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Down-regulation of DLG2 predicts an unfavorable prognosis and promotes the proliferation and migration of Hepatocellular Carcinoma.

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Abstract

Background Discs large MAGUK scaffold protein 2 (DLG2), a member of the MAGUK family, has been associated with certain tumor suppressing processes. In this study, we aim to identify the prognosis value and specific function of DLG2 in hepatocellular carcinomas (HCCs).

Methods Expression of DLG2 in HCCs and adjacent normal tissues (NTs) was analyzed with transcriptomic datasets from the Integrative Molecular Database of Hepatocellular Carcinoma (HCCDB) and immunohistochemical (IHC) staining of a tissue microarray (TMA). Prognostic roles of DLG2 in HCCs were investigated in the TMA cohort and validated in two cohorts from HCCDB. The in vitro activities of DLG2 were investigated in cultured HCC cells with lentiviruses. The underlying mechanism was explored using Gene Set Enrichment Analysis (GSEA) and gene-gene correlation analyses with The Cancer Genome Atlas Liver Hepatocellular Carcinoma (TCGA-LIHC) dataset.

Results The expression of DLG2 was significantly decreased in HCCs compared to that in NTs. Down-regulation of DLG2 in HCCs was associated with unfavorable prognosis. Overexpression of DLG2 inhibited, while knockdown of DLG2 prompted proliferation and migration of cultured HCCs. Mechanistically, DLG2 may inhibited cell growth of HCCs by interacting with key molecules that regulate cell cycles.

Conclusion DLG2 inhibited HCC progression and may be a novel prognosis biomarker and therapeutic target for HCC.

Background

According to the data from the International Agency for Research on Cancer assessed in 2018, the morbidity and mortality rate of liver cancer worldwide is sixth and fourth among all tumor types respectively(1). Hepatocellular carcinoma (HCC) which mainly develops in patients with underlying chronic liver disease, such as hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, alcohol abuse and nonalcoholic fatty liver disease, is the most common form of liver cancer. Although many kinds of prevention and therapies like HBV vaccination, antiviral therapy and surgical resection have been used upon this intractable disease, the 5-year survival rate of HCC in China is still at a low level. This is partially due to the high probability of metastasis and poor patient prognosis. Different hypotheses like clonal selection or neutral and punctuated acquisition of genetic alternations have been proposed to explained tumor occurrence and development(2). It is crucial to further characterize the underlying mechanisms and key biomarkers of HCC metastasis to develop effective therapeutic measures and improve patient survival.

Discs large MAGUK scaffold protein 2 (DLG2), also known as PSD93, PSD-93, PPP1R58 and chapsyn-110, is identified as a member of the membrane-associated guanylate kinase (MAGUK) family. One of the basic function of MAGUKs is to bind to and stabilize proteins at synapses. Several studies have demonstrated that MAGUKs regulate the synaptic expression of glutamate receptors(3). Being a member

of this family, DLG2 is also extensively participated in plenty of processes regulating synaptic expression of receptors and synaptic plasticity. In addition, DLG2 is essential for certain neurological and psychiatric disorders like schizophrenia(4), Parkinson's disease(5), and ischemic cerebral injury(6) etc. Many of the early studies about DLG tried to figure out the physical and pathological functions of this protein through studying Drosophila larvae, in which DLG showed tumor suppressor properties. Loss of DLG function was associated with excessive over-proliferation and neoplastic transformation of epithelial cells within the imaginal discs(7). Disruption of Drosophila DLG also resulted in acute disorganization of epithelial structure with disruption of intercellular junction formation and a loss of apico-basal cell polarity(8, 9).

While in mammalian epithelium, the members of DLG2 play a crucial role in epithelial polarity and polarity during cell division(10). Another member of this MAGUK family, DLG1, exhibited a decrease expression in several solid tumors including cervical, colon and breast cancers(11–14). The breast tumor associated DLG1 mutations diminished binding between DLG1 and two of its tumor suppressor targets adenomatous polyposis coli (APC) and phosphatase and tensin homologue (PTEN)(14, 15). These findings strongly indicated that DLGs possess a tumor suppressor function in mammals and some DLG functions are conserved between species(16). Contradictorily, there are also evidences that specific cellular pool of DLG1 will occur and show certain oncogenic functions in the presence of viral oncoproteins(17, 18). Therefore, the functions of DLGs in different tissues may vary and still needs further study.

Up till now, the roles of DLG2 in cancers such as HCC remain unclear. Herein, we reported the expression, prognosis value and tumor suppressor role of DLG2 in HCCs for the first time. We also investigated the *in vitro* activities of DLG2 in cultured HCC cells and tried to figure out the potential underlying molecular mechanisms of DLG2's tumor suppressor role in HCCs.

Methods

Access to public datasets

Fifteen datasets including GSE6764(19), GSE10143(20), GSE14323(21), GSE14520(22, 23), GSE22058(24, 25), GSE25097(26–28), GSE36376(29), GSE46444(30), GSE54236(31), GSE63898(32), GSE76427(33), GSE112790(34), GSE64041(35), the Liver Hepatocellular Carcinoma Project of The Cancer Genome Atlas (TCGA-LIHC)*, and the Liver Cancer-RIKEN, JP Project from the International Cancer Genome Consortium (ICJC-LIRI-JP)(36) were retrieved from the Integrative Molecular Database of Hepatocellular Carcinoma (HCCDB)(37), and analyzed for DLG2 mRNA expression in HCCs and adjacent hepatic normal tissues (NTs). These datasets included 1431NTs and 2,321 HCCs. Four of these datasets compared gene transcription between HCC and adjacent cirrhotic tissues (GSE10143, GSE14323, GSE25097, and GSE6764), two of them included liver tissues from healthy volunteers (GSE64041 and GSE25097), and one of them also included tissues from different disease stages (GSE6764). The TCGA-LIHC dataset and ICJC-LIRI-JP dataset were used to validate survival analyses from a tissue microarray

(TMA) described below. To explore potential mechanisms of DLG2 in HCC progression, a GSEA was employed using the TCGA-LIHC dataset*.

Tissue microarray and immunohistochemistry (IHC)

Microarray sections of HCCs and neighboring NTs containing 90 paired HCCs and NTs from patients [the TMA cohort] were prepared by Shanghai Tufei Biotech Co., Ltd (Cat. No. TFHCC-01; Shanghai, China). The clinicopathological characteristics of these patients are shown in Table 1.

Table 1

Clinical and pathologic features of HCC patients* (n = 90).

Variable	No. of patients (%)
Sex	
Male	70(77.8)
Female	20(22.2)
Age	
≤ 60	64(71.1)
> 60	26(28.9)
Differentiation	
G1/2	54(60)
G3	36(40)
Tumor size (cm)	
≤ 5	47(52.2)
> 5	43(47.8)
HBV infection	
Negative	19(21.1)
Positive	71(78.9)
AFP(μg/L)	
≤ 200	45(50)
> 200	45(50)
Tumor stage	
T1	58(64.5)
T2	17(18.9)
T3	3(3.3)
T4	12(13.3)
Nodal stage	
N0	85(94.4)

*Data shown here may be duplicated with those from other published resources that are based on the same cohorts. Abbreviations: NA, information not available.

Variable	No. of patients (%)
N1	5(5.6)
M stage	
M0	88(97.8)
M1	2(2.2)
TNM stage	
I	56(62.2)
II	17(18.9)
III	16(17.8)
NA	1(1.1)
Vessel invasion	
No	50(55.6)
Yes	25(27.8)
NA	15(16.6)

*Data shown here may be duplicated with those from other published resources that are based on the same cohorts. Abbreviations: NA, information not available.

As described previously(38), after deparaffination, rehydration in graded ethanol, antigen retrieval with citrate buffer pH 6.0 (1:300 dilution; cat. no. Zli-9065; oriGene Technologies, inc.) and blocking with goat serum (1:20 dilution; cat. no. C0265; Beyotime Institute of Biotechnology) at room temperature for 1 h, slides were stained with a rabbit polyclonal antibody against human DLG2 (1:50 dilution; cat. no. abs141074; Absin Bioscience Inc, Shanghai, China) at 4˚C overnight. Normal rat immunoglobulin G (1:50 dilution; cat. no. D110504; Sangon Biotech Co., Ltd.) instead of the primary antibody was used as a control.

Subsequently, after washing with PBS, a horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000; goat anti-rabbit, cat. no. A0208; Beyotime Institute of Biotechnology, Haimen, China) was added and incubated at room temperature for 1 h. Then, these sections were stained using 3,3'-diaminobenzidine (DAB) (cat. no. GK500705; Shanghai GeneTech Co.,Ltd., Shanghai, China) at room temperature for 5 min and counterstained with 100% hematoxylin (cat. no. C0107; Beyotime Institute of Biotechnology) at room temperature for 2 min. A modifed H score system was used to semiquantitate DLG2 expression, as previously described(39). Briefly, the maximal intensity of staining (0, negative; 1, weak; 2, moderate; and 3, strong) was multiplied by the percentage of positive tumor cells (0100%) to generate the modifed H score (range, 0300). DLG2 expression was categorized as high or low based on the median H score.

Cell lines and culture conditions

A hepatic cell line (L02) and four HCC cell lines (HCCLM3, MHCC97-H, SMMC-7721, Huh7) were kindly provided by Dr. Lijie Ma at Zhongshan Hospital, Fudan university (Shanghai, China). HCC cell lines SK-HEP-1 and Hep3B2.1-7 were kind gifts from Prof. Yongzhong Liu at the Cancer Research Institute, Shanghai Jiao Tong University (Shanghai, China); HCC cell line HepG2 was kindly provided by Prof. Xiuping Liu at the Shanghai Cancer Hospital, Fudan university (Shanghai, China). All cell lines used in this study were regularly authenticated by morphological observation and tested for the absence of mycoplasma contamination (MycoAlert; Lonza Rockland, Rockland, ME, USA).

Cells were cultured in Dulbecco's Modified Essential Medium (DMEM; BBI Life Sciences, Shanghai, China) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin, and 100 mg/mL streptomycin at 37 °C with 5% CO_2 in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA).

Construction of DLG2-expressing plasmids and DLG2targeting shRNAs and packaging of lentiviruses

GFP-expressing lentivirus particles were prepared by Genechem (Shanghai, China). The vectors used were GV358 (for DLG2 and the vector control) and GV248 (for shDLG2 and the scrambled control). The target sequences for shDLG2 were GCCAGTACAATGACAATTT (shDLG2-1), GATCAATGACGACTTGATA (shDLG2-2), GTGAACAAACTATGTGATA (shDLG2-3) and TTGTGGAAATCAAACTGTT (shDLG24). Lentiviral stocks were prepared and purifed as previously described(40).

Infection of HCC cells with lentiviruses

Cells were seeded in 6-well plates at a density of 2 × 105/ml and cultivated for 24 h. Then, 20 μl of lentivirus solution and 1 ml fresh medium containing 10 μg/ml polybrene (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were added to each well. The medium was changed after 24 h and an effcient lentiviral transduction was confirmed by a fluorescence microscope at 48 h and 72 h after infection.

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from cell cultures using RNAiso Plus (Takara Bio, Kusatsu, Japan) according to the manufacturer's instructions, and reverse-transcribed and subjected to real-time reverse transcription-PCR using the 2-ΔΔCT method(41). Each sample was determined in duplicate. All PCR products were confirmed by 2.0% agarose gel electrophoresis. The sequences for RT-PCR primers were the following: DLG2 forward primer, 5'- ATGTTCGGCACCTGTCTGTG-3'; and DLG2 reverse primer, 5'-CCTCCAAAACAAAACCCTTTGG-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control. Experiments were repeated three times, in duplicate.

Western blotting

Total protein was extracted and quantitated following the established protocol described previously (42). Western blotting was performed using a rabbit anti-DLG2 polyclonal antibody (1:1,000 dilution; cat. no. abs141074; Absin Bioscience Inc, Shanghai, China). GAPDH (1:2,000 dilution, rabbit anti-human; Beyotime Biotechnology) was detected as a loading control(43).

Cell proliferation assay

Briefly, stably transfected SMMC-7721 or HCCLM3 cells (2×10^3 cells/well) were seeded in 96-well plates and cultivated for 24, 48, 72, or 96 h. Then, 10 μL cholecystokinin octapeptide-8 reagent (CCK-8) [10% (v/v) in serum-free DMEM; Beyotime Biotechnology] was added to each well and incubated at 37 °C for 1 h. The absorbance at 450 nm was measured using a microplate reader (BioTek Synergy 2; BioTek, Winooski, VT, USA). The detailed protocol of cell proliferation assays could be retrieved from the previous article(42).

Cell colony formation assay

As previously described(42), stably transfected SMMC-7721 or HCCLM3 cells (2×10^3 cells/well) were seeded in 12-well plates and cultivated in DMEM complete medium at 37˚C for 14 days. The cell colonies were washed with phosphate-buffered saline (PBS) twice, fixed with methanol for 20 min, and stained with crystal violet solution (C0121; Beyotime Institute of Biotechnology) for 15 min. The plates were scanned and colonies containing > 50 cells were counted manually, and the experiments were performed in triplicate.

Scratch wound healing assay

Following the previously described protocol(42), stably transfected SMMC-7721 or HCCLM3 cells (4×10^5 cells/well) were seeded in 12-well plates and grown to nearly 100% confluence. After 24 h of serum starvation, a scratch wound was generated with a 200 μL pipette tip. Wound closure was photographed at 0 and 24 h under a microscope using the ImageScope software (Leica Biosystems Nussloch GmbH) with a magnification of x40.

Gene set enrichment analysis

A Gene Set Enrichment Analysis (GSEA) was performed as previously described(42). Briefly, the TCGA LIHC RNA-seq data were loaded into GSEA and run with the latest version of Hallmarks gene sets. Patient data were divided into DLG2 high and low groups by the median of DLG2 expression. Then GSEA was performed according to the default weighted enrichment statistics and genes were ranked by the Pearson method. Gene sets were considered significantly enriched if $P < 0.05$ and $FDR < 0.25$. Those gene sets that

were significantly enriched in the DLG2 high group were designated as DLG2-positively related gene sets, while those that were significantly enriched in the DLG2 low group were designated as DLG2-negatively correlated gene sets.

Statistical analysis.

Statistical analyses were performed using GraphPad Prism7 (GraphPad Software Inc., San Diego, CA, USA), SPSS statistical software for Windows, version 22 (IBM Corp., Armonk, NY, USA), and Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). Student's t-tests were performed for continuous variables between two groups and Wilcoxon signed-rank tests were used if the differences between pairs of data did not follow a Gaussian distribution. One-way analysis of variance (ANOVA) was performed for statistical comparisons among multiple groups along with Bonferroni's post hoc test. Pearson's $\chi 2$ test and Fisher's exact test were used for categorical comparisons. Pearson's R correlation test was used to describe correlations between continuous variables. Survival analyses were conducted using the Kaplan-Meier method and differences in survival were examined using the log-rank test. Univariate and multivariate survival analyses were conducted using the Cox proportional hazards regression model. Statistical significance was defined as a value of $P < 0.05$. All statistical tests were two-sided.

Results

DLG2 is down-regulated in HCCs

DLG2, as far as we know, encodes a kind of protein that has not been studied much in carcinogenesis and the role of DLG2 in HCCs remains unclear now. So, we first searched the online database HCCDB, which integrates 15 independent gene profile datasets about HCC. The search results showed that the expression level of DLG2 was down-regulated in HCC tissues compared with that in adjacent NTs in 10 independent datasets (GSE14520, GSE22058, GSE36376, GSE63898, GSE76427, GSE112709, GSE64041, GSE6764, TCGA-LIHC, and ICGC-LIRI-JP) (Fig. 1A, B, D, G, H, I, J, K, L, O). The expression of DLG2 was comparable between HCCs and NTs in two datasets (GSE10143 and GSE46444) (Fig. 1E and F). In 4 of these datasets which included adjacent cirrhotic tissues as control, no significant difference was observed between HCCs and cirrhotic tissues with regard to DLG2 expression (GSE10143, GSE14323, GSE25097, and GSE6764) (Fig. 1C, M, N, O). In one dataset that compared gene expression among different disease stages, the expression of DLG2 gradually decreased as disease became more invasive (GSE6764) (Fig. 1O). Therefore, we had enough reason to believe that DLG2 was down-regulated in HCCs.

To further validate this discovery, we used a tissue microarray which contains 90 paired HCCs and NTs from patients with primary HCCs. We found that DLG2 was mainly expressed in the cytoplasm of cancer cells, and its expression was stronger in NTs than that in HCCs, as quantified by H-score (Fig. 2A-B). Taken together, these results indicated that DLG2 expression was down-regulated in HCCs compared with NTs.

Down-regulation of DLG2 in HCCs correlates with poor prognoses

To figure out the relationship between the low expression level of DLG2 and its prognostic value in HCC patients, we summarized the associations between DLG2 expression and clinicopathological variables of the patients included in the tissue microarray. As shown in Table 2, low expression level of DLG2 was significantly associated with larger tumor size, poor differentiation, pT stage and pStage.

Table 2

Association between DLG2 expression and clinicopathological variables in HCC patients (n = 90).

Clinicopathological features	N	DLG2 expression		
		Low (45)	High (45)	*P*-value
Sex				
Male	70	34(48.6)	36(51.4)	
Female	20	11(55.0)	9(45.0)	0.800
Age				
≤ 60	64	33(51.6)	31(48.4)	
> 60	26	12(46.2)	14(53.8)	0.816
Histological grade				
G1/G2	54	21(38.9)	33(61.1)	
G3	36	24(66.7)	12(33.3)	**0.017**
Tumor size (cm)				
≤ 5	47	17(36.2)	30(63.8)	
> 5	43	28(65.1)	15(34.9)	**0.011**
HBV infection				
Negative	19	9(47.4)	10(52.6)	
Positive	71	36(50.7)	35(49.3)	1.000
AFP(μg/L)				
≤ 200	45	22(48.9)	23(51.1)	
> 200	45	23(51.1)	22(48.9)	1.000
pT stage				
T1	58	22(37.9)	36(62.1)	
T2/T3/T4	32	23(71.9)	9(28.1)	**0.004**
pN stage				
N0	85	40(47.1)	45(52.9)	

Bold type indicates significance.

Clinicopathological features	N	DLG2 expression		
N1-N3	5	5(100.0)	0(0.0)	0.056
pM stage				
M0	88	43(49.4)	45(50.6)	
M1	2	1(50.0)	1(50.0)	0.737
pStage*				
I	56	21(37.5)	35(62.5)	
III/IV	33	23(69.7)	10(30.3)	**0.004**
Vessel invasion#				
No	50	17(34.0)	33(66.0)	
Yes	25	17(68.0)	8(32.0)	**0.007**
Bold type indicates significance.				

Next, we determined the association between DLG2 expression and patient prognosis. Kaplan-Meier survival analysis of the TMA cohort revealed that patients with low expression level of DLG2 had a shorter overall survival (OS) time compared with those with high expression [hazard ratio (HR), 0.38; 95% CI, 0.21–0.70; logrank test, P = 0.002; Fig. 2C], as well as recurrence-free survival (RFS) time [hazard ratio (HR), 0.34; 95% CI, 0.14–0.83, logrank test, P = 0.018; Fig. 2D]. A multivariate analysis with a Cox proportional hazards model indicated that the low expression of DLG2 was significantly associated with a shorter OS [hazard ratio (HR), 0.42; 95% CI, 0.19–0.92; P = 0.030], after adjustment for tumor size, tumor histological grade, pT stage and pStage (Table 3).

Table 3

Univariate and multivariate Cox proportional hazard models for overall survival in HCC patients (n = 90).

Clinicopathological features	Univariate analysis		Multivariate analysis	
	HR[95% CIs]	*P*-value	HR [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]	

Bold type indicates significance. CI, confidence interval; HR, hazard ratio.

Clinicopathological features	Univariate analysis		Multivariate analysis	
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**
Bold type indicates significance. CI, confidence interval; HR, hazard ratio.				

To further confirm the beneficial prognostic role of DLG2 in patients with HCC, we utilized the TCGA-LIHC dataset. The survival analyses showed that low DLG2 expression was correlated with worse OS (Fig. 2E). To eliminate potential influences from confounding factors, we stratified patients in the TCGA dataset by tumor stage, grade, alcohol drinking and HBV infection status. As indicated by Fig. 2F-M, low DLG2 expression was associated with unfavorable OS in HCC patients with advanced stage, low differentiation, drinking and HBV infection. Survival analysis of the ICGC-LIRI-JP dataset also support the beneficial prognostic roles of DLG2 in HCCs (Fig. 2N). In addition, Survival analysis of the TCGA-LIHC datasets demonstrated that low DLG2 expression was also associated with tumor relapse (Fig. 2O).

Taken together, these findings indicated that DLG2 could be a prognostic marker in HCCs and correlated with a favorable prognosis.

DLG2 inhibits the proliferation and migration of cultured HCC cells

To further illustrate the potential role of DLG2 in HCCs, we first used qRT-PCR and western blotting to examine the intrinsic expression of DLG2 in normal and tumorous hepatic cell lines. The results indicated that DLG2 expression was significantly decreased in HCC cells compared to normal cells (Fig. 3A and B).

We then selected HCCLM3 cells to overexpress DLG2 and SMMC-7721 cells to silence the expression of DLG2 with lentiviruses (Fig. 3C and D) to explore the in vitro activities of DLG2 in these cells.

In cultured SMMC-7721 cells, CCK-8 assays and colony formation assays showed that silencing DLG2 expression significantly prompted cell proliferation, which can be reversed by re-expressing DLG2 again. On the contrary, overexpressing DLG2 in cultured HCCLM3 cells showed a reduced cell proliferation, which was also reversed by introducing DLG2-specific shRNAs (Fig. 4A-D). Moreover, knockdown of DLG2 expression increased cell migration in the scratch wound healing assay while overexpression of DLG2 in HCCLM3 cell decreased cell migration, which were reversed in respective recovery experiments (Fig. 4E and D).

Taking all these results into consideration, we believe that DLG2 may antagonize carcinogenesis by inhibiting the proliferation and migration in HCCs.

DLG2 may inhibit HCC progression by modulating cell cycle process

We used the RNA-seq data of HCC samples from TCGA-LIHC dataset to conduct a gene set enrichment analysis (GSEA) (44) to further explore the potential mechanism of DLG2 in HCC development and progression. The result showed that the expression level of DLG2 was negatively correlated with HALLMARK_E2F_TARGETS, HALLMARK_MYC_TARGETS_V1 and HALLMARK_G2M_CHECKPOINT gene sets (Fig. 5A-C), which contain key genes related to regulation of cell cycle process. To verify these findings, we tested the relationship of DLG2 with several cell cycle markers. As shown in Fig. 5D-O, the expression level of DLG2 was significantly and negatively correlated with CDK1, CDK4, CDK7, CCNA2, CCNB1, CCNB2, CCNE2, CCNF, E2F1, E2F2, E2F6 and PCNA. These results suggested that DLG2 may inhibit HCC progression by regulating the cell cycle process.

Discussion

In the present study, through analyzing the gene profile of HCC patients from online datasets, we found that DLG2 was down-regulated in HCC tissues and this was further confirmed in cultured cell lines. Meanwhile, low expression of DLG2 was significantly associated with worse clinical characteristics and unfavorable prognosis in HCC patients, which was drawn from the TMA cohort and further validated by two other patient cohorts. Knockdown of DLG2 prompted the capacity of cell proliferation and migration, while overexpression of DLG2 got an opposite result. A GSEA and subsequent gene-gene correlation analysis connected the tumor inhibition role of DLG2 to regulation of cell cycles.

DLG2 has been studied in several malignant tumors since the beginning of this century. The down-regulation of DLG2 has also been found in other cancer tissues including oropharyngeal squamous cell carcinomas (OPSCCs), ovarian cancer (OC) and osteosarcoma(45–49). DLG2 showed a consistent tumor suppressor role in abovementioned malignancies and the down-regulation of DLG2 was also related with a poor prognosis in OPSCCs and OC(45, 47). These were corresponding with what we found in HCCs, suggesting that the expression level of DLG2 might be an important prognostic factor of certain cancers.

Furthermore, low expression level of DLG2 in these tumor tissues had a close relationship with the increasement of the proliferation, migration and invasion abilities of above mentioned tumor cells. In another word, DLG2 could inhibit the tumor progression process in certain way. In OC cells, the expression level of DLG2 was under the regulation of miR-23a(47). The inhibition of miR-23a in OC cells could increase the expression level of DLG2 and Bax, a member of pro-apoptotic proteins. While another anti-apoptotic protein, Bcl-2 was decreased at the same time. Bcl-2 and Bax had been confirmed to be related to the chemotherapy resistance in various tumors(50). While this kind of regulation of these two proteins in OC cells indicated an accelerated OC cell apoptosis. Meanwhile, Oct-4, Nanog, and CD133, proteins that known as stem cell markers and indicated as invasive and predictive markers in relation to a poor prognosis in OPSCCs(51), were increased simultaneously. This tumorigenesis effect was also associated with the regulation of cell apoptosis.

Another study revealed that the tumor suppressor role of DLG2 in cell division, migration and tumorigenesis in osteosarcoma might have something to do with the negative regulatory role of DLG2 in GTPase signaling and regulation of cell cycle(48). Other members of DLG family, like DLG1 and DLG4, which also contained PDZ domain had early been found to interact with GPCRs, involved in many diseases like cancer(52). Downstream GTPase-activating proteins (GAPs) like GTPase-activating proteins RGS9 (Regulator of G-protein signaling 9) and GARNL3 (GTPase activating Rap/RanGAP domain-like 3), a family of regulatory proteins binding to activated G proteins and functioning as 'switching off' GTPase signaling, were up-regulated when DLG2 expression was overexpressed(53). Therefore, the inhibition role of GAPs was enhanced and the GTPase signaling activity was decreased, which finally lead to the tumor suppressor effect of DLG2 in osteosarcoma.

Apart from OPSCC, OC and osteosarcoma, the alternation of the expression level of DLG2 had been found in many other malignancy cancers, including renal cell carcinoma, glioma, papillary thyroid carcinoma (PTC) and cervical cancer (54–56). Much of these studies pointed out the the differential expression level and potential important tumorigenesis role of DLG2 identified by the protein-protein interaction (PPI) network analysis. However, specific signaling pathway and downstream molecular factors that involved in the regulating process of DLG2 in certain tumors are still lacking. The modulating mechanism of DLG2 already found in osteosarcoma and HCC which was concluded from the GSEA results in this study both pointed to the regulation of cell-cycle process. Based on these studies, it is possible that molecular factors involving in the cell-cycle process may be the potential regulating target that link DLG2 to the proliferation and migration in HCC. This hypothesis needs further investigation.

However, there are still unanswered questions and limits in this study. First, further experiments are required to validate the cell-cycle regulating role of DLG2 in HCC progressing process both *in vivo* and *in vitro*. Second, specific substrates of DLG2 and downstream signaling pathway still remain unknown and need further investigation.

Conclusions

In this study, we revealed that DLG2 is a promising prognostic biomarker for hepatocellular carcinoma (HCC) and it may have a crucial role in inhibiting tumor progression via the regulation of cell cycle-associated process. Targeting DLG2 may be a possible solution to impede HCC progression. To the best of our knowledge, this study is the first to investigate the prognostic value and molecular function of DLG2 in HCCs.

Abbreviations

DLG2
Discs large MAGUK scaffold protein 2
HCC
hepatocellular carcinoma

NT
normal tissue
HCCDB
Integrative Molecular Database of Hepatocellular Carcinoma
IHC
immunohistochemical
TMA
tissue microarray
GSEA
Gene Set Enrichment Analysis
TCGA-LIHC
The Cancer Genome Atlas Liver Hepatocellular Carcinoma
HBV
hepatitis B virus
HCV
hepatitis C virus
MAGUK
membrane-associated guanylate kinase
APC
adenomatous polyposis coli
PTEN
phosphatase and tensin homologue
ICJC-LIRI-JP
JP Project from the International Cancer Genome Consortium
CCK-8
cholecystokinin octapeptide-8 reagent
OS
overall survival
HR
hazard ratio
RFS
recurrence-free survival
OPSCC
oropharyngeal squamous cell carcinomas
OC
ovarian cancer
PTC
papillary thyroid carcinoma

Declarations

Ethics approval and consent to participate

This study was approved by the Institutional Ethics Committee of the Fifth People's Hospital of Shanghai, Fudan University (Ethical approval form no. 2017097) and adhered to the principles in the Declaration of Helsinki. Informed consent was obtained from each patient before tissue collection for experimentation.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Ke CW and Gu JW contributed to designed this study. Gu JW, Niu GM and Hong RQ collected and analyzed the transcriptomic data. Gu JW performed the IHC assay and analyzed the results. Song T, Hu ZQ, Wang X, Chen L and Han SL and Gu JW were responsible for the in vitro experiments. Gu JW was responsible for manuscript drafting. Ren J and Xia J helped to modify the manuscript. Ke CW, Hong L revised the manuscript. All authors read and approved the final manuscript.

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Figures

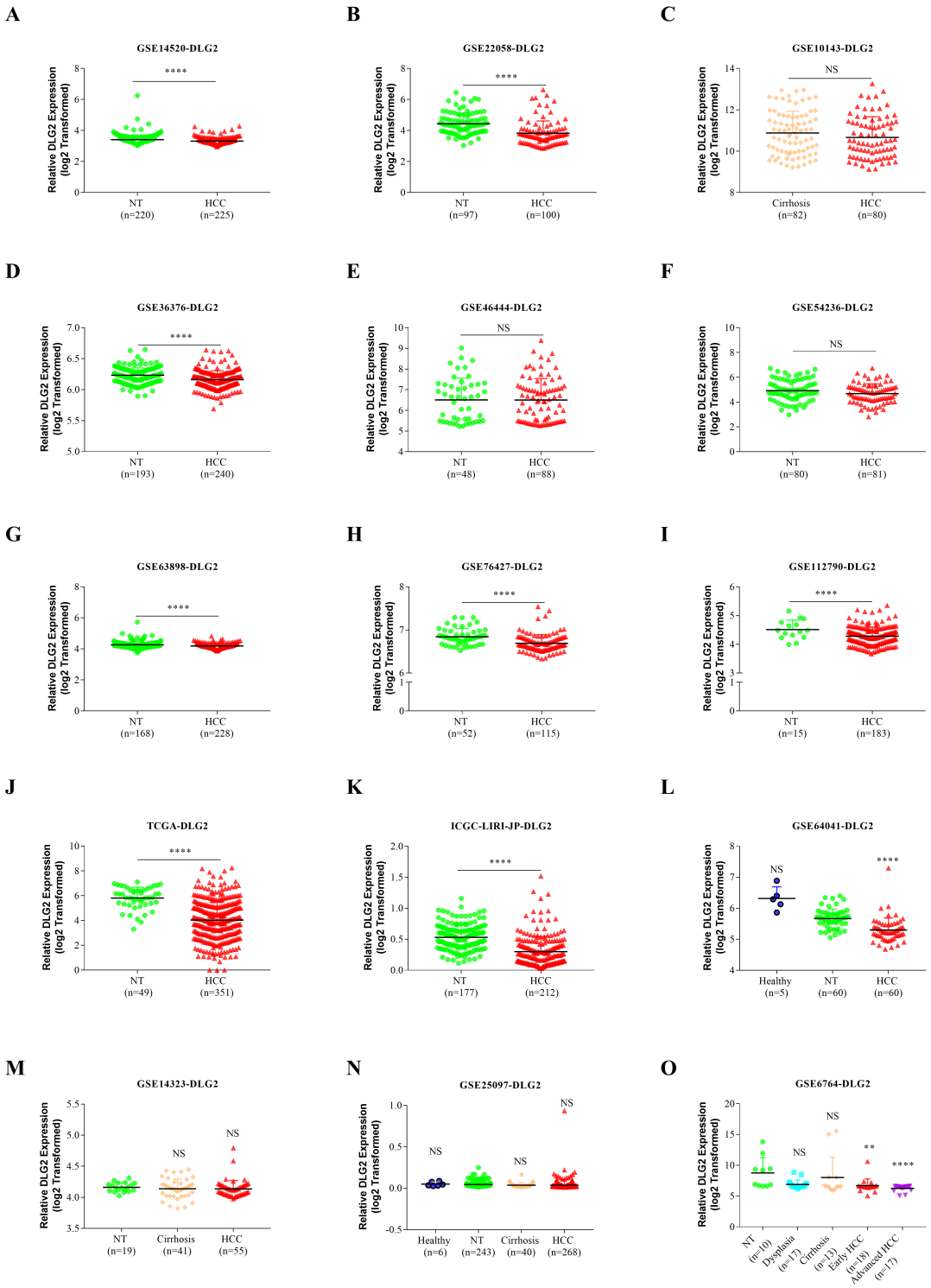

Figure 1

Expression of DLG2 is down-regulated in HCCs. (A, B, D-L) HCCs shows down-regulated expression of DLG2 I compared with adjacent NTs in the gene sets GSE14520, GSE22058, GSE36376, GSE63898, GSE76427, GSE112790,TCGA, ICGC-LIRI-JP and GSE64041, while the difference is not statistical significant in GSE10143, GSE46444 and GSE54236. (C, M, N, O) DLG2 mRNA expression shows no statistical difference between hepatic cirrhosis and HCCs the gene sets GSE10143, GSE14323 and

GSE25097. (O) Compared with NTs, both early and advanced HCCs present with down-regulated expression of DLG2 (GSE6764). *, p < 0.05; **, p < 0.01; ***, p < 0.001；****, p < 0.0001.

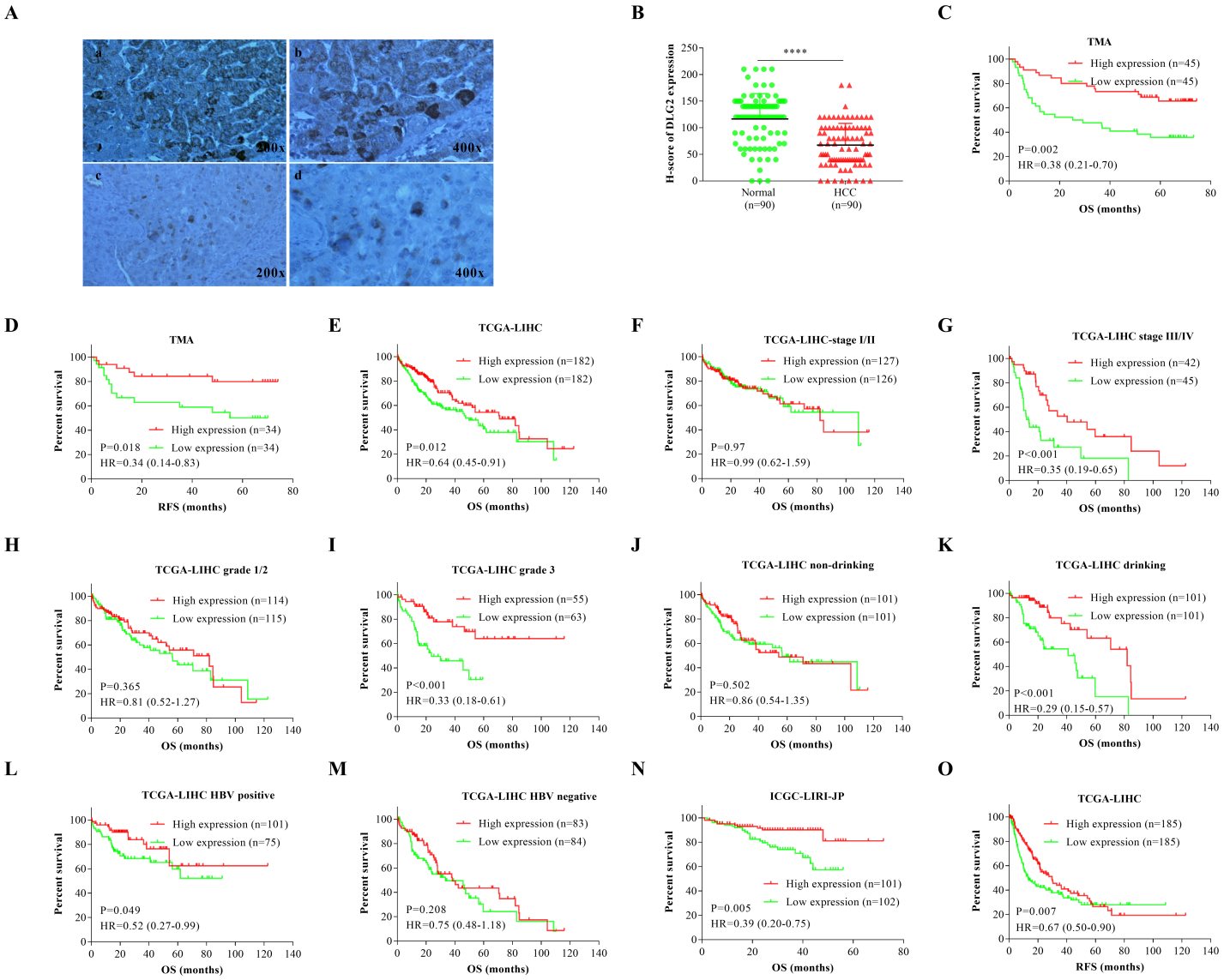

Figure 2

Down-regulation of DLG2 in HCC is associated with poor prognoses. (A) Immunohistochemical staining of DLG2 in HCC tissues and NTs. (A-a) Immunoglobulin G negative control. (A b) DLG2 staining in NT (magnification, x400). (A c) Positive staining of DLG2 in HCC tissue (magnification, x200). (A d) DLG2 staining in HCC tissue (magnification, x400). (B) H scores of DLG2 staining in NTs and HCC. Kaplan Meier plots for the OS (C) and RFS (D) of patients in the TMA cohort. Kaplan Meier plots for OS (E) and RFS (O) in TCGA-LIHC cohort. (F-M) Kaplan Meier plots for OS in HCC patients grouped by different stage, grade, drinking or not, and HBV infection; (N) Kaplan Meier plots for OS in ICGC-LIRI-JP cohort. P-values were obtained using the log-rank test. Censored data are indicated by the + symbol. Patients were stratified into low and high DLG2 expression groups according to DLG2 mRNA expressions (< median vs.

≥ median) in the TCGA-LIHC and ICGC-LIRI-JP cohort, or H scores of DLG2 staining (< median vs. ≥ median) in the TMA cohort. ****, p < 0.0001.

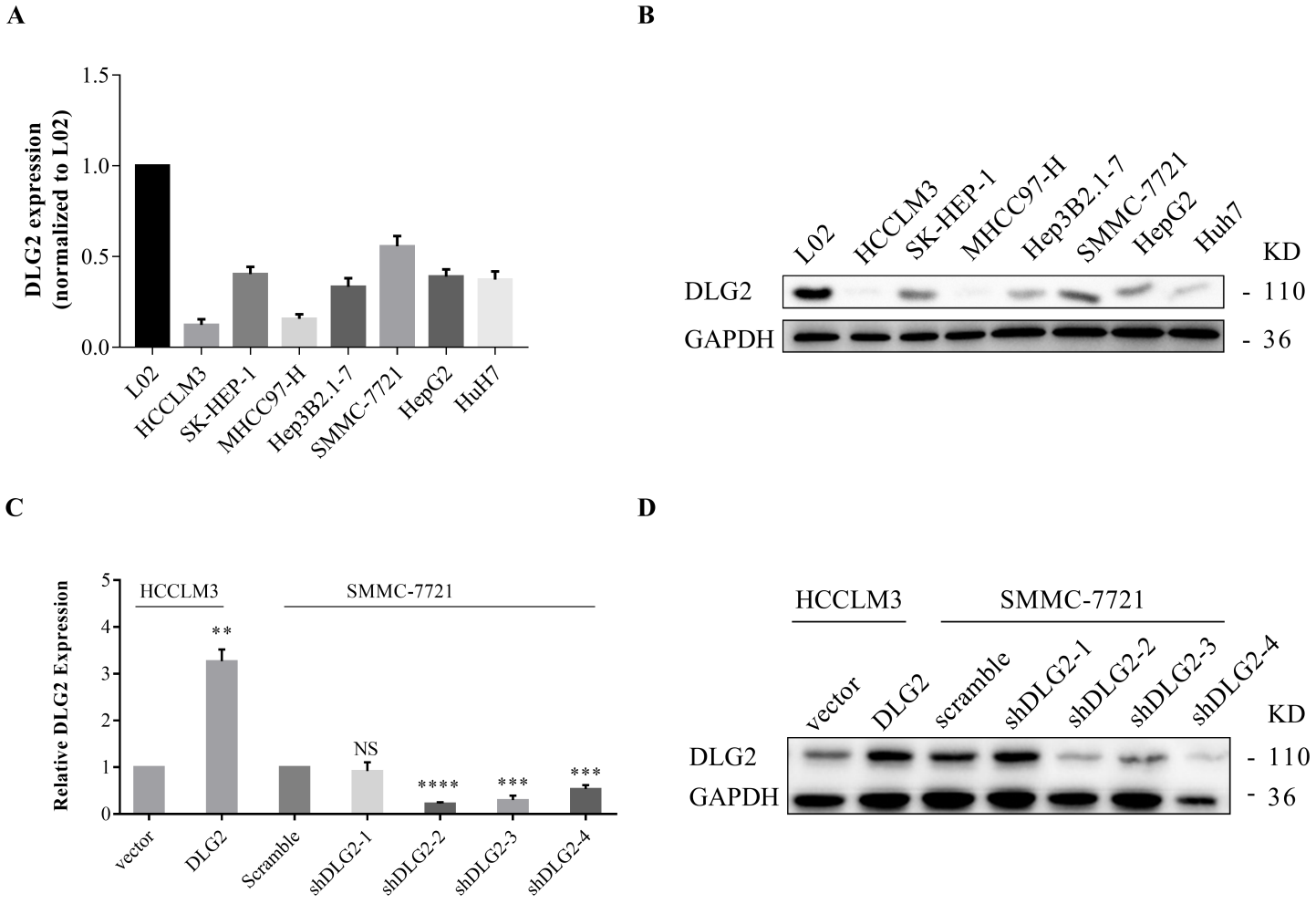

Figure 3

Overexpression and silencing of DLG2 in HCC cells. (A) DLG2 mRNA and (B) DLG2 protein expression levels in a normal HCC cell line (L02) and in seven HCC cell lines. (C and D) HCCLM3 and SMMC-7721 cells were infected with lentiviruses carrying vector control, DLG2, scrambled control shRNA, shDLG2-1, shDLG2-2, shDLG2-3 or shDLG2-4 and then underwent validation by reverse transcription-quantitative PCR and western blot analysis. NS, not significant;*, p < 0.05; **, p < 0.01; ***, p < 0.001 vs. vector control or scramble control.

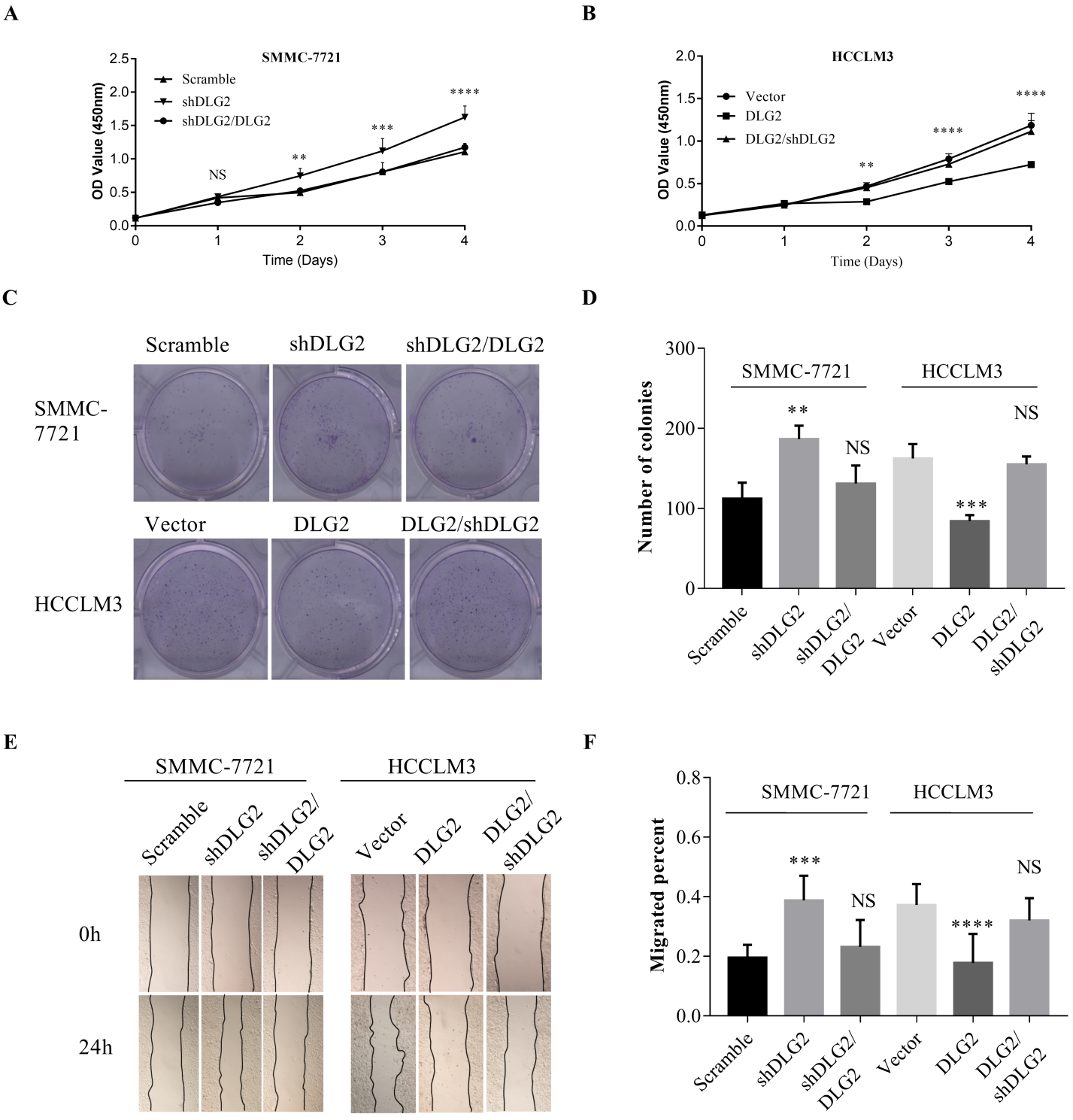

Figure 4

In vitro activities of DLG2 in HCC cells. SMMC-7721 and HCCLM3 cells were infected with lentiviruses carrying scrambled control shRNA, shDLG2, vector control or DLG2 and underwent CCK-8 assay (A and B), colony formation assay (C and D) and scratch wound-healing assay (E and F). DLG2 silencing in these cells significantly inhibited cell proliferation and migration while overexpressing DLG2 showed the

opposite results. Three replicates were conducted for each experiment. NS, not significant; **P<0.05, ***P<0.01, ****P<0.001 vs. the control group.

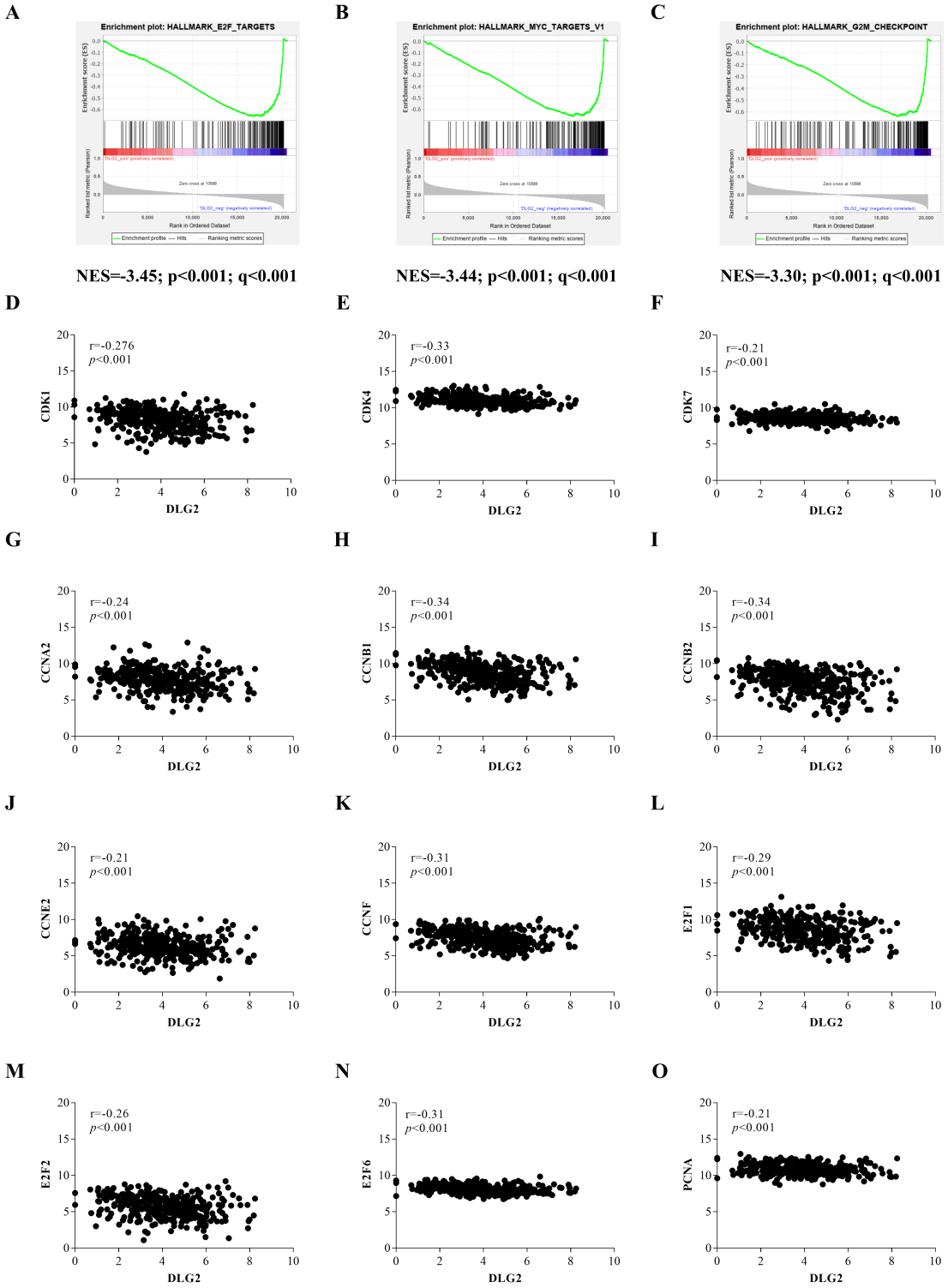

Figure 5

Potential involvement of DLG2 in the regulation of cell cycle process. (A-C) GSEA enrichment plots indicated that DLG2 expression was negatively correlated with cell-cycle associated gene sets

(HALLMARK_E2F_TARGETS, HALLMARK_MYC_TARGETS_V1 and HALLMARK_G2M_CHECKPOINT). (D-L) DLG2 was negatively correlated with several genes associated with cell cycle process.