

Clinical significance of aberrant DEUP1 promoter methylation in hepatocellular carcinoma

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Abstract. Accumulating studies have shown that methylation of tumor suppressor genes plays an important role in tumorigenesis. Deuterosome assembly protein 1 (DEUP1) has been implicated as a suppressor gene in some tumors and promoter methylation led to silencing of its expression. However, the roles of DEUP1 promoter methylation and expression in hepatocellular carcinoma (HCC) are not clear. In the present study, the expression and methylation of the DEUP1 promoter in HCC was investigated and the correlations with HCC occurrence and development were explored. A total of 60 HCC tumor and adjacent non-tumor tissues were included in this study. Reverse transcription-polymerase chain reaction, bisulfite PCR sequencing, immunohistochemistry and western blotting were applied to detect the methylation status of the DEUP1 promoter and its expression, and to analyze their associations with clinicopathological data. The results showed that the mRNA and protein expression of DEUP1 in adjacent non-tumor tissues was significantly increased compared with in the HCC tissues. DEUP1 promoter methylation was detected in 46/60 (76.7%) tumor tissues and there was a negative correlation between promoter methylation and DEUP1 protein expression ($P < 0.05$). Analysis of the clinicopathological data revealed that the mRNA and protein expression

of DEUP1, and its promoter methylation status, was associated with tumor node metastasis stage and tumor differentiation. Taken together, the results of the present study suggested that methylation of the DEUP1 promoter maybe an important mechanism for gene inactivation and has a critical role in the occurrence and development of liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of liver cancer, its survival rate ranks only second to lung cancer and it is a severe threat to human health (1-4). However, the pathophysiological mechanisms involved remain unclear. The occurrence of HCC is a complicated process involving multiple genes and steps. Imbalances in cellular signal transduction pathways, deficiencies in DNA repair-regulating genes, activation of protooncogenes, inactivation of tumor suppressor genes and epigenetic modifications all promote the occurrence of liver cancer (1-4).

Epigenetics refers to the regulation of gene expression by affecting a gene's transcription and translation without changing DNA sequences, including DNA methylation, histone modification and abnormal miRNA expression (5,6). DNA methylation has been widely studied in a number of types of tumor (7). Methylation of DNA leads to the inactivation of tumor suppressor genes and promotes the occurrence and development of tumors (8). Reversion of DNA methylation events has been reported to inhibit the growth of tumor cells and promote tumor cell apoptosis (9).

Deuterosome assembly protein 1 (DEUP1) is a new candidate tumor suppressor gene and is associated with cellular signal transduction during tumor formation. Bioinformatics methods revealed that DEUP1 expression was closely associated with the survival time of patients with HCC. Through database analysis, it was also demonstrated that the inactivation of DEUP1 was correlated with the methylation of its promoter. DEUP1 expression is absent or reduced in malignant tumors, such as gastric and thyroid cancer (10,11). However, its expression in HCC and the association with clinical information have not been reported on, to the best of our knowledge.

The present study was undertaken to explore the effects of DEUP1 in HCC, reverse transcription-polymerase chain reaction (RT-PCR), bisulfite PCR sequencing (BSP),

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Abbreviations: HCC, hepatocellular carcinoma; DEUP1, deuterosome assembly protein 1; RT-PCR, reverse transcription-polymerase chain reaction; BSP, bisulfite PCR sequencing; TNM, tumor node metastasis

Key words: deuterosome assembly protein 1, methylation, hepatocellular carcinoma, bisulfite PCR sequencing, tumor suppressor gene

immunohistochemistry (IHC) and western blotting were conducted to detect methylation of the DEUP1 promoter and DEUP1 expression in 60 cases HCC and adjacent non-tumor tissues, and explore the correlations between DEUP1 and pathological features.

Materials and methods

Clinical information. HCC and adjacent non-tumor tissues (at least 3 cm from the surgical incision) were collected from 60 patients who underwent surgical resection between January 2016 and December 2016 at the First Affiliated Hospital of Zhengzhou University. All specimens were confirmed by pathological diagnosis. No patients underwent radiotherapy or chemotherapy prior to surgery. A total of 45 males and 15 females, aged 31-75 years old (median, 58 years), were recruited to the study. According to tumor node and metastasis (TNM) staging of the AJCC 2018 (12), 32 patients were stage I-II, and 28 were stage III-IV. Informed consent was obtained from each patient and the study protocol was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Relationship between DEUP1 mRNA expression and overall survival. Based on the KM Plotter Online Tool (<http://kmplot.com/analysis/>), 364 patients with HCC were divided into two groups according to the median expression of DEUP1 and Kaplan-Meier survival curve was then plotted. The best cutoff was auto-selected.

DEUP1 mRNA expression detected by RT-PCR. HCC and adjacent non-tumor tissues (100 mg each) were used. Total RNA was extracted using TRIzol (Invitrogen; (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and the RNA concentration and A260/A280 ratio were measured using a Nanodrop 2000 (Thermo Fisher Scientific, Inc.). RNA (1 μ g) was transcribed to cDNA using a reverse transcription kit (cat. no. RR047A; Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. The primers were designed based on the gene coding sequence. DEUP1: 5'-CCTTCGACATTTCAAGCCAAAGA-3' (forward primer) and 5'-GAAATGCTGTGCAGCCAAAGA-3' (reverse primer). GAPDH: 5'-CGCTGAGTACGTCGTGGA GT-3' (forward primer) and CATCACGCCACAGTTTCC CG-3' (reverse primer). TB Green Master Mix kit (Takara Biotechnology Co., Ltd.) was used with a total reaction volume of 20 μ l containing 10 μ l of 2X TB Green Master Mix, 2 μ l of cDNA, 0.8 μ l of upstream and downstream primers each, and 6.4 μ l of ddH₂O. Reaction conditions were as follows: Pre-denaturation at 95°C for 30 sec, denaturation at 95°C for 5 sec and annealing at 59.5°C for 30 sec, for a total of 40 cycles. Human GAPDH was used as an internal reference (5 μ l) and loaded with PCR product (5 μ l) and 6X DNA loading buffer (1 μ l). After 2% agarose gel electrophoresis, the ratio of DEUP1 to GAPDH was compared using the average value of normal tissue as the standard. A ratio higher than the value of the standard or within the range was considered to indicate positive gene expression and no band present or a band lower than the normal range indicated no gene expression. Image J version 1.8.0 (National Institutes

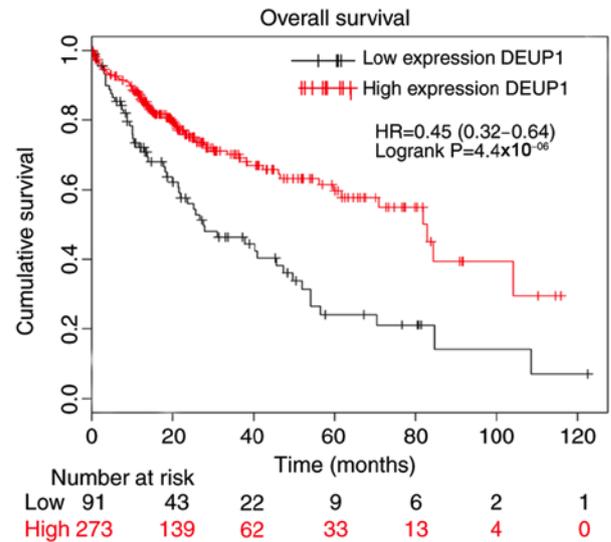


Figure 1. KM plotter analysis is used to predict the correlation between DEUP1 expression and overall survival of HCC patients. Patients with increased expression of DEUP1 had a higher survival rate. HCC, hepatocellular carcinoma; DEUP1, deuterosome assembly protein 1.

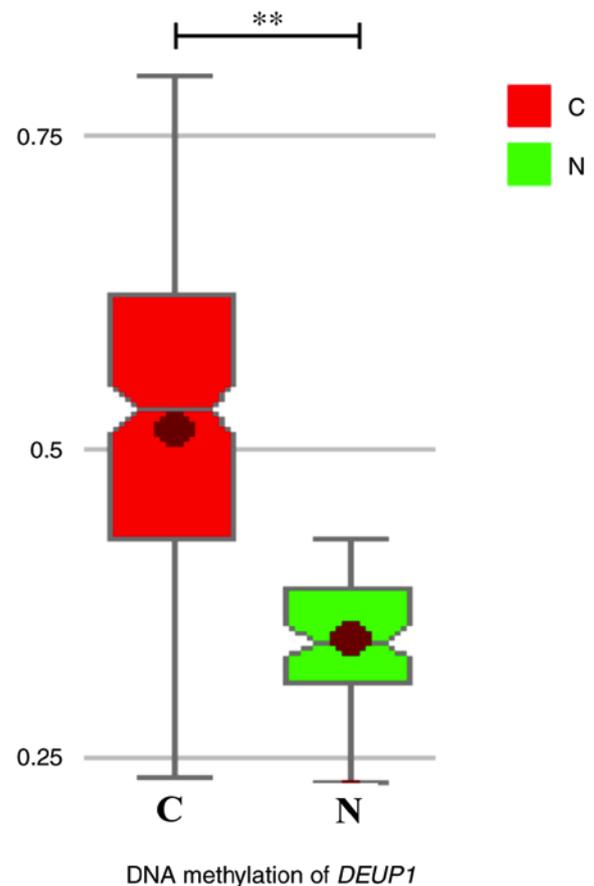


Figure 2. MethHC is used to predict the methylation of DEUP1 promoter in hepatocellular carcinoma. ** $P < 0.01$ vs. adjacent non-tumor tissues. DEUP1, deuterosome assembly protein 1; N, adjacent non-tumor tissues; C, tumor tissues.

of Health) was used to semi-quantitatively analyze the gray scale ratio of target gene and GAPDH.

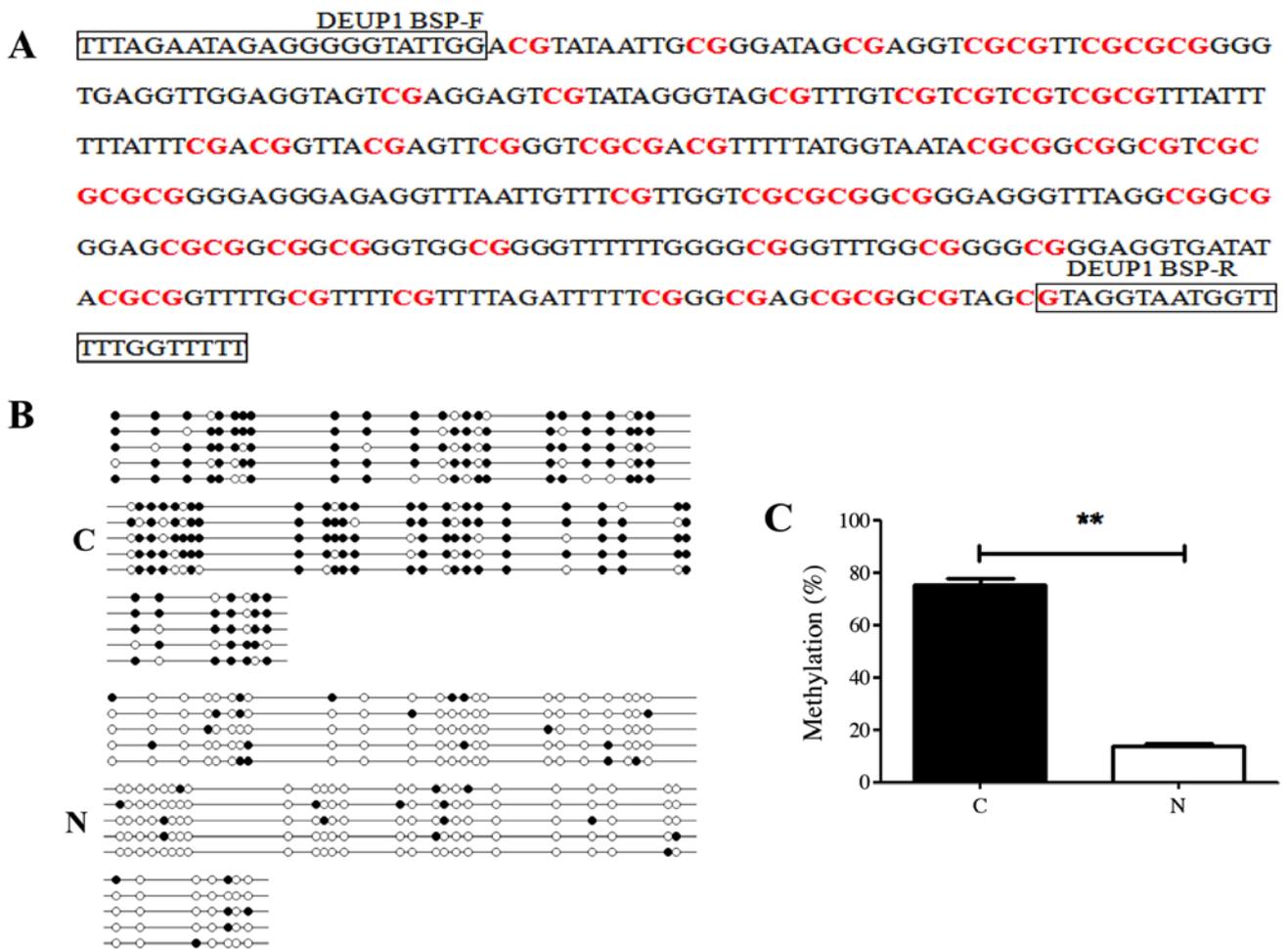


Figure 3. Hypermethylation of the DEUP1 promoter. (A) DEUP1 promoter sequence and CpG islands (red marked). (B) Methylation status of the 56 CpG sites and representative sequences of bisulfite sequencing analysis in HCC tissues. Filled (black) circles correspond to methylated CpG, unfilled (white) circles correspond to unmethylated CpG. (C) Analysis of deup1 methylation. **P<0.01 vs. adjacent non-tumor tissues. DEUP1, deuterosome assembly protein 1; HCC, hepatocellular carcinoma; N, adjacent non-tumor tissues; C, tumor tissues.

DEUP1 promoter methylation detected by BSP. DNA in the tissues was extracted using a TINamp Genomic DNA kit (Sangon Biotech, Co., Ltd.) and resolved via 1% agarose gel electrophoresis. The absorbance (260/280) was measured with a UV spectrophotometer to calculate DNA content. The DNA was modified with sulfite using an EZ DNA Methylation-Gold™ kit D5005 (Zymo Research Corp., Irvine, CA, USA). The primers were designed using Primer Premier 5 (Premier Biosoft International). BSP primers were as follows: Upstream: 5'-TTTAGAATAGAGGGG GTATTGG-3'; downstream: 5'-AAAACCAAACCA TTACTAC-3'. The BSP reaction volume was 20 μl. Cycle parameters were as follows: 95°C pre-denaturation for 5 min, 95°C denaturation for 30 sec, 61°C annealing for 30 sec and 72°C extension for 50 sec, for a total of 35 cycles, followed by extension at 72°C for 8 min. The integrity of the PCR product (5 μl) was established by 1% agarose gel electrophoresis. The PCR product was ligated with a T-vector to generate 10 μl linking product that was transferred to 100 μl SK9307 competent cells using the Rapid Competent Cell Preps kit (cat. no. B529307; Sangon Biotech, Co., Ltd.). After screening using LB culture medium containing ampicillin (cat. no. A600894; Sangon Biotech, Co., Ltd.), five

independent colonies were picked. The target fragment was identified by PCR and the products were sequenced.

DEUP1 protein changes detected by IHC. Paraffin sections of HCC and adjacent non-tumor tissues at 4 μm thickness were dewaxed and rehydrated with graded alcohol. Citric acid buffer was used for antigen retrieval under high temperature (heated to boiling and rested for 15 min at room temperature) Then, the sections were washed with PBS, blocked with normal goat serum (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at room temperature and then incubated with a DEUP1 primary antibody (1:200; cat. no. FLJ25393; Absin) at 4°C overnight. Afterwards, the sections were washed with PBS, incubated with a secondary antibody (1:100; cat. no. SP0021; Beijing Solarbio Science & Technology Co., Ltd.) labeled with biotin for 1 h at room temperature, washed again with PBS, incubated with horseradish peroxidase (HRP)-labeled streptavidin and then a DAB chromogenic reagent, washed with running water, re-stained with hematoxylin at room temperature until nuclei turned blue, dehydrated with gradient ethanol, sealed with gum, and then observed under a light microscope. The IHC results indicated faint yellow or even dark brown granules. Positive cell counting was scored as follows: <5%

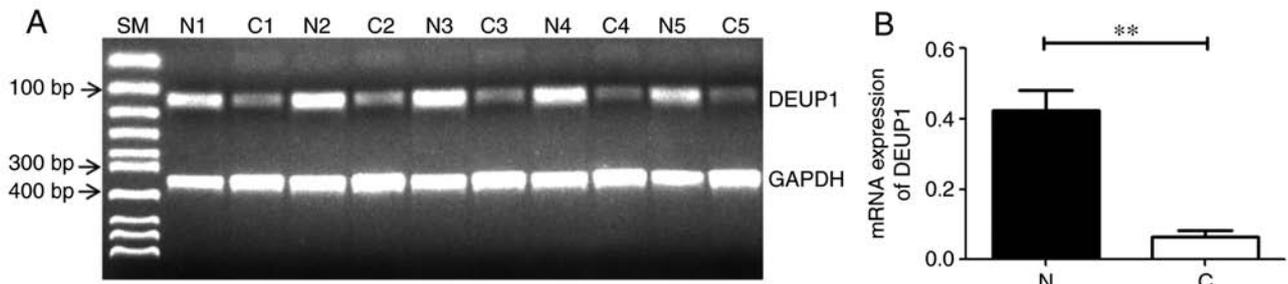


Figure 4. DEUP1 mRNA expression in hepatocellular carcinoma. (A) Representative results of RT-PCR; (B) analysis of mRNA expression. **P<0.01 vs. N. DEUP1, deuterosome assembly protein 1; N, adjacent non-tumor tissues; C, tumor tissues; SM, size marker.

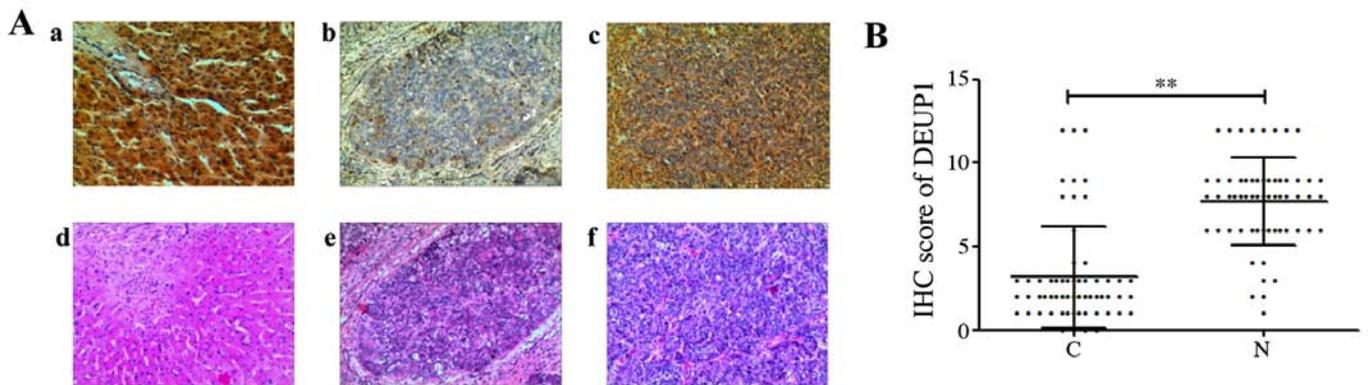


Figure 5. Results of IHC and HE stain (magnification, x100). (Aa) High expression in adjacent non-tumor tissues, (Ab) negative expression of DEUP1 in HCC tissue, (Ac) positive expression of DEUP1 in HCC tissue. (Ad-f) are the corresponding HE stain of adjacent non-tumor and HCC tissues. (B) IHC scores of DEUP1 in tumor tissues and adjacent non-tumor tissues. C vs. N, **P<0.01. DEUP1, deuterosome assembly protein 1; HCC, hepatocellular carcinoma; HE, hematoxylin and eosin; IHC, immunohistochemistry; N, adjacent non-tumor tissues; C, tumor tissues.

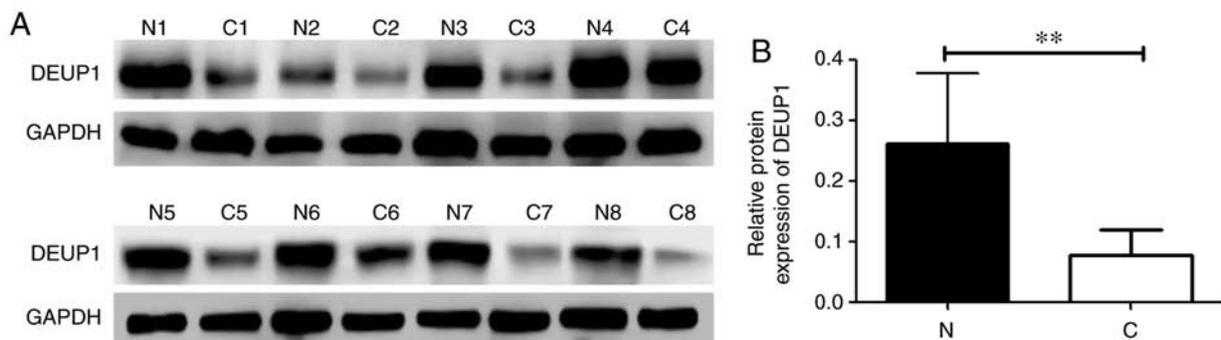


Figure 6. DEUP1 protein expression in HCC (A) Representative western blotting results in HCC and adjacent non-tumor tissues, (B) analysis of western blot. **P<0.01 vs. N. N, adjacent non-tumor tissues; C, tumor tissues; DEUP1, deuterosome assembly protein 1; HCC, hepatocellular carcinoma.

was scored as 0, 5-25% as 1, 25-50% as 2, 50-75% as 3 and >75% as 4. Color intensity was scored as follows: No color was scored as 0, faint yellow as 1, pale brown as 2 and dark brown as 3. If the product of the two scores was ≥ 4 , it was deemed positive.

DEUP1 protein expression detected by western blotting. A total of 100 mg of liver tissue, 1 ml RIPA (Beijing Solarbio Science & Technology Co., Ltd.) and 10 μ l PMSF were placed into an EP tube and fully broken with a tissue breaker. The protein was extracted using RIPA buffer and the concentration was measured using a bicinchoninic acid Protein Assay kit (Beijing Solarbio Science & Technology Co., Ltd.). Then,

40 μ g of total protein was resolved by SDS-PAGE (10%) and transferred onto 0.45 μ m nitrocellulose membranes. The membranes were blocked with 5% non-fat milk at room temperature for 1 h, following which primary antibodies against DEUP1 (1:1,000; cat. no. ab102688; Abcam) and GAPDH (1:1,000; Cell Signaling Technology, Inc.; cat. no. 5174) were added and incubated at 4°C overnight. The membranes were washed three times with 1X TBS containing 1% Tween-20 (10 min each time) before and after incubation with the secondary antibody (goat anti rabbit IgG-HRP; Cell Signaling Technology, Inc.; cat. no. 7074) diluted with 1X TBST at 1:2,000. The blots were visualized using ECL (cat. no. 32106; Thermo Fisher Scientific, Inc.) in a dark room. The

Table I. Correlation of deuterosome assembly protein 1 mRNA expression with clinicopathological features in hepatocellular carcinoma.

Clinical data	Number	Expression of mRNA		Positive rate (%)	P-value
		Positive	Negative		
Sex					
Man	45	10	35	22.3	0.606
Woman	15	5	10	33.3	
Age					
≤50	40	8	32	20.0	0.206
>50	20	7	13	35.0	
Tumor size					
≤5 cm	27	6	21	22.2	0.653
>5 cm	33	9	24	27.3	
HBsAg					
+	44	9	35	20.5	0.312
-	16	6	10	37.5	
TNM stage					
I+II	32	12	20	37.5	0.017
III+IV	28	3	25	10.7	
Portal tumor thrombosis					
No	52	11	41	21.2	0.188
Yes	8	4	4	50.0	
AFP					
≤400 μg/l	41	12	29	29.3	0.423
>400 μg/l	19	3	16	15.8	
Tumor differentiation					
Poor	35	5	30	14.3	0.023
moderate-well	25	10	15	40.0	

TNM, tumor node metastasis; AFP, alpha fetoprotein.

protein expression levels for each specimen were calculated using Quantity-One 4.6.6 (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data are presented as the mean ± standard deviation. SPSS 22.0 software (IBM, Corps.) was used to analyze the data. The statistical significance between two groups of quantitative data were calculated by Student's t-test. A comparison of constituent ratios was conducted using the χ^2 test and χ^2 test of paired quadrilaterals. Correlations in the data were identified and evaluated using correlation analysis of paired quadrilaterals. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Promoter DEUP1 hypermethylation in HCC tissues. The bioinformatic analysis indicated that increased expression of DEUP1 was associated with a higher rate of patient overall survival (Fig. 1) and the degree of promoter methylation in HCC tissues was significantly increased compared with the adjacent non-cancerous tissues ($P < 0.01$; Fig. 2). This suggests

that DEUP1 may be a tumor suppressor gene and promoter methylation may play an important role in the development of HCC occurrence. The results of BSP demonstrated that DEUP1 promoter hypermethylation was detected in 46 of 60 (76.7%) tumors tissues, while only 5 of 60 in adjacent non-tumor tissues. DEUP1 promoter methylation levels in HCC were tissues significantly increased compared with the adjacent non-tumor tissues ($P < 0.01$; Fig. 3).

DEUP1 mRNA expression in HCC tumor and adjacent non-tumor tissues. RT-PCR results demonstrated that 45 of the 60 HCC tissues had reduced or absent DEUP1 mRNA expression compared with adjacent non-cancerous tissues. All adjacent non-tumor tissues showed DEUP1 expression. Representative results are shown in Fig. 4; the expression of DEUP1 mRNA in the adjacent non-cancerous tissues was significantly increased compared with the HCC tissues ($P < 0.01$).

DEUP1 protein expression in HCC tumor and adjacent non-tumor tissues. The expression of DEUP1 protein was

Table II. Correlation of deuterosome assembly protein 1 protein expression with clinicopathological features in hepatocellular carcinoma.

Clinical data	Number	Expression of protein		Positive rate (%)	P-value
		Positive	Negative		
Sex					
Man	45	8	37	17.8	0.709
Woman	15	4	11	26.7	
Age					
≤50	40	7	33	17.5	0.732
>50	20	5	15	25.0	
Tumor size					
≤5 cm	27	4	23	14.8	0.364
>5 cm	33	8	25	24.2	
HBsAg					
+	44	7	37	15.9	0.343
-	16	5	11	31.3	
TNM stage					
I+II	32	10	22	31.3	0.020
III+IV	28	2	26	7.1	
Portal tumor thrombosis					
No	52	9	43	17.3	0.393
Yes	8	3	5	37.5	
AFP					
≤400 μg/l	41	10	31	24.4	0.367
>400 μg/l	19	2	17	10.5	
Tumor differentiation					
Poor	35	3	32	8.6	0.009
moderate-well	25	9	16	36.0	

TNM, tumor node metastasis; AFP, alpha fetoprotein.

further analyzed in the 60 HCC tumor and adjacent non-tumor tissues. IHC results revealed that the positive expression of the DEUP1 protein was mainly located in the cytoplasm, represented by yellow or pale-brown granules (Fig. 5). A total of 48 out of the 60 HCC tumor tissues showed low or no DEUP1 protein expression. Both IHC and western blotting indicated that the expression of DEUP1 in adjacent non-tumor tissues was significantly increased compared with in HCC tissues ($P < 0.01$; Fig. 6).

Association between DEUP1 promoter methylation and expression, and clinicopathologic parameters in HCC. Downregulated expression of DEUP1 mRNA and protein were significantly associated with TNM stage and tumor differentiation ($P < 0.05$; Tables I and II). The DEUP1 promoter hypermethylation were associated with TNM stage and tumor differentiation. (Table III). DEUP1 mRNA, protein and the promoter methylation status had no association with other clinicopathological parameters.

Correlation between DEUP1 promoter methylation and protein expression. Out of the 60 patients with HCC, 46 had

positive DEUP1 promoter methylation and six had positive protein expression. Among the 14 patients with a negative methylation status, six showed protein expression (Table IV). The protein expression of DEUP1 was negatively correlated with promoter methylation. Correlations were statistically significant ($P < 0.05$).

Discussion

DNA methylation is an epigenetic phenomenon. It is considered the second strike for inactivation of tumor suppressor genes after mutation and allele loss (13). DEUP1, also known as coiled-up coil coiled-coil domain-containing 67 (CCDC67), is located on human chromosome 11q2.1, encoding 604 amino acids (11). It is a member of CCDC protein family. CCDC protein is composed of 180-220 amino acids and the quaternary structure in the coiled coil may be associated with angiogenin and other protein features and exhibit diverse functions related to their highly versatile folding motif (10,11), but little is known about DEUP1 function (14-16). The results of a bioinformatic predictive analysis indicated that inactivation of DEUP1 in HCC

Table III. Correlation between Methylation of DEUP1 and clinicopathological features in HCC.

Clinical data	Number	Methylation of DEUP1		Positive rate (%)	P-value
		Methylated	Unmethylated		
Sex					
Man	45	37	8	82.2	0.159
Woman	15	9	6	60.0	
Age					
≤50	40	33	7	82.5	0.235
>50	20	13	7	65.0	
Tumor size					
≤5 cm	27	22	5	81.5	0.425
>5 cm	33	24	9	72.7	
HBsAg					
+	44	36	8	81.8	0.223
-	16	10	6	62.5	
TNM stage					
I+II	32	21	11	65.6	0.031
III+IV	28	25	3	89.3	
Portal tumor thrombosis					
No	52	41	11	78.8	0.570
Yes	8	5	3	62.5	
AFP					
≤400 μg/l	41	32	9	78.0	0.965
>400 μg/l	19	14	5	73.7	
Tumor differentiation					
Poor	35	31	4	88.6	0.010
Moderate-well	25	15	10	60.0	

HCC, hepatocellular carcinoma; TNM, tumor node metastasis; DEUP1, deuterosome assembly protein 1; AFP, alpha fetoprotein.

could be caused by methylation of a DNA CpG island. The expression of DEUP1 is associated with a high rate of patient survival. Furthermore, the expression of DEUP1 has been reported to be absent or significantly reduced in a number of tumors (10,11). Epigenetic changes, especially the methylation of DNA CpG islands, are one of the most important mechanisms behind low or non-expression of mRNA (17,18). Whether DEUP1 functions as a tumor suppressor gene in HCC as well as an inactivation mechanism in HCC has not been reported.

DEUP1 mRNA expression was increased in HCC tissues compared with in adjacent non-tumor tissues in the present study. Statistical analysis of DEUP1 mRNA expression and clinicopathological parameters indicated that DEUP1 mRNA expression in TNM stage I+II and III+IV was 37.5% (12/32) and 10.7% (3/28), respectively. In the poor and moderate-well differentiation groups, the expression of DEUP1 mRNA was 14.3% (5/35) and 40.0% (10/25), respectively, suggesting that mRNA expression is associated with the degree of malignancy of HCC.

The methylation of the DEUP1 promoter in HCC and adjacent non-tumor tissues was detected by BSP. The results indicated that methylation levels in HCC were increased

Table IV. Correlation of deuterosome assembly protein 1 promoter methylation with protein expression in hepatocellular carcinoma.

Protein	Methylation		Total
	Positive	Negative	
Positive	6	6	12
Negative	40	8	48
Total	46	14	60

P=0.039.

compared with in the corresponding para-carcinoma tissues, indicating that methylation might be involved in the occurrence and development of HCC. Methylation levels in TNM stage I+II and III+IV were 65.6% (21/32) and 89.3% (25/28), respectively, and 88.6% (31/35) and 60.0% (15/25) in the low and moderate-well groups, respectively. These data indicated that the methylation status of DEUP1 has the potential to

guide prognostic evaluation for HCC. Yin *et al* (10) reported that, as a tumor suppressor gene, the methylation of the gene promoter led to its inactivation, playing an important role in the occurrence and development of papillary thyroid carcinoma. Park *et al* (11) found that methylation of the DEUP1 promoter led to a decrease in DEUP1 expression and had an important role in gastric cancer. To further confirm the influence of DEUP1 expression on the development of HCC, the expression of the DEUP1 protein in HCC was detected by IHC and western blotting. The DEUP1 protein is located in the cytoplasm and its expression in HCC tissues was decreased compared with in adjacent non-tumor tissues. Furthermore, DEUP1 protein expression was associated with TNM stage and tumor differentiation.

It was also found that in the 46 HCC patients with promoter methylation, 40 did not have DEUP1 protein expression. The analysis indicated that methylation of the DEUP1 promoter had a negative correlation with protein expression, suggesting that gene promoter methylation may be an important mechanism underlying non-expression of the protein.

Methylation of one or more tumor suppressor gene CpG islands occurs in a number of malignant tumors (19-23). The inactivation of these genes has multiple effects on cellular processes such as apoptosis and cell cycle regulation, leading to tumorigenesis. CpG island methylation is a reversible epigenetic gene modification process (24). In healthy individuals, genes are in a low-methylation status and methylation inhibition does not influence gene expression in normal cells. Methylation of tumor suppressor gene CpG islands can render normal cells cancerous and demethylation can revert the phenotype of tumor cells back to normal, therefore providing new avenues for the therapy of tumors (25).

In conclusion, DEUP1 is a new tumor suppressor gene in HCC, with important regulatory effects on its occurrence, development and prognosis. This study lays a foundation for future studies on DEUP1 functions and the mechanisms of gene silencing in HCC, and may provide insights into demethylation drugs and new therapeutic targets. However, if the aim is to better prove that DEUP1 is a suppressor gene in HCC and its promoter methylation results in low expression, cell experiments and animal experiments can be performed. The lack of further validation makes this study imperfect and the authors will follow up on cell and animal experiments to improve this study.

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Availability of data and materials

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

Authors' contributions

QWY, SLC and HWT performed the experiment, SJZ, WZG and JL designed the study, QWY and SLC prepared and wrote the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent was obtained from each patient and the study protocol was approved by the Medical Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Patient consent for publication

Informed consent was obtained from each patient.

Competing interests

The authors declare that they have no competing interests.

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