

Desmoglein-2 overexpression predicts poor prognosis in hepatocellular carcinoma patients

C.-P. HAN^{1,2}, Y.-H. YU¹, A.-G. WANG², Y. TIAN³, H.-T. ZHANG²,
Z.-M. ZHENG², Y.-S. LIU²

¹Department of Radiation Oncology, Shandong Cancer Hospital and Institute Affiliated to Shandong University, Jinan, P.R. China

²Department of Oncology, Qianfoshan Hospital Affiliated to Shandong University, Jinan, P.R. China

³Department of Radiation Oncology, Qianfoshan Hospital Affiliated to Shandong University, Jinan, P.R. China

Abstract. – **OBJECTIVE:** Desmoglein-2 (Dsg2) plays a crucial role in the assembly and adhesion of desmosomes. The absent or aberrant expression of Dsg2 was reported to be associated with the progression of various human cancers. However, the expression of Dsg2 in hepatocellular carcinoma (HCC) and its association with tumor prognosis is still unknown. The aim of this study was to evaluate the expression level of Dsg2 in HCC and of the correlation between Dsg2 expression and clinicopathological variables.

PATIENTS AND METHODS: A total of 104 patients diagnosed with HCC were enrolled in this study. Real time-quantitative PCR (RT-qPCR) and Western blot were performed to determine the expression level of Dsg2 in HCC tumor tissues and matched noncancerous tissues. Cell proliferation and cell cycle were measured by cell counting kit-8 (CCK-8) assay and flow-cytometry assay, respectively.

RESULTS: Our results revealed that Dsg2 expression was significantly higher in HCC tumor tissues than in matched noncancerous tissues ($p < 0.01$), positively correlated with tumor size ($p = 0.035$) and tumor stage ($p = 0.021$). Univariate and multivariate analyses demonstrated Dsg2 expression was an independent prognostic factor for overall survival. Meanwhile, we found knockdown the expression of Dsg2 using small interfering RNA (siRNA) could efficiently impair HCC cell proliferation rate and cell cycle progression ($p < 0.05$).

CONCLUSIONS: Taken together, our results suggest that increased Dsg2 expression was associated with tumor progression in HCC and may function as a promising biomarker for unfavorable prognosis of HCC.

Key Words:

Desmoglein-2, Hepatocellular carcinoma, Prognosis, Biomarker.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancer types, with an increasing incidence and mortality worldwide¹⁻³. In 2006, 466,100 new diagnosed HCC patients and 422,100 HCC-related death were estimated for 2015 in China⁴. Liver transplantation and surgical resection were still the most effective treatment methods for HCC^{5,6}. However, the overall survival of HCC patients remains poor because the vast majority of patients were diagnosed at an advanced stages⁷. Therefore, efforts to elucidate the molecular mechanisms underlying the progression of HCC are urgently needed.

Desmosomes are multi-protein complexes and also a type of cell-cell anchoring junction in tissues⁸⁻¹⁰. The transmembrane protein core of the desmosome is constituted by desmosomal cadherins, desmogleins, and desmocollins^{11,12}. In humans, four desmoglein isoforms and three desmocollin isoforms have been identified¹³. A study¹⁴ revealed the palmitoylation of Desmoglein-2 (Dsg2) plays a vital role in the assembly of desmosome. Also, researches¹⁵⁻¹⁷ demonstrated that altering junction stability or assembly state can lead to inherited disorders, blistering diseases, and cancer. Dsg2 is a cell surface expressed adhesion protein and belongs to the cadherin family¹⁸. However, to date, the role of Dsg2 in cancer is still uncertain. Emerging studies indicated Dsg2 expression was downregulated in gastric cancer¹⁹, prostate cancer²⁰, melanoma²¹, pancreatic cancer²², and colon cancer²³, which showed Dsg2 functions as a tumor suppressor. In contrast, other investigations reported that Dsg2 was overexpressed in skin

SCC and basal cell carcinoma²⁴ and non-small cell lung cancer²⁵, while implied the oncogenic function of Dsg2 protein. However, its expression pattern and clinical significance in hepatocellular carcinoma (HCC) remains unknown.

In this work, we measured Dsg2 expression in HCC tumor tissues and adjacent noncancerous tissues using quantitative Real Time-Polymerase Chain Reaction (RT-qPCR) and Western blot. Also, we investigated the associations of Dsg2 expression with clinicopathological variables and overall survival data of patients with HCC. The effects of Dsg2 knockdown on HCC cell proliferation were also investigated *in vitro*.

Patients and Methods

Patients and Tissue Samples

This investigation was approved by the Ethics Committee of Shandong Cancer Hospital and Institute Affiliated to Shandong University. A total of 104 patients who diagnosed as HCC and went through surgical resection at Shandong Cancer Hospital and Institute Affiliated to Shandong University between Mar 2009 and Mar 2011 were enrolled in this study. The written informed consent was obtained from all the patients. 104 pairs of HCC tumor tissues and adjacent normal tissues were immediately snap-frozen in liquid

nitrogen after surgical resection. The clinicopathological variables including age, gender, HBsAg, smoking status, tumor size, and tumor stage collected from patients were summarized in Table I. Overall survival (OS) was defined as the time that elapsed between the date of surgery and the patient's death from HCC.

Cell Lines and Cultures

Human normal liver cell line HL-7702 and HCC cell lines (QGY-7404 and Bel-7402) were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37°C, humidified incubator containing 5% CO₂.

siRNA Transfection of Cell Lines

The siRNA sequences we used were the same as the previous study²⁵. The detailed siRNA sequences were as follows: Dsg2: 5'-CCUCCAGU-GUUCUACCUAATT-3' (sense) and 5'-UUAG-GUAGAACACUGGAGGTT-3' (antisense), negative control: 5'-UUCUCCGAACGUGUCAC-GUTT-3' (sense) and 5'-ACGUGACACGUUCG-GAGAATT-3' (antisense). These double-stranded siRNAs were synthesized by GenePharma

Table I. Correlations of clinicopathological variables and Dsg2 expression.

Variables	N	Dsg2 expression		p-value
		Low	High	
Age (years)				0.410
≥ 50	51	19	32	
< 50	53	17	36	
Gender				0.728
Male	53	18	35	
Female	51	18	33	
HBsAg				0.087
Positive	54	16	38	
Negative	50	20	30	
Smoking status				0.277
Non-smoker	48	17	31	
Smoker	56	19	37	
Tumor size (cm)				0.035
≥ 5	59	20	39	
< 5	45	16	29	
Tumor stage				0.021
I-II	43	15	28	
III	61	21	40	

Dsg2: Desmoglein-2; HCC: hepatocellular carcinoma; HBsAg: Hepatitis B surface antigen.

(Shanghai, China) and transfected into HCC cell lines by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per the manufacturer's recommendations.

Cell Proliferation Assay

The rate of cell proliferation was determined by the Cell Counting Kit-8 (CCK-8) assay following the protocols provided by the supplier (Beyotime, Haimen, Jiangsu, China). Briefly, human normal liver cell line HL-7702, HCC cell lines (QGY-7404 and Bel-7402), Dsg2 specific siRNA (si-Dsg2) or negative control siRNA (NC siRNA) transfected HCC cell lines were seeded into 96-well plate at a density of 2×10^3 cells/well and cultured in the aforementioned culture conditions. Subsequently, 20 μ l of CCK-8 solution was added to the each well at indicated time points (0 d, 1 d, 2 d, and 3 d) and incubated at 37°C for another 2 h. The absorbance was measured at 450 nm using an epoch microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). Each sample was repeated in triplicates.

Flow-Cytometry Assay

For cell cycle analysis, the cells cultured to logarithmic phase were harvested and washed with PBS. Then, the cells were treated with trypsin and fixed using pre-cold 75% ethanol at 4°C for 2 h. Following, the cells were stained with propidium iodide (PI)/RNase A mixture (Beyotime, Haimen, Jiangsu, China) at darkness for 15 min at room temperature. Cell cycle was analyzed using FACSCalibur™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of cells at G0/G1, S, and G2/M phase were analyzed using ModFit 3.2 software (Verity Software House Company, Topsham, ME, USA).

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from the tissues and cell lines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration was quantified by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). 100 ng RNA was used to synthesize the first strand cDNA using the BeyoRT™ II First Strand cDNA Synthesis Kit (Beyotime, Haimen, Jiangsu, China) following the manufacturer's instructions.

RT-qPCR Assay

RT-qPCR was performed to measure the mRNA expression level of *Dsg2* using the BeyoFast™ SYBR Green qPCR Mix Kit (Beyotime, Haimen, Jiangsu, China) at an ABI 7300 Real Time-PCR System (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany). The following PCR reaction was used: step 1: 95°C for 10 min, 1 cycle; step 2: 95°C for 15 sec, 60°C for 1 min, 40 cycles. The primers used in this study were the same as the previous one²¹ and listed as follows: *Dsg2*: forward 5'-TGGACACCCAAACAGTG-GCCCT-3', reverse 5'-CTCACTTTGTTGCAG-CAGCACAC-3'; β -actin: forward 5'-GGCACCA-CACCTTCTACAATGA-3', reverse 5'-TCTCCT-TAATGTCACGCACGAT-3'. All samples were run in triplicates, and samples were normalized against an endogenous internal control, β -actin. Levels of *Dsg2* mRNA were quantified using $2^{-\Delta\Delta C_q}$ method²⁶.

Western Blot Assay

Total protein was extracted from the tissues and cell lines using the RIPA buffer consisting of 50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholic acid, and 0.1% sodium dodecyl sulfate as reported²⁷. The protein sample was quantified using the Bradford Protein Concentration Determination Kit (Beyotime, Haimen, Jiangsu, China). The same amount of protein sample was separated using a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene difluoride membrane (PVDF, EMD Millipore Corporation, Billerica, MA, USA) at 80 V for 2 h at 4°C. The membrane was blocked with fat-free milk for 1 h at 4°C, and subsequently incubated with the primary antibodies (*Dsg2*: ab14445, 1:5,000, Abcam, Cambridge, MA, USA; β -actin: ab8226, 1:5,000, Abcam, Cambridge, MA, USA). On the next day, the membrane was washed with Tris Buffered Saline and Tween (TBS-T) for three times and incubated with a horseradish peroxidase-conjugated secondary antibody (ab97023, 1:2,000, Abcam, Cambridge, MA, USA) for 2 h at room temperature. Protein bands were visualized using a BeyoECL Plus Kit (Beyotime, Haimen, Jiangsu, China) and quantified using the Tanon Automatic Chemiluminescence Western Blot Imaging system (Tanon, Shanghai, China).

Statistical Analysis

All data were analyzed using SPSS 19.0 statistical software package (SPSS Inc., Chicago, IL,

USA). The chi-square test was used to analyze the association between Dsg2 expression and clinicopathologic variables. The Kaplan-Meier survival curve was used to assess the association of Dsg2 expression with OS of patients with HCC. Multivariate analysis including Dsg2 expression and clinicopathological variables was performed using Cox proportional hazards model in order to assess prognostic factors that were significant in the univariate analysis. The Student's *t*-test was conducted to analyze the difference between two groups. One-way ANOVA and Tukey post-hoc test was performed to analyze the difference among three or above groups. $p < 0.05$ was considered statistically significant.

Results

Dsg2 Expression in HCC Tissues

We found the mRNA expression level of *Dsg2* was significantly elevated in HCC tissues compared to the matched normal noncancerous tissues ($p < 0.01$, Figure 1A). The status of differentially expressed *Dsg2* gene in the HCC tissues was calculated as the ratio of *Dsg2* mRNA expression in tumor tissue to the matched normal tissue (T/N ratio). If T/N was above 1.0-fold, the patients were defined as high Dsg2 expression (65.4%, 68 of 104). Otherwise, the patients were defined as low Dsg2 expression (34.6%, 36 of 104). The Western blot analysis data presented in Figure 1B demonstrated that the protein expression level

of Dsg2 was also significantly elevated in HCC tissues compared to the matched noncancerous tissues ($p < 0.01$, Figure 1B), which is in accordance with the results obtained from RT-qPCR.

Correlation Between Dsg2 Expression and Clinicopathological Variables

To better understand the clinical significance of Dsg2 expression in HCC, we analyzed the correlation between Dsg2 expression and clinicopathological variables. Patients' age, gender, HBsAg, and smoking status were not associated with the Dsg2 expression (all $p > 0.05$). However, Dsg2 expression was strongly associated with tumor size ($p = 0.035$) and tumor stage ($p = 0.021$) (Table I).

Relationship Between Dsg2 Expression and HCC Patients' Survival

To analyze the impact of Dsg2 protein expression on prognosis of HCC patients, we employed Kaplan-Meier survival analysis to assess the relationship between Dsg2 expression and patients' survival. The log-rank test revealed that the OS of HCC patients with high Dsg2 expression were markedly shorter than those with low Dsg2 expression ($p = 0.036$, Figure 2).

Univariate analysis showed that variables including Dsg2 expression ($p = 0.027$), tumor size ($p = 0.037$), and tumor stage ($p = 0.025$) had significantly prognostic influences on OS (Table II). In contrast, variables including age, gender, HBsAg, smoking status had no association with

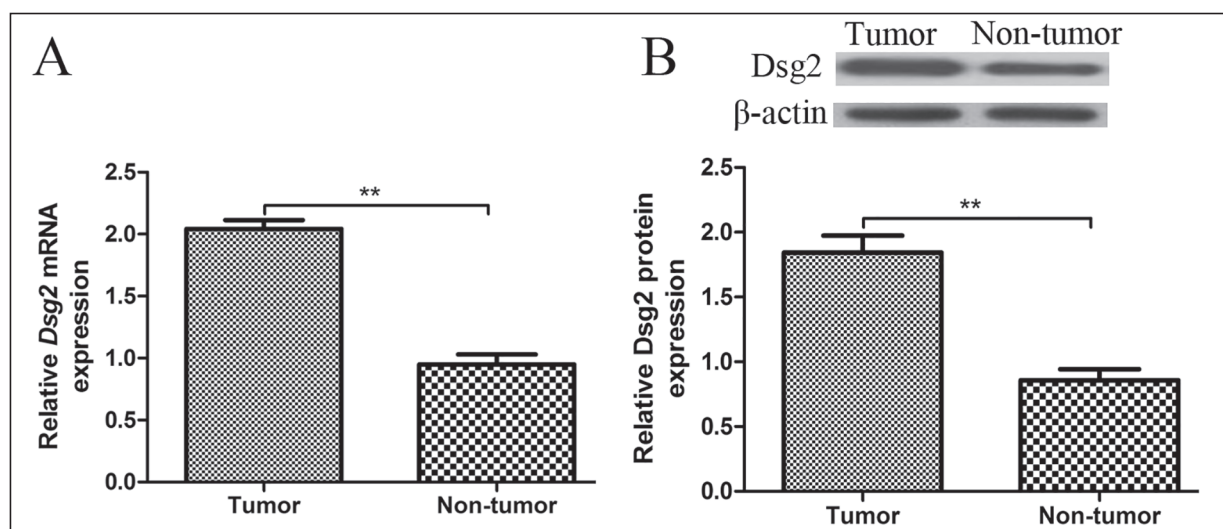


Figure 1. Elevated expression of Dsg2 in HCC tissues. (A) RT-qPCR and (B) Western blot to analyze the expression of Dsg2 in 104 paired HCC tumor tissues and non-tumor tissues. (** $p < 0.01$). RT-qPCR: real time-quantitative PCR; Dsg2: Desmoglein-2; HCC: hepatocellular carcinoma.

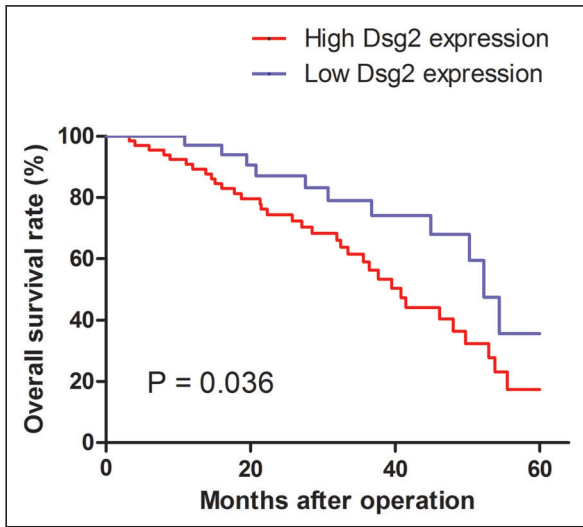


Figure 2. Kaplan-Meier curve of overall survival of HCC patients stratified by Dsg2 expression. Dsg2: Desmoglein-2; HCC: hepatocellular carcinoma.

OS ($p > 0.05$, Table II). Furthermore, multivariate analysis using Cox proportional hazard analyses of variables that were significant in the univariate analyses revealed that Dsg2 expression was an independent prognostic factor for OS ($p = 0.021$), along with tumor size ($p = 0.032$) and tumor stage ($p = 0.019$) (Table II).

Effect of Dsg2 Expression on HCC Cell Proliferation In Vitro

Following the above-presented findings, we then determined whether Dsg2 functions as an oncogene in HCC. The expression levels of Dsg2 in human normal liver cell line HL-7702 and human HCC cell lines QGY-7404 and Bel-7402 were measured. The results presented in Figure 3A indicated that the expression of Dsg2 in HCC cell lines was significantly higher than that in HL-7702 cell line ($p < 0.05$). The Western blot analysis results confirmed the results

Table II. Univariate and multivariate Cox regression analyses of factors associated with overall survival.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	p-value	HR	95% CI	p-value
Dsg2	1.950	1.078-3.527	0.027	2.041	1.113-3.743	0.021
Age	1.466	0.829-2.594	0.189	—	—	—
Gender	1.380	0.785-2.429	0.264	—	—	—
HBsAg	1.560	0.878-2.776	0.130	—	—	—
Smoking status	1.663	0.929-2.977	0.087	—	—	—
Tumor size (cm)	1.893	1.039-3.449	0.037	1.916	1.057-3.472	0.032
Tumor stage	1.955	1.086-3.520	0.025	2.053	1.125-3.744	0.019

HR: hazard ratio; CI: Confidence Index; Dsg2: Desmoglein-2; HCC: hepatocellular carcinoma; HBsAg: Hepatitis B surface antigen.

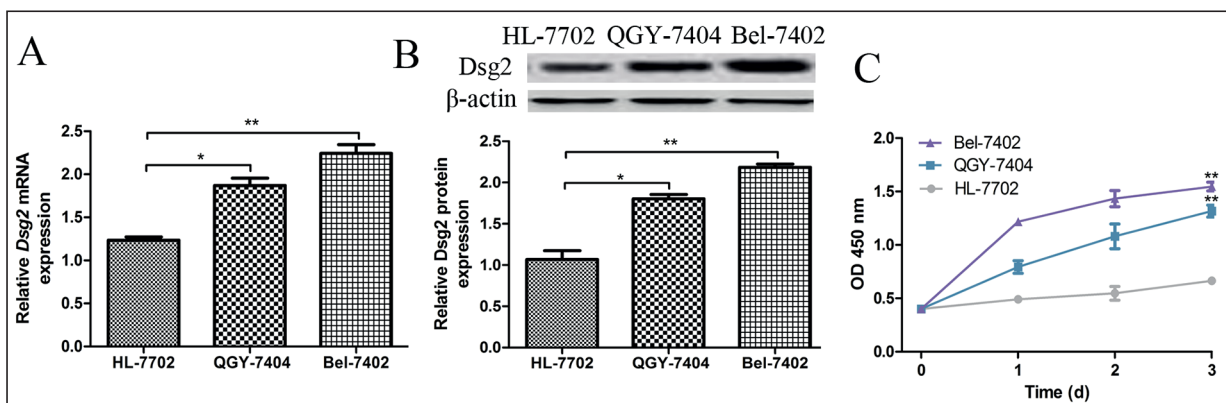


Figure 3. Effect of Dsg2 expression on HCC cell proliferation. **A**, RT-qPCR and **(B)** Western blot to analyze the expression of Dsg2 in HCC cell lines (QGY-7404 and Bel-7402) and normal liver cell line (HL-7702). **C**, CCK-8 assay to analyze the cell proliferation in HCC cell lines (QGY-7404 and Bel-7402) and normal liver cell line (HL-7702). (** $p < 0.01$; * $p < 0.05$). RT-qPCR: real time-quantitative PCR; Dsg2: Desmoglein-2; HCC: hepatocellular carcinoma.

from RT-qPCR (Figure 3B). Subsequently, the cell proliferation rate of these cell lines was examined and our results illustrated that the HCC cell lines had a significant higher cell proliferation rate than HL-7702 cell line ($p < 0.01$, Figure 3C).

Effect of Dsg2 Knockdown on HCC Cell Proliferation Inhibition In Vitro

Next, we evaluated the effect of Dsg2 knockdown on HCC cell proliferation. We found the *Dsg2* mRNA and protein expression level in HCC cell lines was significantly down-regulated post si-Dsg2 transfection ($p < 0.01$, Figure 4A, B). The CCK-8 assay demonstrated that the cell proliferation rate in si-Dsg2 transfected HCC cell lines was markedly impaired compared with the NC siRNA transfected counterparts ($p < 0.01$, Figure 4C). Further analysis of cell cycle distribution in the si-Dsg2 and NC siRNA transfected HCC cell lines revealed the introduction of si-Dsg2 increased the cells at G0/G1 phase but decreased the cells at S phase ($p < 0.05$, Figure 4D).

Discussion

AFP was suggested to be a useful tumor marker for HCC in 1960²⁸ but its limited sensitivity has restricted the use of AFP as a solo predictive marker²⁹. During the past decades, numbers of biomarkers have been identified with the aim to predict HCC clinical outcome and improve the patients' survival quality³⁰⁻³⁸. However, the therapy measures and outcomes for HCC remains poor¹.

Dsg2 is expressed in the basal epidermis, intestinal epithelia, cardiac tissue, and hair follicles³⁹. The role of Dsg2 in desmosome assembly and adhesion has been well recognized, but its role beyond cellular adhesion is poorly understood⁴⁰. Eberts et al⁴¹ showed that Dsg2 was expressed in distinct progenitor cell subpopulations, which was independent from its classical function as a component of desmosomes but played a critical role in the vasculature. This finding suggested the role of Dsg2 in tumor angiogenesis and progression⁴¹. However, the function of Dsg2 in HCC remains to be elucidated.

In this work, we measured the expression of Dsg2 in HCC tissues using RT-qPCR and West-

ern blot to investigate whether the expression of Dsg2 was altered. Our results demonstrated that the expression of Dsg2 was higher in HCC tissues than in matched noncancerous tissues, which suggested Dsg2 might play an important role in HCC. Following, we found high Dsg2 expression was correlated with larger tumor size and advanced tumor stage, the classical signs of tumor malignancy. To further elucidate the significance of Dsg2, we performed Kaplan-Meier curve to analyze the correlations of Dsg2 and overall survival of patients with HCC. We found the patients with high Dsg2 expression had a worse performance in OS time compared to those with low Dsg2 expression. Also, the multivariate analysis revealed that Dsg2 expression was an independent prognostic factor for OS. Taken together, our study validated the overexpression status of Dsg2 and that high Dsg2 expression level predicts a poor prognosis in HCC.

To make a preliminary understanding of how Dsg2 promotes tumor progression, we investigated the biological function of Dsg2 *in vitro*. We found the expression of Dsg2 in HCC cell lines was also significantly higher than the normal liver cell line ($p < 0.05$). Subsequently, we knockdown the expression of Dsg2 in HCC cell lines with the help of siRNA. Then, we found the knockdown of Dsg2 by siRNA had a negative effect on cell proliferation and arrested the cells at G0/G1 phase. However, the detailed mechanisms and the molecular basis involved into this process still need more efforts to elucidate.

Conclusions

For the first time, we revealed the expression pattern of Dsg2 and its clinical signification in HCC. And we may provide a new biomarker for the treatment of HCC. However, the small sample size is a limitation of this study. Therefore, a large, randomized and multicenter study is urgently needed to corroborate the present findings.

Conflict of Interest

The Authors declare that they have no conflict of interests.

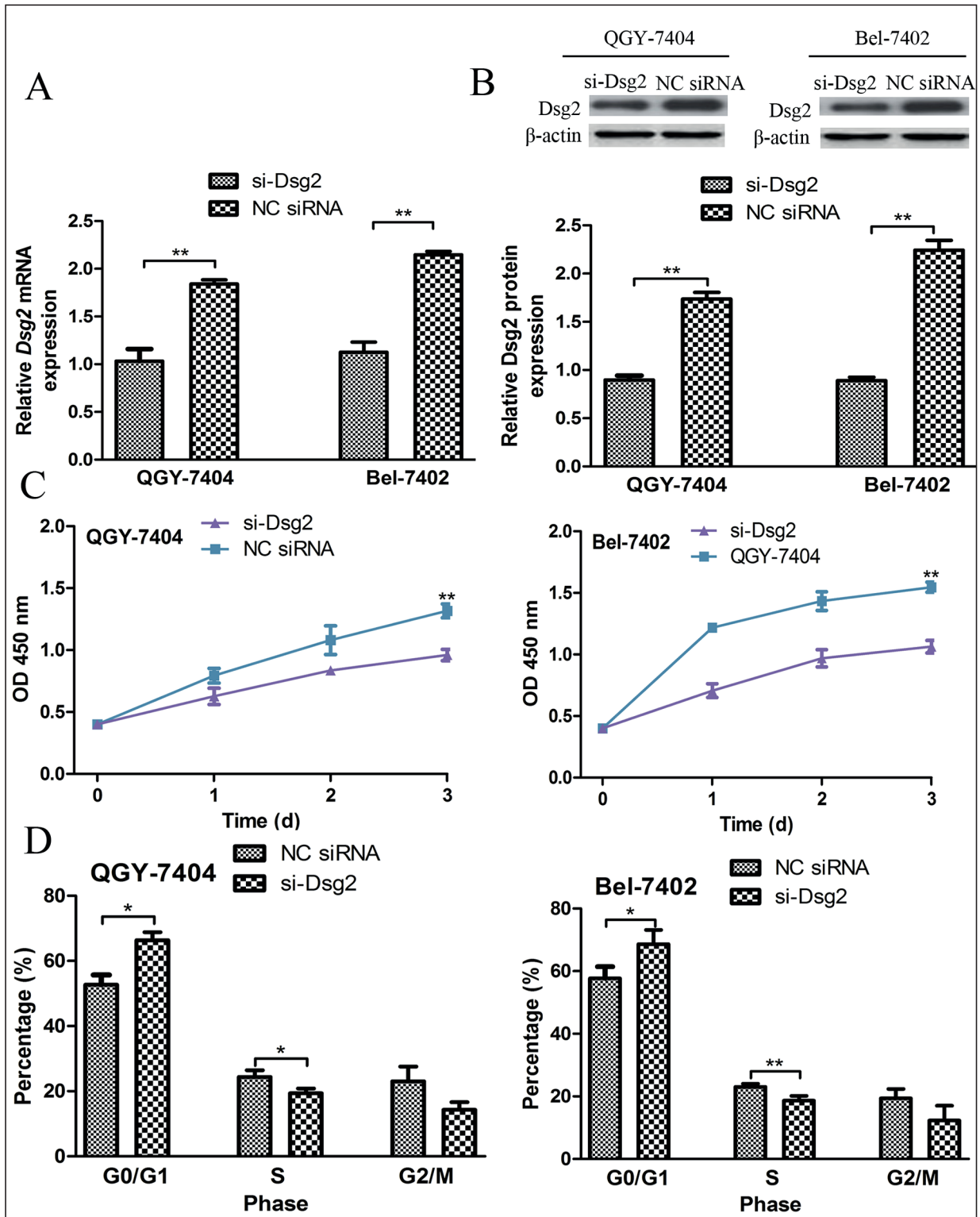


Figure 4. Knockdown of Dsg2 inhibited HCC cell proliferation and arrested cell cycle. **A**, RT-qPCR and **(B)** Western blot to analyze the expression of Dsg2 in HCC cell lines (QGY-7404 and Bel-7402) post si-Dsg2 and NC siRNA transfection. **C**, CCK-8 assay to analyze the cell proliferation in HCC cell lines (QGY-7404 and Bel-7402) post si-Dsg2 and NC siRNA transfection. **D**, Flow cytometry to analyze the cell cycle distribution in HCC cell lines (QGY-7404 and Bel-7402) post si-Dsg2 and NC siRNA transfection. (** $p < 0.01$; * $p < 0.05$). RT-qPCR: real time-quantitative PCR; Dsg2: Desmoglein-2; HCC: hepatocellular carcinoma; siRNA: small-interfering RNA; si-Dsg2: siRNA targeting Dsg2; NC: negative control.

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