shown in Figure 9, besides protein binding, RNA binding, or ubiquitin-protein ligase activity, RPNs still play an important role in biosynthesis: extracellular exosome, translation initiation factor activity, and protein processing in endoplasmic reticulum activity.

Figure 8: Gene-gene interaction network of differently expressed RPNs (GeneMANIA).

3.6. RPN6 and RPN9 are Upregulated in HCC Tissues and Cell Lines. Some RPNs’ oncogenic roles in HCC were uncovered by other researchers [31–35]. To confirm our conclusions, we first conducted (RT-qPCR) to investigate the mRNA levels of RPN6 and RPN9 in 54 paired human HCC tissue specimens and their corresponding non-tumorous tissue samples. The results showed that RPN6 and RPN9 mRNA expression was markedly elevated in HCC tissues relative to normal (Figures 10(a) and 10(b)). An upregulated protein expression levels of RPN6 and RPN9 were further confirmed in 4 paired specimens by immunoblotting assays (Figure 10(c)). To further investigate the oncogene function of RPN6 and RPN9, a panel of human HCC cell lines was evaluated by RT-qPCR and western blot. The PCR results showed that RPN6 and RPN9 were present at higher (Hcclm3 and Huh7) levels than Wrl-68. WB further confirmed an upregulated...
protein expression level of RPN6 and RPN9 in HCC cell lines.

Knockdown of RPN6 and RPN9 inhibits cell proliferation, migration, and invasion by the EMT process. As RPN6 and RPN9 are upregulated in HCC tissues and cell lines, it is necessary to determine whether their suppression could affect the biological activity in HCC cells. We stably downregulated RPN6 and RPN9 in HCC cell lines, Hcclm3 and Huh7, using shRNA. As shown in Figure 11(a), RT-qPCR data indicated a significant loss of mRNA expression in those two HCC cell lines. The CCK-8 proliferation curves demonstrated that RPN6 and RPN9 knockdown remarkably attenuated cell growth in Hcclm3 and Huh7 cells (Figure 11(b)). We further explored the potential impact of RPN6 and RPN9 knockdown on metastatic properties in HCC cells using wound scratch and transwell assays. As shown in Figure 11(c), the results showed that the loss function of RPN6 and RPN9 significantly suppressed the wound closure potential in Hcclm3 and Huh7 cells. In the transwell assay, attenuated RPN6 and RPN9 expression remarkably decreased migration and invasion capabilities in Hcclm3 and Huh7 cells. Thus, our results show that RPN6 and RPN9 expressions are essential for HCC cell proliferation, migration, and invasion. As epithelial-mesenchymal transition (EMT) markers are crucial in cell migration and metastasis, we investigated whether RPN6 and RPN9 affected the EMT process in HCC. As shown in Figure 12(a), western blot analysis documented
that the silencing of RPN6 and RPN9 decreased the expression of mesenchymal markers (N-cadherin, Vimentin, and Snail) in Hcclm3 cells.

3.7. Knockdown of RPN6 Inhibits Tumor Growth in Vivo.
To further validate whether RPN6 could affect tumor growth in vivo, the Hcclm3 cells with NC group or Sh-RPN6 group
(a) Figure 11: Continued.
were inoculated subcutaneously into either side of nude mice. The mice were euthanized 18 days after injection. We found that tumors derived from Sh-RPN6 cells grew more slowly, and the final tumor weight was markedly lower than the NC group (Figures 12(c)–12(e)), which was consistent with the results in vitro.

4. Discussion

Hepatocellular carcinoma, featured by high morbidity and mortality, remains one of the most health threats to people worldwide [1]. Although surgical procedures have become the standard of treatment, most patients are not surgical candidates [4]. Therefore, to conquer HCC, screaming potential molecular biomarkers is a top priority.

By charging 80–90% of protein degradation, UPS is a sophisticated controlled system and responsible for many kinds of cellular procedures and oncogenesis [36, 37]. The Ribophorin family is an essential regulatory subunit of 26s proteasome. Some studies have reported that RPNs' deregulation has robust oncogene functions in multiple cancers [11–14]. Although some RPNs' oncogenic role in HCC has been proven, distinct roles of the RPN family in HCC remained ambiguous. Therefore, we performed a comprehensive bioinformatics analysis of RPNs in this study.

RPN1 is the largest proteasome subunit and functions as the recognition part of the ubiquitin-proteasome system proteins by the proteasome [38]. RPN1 was possessed by two adjacent regions designated as T1 and T2. T1 is the receptor site for specific UBL domain proteins, while T2 binds to USP14, a proteasome-associated deubiquitinating enzyme [39, 40]. As a crucial part of the UPS system, RPN1 was upregulated in lung and kidney cancer [41]. Abnormal RPN1/EVI1 fusion was also popular in myelodysplasia and acute myeloid leukemia [42] and could be one of the relevant genes to predict breast cancer prognostic significance [43]. In our study, data from the online datasets showed that RPN1 was more highly expressed in human HCC tissues than normal tissues. And RPN1 expression is related to tumor grade. Further, highly expressed RPN1 RNA was associated with a worse OS, which was not studied in the previous report.

Located at 20q12-13.1, RPN2 is paralogous RPN1 and a highly conserved protein in the rough endoplasmic reticulum. In 2008, Takahashi et al. reported that overexpressed RPN2 is associated with docetaxel resistance by degenerating GSK3β in breast cancer [11]. Tominaga et al. found the other drug resistance function of RPN2 in breast cancer with a different target of CD63 [44]. Next, RPN2's oncogene role has been found in esophageal squamous cell

![Cell migration](image1)

**Figure 11:** Knockdown of RPN6 and RPN9 suppresses the proliferation, migration, and invasion of HCC cells in vitro. (a). RT-qPCR analysis was employed to examine the efficiency of RPN6 and RPN9 knockdown. (b). CCK-8 assays result for RPN6 and RPN9 knockdown in Hcclm3 and Huh7 cells. (c). Wound-healing assay was performed to measure the migration ability of these cells. (d). Transwell assays were used to measure the migration ability of these cells.
carcinoma [12], osteosarcoma [13], gastric cancer [14], and HCC [32]. The Zhang et al. study showed that RPN2 might serve as a biomarker for colorectal cancer. Recently, Huang et al. identified that RPN2 was upregulated in HCC cell lines and HCC tissues. Overexpressed RPN2 expression increased cell proliferation, metastasis, invasion, and epithelial-mesenchymal transition [32]. RPN2 may precisely interact with STAT3 and NF-κB pathways. Circ_0046599 also promoted HCC development by influencing RPN2 [33]. Moreover, RPN2 can be detected in blood exosomes [45] and urinary exosomes [46], expanding its application in diagnosis.

RPN6 is another essential subunit in the UPS system. Studies have proven that its high expression or phosphorylation may promote proteasome activity [47, 48]. RPN6 could protect pancreatic cancer cells from acute apoptosis [49]. In our work, we indicated that RPN6 was upregulated in HCC and its expression level is correlated with the patient’s tumor grade and OS. Moreover, RPN6 is a sensitivity biomarker for HCC diagnosis. Previous studies thought RPN9 is an aging-related gene [50, 51]. Our study first revealed the oncogene role of RPN9 in cancers. Further work should be done to confirm our results.

Besides being an important subunit of the proteasome, RPN11 is also a robust proteasome deubiquitinase. Luo et al. reported that RPN11 plays an essential role in breast cancer progression [52]. Zhu et al. showed silencing RPN11 reduced the metastasis of esophageal squamous cell carcinoma [53]. Wang et al. indicted that RPN11 dysregulation may correlate with many cancers besides HCC [35]. On the expression level, the RPN11 we uncovered here might serve as an effective diagnostic and prognosis biomarker for HCC.

Our study showed that all members of the RPNs were significantly overexpressed in HCC, and their expression patterns were connected with the patient’s tumor grade. The ROC curve suggested that RPN2, RPN3, RPN6, RPN9, RPN10, RPN11, and RPN12 have sensitivity and specificity in HCC diagnosis. Moreover, the expression of RPN1, RPN2, RPN5, RPN6, RPN9, and RPN11 was negatively correlated with patients’ OS. Furthermore, the level of RPN6’ expression is associated with the level of immune infiltration in HCC. The GGI and functional enrichment of RPNs were analyzed to predict their molecular functions and potential targets. Moreover, we performed western blot and qRT-PCR of RPN6 and RPN9 to verify these results. The silencing of RPN6 and RPN9 significantly influenced HCC cells’...
proliferation, migration, and invasion. An in vivo tumor model further validated the oncogene effect of RPN6 on HCC cell growth. Moreover, RPN6 and RPN9 could promote cell migratory and invasive potential by affecting the epithelial-mesenchymal transition (EMT) process. Our findings showed that the RPN family has the potential to be new prognostic and diagnostic markers and also drug targets for HCC. There are still some limitations in our study. Our findings required a multicenter, randomized, large clinical controlled trial, and the molecule processes behind RPNs should be addressed. As RPNs can be detected in blood and urine, the effect of liquid biopsy may be valued further. Despite the limitations above, our study first investigated the diagnostic and prognostic value of RPNs in HCC. Their results highlighted many RPNs have the potential to be new biomarkers and underlying targets for HCC.

Data Availability
The transcription levels of RPNs and the changes of RPNs’ expression between cancer and normal data were downloaded from the Oncomine database (https://www.oncomine.org) under the accession number (s): n8630, n8887, n8302, n4313, n8472, and n9158. The transcription levels of ITPRs between cancer and normal cells were downloaded from the UALCAN database (https://ualcan.path.uab.edu). Cancer patients’ survival analysis data were downloaded from the Kaplan–Meier Plotter database (https://gepia.cancer-pku.cn). The ITPRs’ gene expression data and clinic information were downloaded from the UCSC Xena database (https://xena.ucsc.edu/). The immune infiltrations data were downloaded from the Timer database (https://cistrome.shinyapps.io/timer/). The ITPRs’ gene alteration data and clinic information were downloaded from the CbioPortal database (https://www.cbioportal.org). The gene relationships network data were downloaded from the GeneMANIA database (https://www.genemania.org). The methylation status of ITPRs’ gene data was downloaded from the Wanderer database (https://maplab.imppc.org/wanderer). The GO and KEGG enrichment data were downloaded from the STRING database (https://string-db.org/).

Disclosure
A preprint of this study has previously been published [54]. Wangyang Zheng and Yuling Zheng are co-first authors.

Conflicts of Interest
The authors have declared that no conflicts of interest exist.

Acknowledgments
This study was funded by National Natural Science Foundation of China (Grant no. 81902431), Special Project of China Postdoctoral Science Foundation (Grant no. 2019T10279), China Postdoctoral Science Foundation (Grant nos. 2018M641849 and 2018M640311), Heilongjiang Postdoctoral Science Foundation (Grant nos. LBH-Z18107 and LBH-Z18112), Natural Science Foundation of Heilongjiang Province (Grant no. YQ2019H007), Postgraduate Innovative Research Project of Harbin Medical University (Grant nos. YJSCX2016-21HYD), Foundation of Key Laboratory of Myocardial Ischemia, Ministry of Education (Grant no. KF201810), Chen Xiaoping Foundation for the Development of Science and Technology of Hebei Province (Grant nos. CXPJJH11800004-001 and CXPJJH11800004-003), The Fundamental Research Funds for the Heilongjiang Provincial Universities (Grant nos. 2018-KYYWF-0511 and 2018-KYYWF-0498), and Hong Kong Scholars Program (Grant no. XJ2020012).

Supplementary Materials
Table S1. Primer sequences oligonucleotides information. Table S2. The ROC test results of RPN subunits. (Supplementary Materials)

References
[1] H. B. El-Serag, “Hepatocellular carcinoma,” New England Journal of Medicine, vol. 365, no. 12, pp. 1118–1127, 2011.
[2] L. A. Torre, F. Bray, R. L. Siegel, J. Ferlay, J. Lortet-Tieulent, and A. Jemal, “Global cancer statistics,” CA: A Cancer Journal for Clinicians, vol. 65, no. 2, pp. 87–108, 2015.
[3] A. Forner, M. Reig, and J. Bruix, “Hepatocellular carcinoma,” The Lancet, vol. 391, no. 10127, pp. 1301–1314, 2018.
[4] M. J. Truty and J. N. Vauthery, “Surgical resection of high-risk hepatocellular carcinoma: patient selection, preoperative considerations, and operative technique,” Annals of Surgical Oncology, vol. 17, no. 5, pp. 1219–1225, 2010.
[5] R. T. P. Poon, S. T. Fan, C. M. Lo, C. L. Liu, and J. Wong, “Long-term survival and pattern of recurrence after resection of small hepatocellular carcinoma in patients with preserved liver function: implications for a strategy of salvage transplantation,” Annals of Surgery, vol. 235, no. 3, pp. 373–382, 2002.
[6] C. M. Wong, L. Wei, C. T. Law et al., “Up-regulation of histone methyltransferase SETDB1 by multiple mechanisms in hepatocellular carcinoma promotes cancer metastasis,” Hepatology, vol. 63, no. 2, pp. 474–487, 2016.
[7] A. M. Weissman, “Themes and variations on ubiquitylation,” Nature Reviews Molecular Cell Biology, vol. 2, no. 3, pp. 169–178, 2001.
[8] A. Devoy, T. Soane, R. Welchman, and R. J. Mayer, “The ubiquitin-proteasome system and cancer,” Essays in Biochemistry, vol. 41, no. 1, pp. 187–203, 2005.
[9] X. Wang, L. Yin, L. Yang et al., “Silencing ubiquitin-conjugating enzyme 2C inhibits proliferation and epithelial-mesenchymal transition in pancreatic ductal adenocarcinoma,” FEBS Journal, vol. 286, no. 24, pp. 4889–4909, 2019.
[10] J. Cloos, M. S. Roeten, N. E. Franke et al., “(Immun) proteasomes as therapeutic target in acute leukemia,” Cancer and Metastasis Reviews, vol. 36, no. 4, pp. 599–615, 2017.
[11] T. X. Jiang, M. Zhao, and X. B. Qiu, “Substrate receptors of proteasomes,” Biological Reviews, vol. 93, no. 4, pp. 1765–1777, 2018.
[12] K. Honma, K. Iwao-Koizumi, F. Takeshita et al., “RPN2 gene confers docetaxel resistance in breast cancer,” Nature Medicine, vol. 14, no. 9, pp. 939–948, 2008.
[13] J. Kurashige, M. Watanabe, M. Iwatsuki et al., “RPN2 expression predicts response to docetaxel in oesophageal squamous cell carcinoma,” British Journal of Cancer, vol. 107, pp. 1233–1238, 2012.

[14] T. Fujiwara, R. U. Takahashi, N. Kosaka et al., “RPN2 gene confers osteosarcoma cell malignant phenotypes and determines clinical prognosis,” Molecular Therapy—Nucleic Acids, vol. 3, 2014.

[15] T. M. Yuan, R. Y. Liang, P. J. Chueh, and S. M. Chuang, “Role of ribophorin II in the response to anticancer drugs in gastric cancer cell lines,” Oncology Letters, vol. 9, no. 4, pp. 1861–1868, 2015.

[16] C. D. Marceau, A. S. Puschnik, K. Majzoub et al., “Genetic dissection of Flaviviridae host factors through genome-scale CRISPR screens,” Nature, vol. 535, no. 7610, pp. 159–163, 2016.

[17] H. B. El-Serag, “Epidemiology of viral hepatitis and hepatocellular carcinoma,” Gastroenterology, vol. 142, no. 6, 2012.

[18] B. Yang, Y. Zhao, L. Wang et al., “Identification of PLOD family genes as novel prognostic biomarkers for hepatocellular carcinoma,” Frontiers in Oncology, vol. 10, 2020.

[19] P. Huang, Y. Liu, and B. Jia, “The expression, prognostic value, and immunological correlation of MCEMP1 and its potential role in gastric cancer,” Journal of Oncology, vol. 2022, Article ID 8167496, 13 pages, 2022.

[20] Y. Xu, Y. Yao, K. Leng et al., “Long non-coding RNA UCA1 indicates an unfavorable prognosis and promotes tumorigenesis via regulating AKT/GSK-3β signaling pathway in cholangiocarcinoma,” Oncotarget, vol. 8, no. 56, pp. 96203–96214, 2017.

[21] M. J. Goldman, B. Craft, M. Hastie et al., “Visualizing and interpreting cancer genomics data via the Xena platform,” Nature Biotechnology, vol. 38, no. 6, pp. 675–678, 2020.

[22] A. Nagy, A. Lánzczky, O. Menyhárt, and B. Győrffy, “Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets,” Scientific Reports, vol. 8, no. 1, 2018.

[23] E. Cerami, J. Gao, U. Dogrusoz et al., “The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data,” Cancer Discovery, vol. 2, no. 5, pp. 401–404, 2012.

[24] D. Warde-Farley, S. L. Donaldson, O. Comes et al., “The GeneMANIA prediction server: biological network integration for gene prioritization and predicting gene function,” Nucleic Acids Research, vol. 38, pp. W214–W220, 2010.

[25] D. Szklarczyk, A. Franceschini, M. Kuhn et al., “The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored,” Nucleic Acids Research, vol. 39, pp. D561–D568, 2011.

[26] S. Roessler, H. L. Jia, A. Budhu et al., “A unique metastasis gene signature enables prediction of tumor relapse in early-stage hepatocellular carcinoma patients,” Cancer Research, vol. 70, no. 24, pp. 10202–10212, 2010.

[27] E. Wurmbach, Y. B. Chen, G. Khitrov et al., “Genome-wide molecular profiles of HCV-induced dysplasia and hepatocellular carcinoma,” Hepatology, vol. 45, no. 4, pp. 938–947, 2007.

[28] C. Guichard, G. Amaddeo, S. Imbeaud et al., “Integrated analysis of somatic mutations and focal copy-number changes identifies key genes and pathways in hepatocellular carcinoma,” Nature Genetics, vol. 44, no. 6, pp. 694–698, 2012.

[29] X. Chen, S. T. Cheung, S. So et al., “Gene expression patterns in human liver cancers,” Molecular Biology of the Cell, vol. 13, no. 6, pp. 1929–1939, 2002.

[30] V. R. Mas, D. G. Maluf, K. J. Archer et al., “Genes involved in viral carcinogenesis and tumor initiation in hepatitis C virus-induced hepatocellular carcinoma,” Molecular Medicine, vol. 15, pp. 85–94, 2009.

[31] Z. Jiang, Q. Zhou, C. Ge et al., “Rpn10 promotes tumor progression by regulating hypoxia-inducible factor 1 alpha through the PTEN/Akt signaling pathway in hepatocellular carcinoma,” Cancer Letters, vol. 447, pp. 1–11, 2019.

[32] L. Huang, Z. Jian, Y. Gao et al., “RPN2 promotes metastasis of hepatocellular carcinoma cell and inhibits autophagy via STAT3 and NF-κB pathways,” Aging (Albany NY), vol. 11, no. 17, pp. 6674–6690, 2019.

[33] Q. Fang, H. Liu, A. Zhou, H. Zhou, and Z. Zhang, “Circ_0046599 promotes the development of hepatocellular carcinoma by regulating the miR-1258/RPN2 network,” Cancer Management and Research, vol. 12, pp. 6849–6860, 2020.

[34] Z. Lu, Q. Guo, A. Shi, F. Xie, and Q. Lu, “Downregulation of NIN/RPN12 binding protein inhibit the growth of human hepatocellular carcinoma cells,” Molecular Biology Reports, vol. 39, no. 1, pp. 501–507, 2012.

[35] R. S. Soong, R. K. Anchoori, R. B. S. Roden et al., “Bisbenzylidine Piperidone RA190 treatment of hepatocellular carcinoma via binding RPN13 and inhibiting NF-κB signaling,” BMC Cancer, vol. 20, no. 1, 2020.

[36] Y. J. Chen, H. Wu, and X. Z. Shen, “The ubiquitin-proteasome system and its potential application in hepatocellular carcinoma therapy,” Cancer Letters, vol. 379, no. 2, pp. 245–252, 2016.

[37] M. Shen, S. Schmitt, D. Buac, and Q. P. Dou, “Targeting the ubiquitin-proteasome system for cancer therapy,” Expert Opinion on Therapeutic Targets, vol. 17, no. 9, pp. 1091–1108, 2013.

[38] R. Rosenzweig, V. Bronner, D. Zhang, D. Fushman, and M. H. Glickman, “Rpn1 and Rpn2 coordinate ubiquitin processing factors at proteasome,” Journal of Biological Chemistry, vol. 287, no. 18, pp. 14659–14671, 2012.

[39] Y. Shi, X. Chen, S. Elsasser et al., “Rpn1 provides adjacent receptor sites for substrate binding and deubiquitination by the proteasome,” Science, vol. 351, no. 6275, pp. 6275, 2016.

[40] V. Brukhin, J. Gheselinck, V. Gagliardi, P. Geschik, and U. Grossniklaus, “The RPN1 subunit of the 26S proteasome in Arabidopsis is essential for embryogenesis,” The Plant Cell Online, vol. 17, no. 10, pp. 2723–2737, 2005.

[41] G. S. Krasnov, O. Ntu, A. A. Dmitriev et al., “Novel reference gene RPN1 for normalization of quantitative data in lung and kidney cancer,” Molecular Biology, vol. 45, no. 2, pp. 238–248, 2011.

[42] B. M. Shearer, W. R. Sukov, H. C. Flynn, R. A. Knudson, and R. P. Ketterling, “Development of a dual-color, double fusion FISH assay to detect RPN1/EVI1 gene fusion associated with inv (3), t (3;3), and ins (3;3) in patients with myelodysplasia and acute myeloid leukemia,” American Journal of Hematology, vol. 85, no. 8, pp. 569–574, 2010.

[43] K. Milde-Langosch, T. Kern, M. Schmidt et al., “Prognostic relevance of glycosylation-associated genes in breast cancer,” Breast Cancer Research and Treatment, vol. 145, no. 2, pp. 295–305, 2014.

[44] N. Tominaga, K. Hagiwara, N. Kosaka, K. Homma, H. Nakagama, and T. Ochiya, “RPN2-mediated glycosylation of tetraspanin CD63 regulates breast cancer cell malignancy,” Molecular Cancer, vol. 13, no. 1, 2014.

[45] B. J. Tauro, D. W. Greening, R. A. Mathias, S. Mathivanan, H. Ji, and R. J. Simpson, “Two distinct populations of exosomes are released from LIM1863 colon carcinoma cell-
derived organoids,” Molecular & Cellular Proteomics, vol. 12, no. 3, pp. 587–598, 2013.

[46] P. G. Moon, J. E. Lee, S. You et al., “Proteomic analysis of urinary exosomes from patients of early IgA nephropathy and thin basement membrane nephropathy,” Proteomics, vol. 11, no. 12, pp. 2459–2475, 2011.

[47] D. Vilchez, L. Boyer, I. Morantte et al., “Increased proteasome activity in human embryonic stem cells is regulated by PSMD11,” Nature, vol. 489, no. 7415, pp. 304–308, 2012.

[48] S. Lokireddy, N. V. Kukushkin, and A. L. Goldberg, “cAMP-induced phosphorylation of 26S proteasomes on Rpn6/PSMD11 enhances their activity and the degradation of misfolded proteins,” Proceedings of the National Academy of Sciences of the United States of America, vol. 112, no. 52, pp. E7176–E7185, 2015.

[49] T. Qi, W. Zhang, Y. Luan et al., “Proteomic profiling identified multiple short-lived members of the central proteome as the direct targets of the addicted oncogenes in cancer cells,” Molecular & Cellular Proteomics, vol. 13, no. 1, pp. 49–62, 2014.

[50] A. Mancini, D. Vitucci, M. B. Randers et al., “Lifelong football training: effects on autophagy and healthy longevity promotion,” Frontiers in Physiology, vol. 10, 2019.

[51] D. Bellizzi, S. Dato, P. Cavalcante et al., “Characterization of a bidirectional promoter shared between two human genes related to aging: SIRT3 and PSMD13,” Genomics, vol. 89, no. 1, pp. 143–150, 2007.

[52] G. Luo, N. Hu, X. Xia, J. Zhou, and C. Ye, “RPN11 deubiquitinase promotes proliferation and migration of breast cancer cells,” Molecular Medicine Reports, vol. 16, no. 1, pp. 331–338, 2017.

[53] R. Zhu, Y. Liu, H. Zhou et al., “Deubiquitinating enzyme PSMD14 promotes tumor metastasis through stabilizing SNAIL in human esophageal squamous cell carcinoma,” Cancer Letters, vol. 418, pp. 125–134, 2018.

[54] https://www.researchsquare.com/article/rs-1071357/v1.