Downregulation of *DENND2D* by promoter hypermethylation is associated with early recurrence of hepatocellular carcinoma

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Abstract. Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide and its prognosis is poor. Novel targets for treating recurrence and progression along with associated biomarkers are urgently required. In this study, the expression and regulatory mechanism of DENN/MADD domain containing 2D (DENND2D) were investigated in an attempt to identify a tumor suppressor gene for HCC regulated by silencing through promoter hypermethylation. The levels of DENND2D expression in HCC cell lines and surgical specimens were determined using a quantitative polymerase chain reaction assay and the relationship between the expression levels of *DENND2D* mRNA and clinicopathological factors was evaluated. The expression and distribution of DENND2D were determined using immunohistochemistry. DNA methylation analysis was performed to determine the regulatory mechanisms of *DENND2D* expression in HCC. Most HCC cell lines (89%) and surgical specimens (78%) expressed lower levels of *DENND2D* mRNA compared with normal liver tissue. In contrast, there was no significant difference in the expression levels of *DENND2D* mRNA between normal tissues of HCC patients with and without cirrhosis. The expression patterns of DENND2D protein and mRNA were consistent. Patients with significantly lower levels of *DENND2D* mRNA in HCC tissues had remarkably earlier recurrences after hepatectomy and their prognosis worsened. The *DENND2D* promoter was methylated in eight out of nine HCC cell lines and DNA demethylation reactivated *DENND2D* mRNA expression. Hypermethylation of *DENND2D* was frequently detected in HCC tissues (75%) and was significantly associated with downregulation of *DENND2D* mRNA expression. *DENND2D* is a candidate tumor suppressor gene that is inactivated by promoter hypermethylation in patients with HCC and may serve as a novel biomarker of early recurrence of HCC.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and third leading cause of cancer-related deaths worldwide and its prognosis is poor despite advances in diagnostic and therapeutic modalities (1,2). A minority of patients with HCC are diagnosed during early stages when the disease is amenable to potentially curative treatments such as surgery and topical therapy (for example, radiofrequency ablation) (3). The prognosis is poor for patients diagnosed at an advanced stage or with recurrent lesions after surgery or topical therapies, owing to underlying liver damage and lack of effective treatment options (1,4). Therefore, investigations that clarify the mechanisms of carcinogenesis and tumor progression in HCC are urgently needed to discover targets for therapy and prognostic markers for recurrence and progression of HCC.

Evidence indicates that the pathogenesis of HCC occurs in a stepwise process driven by oncogene activation and inactivation of tumor suppressor genes (TSGs) (5,6). Epigenetic alterations such as promoter hypermethylation can lead to the transcriptional silencing of TSGs (7,8). We identified several tumor-related genes frequently silenced through promoter hypermethylation in HCC, suggesting that expression status and promoter hypermethylation of these genes can serve as a biomarker for early detection of HCC (9-11).

The differentially expressed in normal and neoplastic cells (DENN) domain proteins regulate Rab GTPases and represent a newly recognized class of membrane trafficking proteins (12-16). The Rab family comprises 70 members in humans and represents the largest family of small GTPases (17). Rab proteins cycle between inactive GDP-bound and active GTP-bound states. In the active state, they recruit effectors that control multiple aspects of membrane trafficking (13,18,19). The DENN domain present in members of the connecdenn family of proteins, interacts directly with Rab35 and functions as a guanine nucleotide exchange factor (GEF) for this GTPase (20). GEFs activate Rabs by mediating the exchange

of GDP for GTP. The human genome encodes eight DENND (DENN domain) proteins that form eight families based on homology and domain structure as follows: DENND1A-1C, DENND2A-2D, DENND3, DENND4A-4C, DENND5A/5B, DENND6A/6B, MTMR5/13 and DENN/MADD. The DENN domain is located toward the N-terminus, except for the DENND2 family, where it is located toward the C-terminus (21-24). There is no significant sequence similarity to other proteins outside of the DENN domain.

Little is known about the functions and expression patterns of DENND family proteins in malignant tumors, although they play important roles in intracellular signaling pathways by integrating the activity of Rab pathways. *DENND2D*, which is located on chromosome 1p13.3, encodes a 53-kDa protein, which suppresses the proliferation and tumorigenicity of non-small cell lung cancer cells (23,25); however, the role of DENND family proteins in gastroenterological cancers has not been reported. Accordingly, we focused on DENND2D and investigated the regulation of its expression in an attempt to identify a TSG regulated by silencing through promoter hypermethylation. Our present study also focused on developing novel epigenetic biomarkers for HCC.

Materials and methods

Sample collection. Nine HCC cell lines (Hep3B, HepG2, HLE, HLF, HuH1, HuH2, HuH7, PLC/PRF/5 HepG2 and SK-Hep1) were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37˚C in an atmosphere containing 5% CO_2. Primary HCC tissues and corresponding non-cancer tissues were collected from 92 patients (75 men, 17 women; mean age, 63.2±9.9 years; range, 34-84 years) who underwent liver resection for HCC at Nagoya University Hospital between January, 1998 and July, 2008. Mean duration of patient follow-up was 48.3±36.9 months (range, 0.8-147 months). Specimens were classified histologically using the 7th edition of the Union for International Cancer Control (UICC) classification (26). Written informed consent for surgery and use of clinical data, as required by the institutional review board, was obtained from all patients. Tissue samples were immediately flash frozen in liquid nitrogen and stored at -80˚C. Microscopic evaluations were performed to ensure that tumor samples contained >80% tumor cells, whereas non-cancer liver tissue samples did not contain regenerative or dysplastic nodules.

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR (qPCR). The levels of DENND2D mRNA expression were analyzed using RT-PCR and qRT-PCR. Total RNAs (10 μg) isolated from each of the HCC cell lines listed above, 92 primary HCC tissues and corresponding non-cancer tissues were used as templates to generate complementary DNAs (cDNAs). PCR primers for *DENND2D* were as follows: sense (S) (5'-CACTGCTCTACCCCTTCAGC-3' in exon 7) and antisense (AS) (5'-TTTTTCATCACCAACCGACA-3' in exons 9 and 10), which amplify a 204-base-pair (bp) product. RT-PCR amplification was performed as follows: 40 cycles at 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec after an initial denaturation step at 94˚C for 5 min. To confirm that equal amounts of cDNA were used as templates, RT-PCR of β-actin was performed. Each RT-PCR product was loaded directly onto 2% agarose gels, electrophoresed, stained with ethidium bromide and visualized with ultraviolet light. The qPCR reactions were performed using a SYBR® Green PCR Core Reagents kit (Life Technologies, Carlsbad, CA, USA) under the following conditions: 1 cycle at 50˚C for 2 min, 1 cycle at 95˚C for 10 min and 45 cycles at 95˚C for 15 sec and 60˚C for 30 sec. Real-time detection of the SYBR-Green emission was conducted with an ABI PRISM® 7000 Sequence Detection System (Life Technologies). The primers were those described above. For standardization, GAPDH (TaqMan®, GAPDH Control Reagents, Life Technologies) was amplified in each sample. Nine HCC cell lines and 92 pairs of clinical samples and negative control reactions without templates were analyzed. Reactions were performed in triplicate. The amount of amplified *DENND2D* DNA in each sample was normalized to that of *GAPDH*. The expression of *DENND2D* mRNA was defined as downregulated in tumor tissues when its level was less than one-third that of the corresponding non-cancer tissues.

Analysis of the promoter region of DENND2D. The nucleotide sequence of the *DENND2D* promoter region was analyzed to determine the presence or absence of CpG islands defined as: ≥200-bp region of DNA with a high GC content (>50%) and an Observed CpG/Expected CpG ratio ≥0.6 (27). We used CpG Island Searcher software (http://cpgislands.usc.edu/) to determine the locations of CpG islands (28).

Methylation-specific PCR (MSP). DNA samples from HCC cell lines, HCC tissues and corresponding non-cancer tissues were treated with bisulfite. Briefly, 2 μg of DNA was denatured with NaOH, reacted with sodium bisulfite and purified using the Wizard® PCR Preps DNA Purification System resin (Promega, Madison, WI, USA), treated again with NaOH, precipitated with ethanol and resuspended in water. The sequences of the unmethylated primer pairs that amplify a 102-bp product were derived from the *DENND2D* promoter region upstream of exon 1 are: S (5'-GATATGTGTTTTTGTGGATT-3') and AS (5'-ACACATCCAAAACTAAAC-3'). Primer sequences derived from the *DENND2D* promoter region used to detect methylated DNA amplify a 193-bp product and are: S (5'-AGGTGGCGTCGTTTAGTTTC-3') and AS (5'-GCGAATCCGACACTTTCACT-3'). DNA was amplified as follows: 45 cycles of 94˚C for 30 sec 58˚C for 30 sec and 72˚C for 30 sec after an initial denaturation step at 94˚C for 5 min. Each PCR product was loaded directly on 2% agarose gels, electrophoresed, stained with ethidium bromide and visualized with ultraviolet light.

Bisulfite sequence analysis. Genomic bisulfite-treated DNAs from HCC cell lines were sequenced to verify the MSP results. The sequences of the primer pair used to generate a fragment for sequencing was derived from the *DENND2D* promoter region: S (5'-GGAGGTTAAGGATAGGGG-3') and AS (5'-ACACTAACCCCCATAACC-3'), which amplify a 133-bp product. DNA was amplified as follows: 50 cycles at 94˚C for 30 sec, 63˚C for 30 sec and 72˚C for 30 sec following an initial denaturation step at 94˚C for 5 min. PCR products were puri-

fied directly using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany). Purified DNA fragments were subcloned into the TA cloning vector (Life Technologies). Each DNA sample was mixed with 3 μl of specific primer (M13) and 4 μl of Cycle Sequence Mix (BigDye® Terminator v1. 1 Cycle Sequencing kit, Life Technologies, Grand Island, NY, USA). Sequences were analyzed using an Applied Biosystems ABI PRISM 310 DNA Analyzer and sequence electropherograms were generated using ABI Sequence Analysis 3.0 software (Life Technologies).

5-Aza-2'-deoxycytidine (5-aza-dC) treatment. To assess the relation of promoter hypermethylation to *DENND2D* expression, HCC cells ($1.5x10^6$) were treated with 5-aza-dC (Sigma-Aldrich, St. Louis, MO, USA) to inhibit DNA methylation and were cultured for 6 days with medium changes on days 1, 3 and 5. RNA was extracted and RT-PCR was performed as described above.

Immunohistochemistry (IHC). We used IHC to investigate DENND2D localization in 30 representative sections of well preserved HCC tissues. Formalin-fixed, paraffin-embedded tissues were treated with 3% H_2O_2 to inhibit endogenous peroxidase, followed by epitope retrieval using five incubations in 10 mM citrate buffer at 95˚C, 5 min each. The samples were incubated with Histofine SAB-PO(R) (Nichirei, Tokyo, Japan) for 5 min to limit non-specific reactivity and were then incubated for 1 h at room temperature with a rabbit antibody against DENND2D (PA5-24032, Thermo Fisher Scientific Inc., Rockford, IL, USA) diluted 1:100 in Antibody Diluent (Dako). Sections were developed for 2 min using liquid 3, 3'-diaminobenzidine as the substrate (Nichirei). Staining properties were determined using surrounding hepatic veins as internal controls and staining patterns were compared between HCCs and the corresponding non-cancer tissues. To avoid subjectivity, specimens were randomized and coded before analysis by two independent observers blinded to the status of the samples. Each observer evaluated all specimens at least twice within a given time interval to minimize intra-observer variation.

Statistical analysis. The relative mRNA expression levels (*DENND2D*/*GAPDH*) between HCC and non-cancer tissues were analyzed using the Mann-Whitney U test. The χ^2 test was used to analyze the association between the expression and methylation status of *DENND2D* and clinicopathological parameters. Overall and disease-free survival rates were calculated using the Kaplan-Meier method and the difference in survival curves was analyzed using the log-rank test. We performed multivariable regression analysis to detect prognostic factors using the Cox proportional hazards model and variables with a P<0.05 were entered into the final model. All statistical analysis was performed using JMP® 10 software (SAS Institute Inc, Cary, NC, USA). P<0.05 was considered statistically significant.

Results

DENND2D mRNA expression in HCC cell lines and tumor tissues. The levels of *DENND2D* mRNA detected using qPCR were reduced significantly relative to median value of normal liver controls in all HCC cell lines except HuH1 (Fig. 1A). In particular, the *DENND2D* mRNA levels were 20-times lower in Hep3B, HLE, HuH2 and PLC-PRF5 cells. When we compared the levels of *DENND2D* mRNA in non-cancer tissues of HCC patients with (n=35) or without (n=57) cirrhosis, no significant difference was observed, suggesting that the expression of *DENND2D* mRNA in non-cancer liver tissue was not affected by liver fibrosis (Fig. 1B). The expression level of *DENND2D* mRNA in 72 (78%) of 92 patients was lower in HCC tissues than in the corresponding normal tissues. Further, the mean expression of *DENND2D* mRNA was 2-fold lower in HCC tissues than in corresponding normal tissues (P<0.001, Fig. 1C).

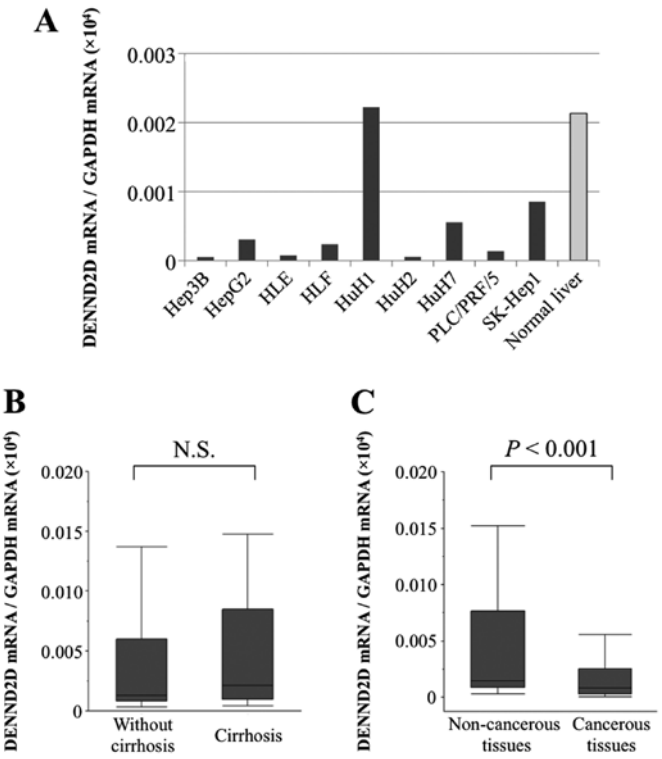

Figure 1. (A) *DENND2D* mRNA expression in HCC cell lines and median value of normal liver tissues (without chronic hepatitis and cirrhosis) using qPCR. Reduced expression was detected in all HCC cell lines compared with controls, except for HuH1. (B) There were no significant differences in *DENND2D* mRNA expression level in livers of patients with and without cirrhosis. (C) The mean expression level of *DENND2D* mRNA was significantly higher in HCC tissues than in corresponding normal tissues. NS, not significant.

Identification of a CpG island in the DENND2D promoter. A CpG island was identified at the *DENND2D* promoter region using the CpG Island Searcher. The properties of the CpG island are: 1214 bp, 68.7% GC and 0.68 Observed CpG/Expected CpG ratio (Fig. 2A). Therefore, we hypothesized that hypermethylation of the CpG islands regulates the expression of DENND2D in HCC tissue.

MSP analysis of HCC cell lines. MSP was conducted to verify the hypothesis described above. We first determined the methylation status of *DENND2D* in nine HCC cell lines. Bands

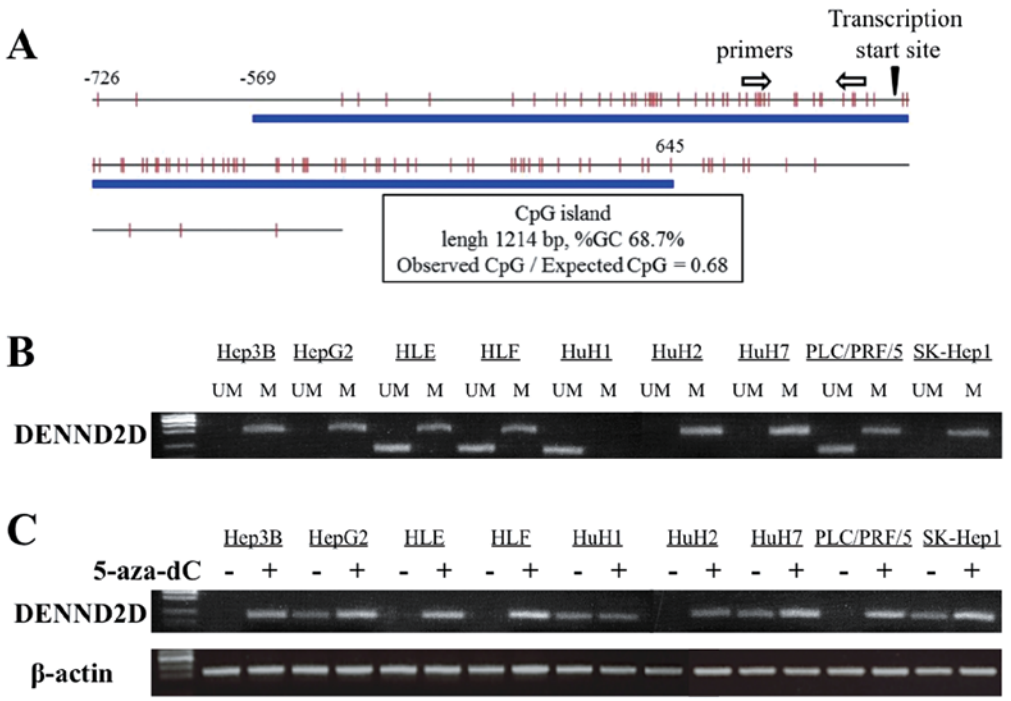

Figure 2. (A) The CpG island indicated by the blue line was centered around the *DENND2D* transcription initiation site extending upstream into the promoter region. (B) MSP analysis. The *DENND2D* promoter was completely methylated in Hep3B, HepG2, HuH2, HuH7 and SK-Hep1 cells, in contrast, methylation was partial in HLE, HLF and PLC/PRF/5 cells and was not detected in HuH1 cells. (C) RT-PCR analysis before and after 5-aza-dC treatment. Reactivation or an increase if *DENND2D* expression was detected in all HCC cell lines except HuH1.

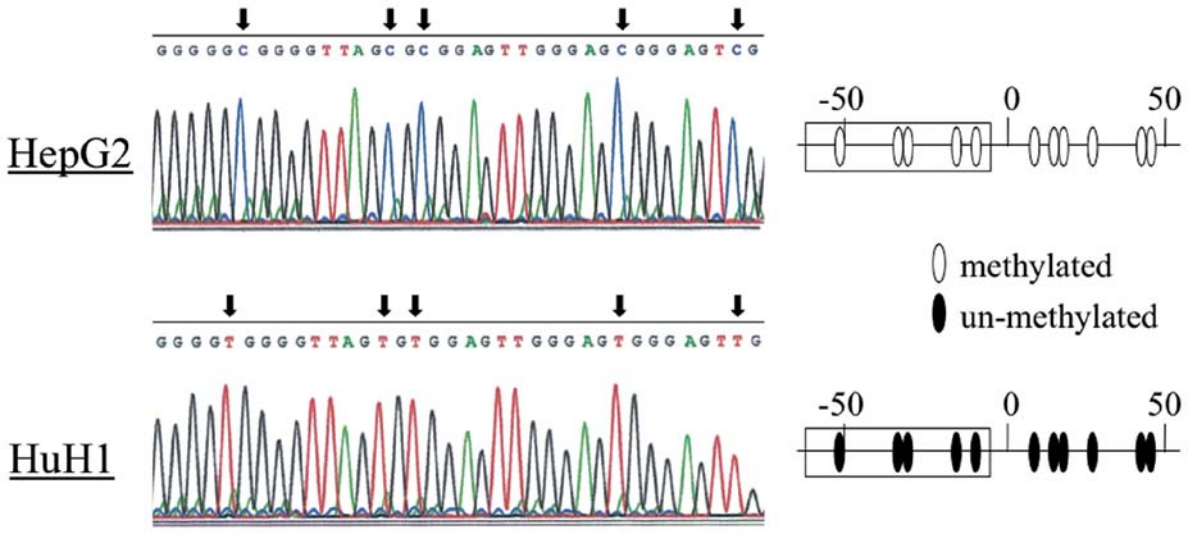

Figure 3. Bisulfite sequence analysis of genomic DNA isolated from HepG2 and HuH1 cells. All CpGs in HepG2 were CG and TG was present at the same position in HuH1.

consistent with methylated DNA were detected in all HCC cell lines except HuH1. PCR using unmethylated primers, amplified bands from HLE, HLF, HuH1 and PLC/PRF/5 *DENND2D* DNA (Fig. 2B). We conclude that methylation of the *DENND2D* promoter was complete in Hep3B, HepG2, HuH2, HuH7 and SK-Hep1; partial in HLE, HLF and PLC/PRF/5 cells and undetectable in HuH1 cells.

Transcription of DENND2D in cells treated with 5-aza-dC. To determine whether promoter hypermethylation leads to the suppression of *DENND2D* transcription, we analyzed HCC cell lines before and after treatment with the DNA methylation inhibitor, 5-aza-dC. Using semi-quantitative RT-PCR, reactivation or an increase in *DENND2D* expression was detected in all HCC cell lines except HuH1, consistent with the results of the MSP analysis (Fig. 2C).

Bisulfite sequence analysis. To confirm the results of the MSP experiments, we directly sequenced the *DENND2D* promoter in HepG2 (complete methylation) and HuH1 (undetectable

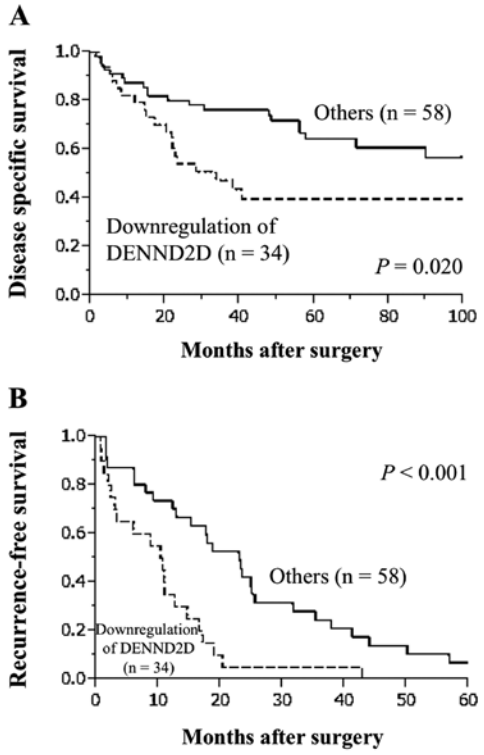

Figure 4. Kaplan-Meier survival curves for 94 patients categorized according to downregulation of *DENND2D* mRNA in HCC tissues. The overall (A) and recurrence-free (B) survivals were significantly shorter in patients with downregulation of *DENND2D*.

methylation) cells and found that all CpGs in the HepG2 fragment were CG, while TG was present at the corresponding position in HuH1 cells (Fig. 3), thus confirming the MSP data.

Prognostic value of DENND2D expression in 92 patients with HCC. Downregulation of *DENND2D* mRNA was detected in tumor samples from 34 of 92 (37.0%) patients with HCC. Median disease specific survival (32.6 versus 112 months, P=0.020, Fig. 4A) and relapse-free survival (10.3 versus 23.0 months, P<0.001, Fig. 4B) were significantly shorter in patients with downregulation of *DENND2D* mRNA. Tumor size ≥3.0 cm and vascular invasion, but not *DENND2D* expression, were identified as independent prognostic factors for disease specific survival using multivariate analysis (Table I). In contrast, univariate analysis for recurrence-free survival showed that serosal infiltration, vascular invasion and downregulation of *DENND2D* mRNA were significantly prognostic of adverse outcomes. Multivariate analysis identified downregulation of *DENND2D* mRNA as an independent prognostic factor for recurrence-free survival (hazard ratio 2.86, P=0.002, Table II). Downregulation of *DENND2D* mRNA was not significantly associated with other clinicopathological parameters.

Methylation status of DENND2D in 92 clinical HCC samples. MSP analysis revealed that 69 (75.0%) out of 92 HCC tissue samples and only 4 (4.3%) of 92 corresponding non-cancer tissues showed hypermethylation of the *DENND2D* promoter.

Table I. Prognostic factors for disease-specific survival in 92 patients with hepatocellular carcinoma.

		Univariate			Multivariate		
Variable	n	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age (≥65)	47	1.61	0.87-3.03	0.130			
Gender (male)	77	1.18	0.55-2.92	0.695			
Background liver (cirrhosis)	36	1.44	0.77-2.67	0.250			
Pugh-Child's classification (B)	7	1.28	0.31-3.57	0.692			
AFP (>20 ng/ml)	47	1.90	1.03-3.57	0.042[a]	1.70	0.87-3.36	0.121
PIVKA II (>40 mAU/ml)	54	2.25	1.18-4.51	0.013[a]	1.36	0.65-2.99	0.415
Tumor multiplicity (multiple)	24	1.97	1.00-3.69	0.049[a]	2.26	1.08-4.62	**0.032[a]**
Tumor size (≥3.0 cm)	67	2.41	1.16-5.67	0.017[a]	1.18	0.49-3.07	0.712
Tumor differentiation (well)	28	0.51	0.23-1.03	0.060			
Growth type (invasive growth)	17	1.16	0.51-2.35	0.705			
Serosal infiltration	25	2.33	1.16-4.49	0.018[a]	1.05	0.46-2.34	0.898
Formation of capsule	68	0.98	0.51-2.00	0.952			
Infiltration to capsule	54	1.07	0.58-2.03	0.822			
Septum formation	34	0.91	0.49-1.75	0.776			
Vascular invasion	23	3.33	1.73-6.23	<0.001[a]	2.35	1.08-5.11	**0.032[a]**
Margin status (positive)	24	2.23	1.17-4.15	0.016[a]	1.89	0.96-3.60	0.064
Hypermethylation of *DENND2D*	69	1.22	0.61-2.73	0.588			
Downregulation of *DENND2D* mRNA	34	2.08	1.10-3.89	0.025[a]	1.79	0.89-3.59	0.101

[a]Statistically significant (P<0.05). CI, confidence interval; AFP, α-fetoprotein; PIVKA, protein induced by vitamin K antagonists. Univariate and multivariate analyses were performed using the log-rank test and the Cox proportional hazards model, respectively.

Table II. Prognostic factors for recurrence-free survival in 92 patients with hepatocellular carcinoma.

		Univariate			Multivariate		
Variable	n	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age (≥65)	47	1.72	0.96-3.08	0.068			
Gender (male)	77	1.41	0.64-3.73	0.418			
Background liver (cirrhosis)	36	1.68	0.86-3.10	0.124			
Pugh-Child's classification (B)	7	0.93	0.28-2.32	0.889			
AFP (>20 ng/ml)	47	1.58	0.89-2.82	0.116			
PIVKA II (>40 mAU/ml)	54	1.10	0.61-2.03	0.759			
Tumor multiplicity (multiple)	24	1.06	0.54-1.96	0.861			
Tumor size (≥3.0 cm)	67	1.93	0.95-4.47	0.073			
Tumor differentiation (well)	28	1.05	0.51-2.02	0.879			
Growth type (invasive growth)	17	1.33	0.57-2.75	0.483			
Serosal infiltration	25	2.62	1.35-5.00	0.005[a]	2.06	1.04-4.02	**0.039**[a]
Formation of capsule	68	0.57	0.29-1.22	0.139			
Infiltration to capsule	54	0.84	0.47-1.55	0.577			
Septum formation	34	0.68	0.38-1.26	0.214			
Vascular invasion	23	2.41	1.27-4.39	0.008[a]	2.12	1.08-4.01	**0.029**[a]
Margin status (positive)	24	1.79	0.97-3.23	0.064			
Hypermethylation of *DENND2D*	69	1.11	0.59-2.24	0.749			
Downregulation of *DENND2D* mRNA	34	2.86	1.52-5.35	0.001[a]	2.86	1.49-5.44	**0.002**[a]

[a]Statistically significant (P<0.05). CI, confidence interval; AFP, α-fetoprotein; PIVKA, protein induced by vitamin K antagonists. Univariate and multivariate analyses were performed using the log-rank test and the Cox proportional hazards model, respectively.

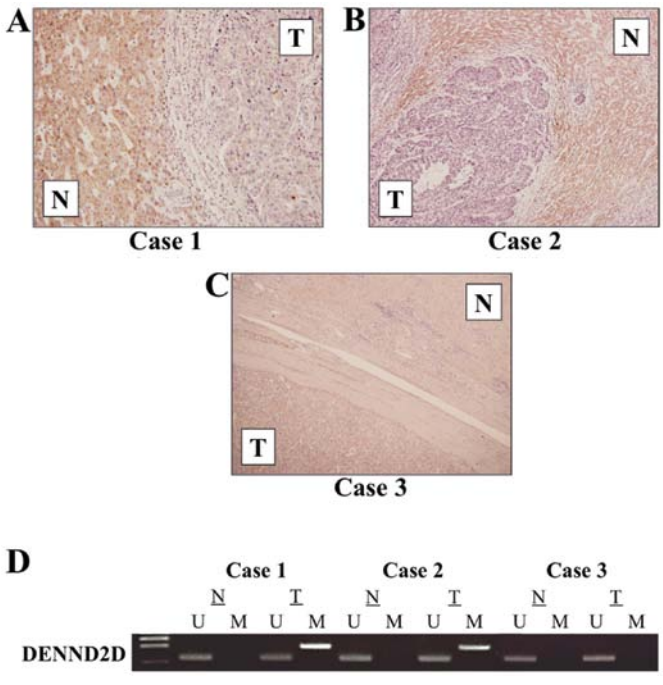

Figure 5. IHC analysis of DENND2D expression in representative patients with HCC. (A) Case 1; (B) Case 2, cancer tissue cells showed downregulation levels of DENND2D compared with adjacent non-cancer tissue cells (x100). (C) Case 3, equivalent expression of DENND2D was detected in cancer and non-cancer tissue cells in patients without downregulation of *DENND2D* mRNA expression (x40). (D) MSP results for cases 1-3 whose IHC data are shown in (A-C). N, non-cancer tissue cells; T, tumor tissue cells.

There was no significant association between hypermethylation of *DENND2D* in HCC tissues and overall or recurrence-free survival (Tables I and II). Analysis of the associations between the methylation status of *DENND2D* and clinicopathological factors, including demographics, background liver status, pathological findings and expression level of *DENND2D* mRNA showed that promoter hypermethylation of DENND2D in HCCs was significantly associated with tumor size ≥3 cm, serosal infiltration and downregulation of DENND2D (P=0.048, 0.049 and 0.004, respectively, Table III).

IHC. The expression of DENND2D was determined using IHC in 30 cases showing relative overexpression, underexpression, or equivalent *DENND2D* mRNA expression in HCC tissues compared with the corresponding non-cancer tissues. Two representative cases with the lowest expression level of *DENND2D* mRNA in HCC tissues showed downregulation of DENND2D in the membrane and cytoplasm of tumor cells compared with adjacent non-cancer tissues (Fig. 5A and B). In contrast, equivalent expression of DENND2D protein in tumor and normal cells was detected in the case without reduced *DENND2D* mRNA expression in HCC tissues (Fig. 5C). The MSP analysis of these cases is shown in Fig. 5D.

Discussion

Research conducted over the past decade has demonstrated that certain TSGs are epigenetically inactivated in HCC, indi-

Table III. Association between methylation status of *DENND2D* and clinicopathological parameters in 92 HCC patients.

Clinicopathological parameters	Methylation positive in tumor tissue (n)	Methylation negative in tumor tissue (n)	P-value
Age			
<65 year	31	15	0.090
≥65 year	38	8	
Gender			
Male	59	16	0.101
Female	10	7	
Background liver			
Normal liver	4	5	0.080
Chronic hepatitis	36	12	
Cirrhosis	29	6	
Pugh-Child's classification			
A	64	21	0.823
B	5	2	
Hepatitis virus			
Absent	9	5	0.617
HBV	19	6	
HCV	41	12	
AFP (ng/ml)			
≤20	34	13	0.547
>20	35	10	
PIVKA II (mAU/ml)			
≤40	32	7	0.175
>40	37	16	
Tumor multiplicity			
Solitary	53	16	0.493
Multiple	16	7	
Tumor size			
<3.0 cm	23	3	**0.048**[a]
≥3.0 cm	46	20	
Differentiation			
Well	21	6	0.689
Moderate to poor	48	17	
Growth type			
Expansive growth	58	18	0.533
Invasive growth	11	5	
Serosal infiltration			
Absent	54	13	**0.049**[a]
Present	15	10	
Formation of capsule			
Absent	19	7	0.790
Present	50	16	
Infiltration to capsule			
Absent	29	11	0.628
Present	40	12	
Septum formation			
Absent	26	8	0.803
Present	43	15	
Vascular invasion			
Absent	53	17	0.779
Present	16	6	
Margin status			
Negative	51	17	1.000
Positive	18	6	
UICC pathological stage			
I, II	44	10	0.089
III, IV	25	13	
Downregulation of DENND2D			
Absent	38	20	**0.004**[a]
Present	31	3	

UICC, Union for International Cancer Control; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, α-fetoprotein; PIVKA, protein induced by vitamin K antagonists; S, serosal infiltration; Fc, formation of capsule; Fc-inf, infiltration to capsule. [a]Statistically significant difference (P<0.05).

cating that this is one of the major molecular alterations that occurs during hepatocarcinogenesis (7,29). Moreover, there is growing evidence that a major mechanism of epigenetic silencing in cancers involves hypermethylation of the TSG promoter (10,30,31). Promoter hypermethylation can be used as a sensitive marker for TSG identification, cancer diagnosis and predicting prognosis. In the present study, *DENND2D* was identified as another candidate TSG that is epigenetically inactivated in HCC.

We show here that the expression level of *DENND2D* mRNA was independent of fibrosis of the liver, because there was no significant difference in *DENND2D* mRNA levels between patients with and without cirrhosis. In contrast, the level of *DENND2D* mRNA was reduced in 8/9 HCC cell lines and in 72/92 surgically resected specimens and the mean expression level was significantly lower in cancer tissues than in corresponding normal tissues. This result indicates that DENND2D plays an important role in hepatocarcinogenesis but not in the fibrous response of the liver. Further,

the expression pattern of DENND2D was consistent with that of its mRNA according to the results of qPCR and IHC analyses. A striking discovery was that a significant reduction in *DENND2D* mRNA level in HCC tissues correlated with remarkably earlier recurrence (hazard ratio 2.86, P=0.002, Table I) and subsequent adverse prognosis. These findings suggest that *DENND2D* acts as a TSG and are consistent with the results of a study on DENND2D expression in lung cancer (25). HCC frequently relapses after hepatectomy, becoming multicentric metastatic within the liver. Downregulation of *DENND2D* expression in HCC tissues could therefore be a biomarker of HCC recurrence.

To understand the regulation of *DENND2D* transcription, we performed methylation analysis of *DENND2D* after we identified a CpG island within its promoter. The *DENND2D* promoter was hypermethylated in 8/9 HCC cell lines and *DENND2D* transcription could be reactivated in cells treated with an inhibitor of methylation. Hypermethylation was frequently (75%) detected in HCC tissues and significantly associated with substantial (≥3-times) reduction of *DENND2D* mRNA levels. Therefore, we consider promoter hypermethylation as a potent regulatory factor of *DENND2D* transcription in HCC. Further, hypermethylation of the *DENND2D* promoter in HCC tissues significantly associated with tumor size and serosal infiltration, indicating the importance of *DENND2D* in tumor-cell growth and migration. Therefore, hypermethylation of *DENND2D* should provide an important new biomarker of HCC progression.

The DENND2 family is the only example where the DENN domain is located within the C-terminal region (23,32). Each DENND2 protein acts as a GEF for Rab9a/b. DENND2D is composed of only a DENN domain (33); therefore, the DENN domain mediates GEF activity. Rab9 functions in retrograde trafficking of the mannose phosphate receptor from late endosomes to the trans-Golgi network and depletion of DENND2A, but not other DENND2 family members, disrupting trafficking (34,35). This suggests that DENND2 proteins activate other Rab9 functions, such as biogenesis of lysosome-related organelles (36).

This study is limited by its lack of sufficient functional analysis of DENND2D, which tempers the conclusion that it acts as a tumor suppressor for HCC. Further studies will be required to clarify the molecular mechanisms underlying the biological activities of DENND2D in HCC.

In conclusion, we propose *DENND2D* as a candidate tumor suppressor gene that is inactivated by promoter hypermethylation in HCC and shows promise as a novel biomarker of early recurrence of HCC.

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