NPRL2 down-regulation facilitates the growth of hepatocellular carcinoma via the mTOR pathway and autophagy suppression

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Abstract
Hepatocellular carcinoma (HCC) is a highly invasive malignancy. Recently, GATOR1 (Gap Activity TOward Rags 1) complexes have been shown to play an important role in regulating tumor growth. NPRL2 is a critical component of the GATOR1 complex. Therefore, this study used NPRL2 knockdown to investigate how GATORC1 regulates the prognosis and development of HCC via the mammalian target of rapamycin (mTOR) and autophagy signaling pathways. We established HepG2 cells with NPRL2 knockdown using small interfering RNA (siRNA) and short hairpin RNA (shRNA) systems. The siRNA-mediated and shRNA-mediated NPRL2 down-regulation significantly
INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly invasive malignancy and is the second leading cause of cancer-related mortality worldwide.\cite{1-4} Hepatocarcinogenesis is a multimodal and complicated process facilitated by genetic changes. Oncogenic mutations disrupt signaling systems and cause aberrant regulation of signal transduction.\cite{5-7} Aberrant regulation of signal transduction in liver cancer can provide us with a potential strategy for preventing and treating HCC. Moreover, most patients are diagnosed late, and options for effective treatments are limited.\cite{1-4} In advanced-stage HCC, phenotypic and molecular heterogeneities mediate resistance to targets and chemotherapy.\cite{8-10} Hence, there is an urgent need to understand the mechanisms of HCC carcinogenesis and find novel targets for the prevention and effective treatment of HCC.

GATOR1 (Gap Activity Toward Rags 1) complexes consist of NPRL2, NPRL3, and DEPDC5 and exhibit GTPases activity protein (GAP) activity to inactivate Rag GTPases under amino acid-deficient conditions to inhibit mammalian target of rapamycin C1 (mTORC1) signaling.\cite{11-14} Tumors with the activation of the mTOR pathway are less differentiated and associated with a poorer prognosis and earlier recurrence than other tumors, regardless of the tumor type.\cite{15-17} Recent studies revealed that GATOR1, through DEPDC5 and mTOR, affects tumorigenesis in vitro and in vivo.\cite{18-20} Several cancers, including breast cancer, ovarian cancer, prostatic cancer, and gastrointestinal stromal tumors, were analyzed and found to be associated with DEPDC5 and NPRL2, but the analysis of NPRL3 remains incomplete.\cite{11,18,19,21-24} A previous study showed that high NPRL2 messenger RNA (mRNA) expression significantly reduces the overall survival (OS) of patients with human HCC.\cite{25} There was a significant association between the DEPDC5 polymorphism and the risk of hepatitis B (HBV) and hepatitis C (HCV)–related HCC.\cite{20,26,27} In addition, our previous studies showed that high LC3 expression in the tumors was significantly associated with disease-free survival and overall survival in 300 patients with HCC after surgical resection. Conclusion: The efficient down-regulation of NPRL2 significantly increased HCC proliferation, migration, and colony formation in vitro, and increased HCC growth in vivo. Low NPRL2 protein expression in the tumors was closely correlated with poorer clinical outcomes in patients with HCC. These results provide a mechanistic understanding of HCC and aid the development of treatments for HCC.

METHODS

Cell lines and cell culture

HepG2, Hep3B, and Huh7 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO₂ at 37°C.
**Lentiviral transduction (shRNA)**

Lentiviruses containing NPRL2 shRNAs (PLKO.1 vector, target sequence: sh1- AGTATGATGTACCTGTCTTTA; pLKO_TRC005 vector, target sequence: sh2- GCAG CATGTGTAGAAGCGAA) were obtained from the RNAi core facility at Academia Sinica, Taiwan. Viral transduction was performed in 6-well plates using $2 \times 10^5$ HepG2 cells in a total volume of 1.5 ml of lentiviral supernatant containing 8 μg/ml polybrene (Sigma-Aldrich). After overnight infection, 5 μg/ml puromycin was added to the media for selection for 4–6 days. The expression levels of the target genes were determined by western blotting.

**siRNA transfection**

HepG2 and Hep3B cell lines were cultured in DMEM containing 10% FBS, penicillin, and streptomycin. The cells were reverse transfected with scrambled siRNA (12935-112; Life Technologies) or siRNA against NPRL2, NPRL3, or DEPDC5 (20,218, s23245, s23246; Life Technologies) for 72h using the transfection reagent RNAiMAX (13778-150; Life Technologies). The cells were harvested for either protein expression analysis by western blotting or subsequent experiments.

**Cell proliferation assay**

HepG2 and Hep 3B cells with or without NPRL2 KD, NPRL3 KD, or DEPDC5 KD were seeded into each well of a 96-well white plate containing 500 cells/well. Every 3 days for 9 days, the cells were mixed with Cell-Titer Glo (G7572; Promega), and the luminescent signal was monitored to reflect cell proliferation (as indicated by the adenosine triphosphate level).

**Colony formation assay**

HepG2 and Hep3B cells with or without NPRL2 KD, NPRL3 KD, or DEPDC5 KD were seeded into 12-well plates at 2000 cells/well in triplicate and cultured for 14 days. The colonies were fixed with 10% formaldehyde for 5 min and were then stained with crystal violet (0.4 g/L; Sigma-Aldrich). The number of colonies was counted.

**Cell migration assay**

NPRL2, NPRL3, or DEPDC5-silenced HepG2 and Hep3B cells ($2 \times 10^5$ cells/well of 35 × 11-mm dishes) were seeded and incubated for 24 h at 37°C. After the cells reached 100% confluence, the cell monolayer of each plate was scratched using a plastic pipette tip. The migration of the cells at the edge of the scratch was analyzed at 0 h and 72 h after microscopic images of the cells were captured.

**Subcutaneous and orthotopic xenograft mouse models**

Male BALB/c nude mice (7 weeks old) were subcutaneously inoculated in the left flank with 200 μl ($1 \times 10^7$ cells) of NPRL2-KD HepG2 cells (shRNA-NPRL2-transfected HepG2 cells) or control cells. Furthermore, in the orthotopic xenograft mouse model, 200 μl ($1 \times 10^7$ cells) of NPRL2-KD HepG2 cells (shRNA-NPRL2-transfected HepG2 cells) or control cells were implanted into the liver of BALB/c nude mice. The animals developed palpable tumors (in approximately 3 weeks) in the subcutaneous xenograft model and tumors (in 3 weeks) in the orthotopic xenograft model after exposing the internal organs of the abdominal cavity. The tumor size was recorded by measuring the tumor length (L) and width (W). The tumor volume (V) was calculated according to the Equation $V = 1/2 (L \times W^2)$. The tumors were collected for the histological analysis and immunohistochemistry (IHC). All animals were sacrificed 7 weeks (in the subcutaneous model) and 3 weeks (in the orthotopic model) after inoculation.

**Western blot analysis**

The cells were lysed by PRO-PREP TM Protein Extraction Solution (Intron Biotechnology, Gyeonggi-do, Korea) according to the manufacturer's instructions. The protein concentration was determined, and proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with BlockPro 1 min protein-free blocking buffer and incubated with the following primary antibodies at 4°C overnight: anti-NPRL2 (sc-376986; Sigma-Aldrich), anti-NPRL3 (ab121346; Abcam, Cambridge, MA), anti-DEPDC5 (GTX133570; GeneTex), anti-Rag A (D8B5; Cell Signaling Technology), anti-Rag C (D8H5; Cell Signaling Technology), anti-phospho-mTOR (Ser2448; Cell Signaling Technology), anti-phospho–4E-binding protein (4E-BP1) (Thr37/46; 2855; Cell Signaling Technology), anti-phospho-S6K (Cell Signaling Technology), anti-LC3 (NB-100-2220; Novus Biologicals), anti-p62 (H0008878-M01; Abnova), and anti-GAPDH (NB-300-221; Novus Biologicals) antibody.

**Patients and follow-up**

This prospective cohort included 300 patients with HCC who underwent resection from 2012 to 2018 at
E-Da Hospital (Taiwan). Our study was approved by the Institutional Review Board of E-Da Hospital. We collected data regarding the clinicopathological features, including demographic characteristics, liver cirrhosis, mortality, tumor features, metastasis, vascular invasion, and HCC recurrence.

Patients were followed up every 3–6 months by abdominal ultrasound, computed tomography, or magnetic resonance imaging. DFS or OS was defined as the time from the date of HCC diagnosis to recurrence or mortality, the last follow-up, or study completion in December 2019, whichever came first.

IHC staining and scoring

Both tumor tissues and adjacent nontumor tissues (ANTs) obtained from the patients were formalin-fixed and paraffin-embedded, and the status (tumor vs. nontumor) of each tissue sample was confirmed using hematoxylin and eosin–stained sections. We stained the tissues with anti-NPRL2 (HPA038196; Sigma-Aldrich), anti-NPRL3 (ab121346; Abcam), anti-DEPDC5 (HPA055619; Sigma-Aldrich), anti-Rag A (D885; Cell Signaling Technology), anti-Rag C (D8H5; Cell Signaling Technology), anti-phospho-mTOR (Ser2448; Cell Signaling Technology), anti-phospho-4E-BP1 (Thr37/46) (2855; Cell Signaling Technology), anti-NPRL2 (HPA038196; Sigma-Aldrich), anti-Rag C (D8H5; Cell Signaling Technology), anti-Rag A (D8B5; Cell Signaling Technology), anti-LC3 (NB100-2220; Novus Biologicals), and anti-p62 (H0008878-M01; Abnova) antibody. The expression of NPRL2 and other proteins was quantitated by a semiquantitative immunoreactive scoring system as previously described. The percentage and intensity scores were combined, and the samples were defined as having negative or positive expression based on an immunoreactivity score cutoff of 2 (Figure S2). All slides were independently scored by two investigators.

Data statistics

Categorical data are presented as numbers and percentages. The chi-squared test was used to compare categorical variables. The results of multiple observations are presented as the mean±SEM. For analysis of multiple groups, group differences were assessed using t-tests and one-way analysis of variance. DFS and OS were analyzed by the Kaplan–Meier method, and significant differences were analyzed by the log-rank test. Multivariate analyses were conducted via Cox regression models. Differences with a p-value of <0.05 were regarded as statistically significant. All statistical analyses were performed with SPSS version 23.0 (SPSS, Inc.).

RESULTS

Efficient down-regulation of the NPRL2 protein significantly promotes the proliferation, migration, and colony formation of HCC cells

We found that the protein expression of NPRL2, NPRL3, and DEPDC5 was relatively high and similar in the HepG2, Hep3B, and Huh7 cell lines by western blotting (Figure 1A). Hence, these three cell lines were considered ideal for investigating the physiological and biological features of NPRL2, NPRL3, and DEPDC5. Furthermore, NPRL2 (an important protein of GATOR1) down-regulation was successfully performed and significantly decreased the NPRL2 protein expression in the HepG2, Hep3B, and Huh7 cells using the siRNA system. The NPRL2 protein expression significantly reduced in the HepG2 cells compared with that in the Hep3B and Huh7 cells (Figure 1B). To elucidate the role of GATOR1 in the progression of HCC, we performed knocked down NPRL2, NPRL3, or DEPDC5 in the HepG2 cells using siRNA. The siRNA sequences successfully and significantly reduced the expression of NPRL2, NPRL3, and DEPDC5 in the HepG2 cells with NPRL2 down-regulation compared to those with NPRL3 and DEPDC5 down-regulation (Figure 1C). The protein expression of NPRL2 (0.1-fold) and NPRL3 (0.1-fold) in the HepG2 was significantly decreased in the HepG2 cells with NPRL2 down-regulation compared to those with NPRL3 and DEPDC5 down-regulation (Figure 1C).

Moreover, two shRNA sequences also significantly reduced the expression of NPRL2, NPRL3, and DEPDC5, producing two lines (lines 1 and 2) of HepG2 cells with NPRL2 down-regulation (Figure 1D). The protein expression of NPRL2, NPRL3, and DEPDC5 was reduced but similar to that following the NPRL2, NPRL3, and DEPDC5 down-regulation in the Hep3B cells (Figure 1D). Moreover, two shRNA sequences also significantly reduced the expression of NPRL2, NPRL3, and DEPDC5, producing two lines (lines 1 and 2) of HepG2 cells with NPRL2 down-regulation (Figure 1E).

We next confirmed the effects of shRNA-mediated NPRL2 down-regulation on HCC cell proliferation. Compared with the control conditions, NPRL2 down-regulation in the HepG2 cells significantly increased the cell proliferation rate on days 3, 6, and 9. Cell proliferation increased up to 6-fold on day 9 (Figure 2A). Furthermore, the migration of HepG2 cells increased significantly after NPRL2 down-regulation (Figure 2B). We performed a colony formation assay to investigate the effects of NPRL2 on the anchoring growth ability of HepG2 HCC cells. We observed that HepG2 cell loss of NPRL2 increased colony formation ability to 3–4-fold that of the vehicle group (Figure 2C,D). Moreover, siRNA-mediated NPRL2, NPRL3, and DEPDC5 down-regulation significantly promoted the cell proliferation, migration, and colony formation compared with those in...
Efficient down-regulation of NPRL2 significantly increased the activity of the rag GTPases via the mammalian target of rapamycin (mTOR) pathway and inhibited autophagy in hepatocellular carcinoma (HCC) cells. (A) The protein expression levels of NPRL2, NPRL3, and DEPDC5 were relatively high and similar in HepG2, Hep3B, and Huh7 cell lines, as determined by western blotting (n = 3). (B) NPRL2 down-regulation was successfully performed and significantly decreased the NPRL2 protein expression in HepG2, Hep3B, and Huh7 cells using the small interfering RNA (siRNA) system. The NPRL2 protein expression significantly reduced in HepG2 cells compared with that in Hep3B and Huh7 cells. (C) We performed knocked down NPRL2, NPRL3, and DEPDC5 in the HepG2 cells using the siRNA system. The siRNA sequences successfully and significantly reduced the expression of NPRL2, NPRL3, and DEPDC5 in the HepG2 cells with NPRL2, NPRL3, and DEPDC5 down-regulation compared to those with NPRL3 and DEPDC5 down-regulation. The protein expression of NPRL2 and NPRL3 in the HepG2 was significantly decreased in the HepG2 cells with NPRL2 down-regulation compared to those with NPRL3 and DEPDC5 down-regulation. (D) The protein expression of NPRL2, NPRL3, and DEPDC5 was reduced but similar to that following the NPRL2, NPRL3, and DEPDC5 down-regulation in the Hep3B cells. (E) We knocked down NPRL2 in HepG2 cells using the short hairpin RNA (shRNA) systems. NPRL2, NPRL3, and DEPDC5 protein expression was significantly reduced in the HepG2 cells (lines 1 and 2). (F) Activation of rag a, rag C, mTOR, and 4E-binding protein (4E-BP1) but not p-S6K in the HepG2 cells with NPRL2 knockdown (n = 3). (G) LC3-II protein expression significantly decreased and p62 protein expression significantly increased in the HepG2 cells with NPRL2 knockdown (n = 3). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; KD, knockdown.
Knockdown of NPRL2 significantly promoted the proliferation, migration, and colony formation in HCC cells. (A) NPRL2 down-regulation of HepG2 cells by the shRNA system significantly increased the cell proliferation rate compared with that in the vehicle group on days 3, 6, and 9. Cell proliferation increased up to 6-fold on day 9. The growth curves and their derivatives were determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay ($n=3$). (B) The migration of HepG2 cells significantly increased after NPRL2 down-regulation by shRNA for 72 h (scale bar: 200 μm; $n=3$). (C,D) HepG2 cells with NPRL2 down-regulation by the shRNA system exhibited 3-fold to 4-fold increased colony formation ability compared with that in the vehicle group ($n=3$). (E–H) NPRL2, NPRL3, and DEPDC5 down-regulation in the HepG2 cells by the siRNA system significantly increased the cell proliferation, migration, and colony formation ability compared with that in the vehicle group ($n=3$). The cell proliferation, migration, and colony formation were significantly increased by siRNA-mediated NPRL2 down-regulation compared with those following the NPRL3 and DEPDC5 down-regulation in the HepG2 cells. (I–L) NPRL2, NPRL3, and DEPDC5 down-regulation in the Hep3B cells by the siRNA system significantly promoted the cell proliferation, migration, and colony formation ability compared with that in the vehicle group ($n=3$). The cell proliferation, migration, and colony formation were significantly increased by the siRNA-mediated NPRL2 down-regulation compared with those following the NPRL3 and DEPDC5 down-regulation in the Hep3B cells. Statistical analysis was performed by one-way analysis of variance (ANOVA). *$p<0.05$; **$p<0.01$ compared with the counterpart group.
the vehicle group in the HepG2 cells (Figure 2E–H) and Hep3B cells (Figure 2I–L). The cell proliferation, migration, and colony formation were significantly increased by the siRNA-mediated NPRL2 down-regulation with those following the NPRL3 and DEPDC5 down-regulation in the HepG2 cells and Hep3B cells (Figure 2E–I,L). Taken together, these results imply that NPRL2, NPRL3, and DEPDC5 down-regulation in HCC cells promotes HCC cell proliferation, migration, and colony formation, especially NPRL2 down-regulation. We wondered whether GATOR1 could play an important role in HCC cell proliferation and metastasis through NPRL2 down-regulation.

NPRL2 down-regulation significantly increased the activity of the rag GTPases via the mTOR pathway and inhibited autophagy in HCC cells

The mTOR signaling pathway is an important regulator associated with cell growth, proliferation, and cancer development. Given the important role of GATOR1 in cancer development, we assessed whether GATOR1 is involved in cancer formation through the mTOR signaling pathway in the HepG2 cells by a western blotting analysis. The loss of GATOR1 function through the KD of NPRL2 significantly induced the Rag A, Rag C, and mTOR activity via the phosphorylation of the initiation factor 4E-BP, but not S6 kinase 1 (Figure 1F). Furthermore, we found that the inhibition of GATOR1 through NPRL2 down-regulation reduced LC3-II levels and increased p62 levels in HepG2 HCC cells (Figure 1G). Thus, these findings indicated that the loss of GATOR1 function through the efficient NPRL2 down-regulation significantly promoted the activation of Rag GTPases and mTOR via the possibility of 4E-BP and inhibited autophagy in HCC cells.

NPRL2 down-regulation promoted HCC tumor growth in vivo

We used a subcutaneous tumor model established in BALB/c nude mice to evaluate the effect of NPRL2 down-regulation on HCC development in vivo. Compared with those in the counterpart groups, the tumor growth curve in the NPRL2 down-regulation group was significantly increased on days 21–49, and the tumor volume was increased by 8-fold on day 49 (Figure 3A,B). In addition, we found that the tumor weight in the NPRL2 down-regulation group was significantly increased by up to 3-fold of that in the vehicle group (Figure 3C,D). The histological analysis of the transplanted tumors indicated malignant tumors (Figure 3E). Moreover, in the orthotopic xenograft mouse model, the tumor diameter (0.8±0.2 cm vs. 2.3±0.4 cm, p<0.001) and intrahepatic metastasis number (0.25±0.5 vs. 13±3.5, p<0.001) were significantly increased in the NPRL2 down-regulation group compared with those in the vehicle group at weeks (Figure 3F–H). The IHC analysis of the subcutaneous transplanted tumors revealed that the protein expression levels of NPRL2, NPRL3, DEPDC5, and LC3 were significantly higher, in the NPRL2 KD group than in the counterpart group (Figure 3I,J). These results along with the in vitro data indicate that NPRL2 down-regulation is potentially involved in HCC tumor growth through the activation of Rag GTPases and mTOR and the suppression of autophagy.

GATOR1 component proteins NPRL2, NPRL3, and DEPDC5 were predictive factors of prognosis in patients with HCC after surgical resection

As revealed by IHC, the protein expression levels of NPRL2, NPRL3, DEPDC5, LC3, and p62 were markedly higher in the tumor tissues and were markedly higher than those in the ANTs (Figure S1A,B). The correlations between NPRL2 protein levels and various clinicopathological features are summarized in Table 1. The NPRL2 level in the tumors was significantly associated with mortality, recurrence, the serum alpha fetoprotein level, the tumor size, the American Joint Committee on Cancer (AJCC) stage, and the Barcelona Clinic Liver Cancer (BCLC) stage (Table 1). Among 300 patients with HCC, 79 patients exhibited recurrence and 62 patients experienced mortality. More importantly, low NPRL2, NPRL3, and DEPDC5 protein levels in the tumors were associated with significantly worse recurrence, mortality, DFS, and OS than high NPRL2, NPRL3, and DEPDC5 protein levels after surgical resection (Figure 4A–C). In the patients with high NPRL2 expression in tumors, the 1-, 3-, and 5-year DFS rates were 89.5%, 87.4%, and 84.1%, respectively, whereas those in the patients with low tumor NPRL2 expression were 71.8%, 49.6%, and 37.5%, respectively (Figure 4B). Moreover, in the patients with high NPRL2 expression in the tumors, the 1-, 3-, and 5-year OS rates were 95.9%, 88.8%, and 80.5%, respectively, whereas those in the patients with low expression were 94.1%, 75.2%, and 59.1%, respectively (Figure 4C). The univariate analysis showed that the tumor size, macrovascular invasion, AJCC stage, BCLC stage, and NPRL2, NPRL3, and DEPDC5 protein expression were significantly associated with tumor recurrence (Table 2), and the multivariate analysis showed that macrovascular invasion and NPRL2 protein expression were significantly associated with tumor recurrence (Table 3). In addition, the univariate analyses showed that antiviral therapy, the tumor size, microvascular invasion, AJCC stage, BCLC stage, and NPRL2, NPRL3,
Knockdown of NPRL2 in human HepG2 cells promoted tumor growth in vivo. (A) HepG2 cells with or without NPRL2 KD were subcutaneously inoculated into BALB/c nude mice. The images show mice with transplanted tumors from the indicated groups at the end of the study. (B) In the NPRL2 down-regulation group compared with the counterpart group, the tumor growth curve was significantly increased on days 21–49, and the tumor volume was increased by up to 8-fold on Day 30. There were 4 and 8 mice in the vehicle group and NPRL2 KD group, respectively. (C) Transplanted tumors from the indicated groups. (D) The tumor weight in the NPRL2 down-regulation group was significantly increased by up to 3-fold of that in the vehicle group at the end of the study. (E) Hematoxylin and eosin staining of the transplanted tumors in both groups indicated malignant tumors. (F–H) In the orthotopic xenograft mouse model, HepG2 cells with or without NPRL2 KD were implanted into the liver of BALB/c nude mice (n = 4). The images show transplanted tumors from the indicated groups. The tumor diameter (0.8 ± 0.2 cm vs. 2.3 ± 0.4 cm, p < 0.001) and intrahepatic metastasis number (0.25 ± 0.5 vs. 13 ± 3.5, p < 0.001) were significantly increased in the NPRL2 down-regulation group compared with those in the vehicle group at 3 weeks. (I) The images show NPRL2, NPRL3, DEPDC5, rag a, rag C, mTOR, 4EBP1, LC3, and p62 in the immunohistochemical (IHC) staining of transplanted tumors from the indicated groups (upper and low panel, ×20). (J) Quantification of the IHC staining results revealed that the protein expression levels of NPRL2, NPRL3, DEPDC5, and LC3 were significantly lower and those of rag a, rag C, mTOR, 4E-BP1, and p62 were significantly higher in the NPRL2 KD group than in the counterpart group. Data are shown as number (%). All data are presented as the mean ± SEM (*p < 0.05; **p < 0.01).
| Characteristics               | Total patients, N (%) | NPRL2 expression              | p-Value<sup>a</sup> |
|------------------------------|-----------------------|-------------------------------|---------------------|
|                              |                       | Low (n = 120) | High (n = 180)       |                     |
| **Sex**                      |                       |                 |                     |                     |
| Male                         | 243 (81.0)            | 95 (79.2)       | 148 (82.2)          | 0.509               |
| Female                       | 57 (19.0)             | 25 (20.8)       | 32 (17.8)           |                     |
| **Age (years)**              |                       |                 |                     |                     |
| <60                          | 105 (35.0)            | 47 (39.2)       | 58 (32.2)           | 0.217               |
| ≥60                          | 195 (65.0)            | 73 (60.8)       | 122 (67.8)          |                     |
| **Alcohol use**              |                       |                 |                     |                     |
| Absent                       | 253 (84.3)            | 105 (87.5)      | 148 (82.2)          | 0.218               |
| Present                      | 47 (15.7)             | 15 (12.5)       | 32 (17.8)           |                     |
| **HBV positive**             |                       |                 |                     |                     |
| Negative                     | 170 (56.7)            | 64 (53.3)       | 106 (58.9)          | 0.321               |
| Positive                     | 130 (43.3)            | 56 (46.7)       | 74 (41.1)           |                     |
| **HCV positive**             |                       |                 |                     |                     |
| Negative                     | 225 (75.0)            | 95 (79.2)       | 130 (72.2)          | 0.174               |
| Positive                     | 75 (25.0)             | 25 (20.8)       | 50 (27.8)           |                     |
| **Cirrhosis**                |                       |                 |                     |                     |
| Absent                       | 190 (63.3)            | 72 (60.0)       | 118 (65.6)          | 0.328               |
| Present                      | 110 (36.7)            | 48 (40.0)       | 62 (34.4)           |                     |
| **Antiviral therapy**        |                       |                 |                     |                     |
| Absent                       | 158 (52.7)            | 66 (55.0)       | 92 (51.1)           | 0.509               |
| Present                      | 142 (47.3)            | 54 (45.0)       | 88 (48.9)           |                     |
| **Edmondson-Steiner grade** |                       |                 |                     |                     |
| I–II                         | 205 (68.3)            | 77 (64.2)       | 128 (71.1)          | 0.205               |
| III                          | 95 (31.7)             | 43 (35.8)       | 52 (28.9)           |                     |
| **Tumor size**               |                       |                 |                     |                     |
| <5 cm                        | 174 (58.0)            | 61 (50.8)       | 113 (62.8)          | 0.04                |
| ≥5 cm                        | 126 (42.0)            | 59 (49.2)       | 67 (37.2)           |                     |
| **Tumor number**             |                       |                 |                     |                     |
| Single                       | 279 (93.0)            | 110 (91.7)      | 169 (93.9)          | 0.46                |
| Multiple                     | 21 (7.0)              | 10 (8.3)        | 11 (6.1)            |                     |
| **AFP (ng/ml)**              |                       |                 |                     |                     |
| <200                         | 221 (73.7)            | 79 (65.8)       | 142 (78.9)          | 0.012               |
| ≥200                         | 79 (26.3)             | 41 (34.2)       | 38 (21.1)           |                     |
| **Resection**                |                       |                 |                     |                     |
| R0                           | 195 (65.0)            | 77 (64.2)       | 118 (65.6)          | 0.805               |
| R1/2                         | 105 (35.0)            | 43 (35.8)       | 62 (34.4)           |                     |
| **Microvascular invasion**   |                       |                 |                     |                     |
| Absent                       | 200 (66.7)            | 84 (70.0)       | 116 (64.4)          | 0.317               |
| Present                      | 100 (33.3)            | 36 (30.0)       | 64 (35.6)           |                     |
| **Macrovacular invasion**    |                       |                 |                     |                     |
| Absent                       | 263 (87.7)            | 102 (85.0)      | 161 (94.4)          | 0.251               |
| Present                      | 37 (12.3)             | 18 (15.0)       | 19 (10.6)           |                     |
| **Lympho nodule metastasis** |                       |                 |                     |                     |
| Absent                       | 298 (99.3)            | 119 (99.2)      | 179 (99.4)          | 0.772               |

(Continues)
and DEPDC5 protein expression were significantly associated with mortality (Table 2), and the multivariate analysis showed that antiviral therapy and NPRL2 protein expression were significantly associated with mortality (Table 3). These results imply that NPRL2 is an important predictive factor for the clinical prognosis of HCC.

Moreover, the combination of low LC3 and high p62 protein expression as revealed by IHC were significantly associated with worse DFS and OS (Figure 4D,E). High mTOR protein expression was significantly associated with worse DFS and OS in patients with HCC (Figure 4D,E).

**DISCUSSION**

Our study demonstrated that NPRL2 might be negatively associated with HCC recurrence and progression and that NPRL2 could serve as a predictive factor and therapeutic target in HCC. First, efficient down-regulation of NPRL2 protein expression in HCC cells significantly promoted HCC cell proliferation, migration, and colony formation. Second, NPRL2 down-regulation significantly increased HCC tumor growth in the subcutaneous and orthotopic xenograft mouse models. More importantly, the down-regulation of NPRL2 protein expression promoted HCC progression via the activation of Rag GTPases and mTOR and the suppression of autophagy in vitro and in vivo. Third, NPRL2 protein expression in the tumors is an important predictive factor of the clinical prognosis of human HCC. As illustrated in Figure 5, the down-regulation of NPRL2 increased cell proliferation, migration, and colony formation in vitro and HCC tumor growth in vivo, and decreased DFS and OS in human HCC through the activation of the mTOR pathways and the suppression of autophagy. These results provide a mechanistic understanding of HCC and could aid the development of treatments for HCC.

Previous studies reported that NPRL2 is associated with tumor growth in vitro and in vivo in different cancers. However, the study of NPRL2 in hepatocarcinogenesis in vitro or in vivo has not been reported in the literature. Our study demonstrated that the efficient down-regulation of NPRL2 protein in HCC cells decreased the NPRL2, NPRL3, and DEPDC5 protein levels, promoted cell proliferation, colony formation and migration, and increased HCC tumor growth in the subcutaneous and orthotopic tumor mouse model through the mTOR pathway and regulation of autophagy. These findings indicate that NPRL2 plays an important role in hepatocarcinogenesis in vitro and in vivo.

The GATOR1 complex consists of NPRL2, NPRL3, and DEPDC5 and plays an integrated role in the amino acid-induced mTOR signaling pathway. Tumors with the activation of the mTOR pathway are less differentiated and associated with a poorer prognosis and earlier recurrence than other tumors, regardless of the tumor type. Our study demonstrated that low NPRL2 protein expression in the tumors is significantly associated with worse DFS and OS in patients with HCC after resection. Our results were different from those of a previous study that showed that high NPRL2 mRNA expression is significantly associated with poor OS in patients with HCC.

Furthermore, our study revealed that low DEPDC5 protein expression is significantly associated with worse...
FIGURE 4  NPRL2, NPRL3, DEPDC5, LC3, p62, and mTOR were predictive factors for disease-free survival (DFS) and overall survival (OS) in patients with HCC after surgical resection. (A) Among 300 patients with HCC, 79 exhibited recurrence and 62 experienced mortality. Low NPRL2, NPRL3, and DEPDC5 protein expression in the tumors was significantly associated with higher recurrence and mortality ($p<0.05$). Data are shown as numbers (%). (B) In patients, low NPRL2, NPRL3, and DEPDC5 protein expression was significantly associated with worse DFS as revealed by Kaplan–Meier analysis ($p<0.05$). (C) Low NPRL2, NPRL3, and DEPDC5 protein expression in patients was significantly associated with worse OS, as determined by Kaplan–Meier analysis ($p<0.05$). (D) Low LC3, high p62, and high mTOR protein expression in patients was significantly associated with worse DFS according to Kaplan–Meier analysis ($p<0.05$). (E) In patients, low LC3, high p62, and high mTOR protein expression was significantly associated with worse OS as determined by Kaplan–Meier analysis ($p<0.05$).
| Characteristics | Recurrence | | | | Mortality | | | |
|-----------------|------------|----------|------------|----------|------------|----------|------------|----------|
| | Without recurrence | With recurrence | p-Value | Without mortality | With mortality | p-Value |
| Gender | | | | | | | | |
| Female | 41 (18.6) | 16 (20.3) | 0.741 | 45 (18.9) | 12 (19.4) | 0.936 |
| Male | 180 (74.1) | 63 (79.7) | | 193 (81.1) | 50 (80.6) | |
| Age (years) | | | | | | | | |
| <65 | 76 (34.4) | 29 (36.7) | 0.711 | 83 (34.9) | 22 (35.5) | 0.929 |
| ≥65 | 145 (65.6) | 50 (63.3) | | 155 (65.1) | 40 (64.5) | |
| HCC etiology | | | | | | | | |
| Non-HBV/HCV | 63 (28.5) | 23 (29.1) | 0.116 | 64 (26.9) | 22 (35.5) | 0.162 |
| Alcohol | 13 (5.9) | 1 (1.3) | | 14 (5.9) | 0 (64.5) | |
| HBV | 88 (39.8) | 36 (45.6) | | 97 (40.8) | 27 (43.5) | |
| HCV | 55 (24.9) | 16 (20.3) | | 58 (24.4) | 13 (21.0) | |
| HBV+HCV | 2 (0.9) | 3 (3.8) | | 5 (2.1) | 0 (0) | |
| AFP (ng/dl) | | | | | | | | |
| <200 | 165 (74.7) | 56 (70.9) | 0.513 | 180 (75.6) | 41 (66.1) | 0.130 |
| ≥200 | 56 (25.3) | 23 (29.1) | | 58 (24.4) | 21 (33.9) | |
| Liver cirrhosis | | | | | | | | |
| Absent | 143 (75.3) | 47 (59.5) | 0.409 | 151 (63.4) | 39 (62.9) | 0.937 |
| Present | 78 (35.3) | 32 (40.5) | | 87 (36.6) | 23 (37.1) | |
| Antiviral therapy | | | | | | | | |
| Absent | 110 (49.8) | 48 (60.8) | 0.093 | 117 (49.2) | 41 (66.1) | 0.017 |
| Present | 111 (50.2) | 31 (39.2) | | 121 (50.8) | 21 (33.9) | |
| Edmondson-Steiner grade | | | | | | | | |
| I–II | 151 (68.3) | 54 (68.4) | 0.996 | 166 (69.7) | 42 (67.7) | 0.872 |
| III | 70 (31.7) | 25 (31.6) | | 72 (30.3) | 20 (32.3) | |
| Macrovascular invasion | | | | | | | | |
| Absent | 200 (90.5) | 63 (79.7) | 0.013 | 213 (89.5) | 50 (80.6) | 0.059 |
| Present | 21 (9.5) | 16 (20.3) | | 25 (10.5) | 12 (19.4) | |
| Microvascular invasion | | | | | | | | |
| Absent | 153 (69.2) | 47 (59.5) | 0.115 | 167 (70.2) | 33 (53.2) | 0.012 |
| Present | 68 (30.8) | 32 (40.5) | | 71 (29.8) | 29 (46.8) | |
| Tumor number | | | | | | | | |
| Single | 209 (94.6) | 70 (88.6) | 0.075 | 222 (93.3) | 57 (91.9) | 0.712 |
| Multiple | 12 (5.4) | 9 (11.4) | | 16 (6.7) | 5 (8.1) | |
| Tumor size | | | | | | | | |
| <5 cm | 137 (62.0) | 37 (46.8) | 0.019 | 149 (62.6) | 25 (40.3) | 0.002 |
| ≥5 cm | 84 (38.0) | 42 (53.2) | | 89 (37.4) | 37 (59.7) | |
| AJCC stage | | | | | | | | |
| I–II | 170 (82.9) | 52 (69.3) | 0.013 | 185 (84.1) | 37 (61.7) | <0.001 |
| III | 35 (17.1) | 23 (30.7) | | 35 (15.9) | 23 (38.3) | |
| BCLC stage | | | | | | | | |
| 0–A | 141 (63.8) | 37 (46.8) | 0.008 | 151 (63.4) | 27 (43.5) | 0.004 |
| B–C | 80 (36.2) | 42 (53.2) | | 87 (36.6) | 35 (56.5) | |
### TABLE 2 (Continued)

| Characteristics | Recurrence Univariate analyses | Mortality Univariate analyses |
|-----------------|-------------------------------|-----------------------------|
|                 | Without recurrence (n = 221) | With recurrence (n = 79) | p-Value | Without mortality (n = 238) | With mortality (n = 62) | p-Value |
| NPRL2 in tumor  | Low | 64 (29.0) | 56 (70.9) | <0.001 | 120 (40.0) | 40 (64.5) | <0.001 |
|                 | High | 157 (71.0) | 23 (29.1) | 180 (60.0) | 22 (35.5) | 180 (60.0) | 22 (35.5) |
| NPRL3 in tumor  | Low | 76 (34.4) | 60 (75.9) | <0.001 | 136 (45.3) | 47 (75.8) | <0.001 |
|                 | High | 145 (65.6) | 19 (24.1) | 164 (54.7) | 15 (24.2) | 164 (54.7) | 15 (24.2) |
| DEPDC5 in tumor | Low | 83 (37.6) | 58 (73.4) | <0.001 | 141 (47.0) | 46 (74.2) | <0.001 |
|                 | High | 138 (62.4) | 21 (26.6) | 159 (53.0) | 16 (25.8) | 159 (53.0) | 16 (25.8) |

Note: Data are shown as number (%).

### TABLE 3  Multivariate analyses of factors associated with recurrence and mortality

| Characteristics | Recurrence Multivariate analyses | Mortality Multivariate analyses |
|-----------------|---------------------------------|---------------------------------|
|                 | HR (95% CI) | p-Value | HR (95% CI) | p-Value |
| NPRL2 in tumor  | Low | 1 | | 1 | 1 |
|                 | High | 0.38 (0.22–0.65) | <0.001 | 0.39 (0.21–0.73) | <0.001 |
| NPRL3 in tumor  | Low | 1 | | 1 | 1 |
|                 | High | 0.64 (0.21–1.99) | 0.439 | 0.42 (0.25–1.33) | 0.141 |
| DEPDC5 in tumor | Low | 1 | | 1 | 1 |
|                 | High | 0.51 (0.95–3.85) | 0.153 | 0.62 (0.29–1.93) | 0.413 |
| Tumor size      | <5 cm | 1 | | 1 | 1 |
|                 | ≥5 cm | 1.13 (0.53–2.43) | 0.741 | 1.49 (0.66–3.34) | 0.332 |
| AJCC stage      | I–II | 1 | | 1 | 1 |
|                 | III | 1.11 (0.55–2.22) | 0.771 | 1.73 (0.83–3.64) | 0.332 |
| BCLC stage      | 0–A | 1 | | 1 | 1 |
|                 | B–C | 1.51 (0.69–3.30) | 0.298 | 0.98 (0.42–2.29) | 0.964 |
| Macrovascular invasion | Absent | 1 | | 1 | 1 |
|                 | Present | 1.99 (1.05–3.78) | 0.034 | 1.93 (1.01–3.72) | 0.049 |
| Microvascular invasion | Absent | 1 | | 1 | 1 |
|                 | Present | 1.99 (1.05–3.78) | 0.034 | 1.93 (1.01–3.72) | 0.049 |

Abbreviations: CI, confidence interval; HR, hazard ratio.
NPRL2 DOWN-REGULATION FACILITATES HCC DFS and OS in patients with HCC. A previous study showed that patients with HBV and HCV-related HCC with high DEPDC5 expression had significantly longer DFS and OS. However, some studies showed that the DEPDC5 polymorphism was related to significantly worse DFS and OS in HCV-related HCC and that DEPDC5 did not affect the clinical prognosis of HCV-related HCC. Our study demonstrated that low NPRL3 protein expression is significantly associated with worse DFS and OS in patients with HCC. This report connects NPRL3 with the clinical outcomes of HCC.

Previous studies have shown that NPRL2 promotes chemoresistance in prostate cancer by regulating autophagy. Our study showed that the efficient down-regulation of NPRL2 protein in HCC cells promoted cell proliferation, migration, and colony formation via the regulation of the mTOR pathway and autophagy in vitro and in vivo. Furthermore, autophagy was found to be significantly associated with DFS and OS in patients with HCC after resection. Low NPRL2 protein expression is associated with HCC progression and poor clinical outcomes, as this change regulates homeostasis by altering mTOR pathway activity and suppressing autophagy.

In summary, our study demonstrates that the efficient down-regulation of NPRL2 protein expression promotes HCC cell proliferation, migration, and colony formation in vitro and increases HCC tumor growth in vivo by enhancing the activity of the Rag GTPases via the mTOR pathway and inhibiting autophagy. We found that low NPRL2 protein expression in the tumors was significantly associated with worse DFS and OS rates in patients with HCC after surgical resection. These results provide a mechanistic understanding of HCC and could aid the development of treatments for HCC.

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CONFLICT OF INTEREST Nothing to report.

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Supporting Information

Additional supporting information may be found in the online version of the article at the publisher’s website.