Article

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The Significance of Discs Large Homolog 2 Expression in Hepatocellular Carcinoma: A Laboratory Study

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Background: Hepatocellular carcinoma (HCC) is an aggressive tumor. The discs large homolog 2 (*DLG2*) is expressed in some malignancies, and it may be related to tumor progression. However, its role in HCC has not been explored. The aim of this study was to explore the prognostic value and role of *DLG2* in HCC.

Methods: The data of mRNA (messenger ribonucleic acid) expression from Gene Expression Omnibus (GEO), International Cancer Genome Consortium (ICGC) and The Cancer Genome Atlas (TCGA) databases were used to evaluate *DLG2* expression in HCC tissues. The DLG2 protein level was checked for using immunohistochemistry analysis of a tissue microarray. The univariate and multivariate Cox regression analyses and the Kaplan–Meier analysis were used to evaluated the correlation between *DLG2* expression and overall survival (OS). Gene set enrichment analysis (GSEA) was performed to research potential pathway that DLG2 participates in. *DLG2* overexpression and knockdown cells were constructed using HCCLM3 cell line transfected with *DLG2* overexpression vector and HepG2 transfected with shDLG2 (short hairpin RNA targeting at *DLG2*), respectively.

Results: DLG2 was significantly downregulated in HCC tissues. *DLG2* expression was significantly associated with the pT (primary tumour) stage, tumor size, histological grade, and vessel invasion. More importantly, low *DLG2* expression was significantly related to poor prognosis in advanced pStages (pathological stages), grade 3 HCC, alcoholic, and HBV (hepatitis B virus)-positive HCC patients. The *MYC* targets V2 (alias *TCRGV9*, T cell receptor gamma variable 9), *E2F* targets, and G2M (gap 2 phase and mitosis phase) checkpoint gene sets were differentially enriched in low *DLG2* expression phenotype. *DLG2* significantly correlated with *MCM2* (minichromosome maintenance complex component 2), *MCM5* (minichromosome maintenance complex component 5), *MCM6* (minichromosome maintenance complex component 6), *KPNA2* (karyopherin subunit alpha 2), *CDK4* (cyclin-dependent kinase 4), *H2AFZ* (H2A histone family member Z), *MAD2L1* (mitotic arrest deficient 2 like 1) and *CDC20* (cell division cycle 20) in LIHC (liver hepatocellular carcinoma) patients. *DLG2* silence and overexpression promoted and suppressed HCC proliferation, respectively.

Conclusions: *DLG2* might function as a biomarker of diagnosis and prognosis in HCC, and suppressed HCC cells proliferation.

Keywords: discs large homolog 2; hepatocellular carcinoma; diagnosis; prognosis; proliferation; biomarker

Introduction

Hepatocellular carcinoma (HCC) is the malignant tumor and occurs in liver, and it is primarily caused by chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, alcoholic liver diseases or cirrhosis [1]. The main treatment for early stage HCC is surgical resection and liver transplantation [2]. Unfortunately, patients who appear symptomatic and liver impairment are often diagnosed with advanced HCC [3]. The recent use of immunotherapy for patients with advanced HCC did not improve patient outcomes [4]. In addition, frequent recurrence and metastasis rates lead to unsatisfactory clinical prognosis [5]. The identification of potential predictive, diagnostic and prognostic biomarkers for HCC would be helpful in making the diagnosis and guiding the selection of treatment.

Discs large homolog 2 (*DLG2*) is a member of membrane-associated guanylate kinases (*MAGUKs*) proteins and mainly expressed in brain adult tissues. It is important for cell-cell intercommunication and usually play function as scaffold for surface complexes, including various adhesion proteins, receptors and signaling molecules [6]. Some studies show that, in the adult rodent brain, *DLG2* is largely expressed in many areas, such as hippocampus, striatum, and cortex [7,8]. Previous studies showed that *DLG2* significantly correlated with pathophysiology of various diseases, such as schizophrenia [9], alzheimer's disease [10], hyperglycaemia [11] and Parkinson's disease [12]. For example, in schizophrenia patients,

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de novo function loss mutations of *DLG2* are observed [13,14]. In addition, an genotype analysis in 1185 Taiwanese persons, who included 592 Parkinson's disease patients and 593 controls, showed that *DLG2* influences the Parkinson's disease risk [12].

Recently, some researchers reported abnormally low *DLG2* expression in some malignancies, such as neuroblastoma [15] and ovarian cancer [16]. However, no work was carried out to explore the role and value of *DLG2* in HCC. The aim of this study was to examine the expression and role of *DLG2*, and explore its potential value in the diagnosis as well predicting the prognosis of HCC.

Materials and Methods

Data Collection

Eight HCC mRNA expression datasets (GSE63898, GSE76427, GSE112790, GSE14520, GSE22058, GSE36376, GSE64041, and GSE6764) were downloaded from Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The HCC tissues and adjacent noncancerous tissues (NTs) mRNA expression profiles were obtained from International Cancer Genome Consortium (ICGC, https://icgc.org/) database and The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/repository) database.

Sample and Clinical Data Collection

To evaluate the clinical value of *DLG2* in HCC patients, TMA (tissue microarrays) was commercially obtained from Shanghai Tufei Biotech Co., Ltd. (Shanghai, China) that comprised 90 paired HCC tissues and adjacent NTs. Therefore, data shown in **Supplementary Tables 1,2** might be repeated with other published resources that were based on the same cohorts. The clinicopathological characteristics of these patients, such as gender, age, and pathological grade, were provided by Shanghai Tufei Biotech Co., Ltd. (**Supplementary Tables 1,2**). The following inclusion criteria was used for the specimen selection:

(1) A definite clinical diagnosis and postoperative pathological diagnosis of HCC.

(2) R0 resection of all patients based on histologic examinations.

(3) Relatively complete clinicopathologic and follow-up data.

(4) Absence of treatment before surgery.

Immunohistochemical (IHC) Analysis

Tumor tissue specimens of HCC were used to check for *DLG2* expression. Repeat information, without any need. After fixation with formalin, the specimens were embedded in paraffin. Then, 4-μm slices were obtained for examination on slides and the specimens were deparaffinized in xylene and then rehydrated with alcohol. To quench the intracellular endogenous peroxidase activity, hydrogen peroxide (3%) was used, and goat serum (C0265; Beyotime Biotechnology, Shanghai, China) was used to block nonspecific binding after antigen retrieval, using an autoclave.

After incubation with anti-DLG2 (1:50 dilution; abs141074; Absin-bio, Shanghai, China) overnight at 4 °C, the slides were incubated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:300 dilution, ab205718; Abcam, Cambridge, MA, USA). After washing with phosphate-buffered saline (PBS), slices were treated with 3,3'-diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin (Beyotime, Shanghai, China). The images of slides were captured and processed using Aperio ImageScope v.12.3 (Leica Biosystems, Nussloch, Germany). The staining intensity was evaluated as follows: 0-negative, 1-weak, 2-moderate, and 3-strong. The final H-score explain was computed as staining intensity multiplying the percentage of DLG2 positive tumor cells (0–100%). Scores greater than median H-score explain value were regarded as high *DLG2* expression.

Cell Lines and Culture

Human normal liver cell line L02 and HCCLM3, MHCC97-H, and Huh7 (3 HCC cell lines) as well as HCC cell line HepG2 were obtained from Zhongshan Hospital, which is part of Fudan university, in Shanghai, China. Hep 3B2.1-7 and SK-HEP-1 cell lines were obtained from the Cancer Research Institute, which is part of Shanghai Jiao Tong University, in Shanghai, China. The cell lines were tested for mycoplasma and only mycoplasma negative were used in the study. The cell lines were identified using STR (short tandem repeat) identification. To avoid mycoplasma contamination, good aseptic technique and laboratory practices were used to cultivate and preserve cells according to international guidelines, and a periodical and continuous mycoplasma-testing program was established [17]. The cells were cultured in Dulbecco's modified Eagle's medium, and the DMEM (Dulbecco's modified Eagle medium) contained 10% fetal bovine serum, 100 mg/mL streptomycin, and 100 μg/mL penicillin, at 37 °C with 5% CO_2. The cells from three to five passages were used for subsequent experiments.

DLG2 Knockdown and Overexpression

For DLG2 knockdown, four targeting short hairpin ribonucleic acids (shRNAs) and a non-targeting RNA (scrambled RNA) were synthesized and integrated into the GV248 lentivirus vector (GV248-shDLG2) by Shanghai Genechem Co., Ltd. (Shanghai, China). The *DLG2* construct was also synthesized and integrated into a GV358 lentiviral vector (GV358-DLG2; GeneChem, Shanghai, China) for *DLG2* overexpression. The shDLG2 target sequences were GCCAGTACAATGACAATTT (shDLG2-1), GATCAATGACGACTTGATA (shDLG2-2), GTGAACAAACTATGTGATA (shDLG2-3) and TTGTGGAAAT-

CAAACTGTT (shDLG2-4). The lentivirus particles were prepared by Shanghai Genechem Co., Ltd. (Shanghai, China) via co-transfection of GV358-DLG2, GV248-shDLG2, or corresponding control vectors with lentiviral packaging plasmids into 293T cells. HCCLM3 and SK-HEP-1cells were seeded into 6-well plate at 5×10^6 cells/well, when cells cultured to 70% confluence, GV248-DLG2 and GV358-shDLG2 lentivirus fluid were used to infect HCCLM3 and SK-HEP-1 cells, respectively. The cells were cultured in medium that contained 2 mg/mL puromycin to screen for stable clone cells after 24 h of infection. The knockdown and overexpression of *DLG2* were validated with quantitative real-time polymerase chain reaction (qPCR).

Quantitative Real-Time Polymerase Chain Reaction

The total RNA was extracted with RNAiso Plus (9108, TaKaRa, Beijing, China), and the extracted RNA was reverse transcribed by PrimeScript RT reagent Kit (RR047A, TaKaRa). Then, qPCR (quantitative real-time polymerase chain reaction) was performed according to the instruction of the SYBR Premix Ex Taq kit (DRR041, TaKaRa, Beijing, China). The primers were as follows: *DLG2* Forward 5′-ATGTTCGGCACCTGTCTGTG-3′, *DLG2* Reverse 5′-CCAGATTGTCATCTCCATAGC-3′; *GAPDH* Forward 5′-GTTCGTCATGGGTGTGAACC-3′, *GAPDH* Reverse 5′-CATCCACAGTCTTCTGGGTG-3′. The internal reference was *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), and the relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method, where Ct is cycle threshold.

Cell Counting Kit-8 (CCK-8) Assay

The cells infected with the aforesaid lentivirus were planted into a 96-well plate at 2×10^3 cells/well, and then incubated in standard culture medium. After culture for 0 to 96 h, 10 μL CCK-8 (cell counting kit-8) solutions (E606335; Sangon Biotech, Shanghai, China) were added into each well, and then the incubation of the 96-well plates were performed at 37 °C for 1 h. Then, the absorbance of each well was detected at 450 nm and directly used as OD value to evaluate the viability of the cells.

Colony Formation Assay

Approximately 1.5×10^3 cells per well were cultured in 12-well plates, and then, the cells were washed with PBS (phosphate-buffered saline) 3 times after culture for 14 days. The cells were fixed by 4% phosphate-buffered paraformaldehyde for 10 min, and the cells were stained with 0.1% crystal violet solution. Then clones were pictured and counted after washed and aired. The clone counting was performed using ImageJ (version 1.48; National Institutes of Health, Bethesda, MD, USA), and the following parameters were used: Size min = 40, max = 600, circularity min = 0.85 max = 1.0.

Gene Set Enrichment Analysis (GSEA)

For exploring DLG2-associated pathways in HCC, GSEA (gene set enrichment analysis) was carried out. The permutation tests were run 1000 times to analyze significantly pathways. Normalized enrichment score was calculated, and false discovery rate (FDR) <0.05 and adjusted $p < 0.01$ were considered significantly associated genes.

Statistical Analysis

The GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) and SPSS (statistical package for the social sciences) 22.0 software (IBM Corp., Armonk, New York, NY, USA) were used to analyze data. Continuous data were checked for normality of distribution, using Shapiro-Wilk test. Normally distributed continuous data were described as the man $\pm$ standard deviation and compared using Student's *t* test, resorting to one-way analysis of the variance (ANOVA) when comparing moe than two groups. Continuous data that did not fit a normal distribution were described as the median [inter-quartile range] and compared using Wilkinson signed rank test, resorting to the Mann-Whitney U test for comparing more than two groups. Categorical data were described as frequency (%) and compared using the chi-squared test, resorting to the Mann-Whitney U test when the expected frequencies were <5 in $\geq 20\%$ of cells and Fisher's Exact test for comparisons that fitted 2×2 tables. When a statistically significant difference was detected between more than two groups, each pair was compared. Correlation was checked for using the Pearson's χ^2 test and Yates' continuity corrected chi-square test. Kaplan–Meier was used for survival analysis and the *p* values were computed by log-rank test. Additionally, the univariate and multivariate Cox proportional hazards models were used to gain hazard ratios (HRs) and 95% confidence intervals (CIs) for evaluating independent prognostic factors of overall survival (OS) in HCC. The common genes of GSEA enrichment pathways were screened out with the venny tool (https://bioinfogp.cnb.csic.es/tools/venny/, Accessed date: March 2022). Statistical tests were two-sided and statistical significance was set at 0.05.

Results

DLG2 was Downregulated in HCC Tissues

Compared to adjacent NTs, the *DLG2* mRNA level was significantly downregulated in HCC tissues (Fig. 1A,B). In GEO datasets, including GSE14520, GSE64041, GSE36376, GSE63898, GSE22058, GSE76427, GSE112790, and GSE6764, similar results were also observed (Fig. 1C–J). The positive expression of DLG2 displayed brownish (Fig. 1K and **Supplementary Fig. 1**), and IHC score revealed that *DLG2* expression was significantly reduced in HCC tissues (Fig. 1L). Taken together, *DLG2* expression was diminished in HCC tissues, which might be related to the cancer progression.

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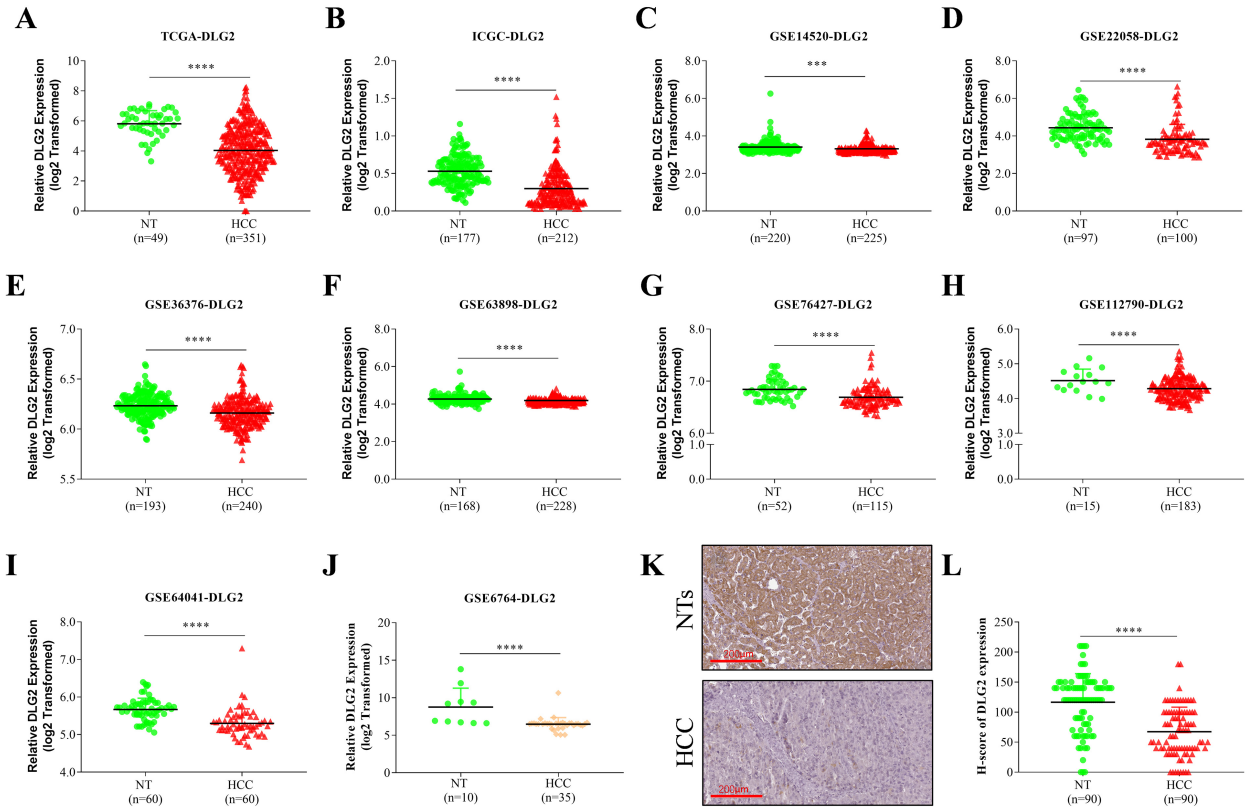

Fig. 1. The expression of *DLG2* (discs large homolog 2) in HCC (hepatocellular carcinoma) tissues. (A–J) Comparison of *DLG2* mRNA expression between HCC tissues and adjacent noncancerous tissues (NTs). (K) Representative DLG2 IHC images of HCC tissues and NTs. (L) The DLG2 protein expression intensity of IHC staining. $^{***}p < 0.001$, $^{****}p < 0.0001$, compared with NTs.

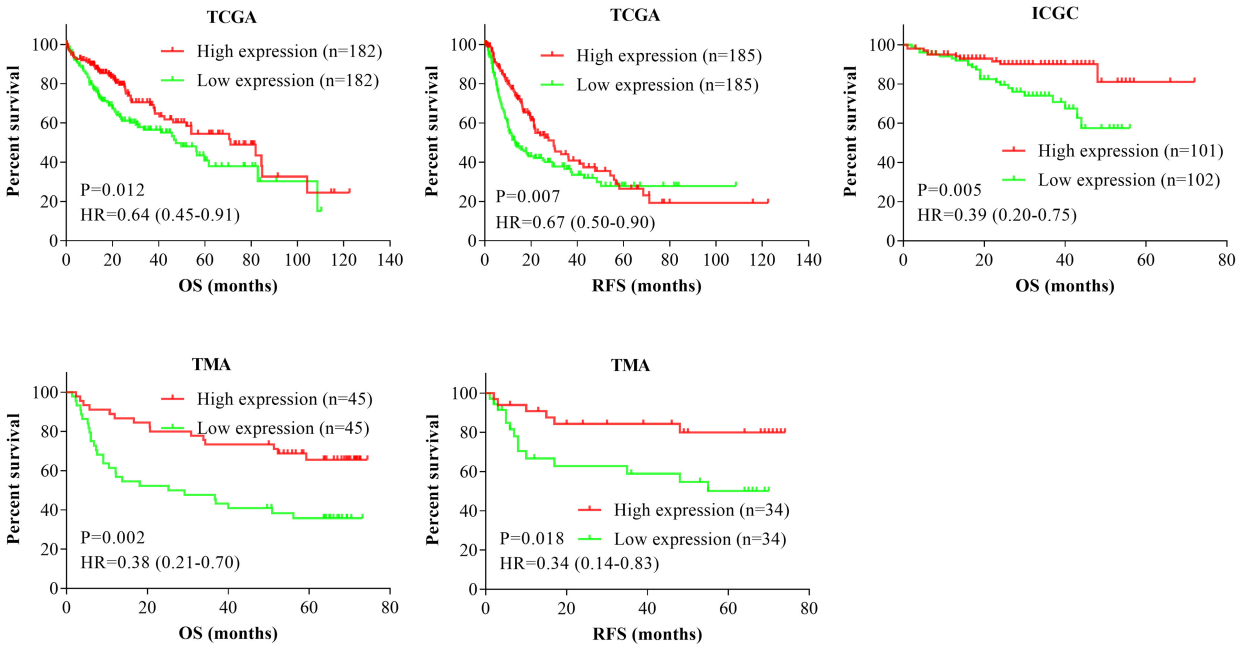

Fig. 2. Kaplan–Meier survival analysis of OS (overall survival) and RFS (recurrence-free survival) of HCC (hepatocellular carcinoma) patients in the TCGA (The Cancer Genome Atlas), ICGC (The International Cancer Genome Consortium), and TMA (tissue microarray) cohort.

DLG2 Might be a Diagnostic Biomarker for HCC

The *DLG2* expression was not significantly associated with gender, age, hepatitis B virus (HBV) infection, alpha-fetoprotein (AFP), lymph node status or metastasis stage, but obviously related to the pT (pathological primary tumor) stage, tumor size, histological grade, and vessel invasion (Table 1). Subsequently, Cox regression analysis results showed that tumor size, AFP, pStage (pathological stages) or DLG2 expression was the significant independent predictors for OS (Table 2). Furthermore, 364 patients with available survival data in the LIHC cohort of TCGA were used to perform survival analysis (Fig. 2). Kaplan–Meier analysis results demonstrated that, compared with low *DLG2* expression patients, the OS time in patients with high *DLG2* expression was significantly longer, and patients that had low *DLG2* expression also had a significantly shorter relapse-free survival (RFS).

Survival analysis of ICGC dataset suggested the possible role of *DLG2* as a significant predictor in HCC. Additionally, patients with low *DLG2* expression had significantly dismal OS and RFS, which was also indicated by the TMA cohort of Kaplan–Meier survival analysis (Table 1, **Supplementary Table 2**).

DLG2 Might be a Better Prognostic Biomarker in Stage III/IV, Grade 3, Alcoholic, and HBV-Positive HCC Patients

Kaplan–Meier survival analysis demonstrated that high and low levels of *DLG2* were not associated with the survival status of patients who was not infected with HBV or did not drink, at stage I/II or grade 1/2 (Fig. 3A,C,E,H). However, patients with lower *DLG2* expression had a shorter OS time at stage III/IV or grade 3 (Fig. 3B,D), and this situation is the same with those who drank or were infected with HBV (Fig. 3F,G), which indicated that *DLG2* may be able to serve as a predictive maker for patients who drank or were infected with HBV (Fig. 3F,G).

DLG2 Expression Might Correlate with Cell Proliferation in HCC

GSEA plot analysis indicated that the hallmark signature of tumor was significantly enriched in *MYC* targets V2 (NES = –3.3, $p < 0.05$), *E2F* targets (NES = –3.45, $p < 0.001$) and G2M checkpoint (NES = –3.44, $p < 0.01$) gene sets (Fig. 4A. Then, the gene sets of hallmark proliferation-related pathways, including *E2F* targets, G2M checkpoint, and *MYC* targets V2, were used to be overlapped by Venny tool. *CDC20, CDK4, GSPT1, H2AFZ, HNRNPD, KPNA2, MAD2L1, MCM2, MCM5, MCM6, MYC, NOLC1, SRSF1, SRSF2, SYNCRIP, TRA2B* and *XPO1* were common genes in these three pathways (Fig. 4B). As shown in Fig. 4C, among these genes, significantly negative correlations between DLG2 and eight genes, including *MCM2, MCM5, MCM6, KPNA2, CDK4, H2AFZ, MAD2L1* and *CDC20*, in mRNA expression were observed. However, other proliferation-related genes had no or low correlation with *DLG2* in mRNA expression (**Supplementary Fig. 2**).

Table 1. The connections between the expression of discs large homolog 2 and clinical pathological parameters in hepatocellular carcinoma patients (n = 90).

Factors	N	DLG2 expression		*p*-value	χ^2
		Low (45)	High (45)		
Sex				0.800	0.064
Male	70	34 (48.6)	36 (51.4)		
Female	20	11 (55.0)	9 (45.0)		
Age				0.816	0.054
≤60	64	33 (51.6)	31 (48.4)		
>60	26	12 (46.2)	14 (53.8)		
Histological grade				**0.018**	5.602
G1/G2	54	21 (38.9)	33 (61.1)		
G3	36	24 (66.7)	12 (33.3)		
Tumor size (diameter, cm)				**0.012**	6.257
≤5	53	26 (49.1)	27 (50.9)		
>5	36	28 (77.8)	8 (22.2)		
HBV infection				1.000	0.000
Negative	19	9 (47.4)	10 (52.6)		
Positive	71	36 (50.7)	35 (49.3)		
AFP (μg/L)				1.000	0.000
≤200	45	22 (48.9)	23 (51.1)		
>200	45	23 (51.1)	22 (48.9)		
pT stage				**0.037**	4.339
T1	58	21 (36.2)	37 (63.8)		
T2/T3/T4	31	4 (12.9)	27 (87.1)		
pN stage				0.244	1.358
N0	85	49 (57.6)	36 (42.4)		
N1–N3	4	4 (100.0)	0 (0.0)		
pM stage				0.653	NA
M0	87	51 (58.6)	36 (41.4)		
M1	2	2 (100.0)	0 (0)		
pStage*				<**0.001**	12.32
I	56	25 (44.6)	31 (55.4)		
III/IV	33	28 (84.8)	5 (15.2)		
Vessel invasion#				**0.036**	4.392
No	50	26 (52.0)	24 (48.0)		
Yes	25	20 (80.0)	5 (20.0)		

*Stages are grouped into I and II/III/IV. #Vessel invasion is grouped into No and Yes. Bold number indicates significance ($p < 0.05$). AFP, alpha-fetoprotein; HBV, hepatitis B virus; NA, not applicable; pT, pathological primary tumor; pN, pathological lymph node; pM, pathological metastasis; pStages, pathological stages.

DLG2 Inhibited Proliferation of HCC Cells

In HCC cell lines, the *DLG2* expression was significantly reduced to various degrees compared to hepatic cell line L02 (Fig. 5A). HCCLM3 and MHCC97-H had significantly the lowest expression of *DLG2*, and HepG2 had significantly the highest expression of *DLG2* among

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Table 2. Univariate and multivariate Cox proportional hazard models analyses for overall survival in hepatocellular carcinoma patients (n = 90).

Clinicopathological features	Univariate analysis		Multivariate analysis	
	HRs [95% Cis]	*p*-value	HRs [95% Cis]	*p*-value
Sex				
Male	1 [Reference]			
Female	1.47 [0.76–2.85]	0.258		
Age				
≤60	1 [Reference]			
>60	0.78 [0.40–1.51]	0.462		
Histological grade				
G1/2	1 [Reference]		1 [Reference]	
G3	2.01 [1.11–3.63]	**0.022**	0.81 [0.39–1.67]	0.559
Tumor size (cm)				
≤5	1 [Reference]		1 [Reference]	
>5	2.32 [1.26–4.27]	**0.007**	2.25 [1.09–4.60]	**0.027**
HBV infection				
Negative	1 [Reference]			
Positive	1.06 [0.51–2.21]	0.872		
AFP (μg/L)				
≤200	1 [Reference]		1 [Reference]	
>200	3.17 [1.67–6.00]	<**0.001**	3.30 [1.38–7.92]	**0.008**
pStage				
I	1 [Reference]		1 [Reference]	
II/III/IV	5.11 [2.73–9.53]	<**0.001**	2.67 [1.12–6.33]	**0.026**
Vessel invasion				
No	1 [Reference]		1 [Reference]	
Yes	4.15 [2.12–8.10]	<**0.001**	1.24 [0.54–2.86]	0.610
DLG2 expression				
Low	1 [Reference]		1 [Reference]	
High	0.37 [0.20–0.70]	**0.002**	0.42 [0.19–0.92]	**0.030**

AFP, alpha-fetoprotein; CI, confidence interval; *DLG2*, discs large homolog 2; HBV, hepatitis B virus; HR, hazard ratio; pStage, pathological stage. Bold number indicates significance ($p < 0.05$).

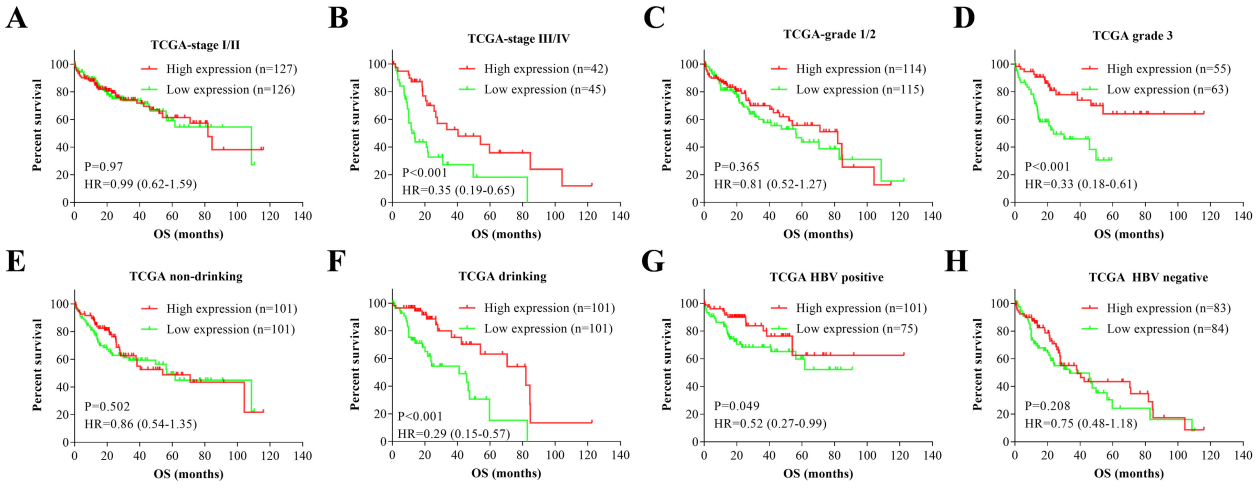

Fig. 3. OS (overall survival) analysis of the high expression and low expression of *DLG2* (discs large homolog 2) in different clinicopathological HCC (hepatocellular carcinoma) groups in the TCGA (The International Cancer Genome Consortium) cohort. (A) Early TNM stages. (B) Late TNM stages. (C) Grade 1/2. (D) Grade 3. (E) Non-drinking. (F) Drinking. (G) HBV-positive. (H) HBV-negative.

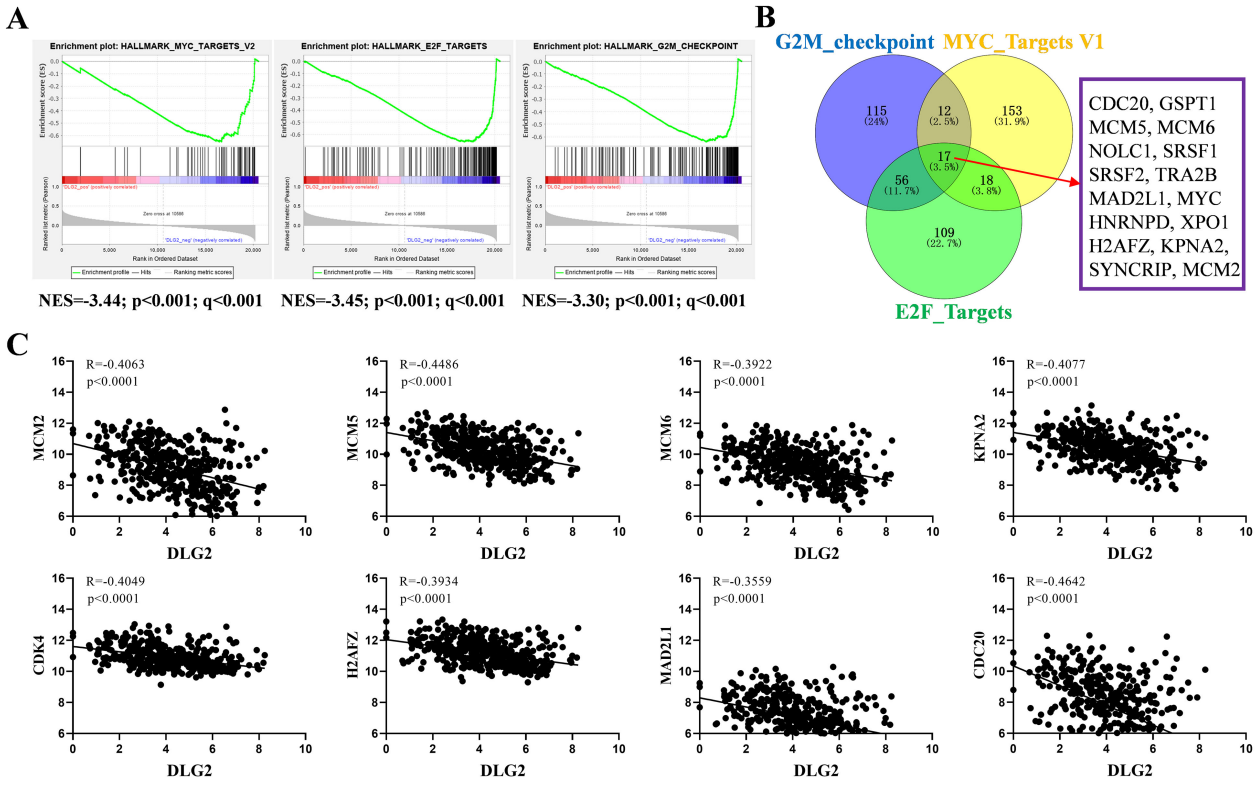

Fig. 4. *DLG2* (discs large homolog 2) was associated with cell proliferation in HCC progression. (A) GSEA showed that low *DLG2* expression was obviously associated with HALLMARK_MYC_TARGETS_V2, HALLMARK_E2F_TARGETS, and HALLMARK_G2M_CHECKPOINT in the TCGA cohort. (B) The common genes of these 3 pathways were overlapped by Venny tool. (C) Correlations of DLG2 with MCM2, MCM5, MCM6, KPNA2, CDK4, H2AFZ, MAD2L1 or CDC20 in expression in LIHC patients of the TCGA cohort.

all cell lines. After validation of *DLG2* expression using qPCR, *DLG2* overexpression HCCLM3 cells and *DLG2*-knockdown HepG2 cells were successfully constructed, where the cells from shDLG2-2 groups showed significantly the lowest *DLG2* level, and therefore selected for further experiments (Fig. 5B). Knockdown of *DLG2* significantly enhanced HepG2 cell viability and proliferation, and this effect was significantly eliminated by overexpressing *DLG2* in cells (shDLG2/DLG2 group) (Fig. 5C,D). Meanwhile, *DLG2* overexpression significantly decreased the viability and proliferation of HCCLM3 cell, and this effect was significantly restored in the cells with *DLG2* overexpressed and then knocked down (DLG2/shDLG2/group) (Fig. 5C,D).

Discussion

The aim of this study was to investigate the role in the progression of hepatocellular carcinoma. The study showed that *DLG2* expression in HCC tissues was significantly decreased, compared to h adjacent NTs, and low *DLG2* level was related to the pT stage, tumor size, histological grade and vessel invasion, which indicated that *DLG2* might be referred to HCC tumorigenesis and progression. In addition, the study showed that *DLG2* was an independent prognostic factor in HCC, and that HCC patients with lower *DLG2* level had significantly worse OS and RFS. In hepatocellular carcinoma, the potential prognostic value and its anti-proliferation role are reported for the first time.

Consistent with this finding, Shao *et al.* [18] demonstrated that *DLG2* might be as a bone tumor suppressor candidate through comparative genomics in idiopathic osteosarcomas in human and dog, and they further demonstrated that osteoblast-specific *DLG2* knockout reduced overall survival and accelerated osteosarcomas development. Likewise, a previous study reported that, in neuroblastoma, patients with lower *DLG2* expression predicted more poor OS time [15]. For the first time, our study certified that *DLG2* might be able to serve as a good predictive biomarker for patients at stage III/IV or grade 3, and who drank or were infected with HBV. These reports were in other types of tumors and this study is the first one to report them in HCC.

The KEGG (Kyoto Encyclopedia of Genes and Genomes) enrichment analysis indicated that low *DLG2* expression in HCC was significantly associated with G2M

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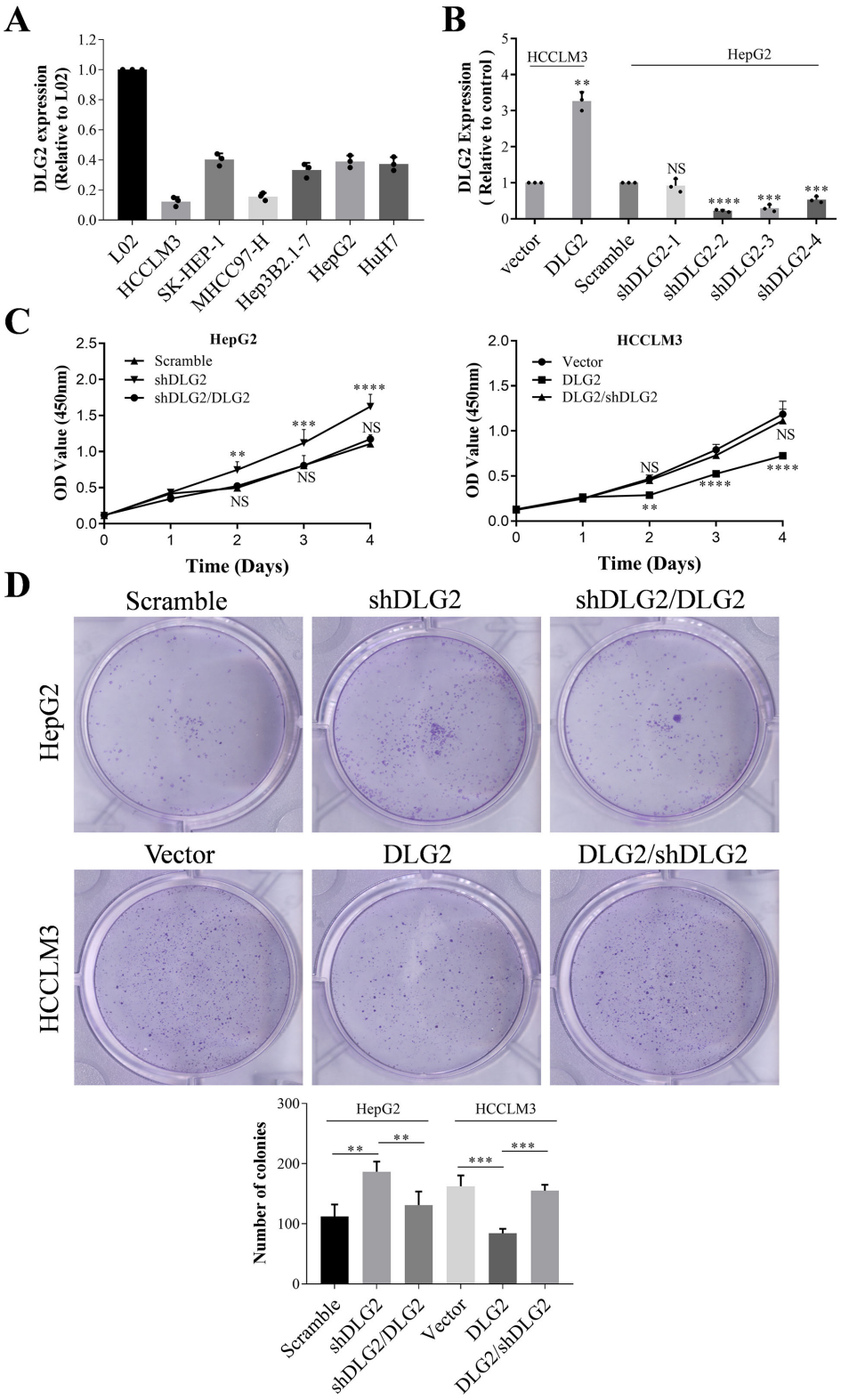

Fig. 5. ***DLG2*** **(discs large homolog 2) inhibited proliferation of HCC (hepatocellular carcinoma).** (A) The *DLG2* mRNA expression in L02 and 6 HCC cell lines. (B) The *DLG2* mRNA level in *DLG2* knockdown or overexpression HCC cell lines. (C) The cell viability of HCCLM3 and HepG2 cells after various treatments This information belongs to the method and did not need to be repeated here. (D) The determination of HCCLM3 and HepG2 proliferation. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, NS, not significant, compared with corresponding control. The dots on the histograms (A & B) are the corresponding individual values.

checkpoint, *E2F* targets, and *MYC* targets V2 pathways. Li *et al.* [19] also reported that these gene sets were significantly enriched in HCC tumor phenotype. In addition, our study showed that *DLG2* was significantly negatively related to the eight common genes (*MCM2, MCM5, MCM6, CDK4, KPNA2, H2AFZ, MAD2L1* and *CDC20*) in these three pathways. In the future, if some studies focus on the exact mechanism mediated by *DLG2* in HCC progression, these genes should be paid more attention to. Therefore, we would like to display some findings about the diagnosis value and/or functions of these genes or their types in cancers through literature research:

In HCC patients, *MCM2-7* (minichromosome maintenance complex 2-7) might be a potential diagnostic biomarker, while, *MCM2* (minichromosome maintenance complex component 2) and *MCM6* (minichromosome maintenance complex component 6) might be the underlying prognostic biomarkers [20]. In the 175 HCC patients of a study, *MCM6* independently predicts poor survival, and the *MCM6* silencing in HCC cells decreases some cell cycle related genes, such as *CCNA1-2*, *CDK2* (cyclin dependent kinase 2) and *CDK4* (cyclin dependent kinase 4), which leads to a delayed S/G2-phase (synthesis/gap 2 phase) progression [21]. In HepG2 and Hep3B cells, the decreased *MCM5* (minichromosome maintenance complex component 6) expression represses colony formation, cell proliferation, and cell cycle progression [22]. MCM proteins have the activity of replicative helicase and unlocks the DNA (deoxyribonucleic acid) template before the replication fork, and they are necessary conserved proteins in DNA replication of eukaryotes [23]. In addition to HCC, MCM also abnormally expresses in other cancer, such as human gliomas and cervical cancer, and it is related to disease prognosis [24,25].

CDK4 is a member of the cyclin-dependent kinase family, and Cdk4 (cyclin-dependent kinase 4)-CyclinD in G1 phase drive cells entry into S phase [26]. In human HCC tissues, *CDK4* is upregulated, and upon glucose induction, *CDK4* mediated transcription may inhibit cell cycle progression and tumor growth [27]. *KPNA2* (karyopherin subunit alpha 2) anticipates nucleocytoplasmic transport in eukaryotes by bounding transport cargo protein and importin-β to form a complex for interacting with the nuclear pore complexes, and then, translocating into the nucleus [28,29]. Downregulation of *KPNA2* inhibits the proliferation of cells in breast cancer and induces the cycle arrest by block of c-Myc nuclear translocation [30]. A previous study indicated that *KPNA2* was significantly increased and its silencing significantly decreased the cell viability, and that the high *KPNA2* level was related to the poor prognosis in HCC [31]. *H2AFZ* (H2A histone family member Z) largely expresses in various cancer, such as non-small cellular lung cancer, breast cancer, and bladder cancer [32–34]. In HCC, *H2AFZ* is associated with the immune infiltrations, and *TP53* mutation regulates the overexpression of *H2AFZ*, leading to rapid proliferation phenotype of HCC cells *in vitro* [35]. *MAD2L1* (mitotic arrest deficient 2 like 1) is an element of spindle assembly checkpoint, and it delays cell division until the cells ensure the precise segregation of chromosome, to keep the stability in genome [36,37].

MAD2L1 (mitotic arrest deficient 1 like 1) directly recruits *MAD2L1* in the early stage of mitosis, and *MAD2L1* interacts with *CDC20* (cell division cycle 20), an anaphase promoting complex regulator, to suppress the complex activation in late stage of mitosis, ultimately resulting in the segregation of sister chromatids [38]. In HCC, *MAD2L1* gene largely expresses in HCC patients, and high *MAD2L1* expression correlates with awful OS [39]. Compared with HCC patients that have low *CDC20* expression, patients of higher *CDC20* expression have poor overall survival [40]. In HCC tissues and HCC cell lines, *CDC20* is highly upregulated, and the knockdown of *CDC20* promotes E-cadherin (epithelial cadherin), and decreases N-cadherin (neural cadherin) and vimentin expression to mediate cell epithelial-mesenchymal transition [41]. A previous study showed that the decreased *CDC20* inhibited cell proliferation, promoted G2/M (gap 2 phase and mitosis phase) arrest and aggravated DNA damage in HCC cells [42].

Based on the above knowledge, we noticed these genes are closely associated with one another instead of simply correlated in expression level. Therefore, we speculate that *DLG2* may regulate the related genes of cell proliferation, such as *MCM2, CDK4, MAD2L1* and *KPNA2*, to affect HCC development, which needs further verification.

Many studies showed that the regulation of *DLG2* in cell proliferation of some tumor. A previous study reported that low *DLG2* expression enhanced cell cycle progression [15]. *DLG2* overexpression inhibits the colorectal cancer cell proliferation and promotes phosphorylation of Yes-associated protein, which is a downstream effector of DLG2 and has a tumor-suppressive capacity [43]. Zhuang *et al.* [16] demonstrated that *DLG2* silence remarkably enhanced viability and cloning ability of ovarian cancer cells, and bigger tumor formation was found in nude mice with siRNA-DLG2 (small interfering ribonucleic acid targeting at *DLG2*) injection in ovarian cancer cells. In addition, a previous study indicated that *DLG2* overexpression obviously reduced the DNA fragmentation of neuroblastoma cells exposed to ultraviolet C radiation, and it induced cell apoptosis mediated by p53 [44]. In our study, *DLG2* knockdown obviously promoted cell viability, proliferation ability of HepG2 cells, and *DLG2* overexpression significantly reduced the viability and proliferation of HCCLM3 cells. Therefore, in HCC, *DLG2* may also inhibit tumor growth by repressing cell proliferation.

In addition to the effect of *DLG2* on cell proliferation, many studies looked at the regulation of *DLG2* other cell biological processes in cancer, and the role of the particular *DLG2* isoform was also explored. A previous study indicated that, in glioma cells, *DLG2* was significantly down-

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regulated, and in addition to affecting the cell proliferation, *DLG2* also regulated the cell migration and invasion [45]. In cell and animal models induced with inflammatory agents, the expression of *DLG2* was significantly increased at the beginning and then decreased, and *DLG2* overexpression promoted the expression of interleukin-1beta, IκBζ (I-kappa-B-zeta) and Bax (B-cell leukemia/lymphoma 2-associated X protein), which enhanced the formation of inflammasome in the gut and colon cancer [46]. Therefore, *DLG2* may be also related to function by regulation *NLRP3* (nucleotide-binding domain and leucine-rich repeat containing family pyrin domain containing 3) inflammasome axis. In the future, the regulation of *DLG2* on inflammatory in HCC may also be considered. In renal oncocytoma, a new *DLG2* isoform highly expresses, but it decreases in normal kidney tissues and other renal cell cancer [47]. A previous study showed that, only two *DLG2* isoforms (isoform 2 and isoform 7/8) are expressed in neuroblastoma, and in high stage neuroblastoma, a decreased expression was only observed in the full length L27 involving *DLG2* isoform 7/8 and the increased isoform 7 level decreased the cell viability and proliferation of neuroblastoma cells [48]. Therefore, in the future, the *DLG2* isoforms should be took into account in further investigation of the function and role of *DLG2* in HCC. After we finish all these issues, we might be able to develop *DLG2* as a potent molecular target for HCC clinical therapy.

There are some limitations in this study. There was no *in vivo* tumorigenesis experiment used to verify the inhibition of *DLG2* on HCC cell proliferation. The effects of *DLG2* on other biological behaviors of HCC cells, such as invasion and cell apoptosis, were not explored. The role of *DLG2* and its effect on the prognosis of different types of HCC was not researched. Overall, the molecular mechanism of proliferation regulation by *DLG2* in HCC still requires more efforts.

Conclusions

The aim of this study was to investigate the potential value in diagnosis and prognosis, and preliminarily reveal its function in HCC. The study showed that *DLG2* was significantly decreased in HCC, and it was possibly a novel potential diagnostic biomarker in HCC. The study also confirmed that *DLG2* might be a good potential prognostic biomarker for stage III/IV, grade 3, alcohol consuming, and HBV infection HCC patients. Additionally, *DLG2* may participate in progression of HCC by regulating proliferation related genes in HCC cells. These findings may provide the basis for *DLG2* becoming a candidate gene to be used in gene therapy of HCC.

Availability of Data and Materials

The data used to support the findings of this study are included within the article.

Author Contributions

JWG and RQH—performed the experiments, interpreted the data, and contributed the most to writing the manuscript, they are listed as the co-first authors; JWG, RQH, YZ, GMN, ZQH, XTZ, and ZPC—analyzed and visualized the data, and they were responsible for data interpretation, literature search, and statistical analysis; XL and CWK—contributed to the design and conception of the study, and were major contributors in critically revising the manuscript. All authors read and approved the final version of the manuscript.

Ethics Approval and Consent to Participate

The written informed consent had been received from each patient before the study. The use of the TMA complied with relevant regulations and was approved by the Ethics Committee of Shanghai Fifth People's Hospital, Fudan University (Approval Number: 2020SDW-012).

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.23812/j.biol.regul.homeost.agents.20223605.132.

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