Objective to identify and verify the regulatory mechanism of DTNBP1 as a prognostic marker for hepatocellular carcinoma

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Although the overall survival of hepatocellular carcinoma (HCC) patients has been significantly improved, prognostic clinical evaluation remains a substantial problem owing to the heterogeneity and complexity of tumor. A reliable and accurate predictive biomarker may assist physicians in better monitoring of patient treatment outcomes and follow the overall survival of patients. Accumulating evidence has revealed that DTNBP1 plays functional roles in cancer prognosis. Therefore, the expression and function of DTNBP1 in HCC was systematically investigated in our study. The expression and prognostic value of DTNBP1 were investigated using the data from Cancer Genome Atlas (TCGA) database, Gene Expression Omnibus (GEO) cohorts and clinical samples. A series of cellular function assays were performed to elucidate the effect of DTNBP1 on cellular proliferation, apoptosis and metastasis. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment and Protein–protein interaction (PPI) network construction were performed to screen the genes with highest interaction scores with DTNBP1. Finally, the underlying mechanism was also analyzed using Gene Set Enrichment Analysis (GSEA) and confirmed using RT-qPCR and western blotting. DTNBP1 was upregulated in many types of cancers, especially in HCC. The DTNBP1 expression levels is associated with clinicopathologic variables and patient survival status. The differential expression of DTNBP1 could be used to determine the risk stratification of patients with HCC. DTNBP1 deficiency inhibited cell proliferation and metastasis, but promoted cell apoptosis. Mechanistically, DTNBP1 regulated the cell cycle progression through affecting the expression of cell cycle-related genes such as CDC25A, CCNE1, CDK2, CDC20, CDC25B, CCNB1, and CDK1. DTNBP1, which regulates the cell cycle progression, may be used as a prognostic marker for HCC.

Globally, hepatocellular carcinoma (HCC) is the second leading cause of cancer-related deaths, thus necessitating development of more advanced treatment options and better treatment monitoring¹. However, due to the combination of adverse factors with a range of different biological and clinical behaviours and the increased resistance to anti-HCC drugs, existing targeted drugs have shown unsatisfactory efficacy². There is a lack of novel biomarkers for the evaluation of the clinical treatment of malignancies in HCC. The lack of specific biomarkers for tumor stages or cancer subtypes represents a key gap during HCC therapy. Thus, it is necessary to develop an independent prognostic biomarker at the molecular level to determine liver cancer prognosis, especially considering the genomic and host factors that drive the progression. Investigation of factors can enhance our understanding of liver cancer biology, enable the development of enhanced screening strategies, and improve patient prognosis.

Dysbindin-1 (dystrobrevin binding protein-1, DTNBP1) is widely identified as a schizophrenia susceptibility gene⁴,⁵. DTNBP1 expression level is significantly reduced in the brain of schizophrenia patients⁶. DTNBP1 expression disrupted both glutamatergic and gamma-aminobutyric acidergic transmission in the cerebral cortex⁷. Loss of DTNBP1 expression may result in development of deficient presynaptic vesicle transmission in the central nervous system⁸. The DTNBP1 mutation affected the GluN2B-GluN2A switch at the synapse in a

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expression level was semi-quantified using the formula: level = intensity score × positive rate × 100.

In brains, single-nucleotide polymorphisms within the gene DTNBP1 were significantly associated with attention, executive functioning, and memory scores in patients with brain tumors. However, the biological function of DTNBP1 in HCC remains unclear, and whether DTNBP1 can serve as a predictor for prognosis need to be determined.

In this study, for the first time, we found that the DTNBP1 levels in HCC tissues were significantly higher than those in normal liver tissues according to the Cancer Genome Atlas (TCGA) database. DTNBP1 deficiency inhibited cell proliferation and metastasis, but promoted cell apoptosis. Mechanistically, DTNBP1 regulated the cell cycle progression through affecting the expression of cell cycle-related genes such as CDC25A, CCNE1, CDK2, CDC20, CDC25B, CCNB1, and CDK1. The present study may reveal a novel biomarker for the prognosis assessment in HCC.

Methods
Data mining from TCGA and GEO databases. Gene expression profiles (https://portal.gdc.cancer.gov/) of 416 LICH patients in the cohort TCGA-LICH were investigated. The clinical and survival status information of 419 LIHC patients were downloaded from the UCSC Xena website (https://tcga.xenabubs.net with cohort: TCGA-LICH). Among them, 339 patients with whole clinical and survival data were subjected to further analysis. The raw counts of RNA were log-normalized and analyzed using the edge R package (3.30.3). The threshold was log2FC |(fold change)| > 0.5 p-value < 0.001. The DTNBP1 gene expression profile was analyzed using tools: GEPIA (http://geopia.cancer-pku.cn/). The GEO dataset GSE45436 (NT41, HCC62), GSE46408 (NT6, HCC6), GSE64041 (normal5, tumor&non-tumor120), GSE76427 (NT52, HCC115), GSE101685 (normal8, HCC24), GSE112790 (normal15, HCC183) were downloaded from the GEO website to evaluate the difference in GNPNAT1 expression in normal tissues compared to that tumor tissues. The Human Protein Atlas (HPA) database (https://www.proteinatlas.org/) was used to investigate the DTNBP1 protein spatial expression in normal tissues and tumor tissues.

Construction of a predictive nomogram. Independent prognostic measures were performed to construct a nomogram using the software R: A Language and Environment for Statistical Computing 2020 (R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/) with the Regression Modeling Strategies package. The calibration plot was generated for estimating the calibration value of the nomogram.

Immunohistochemistry (IHC). The tissue microarray, which contained 75 samples obtained from HCC tissues and 75 samples obtained from corresponding adjacent normal tissues, was purchased from Shanghai Outdo Biotech Company (Shanghai, China). The tissue slides were deparaffinized, treated with 3% H2O2, sub-

Ethics approval and consent to participate. The tissue array was formed from tissues of consenting donors. The use of this array was approved by Ethics Committee of Shanghai Outdo Biotech Company (SHYJS-CP-1904064). The datasets accessed were publicly available and anonymized, and therefore formal ethical approval and informed consent was not required. All experiments were performed in accordance with relevant guidelines and regulations.

Cell lines and cell culture. Normal liver cell line THLE-2 and HCC cell lines (Hep3B, HepG2, Huh7, and PLC/PRF/5) were obtained from Xiamen ImmoCell Biotechnology Co., Ltd (Xiamen, China). All cells were cultured in DMEM (Gibco, Detroit, MI, USA) containing 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), and 2 mM l-glutamine (Gibco) at 37 °C in a 5% CO2 incubator.

shRNA transfection. The shRNA-1, shRNA-2, and shRNA-3 were synthesized by and purchased from GenScript Biotech (Nanjing, China) based on the sequence shown in Supplementary Table 1. The medium was replaced with FBS-free DMEM and then the cells were transfected with shRNA using Lipofectamine 2000 (Inv-}

\[\text{level} = \text{intensity score} \times \text{positive rate} \times 100.\]

\[\text{DTNBP1} \]

\[\text{GNPNAT1} \]
analysed 50 h post-transfection, and DTNBP1 expression was analyzed using RT-qPCR and western blotting. Each experiment was conducted in triplicate.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from cells 50 h post-transfection using the RNA Isolator Total RNA Extraction Reagent (Vazyme, Nanjing, Jiangsu, China) and then was subjected to reverse transcription using Superscript III Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Inc.) at 42 °C for 50 min. The qPCR assay was conducted using the ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.). The thermocycling conditions were as follows: 98 °C for 30 s, followed by 40 cycles of 98 °C for 5 s, 60 °C for 15 s. Each reaction was performed in triplicate, and expression levels were normalized to those of 18S ribosomal RNA. The qPCR primers are listed in Supplementary Table 1.

**Western blotting.** Protein was extracted from the cells using mammalian protein extraction reagent (Sangon Biotech Co., Ltd., Shanghai, China) 50 h post-transfection. Equal amounts of protein (20 μg per lane), as estimated by using a bicinchoninic acid (BCA) protein assay kit (Abcam, Shanghai, China), were loaded into wells of 10% denaturing SDS-PAGE gels and transferred onto a PVDF membrane (Millipore, MA, USA). The blots obtained were probed with a monoclonal antibody, followed by addition of the secondary HRP-conjugated anti-mouse/rabbit antibody. Detailed information on the antibodies is listed in Supplementary Table 2. After washing, the blots were subjected to chemiluminescence, and relative optical densities were analyzed using the image processing software (Image J). Relative protein content was calculated by dividing the optical density of the target band using the optical density of the GAPDH band obtained. Bands on the film from different projects were respectively cropped. Therefore, the original images of blots were not full-length.

**Assessment of cell viability.** These cells were seeded in 96-well plates at a density of 4 × 10⁴ cells per well. Cell proliferation was assessed using an MTT kit (Cat: 40201ES72, Yeasen, Shanghai, China) at each time point or drug concentration for 60 min at 37 °C. Each experiment was performed in sextuple.

**Detection of apoptosis.** Twenty-two hours after performing cell treatment experiments, cells were seeded into 6-well plates at a density of 1 × 10⁵ cells per well. The cells were then collected and stained using Annexin V-fluorescein isothiocyanate (FITC) and 30 mg/mL PI (Cat: A211-02, Vazyme, Nanjing, China), and apoptosis was subsequently determined using a flow cytometer NovoCyte 1300 (ACEA, San Diego, CA, USA).

**EdU incorporation assay.** Twenty-two hours after performing cell treatment experiments, cells were labeled with EdU for 4 h and then fixed to stain the cells using the EdU staining kit BeyoClick™ EdU-488 (Cat: C0071S, Beyotime, Shanghai, China). The EdU-positive cells were then observed and photographed under a fluorescence microscope (MOTIC, Hongkong, China). The number of cells were counted using Image J 1.52v (NIH, Bethesda, MD, USA).

**Cell cycle analysis.** Forty-eight hours after performing cell treatment experiments, cells were harvested and washed in PBS, followed by fixation in chilled 70% ethanol for 30 min at 4 °C. The cells were washed and treated with 20 μL of ribonuclease (100 μg/mL). Addition of 200 μL PI (50 μg/mL stock solution) was performed to stain the cells, and cells were further analyzed by flow cytometry.

**Cell migration and invasion.** Twenty-two hours after performing cell treatment experiments, cells were harvested and suspended with DMEM. Eighty microliters of cell suspensions containing 6 × 10⁴ cells were placed above the filter membrane of Transwell plates (Cat: 3422, Corning, Corning, NY, USA) and incubate for 24 h at 37 °C and 5% CO₂. For invasion assay, additional extracellular matrix materials (Cat: 356234, BD Biosciences, Sparks, MD, USA) were added on the Transwell membrane. For staining, 600 μL 70% ethanol was used to fix the cells and 0.2% crystal violet was applied for staining. The staining results were detected under a light microscope (MOTIC) and the number of cells was counted using Image J 1.52v.

**Functional enrichment analysis and protein–protein interaction (PPI) network construction.** Select DTNBP1 co-expression genes from the CBioPortal database (https://www.cbioportal.org/) with the Spearman’s correlation p-value < 0.001. These genes were taken to DAVID (https://david.ncifcrf.gov/) for enrichment analysis. The Kyoto encyclopedia of genes and genomes (KEGG) pathway categories were identified with p values < 0.05 and enrichment scores > 2 were considered statistically significant and were plotted using the R package ggplot217-19. Genes contributing to the leading-edge subset within the gene set were defined with the Leading Edge analysis (https://www.gsea-msigdb.org/gsea/doc/GSEAUserGuideFrame.html). Then the genes with [Spearman’s Correlation coefficient value]> 0.4 were analyzed using the STRING website (https://string-db.org/) to examine the relationship among these genes, followed by generation of the network map using the Cytospace software.

**Gene set enrichment analysis (GSEA).** GSEA software (v4.0.3) was used to explore the mechanisms of DTNBP1 expression on the progression of HCC. CP (canonical pathways): KEGG gene sets (n = 186) was obtained from MSigDB database V7.2. The Nominal p-value < 0.05 was considered to be significantly enriched.

**DTNBP1 overexpression and transfection.** cDNA of DTNBP1 was amplified using the primers shown in Supplementary Table 1. And was cloned into the vector pCDH-CMV-MCS-T2A-BSD (Antihela, Xiamen,
Fujian, China). Huh7 cells were seeded into 6-well at the density of $2 \times 10^6$ per well. The cells in “Vector” and “DTNBP1 OE” groups were transfected with empty vector (4 μg/well) and pCDH-DTNBP1 plasmid (4 μg/well) using Lipofectamine 2000, respectively. Twenty-four hours after transfection, the cells groups were harvested for analyse of cellular function as above. All experiments were performed in triplicate.

**Statistical analysis.** All statistical analyses were performed using SPSS Statistics version 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 8.0.2 (GraphPad Software, Inc.). Mann–Whitney test was performed for nonparametric data between two groups. Wilcoxon matched–pairs signed rank test was used to compare matched samples for nonparametric data. Kruskal–Wallis one-way ANOVA followed by Dunn’s multiple comparison test were performed for nonparametric data among multiple groups. The chi-square test was used to
analyze the correlation between DTNBP1 expression levels and clinical features of the patients. Survival curves were calculated using the Kaplan–Meier method, and the significance was determined by the log-rank test. The independent indicators related to OS were identified using Cox proportional hazards model, and the hazard ratios (HR) with 95% confidence intervals (CI) were also calculated. Nomograms were constructed based on the results of Cox multivariate analyses in terms of OS. By combined evaluation of the C-index and calibration, the performance of the established nomograms was effectively measured. One way ANOVA followed by Tukey's post-hoc test was used for multiple comparisons among three groups of parametric data. The level of statistical significance was set at $P<0.05$.

**Results**

**DTNBP1 expression is upregulated in HCC patients.** First, we analyzed the differentially expressed genes in TCGA dataset by using Edge R and found that DTNBP1 expression was upregulated in the hepatocellular carcinomas (Fig. S1). Furthermore, we investigated the DTNBP1 gene expression profile in 33 cancer types both in both normal and paired tumor samples. We found that the mRNA expression of DTNBP1, as determined via TPM analysis, was significantly upregulated in TCGA dataset: DLBC, LIHC, PAAD, SKCM, THYM (Fig. 1A). To further validate the differential expression pattern of DTNBP1 in LIHC, normal tissues and tumor tissues were compared in terms of their DTNBP1 mRNA levels. As shown in Fig. 1B, the mRNA levels of DTNBP1 were higher in tumor tissues than those in the normal controls. Additionally, five GEO datasets were used as the testing datasets. Consistent with the findings obtained from TCGA analysis, DTNBP1 mRNA level in tumor tissues was also significantly higher than that in normal liver tissues (Fig. 1C). Furthermore, we investigated the protein expression of DTNBP1 used the Human Protein Atlas (HPA) database. As shown in Fig. 1D,E, The DTNBP1 was mainly expressed in the hepatocytic cells. The positive signal was distributed in the cytoplasm. The HCC tissues displayed higher DTNBP1 level compared to normal liver tissues. In addition, we analyzed the differential expression of DTNBP1 in the subgroup divided by the clinical features. As shown in Fig. 2A, alphafetoprotein (AFP)-positive patients had higher DTNBP1 mRNA level than AFP-negative patients. DTNBP1 mRNA level was lower in patients who were alive than in dead patients (Fig. 2B). Notably, higher mRNA level of DTNBP1 was observed in patients with micro vascular invasion than in non-vascular patients (Fig. 2C). Moreover, higher DTNBP1 mRNA level was observed in TNM stage II and G3 (Fig. 2D,E) than in their corresponding controls. The correlation between clinicopathological variables and DTNBP1 expression level in HCC was summarized in Table 1. Statistical analysis revealed that DTNBP1 level was significantly correlated with age, gender, histologic grade, ATP, and living status. There was no significant correlation between DTNBP1 level and other clinicopathological variables including Family history of cancer, TNM stage, Ishak score, Child–Pugh grade, vascular invasion, residual tumor, and disease status.
| Clinicopathological variables | Total (N = 339) | DTNBP1 expression | p-value *a |
|------------------------------|----------------|-------------------|-----------|
|                             |                | High (N = 110)    | Low (N = 229) |
| **Age (year)**              |                |                   |            |
| < 65                        | 208 (61.4%)    | 78 (70.9%)        | 130 (56.8%) | **0.017** |
| ≥ 65                        | 131 (38.6%)    | 32 (29.1%)        | 99 (43.2%) |
| **Gender**                  |                |                   |            |
| Male                        | 231 (68.1%)    | 66 (60.0%)        | 165 (72.1%) | **0.035** |
| Female                       | 108 (31.9%)    | 44 (40.0%)        | 64 (27.9%) |
| **Family history of cancer**|                |                   |            |
| No                          | 196 (57.8%)    | 69 (62.7%)        | 127 (55.5%) | 0.447     |
| Yes                         | 98 (28.9%)     | 28 (25.5%)        | 70 (30.6%) |
| Unknown                     | 45 (13.3%)     | 13 (11.8%)        | 32 (14.0%) |
| **TNM stage**               |                |                   |            |
| I                           | 170 (50.1%)    | 46 (41.8%)        | 124 (54.1%) | 0.186     |
| II                          | 84 (24.8%)     | 32 (29.1%)        | 52 (22.7%) |
| III                         | 81 (23.9%)     | 30 (27.3%)        | 51 (22.3%) |
| IV                          | 4 (1.2%)       | 2 (1.8%)          | 2 (0.9%)   |
| **Histologic grade**        |                |                   |            |
| G1–G2                       | 212 (62.5%)    | 53 (48.2%)        | 159 (69.4%) | < **0.001** |
| G3–G4                       | 125 (36.9%)    | 56 (50.9%)        | 69 (30.1%) |
| Unknown                     | 2 (0.6%)       | 1 (0.9%)          | 1 (0.4%)   |
| **Ishak score**             |                |                   |            |
| 0–4                         | 124 (36.6%)    | 33 (30.0%)        | 91 (39.7%) |
| 5–6                         | 74 (21.8%)     | 25 (22.7%)        | 49 (21.4%) |
| Unknown                     | 141 (41.6%)    | 52 (47.3%)        | 89 (38.9%) |
| **Child–pugh grade**        |                |                   |            |
| A                           | 207 (61.1%)    | 63 (57.3%)        | 144 (62.9%) | 0.128     |
| B–C                         | 21 (6.2%)      | 4 (3.6%)          | 17 (7.4%)  |
| Unknown                     | 111 (32.7%)    | 43 (39.1%)        | 68 (29.7%) |
| **Vascular invasion**       |                |                   |            |
| None                        | 193 (56.9%)    | 52 (47.3%)        | 141 (61.6%) | 0.094     |
| Micro                       | 84 (24.8%)     | 33 (30.0%)        | 51 (22.3%) |
| Macro                       | 14 (4.1%)      | 5 (4.5%)          | 9 (3.9%)   |
| Unknown                     | 48 (14.2%)     | 20 (18.2%)        | 28 (12.2%) |
| **Alpha fetoprotein**       |                |                   |            |
| Negative                    | 143 (42.2%)    | 34 (30.9%)        | 109 (47.6%) | < **0.001** |
| Positive                    | 120 (35.4%)    | 54 (49.1%)        | 66 (28.8%) |
| Unknown                     | 76 (22.4%)     | 22 (20.0%)        | 54 (23.6%) |
| **Residual tumor**          |                |                   |            |
| R0                          | 301 (88.8%)    | 97 (88.2%)        | 204 (89.1%) | 0.777     |
| R1–R2                       | 12 (3.5%)      | 5 (4.5%)          | 7 (3.1%)   |
| Unknown                     | 26 (7.7%)      | 8 (7.3%)          | 18 (7.9%)  |
| **Living status**           |                |                   |            |
| Alive                       | 224 (66.1%)    | 60 (54.5%)        | 164 (71.6%) | **0.003** |
| Dead                        | 115 (33.9%)    | 50 (45.5%)        | 65 (28.4%) |
| **Disease status**          |                |                   |            |
| No                          | 163 (48.1%)    | 54 (49.1%)        | 109 (47.6%) | 0.967     |
| Yes                         | 132 (38.9%)    | 42 (38.2%)        | 90 (39.3%) |
| Unknown                     | 44 (13.0%)     | 14 (12.7%)        | 30 (13.1%) |
| **Disease specific**        |                |                   |            |
| No                          | 266 (78.5%)    | 79 (71.8%)        | 187 (81.7%) | 0.106     |
| Yes                         | 65 (19.2%)     | 27 (24.5%)        | 38 (16.6%) |
| Unknown                     | 8 (2.4%)       | 4 (3.6%)          | 4 (1.7%)   |

Table 1. Correlation between clinicopathological variables and DTNBP1 expression in HCC. *Chi-square test. Statistically significant p-value was given in bold italic.
To further validate the clinical value of DTNBP1 in clinical applications, we performed immunohistochemistry to stain DTNBP1 in commercial HCC microarray (Fig. 3A). The DTNBP1 expression levels were higher in tumor tissues than those in adjacent normal tissues (Fig. 3B). No significant differences were observed in clinical factors, such as age and sex (Fig. 3C,D), while DTNBP1 protein levels were significantly different in the subgroups when groups were divided by the histological stage and TNM stage (Fig. 3E,F).

Taken together, these findings demonstrated that DTNBP1 is overexpressed in HCC patients and is further increased as the patient’s progress moves to a more lethal stage, indicating that DTNBP1 may serve as an oncogenic factor determining HCC progression.

**Higher DTNBP1 level indicated shorter OS time of HCC patients.** Additionally, we investigated the relationship of DTNBP1 level with OS, disease-specific survival (DSS), and disease-free interval (DFI). As shown in Fig. 4A, patients in the high DTNBP1 group had a shorter OS. However, there was no significant difference in DSS (Fig. 4B) or DFI (Fig. 4C) found between the high DTNBP1 and low DTNBP1 groups. Furthermore, we did more analyses on OS with other clinicopathological variables. Stratified analyses also illustrated that high DTNBP1 level served as a factor for poor prognosis, when patients were classified as age > 65 years (Fig. 4D, left, p = 0.0450), age ≤ 65 years (Fig. 4D, right, p = 0.0330), male (Fig. 4E, left, p = 0.0057), TNM stage I + II (Fig. 4F, left, p = 0.0042), G3 + G4 (Fig. 4G, right, p = 0.0073), and AFP positive (Fig. 4H, right, p = 0.0330). Notably, the DTNBP1 level failed to predict the OS of patients categorized as female (Fig. 4E, right, p = 0.2350), TNM stage III + IV (Fig. 4F, right, p = 0.2590), G1 + G2 (Fig. 4G, left, p = 0.4420), AFP negative (Fig. 4H, left, p = 0.6500), micro vascular invasion (Fig. 4I, left, p = 0.0610), and micro vascular invasion (Fig. 4I, right, p = 0.0710). In addition, using univariate and multivariate Cox analyses, DTNBP1 and TNM stage were observed to also function as significant independent predictors of OS (Table 2). Taken together, these findings suggested that DTNBP1 is a useful prognostic factor of HCC.

**Figure 3.** Validation of the clinical significance of DTNBP1 using HCC microarray. (A) Representative image of DTNBP1 IHC staining. (B) The DTNBP1 protein level was higher in tumor tissues, compared with ANT. Statistical analysis: left panel, Mann–Whitney test; right panel, Wilcoxon matched-pairs signed rank test. (C–D) Violin plot illustrating the differential expression of DTNBP1 in subgroups of patients divided by gender (C) and age (D). Mann–Whitney test was used for statistical analysis. (E) Plot illustrating the differential expression of DTNBP1 in subgroups of patients divided by historical stage. Kruskal–Wallis one-way ANOVA followed by Dunn’s multiple comparison test was used for statistical analysis. (F) Box plot illustrating the differential expression of DTNBP1 in subgroups of patients divided by TNM stage. The data are analyzed using Kruskal–Wallis one-way ANOVA test followed by Dunn’s multiple comparison test. ns, no significance; *p < 0.05; **p < 0.01; ****p < 0.0001.
Development and validation of a DTNBP1-predicting nomogram. The two independent predictors, DTNBP1 and TNM stage, were used to form a DTNBP1 expression level-related risk estimation nomogram (Fig. 5). The nomogram demonstrated excellent accuracy in estimating the risk of DTNBP1 in OS, with a C-index of 0.63 (95% CI 0.60–0.66) (Fig. 5A). Additionally, calibration plots graphically showed adequate agreement on the presence of DTNBP1 between the risk estimation by the nomogram and histopathologic confirmation (Fig. 5B).

Downregulation of DTNBP1 expression reduces cell proliferation and increases apoptosis. To verify the functional roles of DTNBP1 in cell functions, we first compared its expression level between normal liver cell lines and HCC cell lines. As shown in Fig. S2, both mRNA and protein levels of DTNBP1 in the HCC cell lines except for Huh7 were significantly higher than those in the normal liver cell line THLE-2. Hep3B and PLC-PRF-5, which had the highest levels of DTNBP1, were chosen for further experiments. We used
short hairpin RNAs (shRNAs) against DTNBP1 to knock down DTNBP1 in Hep3B and PLC-PRF-5. Indeed, we observed that the DTNBP1 shRNAs (shDTNBP1-1 and shDTNBP1-2) reduced both mRNA and protein levels of DTNBP1 in Hep3B and PLC-PRF-5 (Fig. 6A,B). Knockdown of DTNBP1 could inhibit cell viability, as evidenced by the results of the MTT assay (Fig. 6C). Furthermore, the EdU staining assay showed that the percentage of cells in replication (the EdU-positive cells) significantly decreased when DTNBP1 was knocked down (Fig. 6D). As shown in Fig. 6E, the DTNBP1-knockdown group showed that the cell cycle was arrested in the G0/G1 phase, which implied that the cell cycle progression was inhibited. Furthermore, the percentage of apoptosis was significantly increased in the DTNBP1-knockdown group (Fig. 6F). Taken together, our results demonstrated that reduced levels of DTNBP1 inhibit cell proliferation and increases apoptosis in HCC cell lines. Downregulation of DTNBP1 expression inhibits the metastasis of HCC cells. Results from the Transwell migration assays demonstrate that the DTNBP1-knockdown group exhibited significantly decreased cell migration compared to the cells transfected with the empty vector (Fig. 7A,B). Additionally, results from the Matrigel invasion assays demonstrate that DTNBP1 knockdown significantly reduced the number of invasive cells (Fig. 7C,D). Taken together, downregulation of DTNBP1 expression suppresses cell metastasis.

| Variables                              | Univariate analysis | Multivariate analysis |
|----------------------------------------|---------------------|-----------------------|
|                                        | HR (95% CI)         | P-value               |
| Age (≥ 65 vs. < 65)                    | 1.23 (0.85,1.78)    | 0.273                 |
| Gender (female vs. male)               | 1.26 (0.87,1.84)    | 0.228                 |
| Family history of cancer (yes vs. no)  | 1.14 (0.76,1.69)    | 0.530                 |
| TNM stage (II vs. I)                   | 1.42 (0.87,2.32)    | **0.160**             |
| TNM stage (III vs. I)                  | 2.72 (1.78,4.15)    | < 0.001               |
| TNM stage (IV vs. I)                   | 5.44 (1.68,17.63)   | **0.005**             |
| Histologic grade (G3–G4 vs. G1–G2)    | 1.14 (0.78,1.67)    | 0.489                 |
| Ishak score (5–6 vs. 0–4)              | 0.87 (0.5,1.5)      | 0.612                 |
| Child–Pugh grade (B–C vs. A)           | 1.66 (0.82,3.36)    | 0.159                 |
| Vascular invasion (micro vs. none)     | 1.16 (0.72,1.88)    | 0.539                 |
| Vascular invasion (macro vs. none)     | 2.52 (1.14,5.58)    | **0.023**             |
| Alpha fetoprotein (positive vs. negative) | 1.45 (0.92,2.28)   | **0.108**             |
| Residual tumor (R1–R2 vs. R0)          | 1.17 (0.43,3.2)     | 0.754                 |
| DTNBP1 (high vs. low)                  | 1.21 (0.97,1.51)    | **0.089**             |

Table 2. Cox proportional hazards regression model analysis of overall survival. Statistically significant P value is given in bold italic. CI: confidence interval. HR: hazard ratio.
DTNBP1 regulatory network analysis. To investigate the biological classification of DTNBP1 in HCC, we conducted functional enrichment analysis of genes that co-expressed with DTNBP1. The top 20 pathways concerning the significantly enriched KEGG pathways showed that DTNBP1 was significantly related with the processes of cell cycle and DNA replication (Fig. 8A), suggesting that DTNBP1 potentially facilitates the growth of HCC cells. The PPI network was illustrated in Fig. 8B, showing the 43 genes which have the highest interaction scores with DTNBP1.

DTNBP1 regulates the expression levels of cell cycle-related proteins. To further investigate the potential signaling pathways involved in DTNBP1-inhibitory effects in cancer cells, we performed GSEA to explore the biological function of DTNBP1 upregulation in HCC, and “cell cycle” pathway was significantly enriched (Fig. 9A). The core genes in the both pathways were listed in the Supplementary Table 3. We further investigated the effect of DTNBP1 expression on the mRNA levels of cell cycle-related genes including CCNB1, CDC25A, CDC20, CDK1, CCNE1, CDC25B, and CDK2. As shown in Fig. 9B, DTNBP1 knockdown significantly...
reduced the mRNA expression levels of these genes. In consistent with the mRNA levels, the protein levels of these genes were also significantly reduced after DTNBP1 deficiency with the exception of CDC25A in group shDTNBP1-2 for PLC-PRF-5 cells (Fig. 9C,D). These data indicated that DTNBP1 regulates cell cycle progression.

Overexpression of DTNBP1 promotes cell cycle progression. To further confirm the regulation of DTNBP1 on cell cycle and growth, we overexpressed DTNBP1 in Huh7 cells. As shown in Fig. 10A,B, DTNBP1 overexpression increased the protein levels of CDC25A, CCNE1, and CDK2. Moreover, high level of DTNBP1 promoted the cell proliferation (Fig. 10C), DNA replication (Fig. 10D,E), and passing through G0/G1 phase (Fig. 10F,G). These data suggested that DTNBP1 acts as an oncogene by promoting cell cycle progression.

Discussion
An independent prognostic biomarker is helpful for the evaluation of the clinical treatment of malignancies in HCC. In this study, a novel and efficient prognostic biomarker, DTNBP1, was investigated in HCC patients. DTNBP1 was significantly upregulated in HCC tissues and demonstrated a high correlation with clinical features and OS of patients. DTNBP1 and TNM stage are both independent predictors for patient prognosis. It seems that TNM stage outperforms DTNBP1 expression in the OS prediction. However, we should use both of them to form a nomogram of OS to estimate the risk. The nomogram was credible and available. The association between high DTNBP1 expression in HCC and unfavourable characteristics (high AFP value, high TNM stage, poor OS, etc.)
makes us wonder whether a high DTNBP1 expression might simply represent an epiphenomenon of a poorly differentiated and aggressive histotype instead of playing a primary causative role. Thus, we investigated the effect of DTNBP1 on the cellular function including cellular proliferation, apoptosis, and metastasis after DTNBP1 knockdown in HCC cell lines Hep3B and PLC/PRF/5. The in vitro experiments confirmed that DTNBP1 acts as an oncogene. Moreover, KEGG enrichment analysis revealed that the enriched pathways were related to DNA replication and cell cycle. Our results of EdU and cell cycle assays showed that DTNBP1 knockdown inhibited the cell replication and induced the arrest at G0/G1 phase, which was consistent with the conclusion of KEGG enrichment analysis. As we know, during the progression of the cell cycle, cyclin E1 (CCNE1) functions as a regulatory subunit of CDK2, which is required for G1/S progression. CDK2 is dephosphorylated to be activated by CDC25A. Thus, downregulation of CDC25A, cyclin E1 (CCNE1), and CDK2 can induce G0/G1 arrest. CDC20, CDC25B, cyclin B1 (CCNB1), and CDK1 are critical for cells entering mitosis. However, our results showed that the reduction of forementioned molecules induced by DTNBP1-deficiency only caused G0/G1 arrest without G2/M arrest. The reason may be that the cells were synchronized by starvation and the G0/G1 phase was the first stage of interphase. Our findings may provide a potential biomarker for malignancy and survival predictions in HCC patients.

Our analysis of bioinformatics revealed that DTNBP1 is upregulated in several other cancer types, indicating that the specificity of DTNBP1 as a diagnostic marker for HCC may be not enough. However, DTNBP1 may be used as a universal predictor for OS in different kinds of cancer, which is need to be further confirmed.

We did a univariate Cox analysis on individual variable and out of those which became significant (p < 0.20) should be subjected to multivariate Cox analysis (Table 2). The threshold of univariate Cox analysis was set at p value < 0.20 rather than 0.05 to prevent from missing the significant independent risk factors.

There are several new biomarkers for HCC have been reported in the recent years. Lai et al. demonstrated that GRPEL2 is highly expressed in the HCC and plays well in predicting OS in patients with the same gender, age, pathological grade or clinical stage. CDT1 and RM12 are also reported to be overexpressed in HCC and act as independent predictors for OS in HCC patient. We suggest using TNM stage, DTNBP1, GRPEL2, CDT1, and RM12 to form a predicting nomogram, which should be more accurate and credible.

It should be noted that there are also several limitations for this study. For example, we need to uncover the mechanism of DTNBP1 on apoptosis and metastasis in the next work. Moreover, in vivo experiments should be performed to validate our conclusion.

Conclusions

In summary, we identified a novel biomarker DTNBP1 in HCC. Furthermore, DTNBP1 expression may reveal relevant cell cycle progression in HCC patients and may also help the prognosis assessment in HCC.
Figure 9. Downregulation of DTNBP1 expression decreased the expression of cell cycle related proteins. (A) GSEA analysis of the top 20 co-expressed genes of DTNBP1. (B) The mRNA levels of CCNB1, CDC25A, CDC20, CDK1, CCNE1, CDC25B, and CDK2 were detected using RT-qPCR. (C) The protein levels of CCNB1, CDC25A, CDC20, CDK1, CCNE1, CDC25B, and CDK2 were detected using western blotting. The bolts were cropped from different gels with different exposure times. (D) Quantitation of band intensity with values normalized to GAPDH. One way ANOVA followed by Tukey’s post-hoc test: ns, no significance; *p < 0.05; **p < 0.01; ***p < 0.001.
Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Figure 10.** DTNBP1 overexpression promotes cell cycle progression. (A) The protein levels of CDC25A, CCNE1, CDK2, and DTNBP1 were detected using western blotting. The bolts were cropped from different gels with different exposure times. (B) Quantitation of band intensity with values normalized to GAPDH. (C) MTT assay. (D) Representative images of EdU incorporation. Bar: 10 μm. (E) Quantification of (D) using the bar plot. (F) Representative FACS images of cell cycle analysis. (G) Quantification of (F) using the bar plot. Unpaired Student's t test: **p < 0.01; ***p < 0.001; ****p < 0.0001.
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Author contributions
W.Z. conceived and designed the study. W.Z., X.C., and D.L. collected the data, analyzed the data, and wrote the manuscript. J.S. and T.Z. performed the experiments. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.
