Multilevel regulation of RUVBL2 expression predicts poor prognosis in hepatocellular carcinoma

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Abstract

Background: Hepatocellular carcinoma (HCC) is the second-most lethal cancer worldwide with a complex pathogenesis. RuvB-like 2 (RUVBL2) was previously found to contribute to hepatocarcinogenesis. However, its expression, regulation and clinical significance have not been systematically evaluated in a large number of clinical samples.

Methods: Here, we performed a comprehensive analysis of RUVBL2 based on multiple datasets from 371 liver cancer patients of The Cancer Genome Atlas (TCGA) and on immunohistochemical staining in 153 subjects. In addition, the aberrant signaling pathways caused by RUVBL2 overexpression were investigated.

Results: We demonstrated that promoter hypomethylation, copy number gain, MYC amplification and CTNNB1 mutation were all responsible for RUVBL2 overexpression in HCC. High levels of RUVBL2 mRNA were associated with shorter recurrence-free survival time (RFS) but not overall survival time (OS). Furthermore, RUVBL2 protein was over-expressed in the nucleus and cytoplasm of HCC samples. Univariate and multivariate survival analyses showed that strong nuclear and cytoplasmic staining of RUVBL2 independently predicted worse OS and RFS with a 2.03-fold and a 1.71-fold increase in the hazard ratio, respectively. High levels of RUVBL2 promoted carcinogenesis through the heat shock protein 90 (HSP90)-Cell Division Cycle 37 (CDC37), AKT serine/threonine kinase (AKT) and mitogen-activated protein kinase (ERK/MAPK) pathways.

Conclusion: The deregulation of RUVBL2 in HCC is influenced at the genomic, epigenetic and transcriptional levels. Our findings highlight the potential roles of RUVBL2 as a promising prognostic marker as well as a therapeutic target for HCC.

Keywords: Liver cancer, Prognostic factor, RuvB-like 2, Regulation, Mechanism
Recently, RuvB-like 2 (RUVBL2) was found to interact with catenin beta 1 (CTNNB1), telomerase reverse transcriptase (TERT), MYC proto-oncogene (MYC), nuclear factor-kappa B1 (NFkB1), etc. to regulate the cancer-related signaling pathways in HCC. RUVBL2 belongs to the conserved ATPases associated with various cellular activities (AAA+) protein subfamily, which is characterized by the presence of conserved Walker A and B motifs that are involved in ATP binding and hydrolysis [6, 7]. Due to its chaperone characteristics, this subfamily helps to assemble multiple complexes to participate in many biological functions, including those regulating nutrient sensing, transcription, chromatin remodeling, telomerase assembly, RNA metabolism, and DNA damage repair [8, 9]. For transcriptional regulation, RUVBL2 can activate MYC- and E2F transcription factor 1 (E2F1)-dependent transcription; however, RUVBL2 represses CTNNB1, hypoxia-inducible factor 1-alpha (HIF1A), TP53, activating transcription factor 2 (ATF2), nuclear factor kappa light chain enhancer of activated B cells (NF-κB) and MYB proto-oncogene (MYB)-dependent transcription [10–15]. This pattern differs from that of its homologous partner RUVBL1 [8]. Thus, RUVBL2 may contribute to tumorigenesis and cancer development; indeed, RUVBL2 overexpression has been reported in HCC, colorectal cancer, renal cell carcinoma, gastric cancer, breast cancer and salivary gland cancer [11, 16–20].

Silencing RUVBL2 in HCC cells reduced cell growth, increased apoptosis and induced cell senescence and migration; therefore, it is associated with poor prognosis and chemoresistance [16, 21–23]. In addition, RUVBL2 controlled glucose and lipid metabolism and contributed to the pathogenesis of insulin resistance and non-alcoholic fatty liver disease via mTOR and PI3K–AKT pathways [24, 25]. However, the previous studies mainly investigated the mRNA expression characteristics of RUVBL2 in limited HCC samples using real-time reverse transcript-PCR, while its protein expression levels was detected in only 20 clinical samples by immunohistochemical staining [16, 23]. In addition, the possible transcriptional and epigenetic regulation mechanism of RUVBL2 remains unclear. In this study, we analyzed the mRNA expression characteristics and expression regulation of RUVBL2 in HCC using multiple datasets from The Cancer Genome Atlas (TCGA) and investigated the function, clinical and prognostic significance of RUVBL2 protein using immunohistochemical staining and functional assays.

Methods

TCGA data mining

The RNA sequencing, somatic copy number alteration, DNA methylation data and clinical information from 371 patients with liver cancer were obtained from TCGA (https://tcga-data.nci.nih.gov). The samples contained 361 HCC, seven hepatocellulargiroma carcinoma (mixed), and three fibrolamellar carcinoma cases. Among them, 355 HCC cases had detailed clinical and follow-up information. The median duration of patient follow-up was 20 months.

Clinical samples

Formalin-fixed paraffin embedded (FFPE) tissue samples were collected after approval from the Institutional Review Board of the Cancer Institute and Hospital of Chinese Academy of Medical Sciences (Beijing, China). All patients were diagnosed as HCC by two senior pathologists and had not received chemo/radiotherapy before surgical operation. A total of 153 HCC tumor samples and paired adjacent nontumor liver tissue samples were collected (143 male, 10 female; median age, 54 ± 11 SD; range 31–83 years) during the period from March 2004 to September 2008. Among them, 81.7% (125/153) patients were HBsAg positive, whereas 8.5% (13/153) patients were HCV positive. Additionally, 9.2% (14/153) of the cases had no histologic evidence of cirrhosis, whereas 43.1% (66/153), 24.2% (37/153) and 23.5% (36/153) of cases showed mild, moderate and severe cirrhosis, respectively. Furthermore, 98.0% (150/153) of cases were Child–Pugh Grade A, whereas 2.0% (3/153) of them were Grade B. The median follow-up time of all patients was 62 months (range 7 months to 165 months).

Immunohistochemistry

The tissue slides were deparaffinized and rehydrated at room temperature, then immersed in methanol containing 3% hydrogen peroxide for 10 min to block endogenous peroxidase. Heat-induced epitope retrieval was performed in a water bath for 30 min in an antigen retrieval solution (0.1 M sodium citrate buffer, pH 6.0). After washing, the sections were incubated overnight with anti-RUVBL2 antibody (1:80 dilution, Cat No. 14195-1-AP; ProteinTech Group Inc., Chicago, IL, USA) at 4 °C. The staining was performed using the Prolink-1 Plus HRP rabbit polymer detection kit (Golden Bridge International Inc., Bothell, WA, USA) according to the manufacturer’s instructions. The images were captured using Aperio ScanScope CS software (Vista, CA, USA).

The results were evaluated separately by two independent pathologists. The RUVBL2 staining intensity and area were quantified as described previously [26]. Briefly, RUVBL2 staining area was scored as follows: 0, <5% of the epithelial cells in the respective lesions; 1, 5–25% of the epithelial cells; 2, 26–50% of the epithelial cells; 3, 51–75% of the epithelial cells; and 4, ≥75% of the epithelial cells. The intensity was graded as follows: 0, negative;
1+, weak (yellow); 2+, moderate (light brown); and 3+, strong (dark brown). A final score between 0 and 12 was achieved by multiplication of the extent of positivity and intensity. A staining index was used in which 0 was considered negative, 1–3 was weak, and ≥4 was considered strong expression.

**Transient transfection and Western blot analysis**

The human liver cancer cell lines HepG2 and Huh7 were purchased from the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences (Shanghai, China) and maintained in recommended media at 37 °C with 5% CO₂. The cells were authenticated via short tandem repeat profile, which was performed by Microread Corporation in Beijing, China.

Two specific siRNA duplexes targeting RUVBL2 mRNA (RefSeq#: NM_006666.2) were designed and synthesized by GenePharma (Shanghai, China). The siRUVBL2-1 and siRUVBL2-2 sequences were 5’-CCGUCCGGGCAG UCCUUAU-3’ and 5’-CCAUCGGCGUUCGCAUC A-3’, respectively. As a control, a scrambled sequence of 5’-UUUCUGAGCUUGUACGU-3’ was used. The siRNAs were transiently transfected into HepG2 and Huh7 cells with Lipofectamine 2000 (Invitrogen, MA, USA) according to the manufacturer’s protocol.

Cells were lysed using a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, and protease inhibitor cocktail (Roche, Germany). Protein samples were loaded to SDS-PAGE gel and then transferred to polyvinylidene difluoride membranes. After blocking with 10% nonfat milk in PBS-T (0.1% Tween-20), the membranes were incubated with the following antibodies: anti-RUVBL2, anti-ERK (anti-MAPK1), anti-p-ERK, anti-AKT, anti-p-AKT, anti-p-CDC37 (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA); anti-p-HSP90, anti-CDC37 (1:1000 dilution; Santa Cruz Biotechnology, Dallas, TX, USA); anti-HSP90 (1:1000 dilution; Abcam, Cambridge, UK); and anti-β-actin (1:5000 dilution; Sigma-Aldrich, St. Louis, MO, USA). Following intensive washing, the membranes were developed and visualized with the ImageQuant LAS4000 system (GE Healthcare, Chicago, IL, USA).

**Cellular proliferation and colony formation assays**

Huh7 and HepG2 cells were transfection with scrambled control or specific siRNA oligonucleotides against RUVBL2 and seeded at a density of 3000 per well in 96-well plates. Cell viability was measured by cell counting kit-8 (CCK-8) assay (Dojindo, Japan). The absorbance at 450 nm was measured using a microplate reader.

The colony formation assay was performed in 6-well plates in which 1000 cells were seeded per well and cultured for 2 weeks. Colonies were counted manually after staining with 0.5% crystal violet.

**Migration and invasion assay**

Twenty-four hours after transfection with siRUVBL2-1, siRUVBL2-2 or scrambled control, 10,000 HuH7 cells or 50,000 HepG2 cells were added to the upper chamber containing 200 μL of serum-free medium; then, 600 μL of complete medium containing 10% FBS was added into the lower chambers as a chemoattractant. After 24 h of incubation, the upper chambers were stained with 0.25% crystal violet. The cells that penetrated through the membrane were observed under microscope and manually counted within eight random 100× fields. Transwell invasion assays were performed in the same protocol as the migration assay with the exception that the inserts were precoated with 30 μg of Matrigel (Corning Incorporated, NY, USA) in culture medium.

**Statistical analysis**

The Mann–Whitney U test, Wilcoxon signed-rank test or Kruskal–Wallis test was used to compare the Read per Million (RPM) values among two or multiple groups. In addition, the correlation coefficients of log2-transformed RPM values were calculated by Spearman’s rank correlation. Chi-square test was used to compare qualitative data. The Kaplan–Meier method combined with log-rank analysis was used to determine the relationship between the levels of RUVBL2 and patient survival. Univariate and multivariate survival analyses were performed using the Cox regression model. P values <0.05 were considered significant. All analyses were performed and visualized using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA, USA).

**Results**

**RUVBL2 mRNA was significantly upregulated in liver cancer tissues**

According to the RNA sequencing data from TCGA, we first observed RUVBL2 expression between primary tumor and paired adjacent noncancerous tissues (n = 50). RUVBL2 mRNA was significantly upregulated in tumor tissues (Fig. 1a, P < 0.0001). Moreover, when the samples of tumor tissues were expanded to 371 cases, RUVBL2 mRNA remained at an approximately 1.3-fold increase in HCC (Fig. 1b, P < 0.0001).

The correlation analyses between the clinical features and the RUVBL2 mRNA levels in the tumors showed that RUVBL2 expression was associated with sex, race, drinking status and differentiation degree (Fig. 1b, c). The male and Asian patients with drinking habits had higher levels of RUVBL2 mRNA than the female and Caucasian patients without alcohol-related
liver diseases (all, \( P < 0.05 \)). In addition, \( RUVBL2 \) levels were higher in poorly differentiated tumors compared with those that were well differentiated (\( P = 0.0344 \)). However, the correlation between \( RUVBL2 \) mRNA and other features, such as age, vascular invasion, Child–Pugh classification, TNM staging, hepatic fibrosis degree, serum AFP levels and hepatic inflammation in adjacent liver tissue, was not observed.

Based on the quartile RPM values of \( RUVBL2 \) in tumor tissues, all 355 HCC cases with available follow-up information were divided into two groups: the high-expression group (top 25%) and the low-expression group (bottom 75%). Kaplan–Meier survival analysis with a log-rank test showed that \( RUVBL2 \) expression was not associated with the overall survival of the patients with liver cancer (\( P = 0.1525 \); Fig. 1d). However, there was a significant correlation between high \( RUVBL2 \) mRNA levels and a shorter recurrence-free survival time (\( P = 0.0132 \); Fig. 1e). The median relapse periods of high- and low-expression groups were 15.2 and 29.3 months, respectively.

**Aberrant transcriptional regulation of \( RUVBL2 \) mRNA in liver cancer**

To clarify why the \( RUVBL2 \) gene is overexpressed in liver cancer, we first observed the methylation of its promoter. As shown in Fig. 2a, the methylation level of \( RUVBL2 \) was weakly inversely correlated with its mRNA levels, suggesting that promoter hypomethylation may participate in the overexpression of \( RUVBL2 \) gene (Pearson correlation coefficient \( = -0.2354; P < 0.0001 \)). When the copy number alterations of \( RUVBL2 \) were compared with its expression levels, a weak positive correlation was observed (Spearman rank correlation coefficient \( = 0.3390, P < 0.0001 \)). The gain of chromosome region near \( RUVBL2 \) gene showed significantly higher mRNA expression than diploid and hemizygous deletion (Fig. 2b, \( P < 0.0001 \)).

The previous studies found that \( RUVBL2 \) physically interacts with a number of transcriptional factors (TFs), including CTNNB1, TATA-box binding protein (TBP), MYC, E2F1, ATF2 and HIF1A [6, 13, 27], and MYC transcriptionally activated \( RUVBL2 \) [28]. Given that MYC amplification and CTNNB1 mutation are the driver alterations in liver cancer, we thus detected whether these
|                          | Nuclear RUVBL2 |                     | P value | Cytoplasmic RUVBL2 |                     | P value |
|--------------------------|----------------|---------------------|---------|---------------------|---------------------|---------|
|                          | Negative | Weak | Strong |  | Negative | Weak | Strong |  |
| Age (year)               |          |       |        | 0.6548              |          |       |        | 0.3616 |
| < 60                     | 55 (53.4)| 32  (31.1)| 16 (15.5)| | 4 (3.9) | 42 (40.8)| 57 (55.3)| |
| ≥ 60                     | 23 (46.0)| 17 (34.0)| 10 (20.0)| | 3 (6.0) | 24 (48.0)| 23 (46.0)| |
| Sex                      |          |       |        | 0.6956              |          |       |        | 0.6332 |
| Male                     | 74 (51.7)| 44 (30.8)| 25 (17.5)| | 7 (4.9) | 60 (42.0)| 76 (53.1)| |
| Female                   | 4 (4.0)  | 5 (5.0)  | 1 (10.0) | | 0 (0.0) | 6 (6.0)  | 4 (4.0)  | |
| Family history           |          |       |        | 0.3719              |          |       |        | 0.4666 |
| Yes                      | 56 (52.8)| 35 (33.0)| 15 (14.2)| | 3 (2.8) | 45 (42.5)| 58 (54.7)| |
| No                       | 22 (46.8)| 14 (29.8)| 11 (23.4)| | 4 (8.5) | 21 (44.7)| 22 (46.8)| |
| Symptomatic presentation |          |       |        | 0.1776              |          |       |        | 0.9539 |
| No                       | 54 (56.8)| 27 (28.4)| 14 (14.7)| | 7 (7.4) | 39 (41.1)| 49 (51.6)| |
| Yes                      | 24 (41.4)| 22 (37.9)| 12 (20.7)| | 0 (0.0) | 27 (46.6)| 31 (53.4)| |
| Drinking                 |          |       |        | 0.0316              |          |       |        | 0.3927 |
| No                       | 59 (52.7)| 30 (26.8)| 23 (20.5)| | 5 (4.5) | 52 (46.4)| 55 (49.1)| |
| Infrequent               | 17 (47.2)| 16 (44.4)| 3 (8.3)  | | 2 (5.6) | 11 (30.6)| 23 (63.9)| |
| Frequent                 | 2 (40.0) | 3 (60.0) | 0 (0.0)  | | 0 (0.0) | 3 (60.0) | 2 (40.0) | |
| HBsAg                    |          |       |        | 0.1774              |          |       |        | 0.3722 |
| Negative                 | 12 (42.9)| 13 (46.4)| 3 (10.7) | | 0 (0.0) | 14 (50.0)| 14 (50.0)| |
| Positive                 | 66 (52.8)| 36 (28.8)| 23 (18.4)| | 7 (5.6) | 52 (41.6)| 66 (52.8)| |
| Anti-HCV                 |          |       |        | 0.9411              |          |       |        | 0.4514 |
| Negative                 | 72 (51.4)| 44 (31.4)| 24 (17.1)| | 7 (5.0) | 58 (41.4)| 75 (53.6)| |
| Positive                 | 6 (46.2) | 5 (38.5) | 2 (15.4) | | 0 (0.0) | 8 (61.5) | 5 (38.5) | |
| Cirrhosis                |          |       |        | 0.0416              |          |       |        | 0.2321 |
| w/o + mild               | 33 (41.3)| 33 (41.3)| 14 (17.5)| | 3 (3.8) | 31 (38.8)| 46 (57.5)| |
| Moderate                 | 23 (62.2)| 7 (18.9)| 7 (18.9) | | 3 (8.1) | 19 (51.4)| 15 (40.5)| |
| Severe                   | 22 (61.1)| 9 (25.0)| 5 (13.9) | | 1 (2.8) | 16 (44.4)| 19 (52.8)| |
| CEA (ng/ml)              |          |       |        | 0.8181              |          |       |        | 0.8920 |
| ≤ 5                      | 72 (51.1)| 48 (34.0)| 21 (14.9)| | 5 (3.5) | 63 (44.7)| 73 (51.8)| |
| > 5                      | 6 (50.0) | 1 (8.3)  | 5 (41.7) | | 2 (16.7) | 3 (25.0)| 7 (58.3)| |
| ALP                      |          |       |        | 0.8388              |          |       |        | 0.9196 |
| Normal                   | 71 (51.1)| 46 (33.1)| 22 (15.8)| | 5 (3.6) | 61 (43.9)| 73 (52.5)| |
| Aberrant                 | 7 (50.0) | 3 (21.4) | 4 (28.6) | | 2 (14.3) | 5 (35.7)| 7 (50.0)| |
| PT(a) (%)                |          |       |        | 0.4977              |          |       |        | 0.2135 |
| ≥ 80                     | 51 (54.3)| 26 (27.7)| 17 (18.1)| | 4 (4.3) | 36 (38.3)| 54 (57.4)| |
| < 80                     | 27 (47.4)| 21 (36.8)| 9 (15.8) | | 3 (5.6) | 28 (30.6)| 26 (63.9)| |
| AFP (ng/ml)              |          |       |        | 0.2972              |          |       |        | 0.1644 |
| ≤ 20                     | 33 (44.0)| 26 (34.7)| 16 (21.3)| | 4 (5.3) | 27 (36.0)| 44 (58.7)| |
| > 20                     | 41 (55.4)| 23 (31.1)| 10 (13.5)| | 3 (4.1) | 37 (50.0)| 34 (45.9)| |
| AFP (ng/ml)              |          |       |        | 0.7712              |          |       |        | 0.5453 |
| ≤ 400                    | 57 (51.4)| 35 (31.5)| 19 (17.1)| | 5 (4.5) | 50 (45.0)| 56 (50.5)| |
| > 400                    | 17 (44.7)| 14 (36.8)| 7 (18.4) | | 2 (5.3) | 14 (36.8)| 22 (57.9)| |
| Differentiation grade    |          |       |        | 0.5340              |          |       |        | 0.0016 |
| Well                     | 15 (57.7)| 8 (30.8)| 3 (11.5) | | 5 (19.2)| 14 (53.8)| 7 (26.9) | |
| Moderate                 | 48 (51.6)| 31 (33.3)| 14 (15.1)| | 1 (1.1) | 44 (47.3)| 48 (51.6)| |
| Poor                     | 15 (44.1)| 10 (29.4)| 9 (26.5) | | 1 (2.9) | 8 (23.5)| 25 (73.5)| |
| Tumor size (cm)          |          |       |        | 0.6152              |          |       |        | 0.6543 |
| ≤ 5                      | 53 (52.5)| 33 (32.7)| 15 (14.9)| | 6 (5.9) | 44 (43.6)| 51 (50.5)| |
| > 5                      | 25 (48.1)| 16 (30.8)| 11 (21.2)| | 1 (1.9) | 22 (42.3)| 29 (55.8)| |
two TFs influenced RUVBL2 expression. Intriguingly, the patients with MYC gain and amplification showed higher levels of RUVBL2 than those without this amplification (Fig. 2c–d). Furthermore, the individuals with CTNNB1 mutation had higher levels of RUVBL2 mRNA than those without the mutation (Fig. 2e, \( P = 0.0076 \)). However, there was an inverse correlation between the expression of RUVBL2 and CTNNB1 (Pearson correlation coefficient \( r = -0.2580; P < 0.0001 \); Fig. 2f). Taken together, RUVBL2 overexpression in liver cancer is caused by a variety of reasons, including promoter hypomethylation, chromosome gain and transcriptional regulation of TFs, etc.

RUVBL2 protein was significantly overexpressed in HCC
To further clarify the expression characteristics of RUVBL2 protein, an immunohistochemistry assay was performed in HCC tumor and adjacent noncancerous tissues \((n=153)\). In the adjacent noncancerous tissues, RUVBL2 was strongly stained in the bile duct epithelial cells (Fig. 3a), while the positive rate of hepatocytes was 80.0% (116/145), and the staining was mainly localized to cytoplasm (Fig. 3a, c and e). In these 116 samples, 65.5% (76/116) and 35.5% (40/116) showed weak and strong expression, respectively. Additionally, 35.2% (51/145) of the cases showed positive nuclear staining of RUVBL2. The weak and strong nuclear staining comprised 84.3% (43/51) and 15.7% (8/53) of cases, respectively.

In contrast, positive cytoplasmic and nuclear immunostaining for RUVBL2 was observed in 95.4% (146/153) and 46.0% (75/153) of the HCC tumors, respectively (Fig. 3b, d and f). In the positive cases that showed cytoplasmic staining for RUVBL2, 66 (45.2%) and 80 (54.8%) had weak and strong expression, respectively. Moreover, with regard to the nuclear staining, 49 (65.3%) and 26 (34.7%) had weak and strong expression, respectively. Collectively, apparent cytoplasmic and nuclear overexpression of RUVBL2 protein was found in HCC tissues (Chi-square test, \( P < 0.0001 \) for the cytoplasm and \( P = 0.0037 \) for the nucleus).

Clinical significance of RUVBL2 protein in HCC
The correlations between the clinicopathological characteristics of HCC patients and cytoplasmic and nuclear expression of RUVBL2 were subsequently analyzed (Table 1). Higher nuclear RUVBL2 levels were associated with drinking alcohol \( (P = 0.0316) \), whereas the patients with moderately and severe cirrhosis had lower levels of nuclear RUVBL2 \( (P = 0.0416) \). Higher cytoplasmic

Table 1 (continued)

|                | Nuclear RUVBL2 |       |       |       | Cytoplasmic RUVBL2 |       |       |       |
|----------------|----------------|-------|-------|-------|--------------------|-------|-------|-------|
|                | Negative       | Weak  | Strong|       | Negative           | Weak  | Strong|       |
|                |                |       |       |       |                    |       |       |       |
| Multinodules   |                |       |       |       |                    |       |       |       |
| No             | 62 (50.8)      | 39 (32.0) | 21 (17.2) |   | 5 (4.1)          | 54 (44.3) | 63 (51.6) |
| Yes            | 16 (51.6)      | 10 (32.3) | 5 (16.1)  |   | 2 (6.5)          | 12 (38.7) | 17 (54.8) |
| Tumor-infiltrating lymphocytes |       |       |       |       |                    |       |       |       |
| No             | 57 (50.4)      | 35 (31.1) | 21 (18.6) |   | 6 (5.3)          | 49 (43.4) | 58 (51.3) |
| Yes            | 21 (52.5)      | 14 (35.0) | 5 (20.7)  |   | 1 (2.5)          | 17 (42.5) | 22 (55.0) |
| Liver capsule invasion |       |       |       |       |                    |       |       |       |
| No             | 48 (52.7)      | 27 (29.7) | 16 (17.6) |   | 5 (5.5)          | 40 (44.0) | 46 (50.5) |
| Yes            | 29 (47.5)      | 22 (36.1) | 10 (16.4) |   | 2 (3.3)          | 26 (42.6) | 33 (54.1) |
| Carcinoma cell embolus |       |       |       |       |                    |       |       |       |
| No             | 66 (48.9)      | 45 (33.3) | 24 (17.8) |   | 7 (5.2)          | 61 (45.2) | 67 (49.6) |
| Yes            | 12 (66.7)      | 4 (22.2)  | 2 (11.1)  |   | 0 (0.0)          | 5 (27.8)  | 13 (72.2) |
| TNM staging    |                |       |       |       |                    |       |       |       |
| I              | 55 (49.1)      | 36 (32.1) | 21 (18.8) |   | 4 (3.6)          | 51 (45.5) | 57 (50.9) |
| II             | 11 (52.4)      | 8 (38.1)  | 2 (9.5)   |   | 2 (9.5)          | 9 (42.9)  | 10 (47.6) |
| III            | 9 (56.3)       | 4 (25.0)  | 3 (18.8)  |   | 0 (0.0)          | 6 (37.5)  | 10 (62.5) |
| IV             | 3 (75.0)       | 1 (25.0)  | 0 (0.0)   |   | 1 (25.0)         | 0 (0.0)   | 3 (75.0)  |
| BCLC staging   |                |       |       |       |                    |       |       |       |
| 0              | 4 (66.7)       | 0 (0.0)   | 2 (33.3)  |   | 0 (0.0)          | 3 (50.0)  | 3 (50.0)  |
| 1              | 57 (47.9)      | 41 (34.5) | 21 (17.6) |   | 7 (5.9)          | 53 (44.5) | 59 (49.6) |
| 2              | 12 (54.5)      | 7 (31.8)  | 3 (13.6)  |   | 0 (0.0)          | 8 (36.4)  | 14 (63.6) |
| 3              | 5 (83.3)       | 1 (16.7)  | 0 (0.0)   |   | 0 (0.0)          | 2 (33.3)  | 4 (66.7)  |
RUVBL2 levels were related to poor pathological differentiation ($P = 0.0016$).

However, there was no correlation between RUVBL2 protein and hepatitis virus infection, AFP, CEA, tumor size, multinodules, liver capsule invasion, carcinoma cell embolus, tumor-node-metastasis (TNM) stages or Barcelona Clinic Liver Cancer (BCLC) stages.

**Prognostic relevance of RUVBL2 protein in HCC**

To investigate the prognostic relevance of RUVBL2 protein in HCC, we performed Kaplan–Meier survival analysis with a log-rank test for nuclear and cytoplasmic expression levels. For nuclear staining, strong RUVBL2 expression was significantly associated with a shorter overall survival time ($P = 0.0050$; Fig. 4a). The median survival times of the strong and the negative/weak expression groups were 58 and 96 months, respectively. However, RUVBL2 expression was not associated with recurrence-free survival ($P = 0.1457$; Fig. 4b). For cytoplasmic staining, RUVBL2 expression had no significant influence on overall survival ($P = 0.0817$; Fig. 4c), whereas the patients with strong cytoplasmic RUVBL2 had significantly lower recurrence-free survival compared with those with negative and weak expression, with a median time to relapse of 26 months vs. 58 months ($P = 0.0074$; Fig. 4d).

**RUVBL2 is an independent prognostic factor for HCC patients**

The subsequent univariate Cox regression analysis showed that patients with the strong nuclear RUVBL2 expression exhibited a 2.03-fold increase of a hazard ratio (HR) with a 95% confidence interval (CI) of 1.22 to 3.37 for overall survival (Table 2; $P = 0.0064$), compared with the negative/weak expression group. Other significant risk factors for overall survival included differentiation grade (HR = 1.30, 95% CI 1.02–1.66; $P = 0.0363$), tumor size (HR = 1.57, 95% CI 1.01–2.44; $P = 0.0459$), carcinoma cell embolus (HR = 2.19, 95% CI 1.23–3.90; $P = 0.0080$), TNM staging (HR = 1.65, 95% CI 1.25–2.19; $P < 0.0001$) and BCLC staging (HR = 2.45, 95% CI 1.49–4.04; $P < 0.0001$). As indicated by the multivariate analysis, strong nuclear RUVBL2 expression (HR = 2.47, 95% CI 1.47–4.14; $P = 0.0007$), carcinoma cell embolus (HR = 1.84, 95% CI 1.01–3.36; $P = 0.0451$) and BCLC staging (HR = 2.32, 95% CI 1.59–3.40; $P < 0.0001$) were independent prognostic factors for overall survival.

For the recurrent-free survival, in the univariate analysis, cytoplasmic RUVBL2 expression (HR = 1.71, 95% CI 1.44–2.56; $P = 0.0088$), differentiation grade (HR = 1.27, 95% CI 1.01–1.60; $P = 0.0409$), tumor size (HR = 1.69, 95% CI 1.13–2.53; $P = 0.0107$), carcinoma cell embolus (HR = 2.66, 95% CI 1.56–4.53; $P = 0.0003$), TNM staging (HR = 1.54, 95% CI 1.18–2.00; $P = 0.0014$) and BCLC staging (HR = 2.10, 95% CI 1.30–3.38; $P = 0.0023$) were associated with the increased risk of
relapse (Table 2). Cytoplasmic RUVBL2 expression (HR = 1.56, 95% CI 1.04–2.36; P = 0.0336) was considered an independent recurrent factor, whereas tumor size (HR = 1.45, 95% CI 0.95–2.23; P = 0.0887), carcinoma cell embolus (HR = 1.75, 95% CI 0.97–3.19; P = 0.0652) and BCLC staging (HR = 1.59, 95% CI 0.94–2.67; P = 0.0811) showed marginal correlations.

**RUVBL2 promotes cell malignant phenotypes through activating HSP90-CDC37, AKT and ERK pathways**

To investigate the potential function of RUVBL2 during hepatocarcinogenesis, we knocked down RUVBL2 expression in multiple cells. As shown in Fig. 5a–f, the short-term growth, long-term survival, migration and invasion abilities were markedly inhibited after the
transfection with specific siRNA against RUVBL2 compared with scrambled-transfected cells.

To gain insight into these mechanisms, we observed the expression levels of phosphorylated-CDC37 (p-CDC37), total CDC37, phosphorylated-HSP90 (p-HSP90), total HSP90, phosphorylated-ERK (p-ERK), total ERK, phosphorylated-AKT (p-AKT) and total AKT in RUVBL2 depleted cells. There was a significant trend of a decrease for p-CDC37, p-HSP90, p-AKT and p-ERK at 24 h after the transfection of RUVBL2 siRNA (Fig. 5g, h). Thus, it appears that RUVBL2 overexpression in HCC strengthens the proliferation, survival, migration and invasion of HCC cells through activating the HSP90-CDC37, AKT and ERK signaling pathways.

**Discussion**

In the present study, we found that RUVBL2 mRNA was upregulated in HCC tissues, and promoter hypomethylation, copy number gain, MYC amplification and CTNNB1 mutation all contributed to its deregulation. High levels of RUVBL2 mRNA were associated with shorter recurrence-free survival time but not overall survival time. Furthermore, RUVBL2 protein that was localized at both the nucleus and cytoplasm was also overexpressed in HCC samples. Strong nuclear staining of RUVBL2 predicted worse overall survival, whereas strong intensity of cytoplasmic RUVBL2 was an independent unfavorable prognostic factor for recurrence-free survival.

Our results showed that high RUVBL2 mRNA expression was associated with poor differentiation of HCC tumor, which is in agreement with a previous report [16]. However, this finding is at odds with previous observations that RUVBL2 mRNA expression is significantly lower in HBV-related HCC [16]. We observed that male patients with an Asian background and a drinking habit had a higher expression of RUVBL2 mRNA. The difference might be a result of the different race and etiology composition of clinical samples. Actually, when the Asian and Caucasian people were separately analyzed, we found that RUVBL2 mRNA levels were indeed lower in
only HBV-infected Caucasian patients (Additional file 1: Figure S1), which is consistent with the previous finding [16]. This result suggested that the expression patterns and roles of RUVBL2 in hepatocarcinogenesis might be different in different ethnic groups.

Additionally, although RUVBL2 is a known transcription factor, the transcriptional regulation of RUVBL2 itself remains unknown. Here, based on the multiple sequencing datasets from TCGA, we found that the methylation status and copy number alterations of RUVBL2 gene, MYC amplification as well as CTNNB1 mutation were all increased the mRNA expression of RUVBL2. Interestingly, RUVBL2 expression negatively correlated with CTNNB1 expression. Further analyses suggested that wild-type CTNNB1 repressed the transcription of RUVBL2, whereas CTNNB1 mutation might lose this function (Additional file 1: Figure S2). To our knowledge, this report is the first observation of transcriptional regulation of RUVBL2 in tumors.

Intriguingly, most studies addressed that RUVBL2 is a nuclear protein. However, accumulating evidence has demonstrated the definite cytoplasmic staining of RUVBL2 protein, especially in malignant cells [6, 8, 16, 18]. We found that both cytoplasmic and nuclear staining of RUVBL2 was significantly increased in HCC. When cytoplasmic and nuclear staining were separately

Table 2 Univariate Cox regression analysis of overall and recurrence-free survival in 153 HCC patients

| Variables                        | Overall survival | Relapse-free survival |
|----------------------------------|------------------|-----------------------|
|                                  | P-value | HR | 95% CI | P-value | HR | 95% CI |
| Nuclear RUVBL2 (strong vs. Neg + weak) | 0.0064   | 2.03 | 1.22–3.37 | 0.1522 | 1.42 | 0.88–2.31 |
| Cytoplasmic RUVBL2 (strong vs. Neg + weak) | 0.0862 | 1.47 | 0.95–2.27 | 0.0088 | 1.71 | 1.44–2.56 |
| Age (> 60 vs. ≤ 60)               | 0.7896   | 1.06 | 0.68–1.66 | 0.6266 | 1.11 | 0.73–1.67 |
| Sex (female vs. male)             | 0.2357   | 0.54 | 0.20–1.49 | 0.0713 | 0.40 | 0.15–1.08 |
| Family history (yes vs. no)      | 0.0943   | 0.66 | 0.41–1.07 | 0.0590 | 0.65 | 0.42–1.02 |
| Symptom presentation (yes vs. no) | 0.0864   | 1.46 | 0.95–2.26 | 0.1258 | 1.36 | 0.92–2.03 |
| Drinking (yes vs. no)            | 0.7888   | 0.94 | 0.62–1.44 | 0.7493 | 0.94 | 0.65–1.37 |
| HBsAg (positive vs. negative)    | 0.1842   | 0.71 | 0.42–1.18 | 0.4802 | 0.84 | 0.51–1.37 |
| Anti-HCV (positive vs. negative) | 0.5942   | 1.23 | 0.57–2.68 | 0.7104 | 1.15 | 0.56–2.37 |
| Cirrhosis (moderate + severe vs. w/o + mild) | 0.0545 | 1.52 | 0.99–2.34 | 0.1645 | 1.32 | 1.89–1.96 |
| CEA (> 5 vs. ≤ 5)                | 0.3999   | 1.40 | 0.64–3.04 | 0.3377 | 1.40 | 0.70–2.78 |
| ALP (aberrant vs. normal)        | 0.9062   | 0.96 | 0.50–1.84 | 0.3606 | 1.29 | 0.75–2.24 |
| AFP (> 20 vs. ≤ 20)              | 0.6581   | 1.10 | 0.71–1.70 | 0.8061 | 1.05 | 0.71–1.56 |
| Differentiation grade (poor vs. well + moderate) | 0.0363 | 1.30 | 1.02–1.66 | 0.0409 | 1.27 | 1.01–1.60 |
| Tumor size (> 5 cm vs. ≤ 5 cm)   | 0.0459   | 1.57 | 1.01–2.44 | 0.0417 | 1.69 | 1.13–2.53 |
| Multinodules (yes vs. no)        | 0.0853   | 1.56 | 0.94–2.58 | 0.1152 | 1.46 | 0.91–2.36 |
| Tumor-infiltrating lymphocytes (yes vs. no) | 0.7268 | 1.09 | 0.67–1.76 | 0.4599 | 1.18 | 0.76–1.84 |
| Liver capsule invasion (yes vs. no) | 0.4503 | 0.84 | 0.54–1.31 | 0.7892 | 0.95 | 0.63–1.42 |
| Carcinoma cell embolus (yes vs. no) | 0.0080 | 2.19 | 1.23–3.90 | 0.0003 | 2.66 | 1.56–4.53 |
| TNM staging (III + IV vs. I + II) | < 0.0001 | 1.65 | 1.25–2.19 | 0.0014 | 1.54 | 1.18–2.00 |
| BCLC staging (2 + 3 vs. 0 + I)   | < 0.0001 | 2.45 | 1.49–4.04 | 0.0023 | 2.10 | 1.30–3.38 |

(See figure on next page.)

Fig. 5 Knockdown of RUVBL2 expression inhibited cell proliferation and survival. a, d Cell proliferation was measured with CCK-8 assay at indicated times after the transfection of specific siRNA duplexes against RUVBL2 in HepG2 (a) and Huh7 (d) cells. The absorbance is shown as the mean ± standard error for each day. b, e The colony formation assay in HepG2 (b) and Huh7 (e) at 24 h after the transfection of specific siRNA duplexes against RUVBL2. Representative dishes are shown in the left panel, and quantitative colony numbers are compared in the right panel. c, f The migration and invasion assays (left panels) were performed in HepG2 (c) and Huh7 (f) at 24 h after the transfection of specific siRNA duplexes against RUVBL2. Cell migration and invasion capability is shown in the right panel by counting cells per field. For (a–f), Scr, scrambled negative control; siR-1, siRUVBL2-1; siR-2, siRUVBL2-2; *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. g, h Western blot analysis of cell proliferation- and survival-associated signaling genes in HepG2 (g) and Huh7 (h) at 24 h post-transfection with specific siRNA duplexes against RUVBL2. Densitometry was performed to quantify each lane, and the ratio of each protein over the loading control β-actin is presented under each blot, with the ratio in the scramble group being the reference value.
assessed, it was found that positive nuclear and cytoplasmic expression had different clinical and prognostic significance. It appears that patients with a cirrhotic background had decreased levels of nuclear RUVBL2, while poorly differentiated tumors showed dramatically increased levels of cytoplasmic RUVBL2. In addition, nuclear and cytoplasmic RUVBL2 independently indicated a worse overall survival and recurrence-free survival, respectively. According to the known functions of RUVBL2, nuclear forms might regulate DNA replication, chromatin remodeling, biogenesis of small nucleolar ribonucleoprotein (RNP) and small nuclear RNP, assembly of the telomerase complex, transcriptional regulation and DNA damage repair [8, 29]. Moreover, cytoplasmic RUVBL2 can interact with phosphatidylinositol 3-kinase-related protein kinase (PIKK) signaling family proteins to sense cellular nutrients and energy levels and to modulate the nonsense-mediated decay of mRNAs [30, 31]. RUVBL2 in the mitochondrion binds to mitochondrial DNA polymerase gamma (POLG) to participate in mitochondrial biogenesis [32]. Therefore, cytoplasmic RUVBL2 protein may play different roles from its nuclear forms in carcinogenesis; however, its cytoplasmic functions so far were not yet determined.

Furthermore, we found that high levels of RUVBL2 facilitated HCC cell proliferation, survival, migration and invasion. Previous studies reported that HSP90 can form complexes with RUVBL2 [31]; thus, we investigated the effect of RUVBL2 knockdown on HSP90-CDC37 complexes and the downstream pathways in HepG2 and Huh7 cells. We found that RUVBL2 depletion significantly attenuated the phosphorylation of HSP90, CDC37, ERK and AKT proteins (Fig. 5g, h). AKT is known as a HSP90 client kinase [33, 34], and CDC37 can stabilize ERK and AKT kinase activities in numerous cancer cells [34–36]. Therefore, these results demonstrated that RUVBL2 contributes to hepatocarcinogenesis via HSP90-CDC37, AKT and ERK pathways.

Conclusions
We systematically investigated the mRNA and protein expression characteristics of RUVBL2 in HCC in a relatively large number of clinical samples. The results showed that promoter hypomethylation, copy number gain, MYC amplification and CTNNB1 mutation were responsible for the overexpression of RUVBL2. The high levels of RUVBL2 promote tumorigenesis through activating HSP90-CDC37, AKT and ERK pathways. RUVBL2 protein was distributed to the nucleus and the cytoplasm of malignant hepatocytes, and its different localization indicated distinct clinical and prognostic features. Therefore, high levels of nuclear and cytoplasmic RUVBL2 could be used as independent prognostic factor for overall survival and recurrence-free survival in HCC patients, respectively. RUVBL2 may also be a promising target for HCC prevention and treatment.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12935-019-0974-z.

Additional file 1: Figure S1. RUVBL2 mRNA expression according to the viruses infection status in Asian (A) and Caucasian (B) liver cancer samples from TCGA. Figure S2. The correlation of RUVBL2 and CTNNB1 mRNA in liver cancer patients with wild-type CTNNB1 (A) and mutant CTNNB1 (B).

Authors’ contributions
TY: Performed the experiments, acquired and analyzed patient data; FL, JG and HL: Performed the experiments and collected the data; JC: Conceptualization; XZ: Study concept and design, funding acquisition; YS: Study concept and design, analysis of data, funding acquisition and drafting of the manuscript. All authors read and approved the final manuscript.

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Please contact the authors for data request.

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Competing interests
The authors declare that they have no competing interests.

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