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Expression of Cancer Stem Cell-associated *DKK1* mRNA Serves as Prognostic Marker for Hepatocellular Carcinoma

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Abstract. *Background/Aim: Cancer stem cells (CSCs) are associated with prognosis of hepatocellular carcinoma (HCC). In our previous study, we created cDNA microarray databases on the CSC population of human HuH7 cells. In the present study, we identified genes that might serve as prognostic markers of HCC by employing existing databases. Materials and Methods: Expressions of glutathione S-transferase pi 1 (GSTP1), lysozyme (LYZ), C-X-C motif chemokine ligand 5 (CXCL5), interleukin-8 (IL8) and dickkopf WNT signaling pathway inhibitor 1 (DKK1), the five most highly expressed genes in the CSC cDNA microarray databases, were examined in 99 patients with HCC by real-time polymerase chain reaction (qRT-PCR), and their clinical significance was analyzed. Results: The Kaplan–Meier analysis showed that both overall and cancer-specific survival were significantly longer in patients with low DKK1 expression than in those with high DKK1 expression. The multivariate analysis revealed that overall survival was negatively associated with albumin and positively associated with alkaline phosphatase (ALP), serosal invasion and stage, and cancer-specific survival was positively associated with ALP, portal vein invasion and DKK1 mRNA. Conclusion: Expression of CSC-associated DKK1 mRNA might be an unfavorable prognostic marker for patients with HCC.*

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Hepatocellular carcinoma (HCC) is the sixth most common cancer, and the third most frequent cause of death worldwide (1). Since biomarkers are useful for early diagnosis and prediction of prognosis (2), they may provide effective treatment options. Although several biomarkers, including alpha-fetoprotein (AFP), protein induced by vitamin K deficiency or antagonist-II (PIVKAII), and glypican-3, were reported as being useful (2, 3), identification of novel biomarkers for HCC is expected to improve prognosis of patients with HCC.

Cancer stem cells (CSCs) are defined as cells that possess the capacity to self-renew and produce heterogeneous lineages of cancer cells (4). CSCs are involved in development, progression, metastasis, recurrence and prognosis of cancer (5). Indeed, it is reported that patients with HCC having a CSC phenotype have a poor prognosis (6). Dysregulation of several specific signaling pathways in CSCs has been associated with stemness (7). CSCs are also 'robust', which encompasses several characteristics including the ability to escape from the effect of cytotoxic agents, resistance to oxidative stress, and a rapid response to and repair of DNA damage (8). Specific genes which confer resistance to chemotherapy and radiotherapy seem to be expressed in CSCs, resulting in poor prognosis for patients with cancer.

In our previous study, we identified CD44 as the best prognostic marker out of four CSC markers, namely CD13, epithelial cell adhesion molecule (EpCAM), CD44 and CD44 variant 9, in HCC (9). In addition, CD44-positive HuH7 HCC cells had CSC properties such as proliferative potential and sphere-forming ability. Importantly, we developed databases for cDNA/miRNA expression of liver CSCs. In the present study, by employing the previously developed cDNA microarray databases, we examined whether the top five most highly expressed genes, glutathione S-transferase pi 1

Table I. *Clinical parameters of patients with hepatocellular carcinoma.*

Characteristic	Value	Characteristic	n
No. of patients	99	Capsular invasion (n=95)	
Gender		Negative	50
Male	83	Positive	45
Female	26	Septum formation	
Age, years	67 (32-88)	Negative	32
Etiology		Positive	67
HBV	14	Serosal invasion	
HCV	62	Negative	87
HBV/HCV	21	Positive	12
Non HBV/C	2	Portal vein invasion	
Total bilirubin (mg/dl)	0.8 (0.1-3.9)	Negative	62
Albumin (g/dl)	3.9 (2.9-5.3)	Positive	37
AST (IU/l)	47 (17-166)	Hepatic vein invasion	
ALT (IU/l)	44 (8-311)	Negative	88
ALP (IU/l)	278 (33-1159)	Positive	11
γ-GTP (IU/l) (n=97)	66 (17-969)	Bile duct invasion	
AFP (ng/ml)	52 (3.2-639256)	Negative	87
PIVKA-II (U/ml) (n=98)	119 (0.02-30100)	Positive	12
Tumor number (n=98)		Stage	
1	70	I	6
2	13	II	43
>3	15	III	28
Tumor size (cm) (n=98)	3.5 (1.0-16)	IV	22
Survival period (days)	1717 (28-4450)		
Differentiation (n=95)			
Well	19		
Moderate	54		
Poor/undifferentiated	22		

HBV, Hepatitis B virus; HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma-glutamyl transpeptidase AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II. Data are the median (range) for continuous variables and absolute numbers for categorical variables.

(*GSTP1*), lysozyme (*LYZ*), C-X-C motif chemokine ligand 5 (*CXCL5*), interleukin-8 (IL8) and dickkopf WNT signaling pathway inhibitor 1 (*DKK1*), in the CSC population can serve as prognostic markers for HCC.

Materials and Methods

Patients and clinical samples. The samples for the gene-expression analysis were obtained from 99 patients with HCC who were admitted to Kyoto University Hospital from 1998 to 2008 and agreed to undergo surgical resection with curative intent under informed consent. Clinicopathological parameters of these patients are summarized in Table I. This study conformed with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of Tottori University Faculty of Medicine and Kyoto University Hospital (approval number: 1619).

Real-time reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA from hNHeps (Lonza, Walkersville, MD, USA), CD44-negative HuH7 (Japanese Collection of Research Bioresources Cell Bank, Osaka, Japan), CD44-positive HuH7 cells (Japanese Collection of Research Bioresources Cell Bank), and 99 HCC clinical specimens were obtained according to the method previously described (9). Total RNA was reverse-transcribed using SuperScript II (Invitrogen, Carlsbad, CA, USA) and oligo (dT) primers. Expression levels of mRNA were measured by Applied Biosystems 7900HT Fast Real Time PCR System using EXPRESS qPCR Supermix with Premixed ROX (Applied Biosystems, Life Technologies, Foster City, CA, USA), universal probes (Roche Applied Science, Basel, Switzerland), and gene-specific primers. Universal probes and gene-specific primers used in this study are summarized in Table II.

Statistical analysis. EXCEL (Microsoft Corporation, Redmond, WA, USA) and PASW statistics (SPSS Inc., Chicago, IL, USA) were used for the statistical calculations in this study. The comparison for statistical differences was performed using Student's *t*-test, or Mann–Whitney *U*-test. The Kaplan–Meier analysis was performed for both overall and cancer-specific survival according to CSC-related genes. The log-rank test was performed to determine the prognostic variables associated with overall survival and cancer-specific survival ratios in patients with HCC. Cox regression model was used for multivariable analysis of variables affecting overall and cancer-specific survival. Diffuses with a value of $p<0.05$ were considered to be statistically significant.

Table II. *Universal probes and primers used in this study.*

Gene	Encoded protein name	Gene ID	Universal probe ID	Forward	Reverse
ACTB	Actin, cytoplasmic 1	NM_001101.3	#64	ccaaccgcgagaagatga	ccagaggcgtacagggatag
GSTP1	Glutathione S-transferase P	NM_000852.3	#56	tctccctcatctacaccaactatg	aggtcttgcctccctggt
LYZ	Lysozyme C precursor	NM_000239.2	#68	ccgctactggtgtaatgatgg	catcagcgatgttatcttgcag
CXCL5	C-X-C Motif chemokine 5 precursor	NM_002994.3	#71	ggtccttcgagctccttgt	gcagctctctcaacacagca
IL8	Interleukin-8 precursor	NM_000584.3	#72	gagcactccataaggcacaaa	atggttccttccggtggt
DKK1	Dickkopf-related protein 1 precursor	NM_012242.2	#4	caggcgtgcaaatctgtct	aatgattttgatcagaagacacacata

Table III. *List of genes exhibiting differential expression patterns between hNHeps, CD44 − HuH7, and CD44+ HuH7 cells.*

Pattern A (hNHeps <CD44$^-$ <CD44$^+$)				Pattern B (CD44$^-$ <CD44$^+$ <hNHeps)				Pattern C (CD44$^-$ <hNHeps <CD44$^+$)			
Symbol	Normalized expression			Symbol	Normalized expression			Symbol	Normalized expression		
	hNHeps	CD44$^-$	CD44$^+$		hNHeps	CD44$^-$	CD44$^+$		hNHeps	CD44$^-$	CD44$^+$
C17orf45	1343	2364	5311	*UCHL1*	1167	370	4169	*HIGD1A*	3595	1551	3144
GSTP1	68	1621	3278	*PSAP*	874	607	1251	*S100A6*	15717	547	1480
LYZ	12	530	2394	*CLPTM1L*	1015	563	1155	*PTGR1*	2431	279	1071
CXCL5	28	565	2191	*MAP2K4*	80	74	519	*ENC1*	884	246	498
IL8	331	832	1767	*DHRS7B*	192	124	505	*UGDH*	1346	167	384
DKK1	12	438	1126	*CEBPD*	182	127	420	*ANKRD1*	385	156	346
USP14	496	499	1003	*ZSCAN5C*	334	183	368	*GPX3*	602	132	331
USP22	237	442	901	*MEST*	182	157	344	*TUBA1*	1739	107	235
TMEM11	174	183	540	*XPO1*	277	11	335	*FAM3C*	398	102	212
MLLT11	57	98	375	*FBXO2*	277	87	301	*SRXN1*	607	87	197
PIR	108	139	316	*LCP1*	202	122	297	*IGFBP3*	235	78	196
HPGD	34	102	297	*AC026412.4*	237	134	284	*BLVRB*	607	84	189
B9D1	44	103	246	*CDKN2B*	195	102	260	*GBP2*	458	74	173
ZNF18	28	40	225	*NFU1*	123	97	202	*TIMP1*	1809	45	159
PKIB	50	65	223	*KIAA1143*	105	79	181	*EMP3*	368	40	149

GSTP1, Glutathione S-transferase pi 1; *LYZ*, lysozyme; *CXCL5*, C-X-C motif chemokine ligand 5; *IL8*, interleukin; *DKK1*, dickkopf WNT signaling pathway inhibitor 1; *USP14*, ubiquitin specific peptidase 14; *USP22*, ubiquitin specific peptidase 22; *TMEM11*, transmembrane protein 11; *MLLT11*, MLLT11, transcription factor 7 cofactor; *PIR*, pirin; *HPGD*, hydroxyprostaglandin dehydrogenase 15-(NAD); *B9D1*, B9 domain containing 1; *ZNF18*, zinc finger protein 18; *PKIB*, cAMP-dependent protein kinase inhibitor beta; *UCHL1*, ubiquitin C-terminal hydrolase L1; *PSAP*, prosaposin; *CLPTM1L*, CLPTM1 like; *MAP2K4*, mitogen-activated protein kinase kinase 4; *DHRS7B*, dehydrogenase/reductase 7B; *CEBPD*, CCAAT/enhancer binding protein delta; *ZSCAN5C*, zinc finger and SCAN domain containing 5C; *MEST*, mesoderm specific transcript; XPO1, exportin 1; *FBXO2*, F-box protein 2; *LCP1*, lymphocyte cytosolic protein 1; *CDKN2B*, cyclin-dependent kinase inhibitor 2B; *NFU1*, NFU1 iron-sulfur cluster scaffold; *HIGD1A*, HIG1 hypoxia-inducible domain family member 1A; *S100A6*, S100 calcium-binding protein A6; *PTGR1*, prostaglandin reductase 1; *ENC1*, ectodermal-neural cortex 1; *UGDH*, UDP-glucose 6-dehydrogenase; *ANKRD1*, ankyrin repeat domain 1; *GPX3*, glutathione peroxidase 3; *TUBA1*, tubulin alpha 4a; *FAM3C*, family with sequence similarity 3 member C; *SRXN1*, sulforedoxin 1; *IGFBP3*, insulin-like growth factor binding protein 3; *BLVRB*, biliverdin reductase B; *GBP2*, guanylate binding protein 2; *TIMP1*, TIMP metallopeptidase inhibitor 1; *EMP3*, epithelial membrane protein 3.

Results

Differential expression patterns of mRNA from CSCs, non-CSCs, and normal hepatocytes. In our previous study, the genes with at least two-fold up-regulation in CD44-positive compared to CD44-negative HuH7 cells were registered in Gene Expression Omnibus (accession number GSE84226) (9). Of these 604 genes, 216 genes in which the global normalization number was over 20 were divided into three patterns of expression as follows: the first pattern included successive increase of expression in the order of normal hepatocytes, CD44-negative cells, and CD44-positive cells. The second pattern included successive increase in the order of CD44-negative cells, normal hepatocytes, and CD44-positive cells. The third pattern included successive increase in the order of CD44-negative cells, CD44-positive cells, and

Table IV. *Univariate and multivariate analyses of clinical variables and cancer stem cells-related mRNA expression for overall and cancer-specific survival.*

Factor	Ref. *vs.* comparator	Overall survival			Cancer-specific survival		
		Univariate	Multivariate		Univariate	Multivariate	
		p-value	HR (95% CI)	*p*-Value	*p*-value	HR (95% CI)	*p*-Value
Gender	Male *vs.* female	0.776			0.447		
Age	≤67 *vs.* >67 years	0.209			0.876		
HBs-Ag	Negative *vs.* positive	0.128			0.006	N/A	0.589
HCV-Ab	Negative *vs.* positive	0.161			0.017	N/A	0.444
AFP	≤57 *vs.* >57 ng/ml	0.004	N/A	0.548	0.001	N/A	0.235
PIVKA-II	≤140 *vs.* >140 U/ml	0.275			0.060		
Total bilirubin	≤0.8 *vs.* >0.8 mg/dl	0.525			0.575		
Albumin	≤3.9 *vs.* >3.9 g/dl	0.015	0.544 (0.312-0.949)	0.032	0.081		
AST	≤51 *vs.* >51 IU/l	0.454			0.256		
ALT	≤45 *vs.* >45 IU/l	0.457			0.374		
ALP	≤283 *vs.* >283 IU/l	<0.001	2.796 (1.592-4.909)	<0.001	<0.001	3.189 (1.522-6.683)	0.002
γ-GTP	≤74 *vs.* >74 IU/l	0.069			0.088		
Tumor number	1 *vs.* ≥2	0.071			0.006	N/A	0.070
Tumor size	≤3.5 *vs.* >3.5 cm	0.023	N/A	0.297	0.016	N/A	0.434
Capsular invasion	Negative *vs.* positive	0.141			0.030	N/A	0.498
Septum formation	Negative *vs.* positive	0.187			0.082		
Serosal invasion:	Negative *vs.* positive	0.012	2.182 (1.020-4.671)	0.044	0.001	N/A	0.389
Portal vein invasion	Negative *vs.* positive	0.004	N/A	0.338	<0.001	3.149 (1.616-6.135)	0.001
Bile duct invasion	Negative *vs.* positive	0.023	N/A	0.604	0.028	N/A	0.320
Hepatic vein invasion	Negative *vs.* positive	0.066			0.003	N/A	0.971
Stage	I/II *vs.* III/IV	0.007	1.840 (1.054-3.212)	0.032	<0.001	N/A	0.132
GSTP1 mRNA	Low *vs.* high	0.842			0.744		
LYZ mRNA	Low *vs.* high	0.664			0.266		
CXCL5 mRNA	Low *vs.* high	0.207			0.638		
IL8 mRNA	Low *vs.* high	0.797			0.819		
DKK1 mRNA	Low *vs.* high	0.016	N/A	0.611	0.002	2.242 (1.093-4.599)	0.028

Ref.: Referent; HR, hazard ratio; CI, confidence interval; N/A, not applicable; HBs-Ag, hepatitis B surface antigen; HCV Ab, hepatitis C virus antibody; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GTP, gamma-glutamyl transpeptidase; GSTP1, glutathione S-transferase pi 1; LYZ, lysozyme; CXCL5, C-X-C motif chemokine ligand 5; IL8, interleukin-8; DKK1, dickkopf WNT signaling pathway inhibitor 1.

normal hepatocytes. Each pattern included 60, 57 and 99 genes, respectively. The top 15 genes in each group are listed in Table III. On the assumption that the genes showing a successive increase of expression in the order of normal hepatocytes, CD44-negative cells, and CD44-positive cells are associated with CSCs of HCC, the individual mRNA expressions of the top five genes were examined in normal hepatocytes, CD44-negative cells, and CD44-positive cells by qRT-PCR. C17orf45 was omitted since this gene is a non-protein coding RNA (10). Expression of *GSTP1, LYZ, CXCL5, IL8*, and *DKK1* was confirmed to be highly expressed in CD44-positive HuH7 cells (Figure 1).

Kaplan–Meier analysis of association of CSC-related genes with overall and cancer-specific survival. The association of expression of *LYZ, CXCR5, DKK1, IL8* and *GSTP1* with overall survival was examined. Both overall survival (p=0.016, Figure 2A) and cancer specific-survival (p=0.002, Figure 2B) were significantly associated with *DKK1* mRNA, but not with that for other genes.

Univariate and multivariate analyses of clinical factors and CSC-related genes for overall and cancer-specific survival. Overall survival was significantly associated with AFP; albumin; ALP; tumor size; invasion of serosa, portal vein and bile duct; tumor stage; and *DKK1* mRNA by univariate analysis, and was associated with albumin, ALP, serosal invasion and stage by multivariate analysis (Table IV). Cancer-specific survival was significantly associated with hepatitis B surface antigen (HBs-Ag), hepatitis C virus antibody (HCV-Ab); AFP; ALP; tumor number; tumor size; invasion of capsule, serosa, portal vein, bile duct and hepatic vein; stage; and *DKK1* mRNA by univariate analysis, and was associated with ALP, portal vein invasion and *DKK1* mRNA by multivariate analysis.

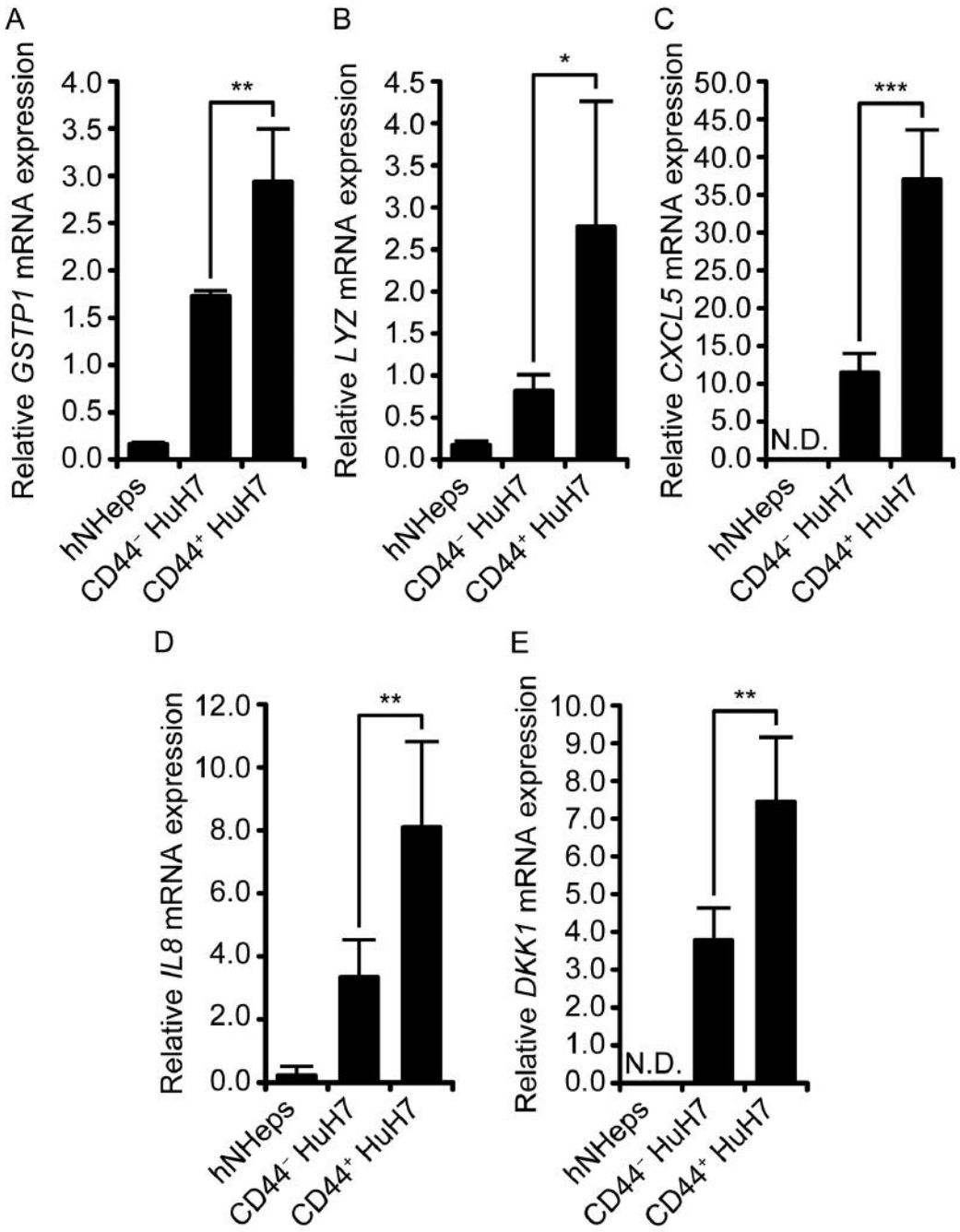

Figure 1. *Gene expression analysis in hNHeps, CD44- HuH7, and CD44⁺ HuH7 cells. Relative expression levels of glutathione S-transferase pi 1 (GSTP1) (A), lysozyme (LYZ) (B), C-X-C motif chemokine ligand 5 (CXCL5) (C), interleukin-8 (IL8) (D), and dickkopf WNT signaling pathway inhibitor 1 (DKK1) (E) mRNA in hNHeps, CD44⁻ HuH7 cells, and CD44⁺ HuH7 cells. mRNA expression levels were normalized by actin beta (ACTB) expression. Data are indicated as means±SD (n=5). Student's t-test was used to determine the statistical significance. Significantly different at *p<0.05, **p<0.01 and ***p<0.001.*

Association of DKK1 mRNA with clinicopathological variables. Expression of *DKK1* mRNA was negatively associated with albumin, but was positively associated with AFP, *GSTP1* mRNA, *CXCL5* mRNA, and *IL8* mRNA (Figure 3A). Expression of *DKK1* mRNA was higher in patients with HBsAg-positive, stage III/IV, or capsular invasion-positive tumor than HBsAg-negative, stage I/II or capsular invasion-negative, respectively (Figure 3B).

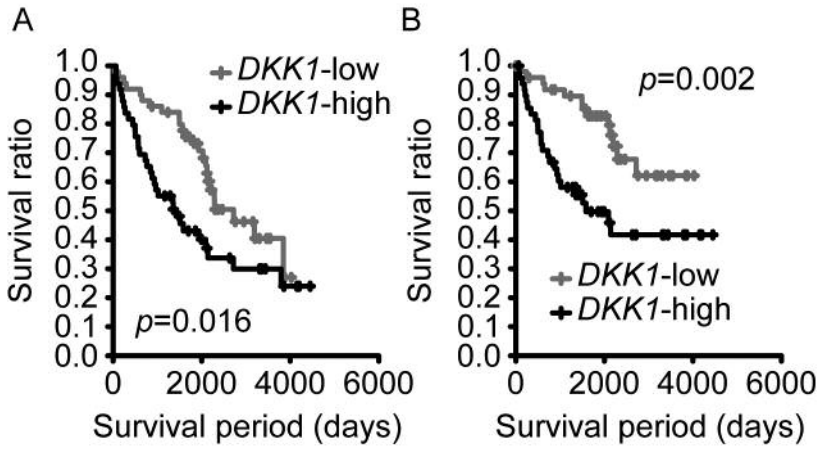

Figure 2. *Analysis of the association between the expression of cancer stem cell-related mRNAs and prognosis of patients with hepatocellular carcinoma (HCC). Overall (A) and cancer-specific (B) survival were assessed by Kaplan–Meier analysis in 99 patients with HCC. Patients were classified into positive and negative groups according to the median expression of dickkopf WNT signaling pathway inhibitor 1 (DKK1) mRNA. The log-rank test was performed to determine the statistical significance of differences in survival.*

Discussion

In the present study, we showed that *DKK1* mRNA is significantly associated with cancer-specific survival in patients with HCC. A meta-analysis of prognostic significance in solid tumors reported that DKK1 overexpression predicted poor overall survival in HCC, ovarian cancer, and other cancer types (11-15). On the other hand, serum DKK1 was reported to be a biomarker useful for diagnosis of HCC by a large-scale and multicenter study (16). These data suggest that DKK1 plays an important role in HCC from viewpoints of biology and clinical settings.

DKK gene family comprise an evolutionary conserved small genes (17). DKK genes encode secreted proteins that antagonize WNT/β-catenin signaling by binding to the WNT co-receptors LRP5 and -6. The human *DKK* family consists of five members, *DKK1, DKK2, DKK3, DKK4*, and a unique *DKK3*-related gene, *DKKL1* (18). Dysregulation of WNT signal activation is thought to play a causative role in several types of cancer and is involved in the acquisition of stem cell-like properties of CSCs. DKK1 is an antagonist of the WNT signaling pathway, and plays crucial roles in tumor growth and progression. DKK1 levels are elevated in a wide variety of cancer types including HCC (11-15), and breast (19), colorectal (20), and pancreatic (21) cancer. Although how DKK1 regulates WNT signaling remains to be solved, one important fact is that DKK1 is a downstream target of WNT signaling, allowing for a negative feedback loop (18). Indeed, activation of canonical WNT signaling causes an increase in *DKK1* mRNA and protein (22). These data suggest that DKK1 overexpression is a result of WNT/β-catenin pathway expression.

In the present study, by employing the previously developed databases of cDNA microarray in CSCs population, we identified *DKK1* mRNA expression as a prognostic marker. Since liver CSCs have been reported to be associated with increased chemo/radioresistance, earlier recurrence after surgical or locoregional treatment, increased invasiveness, metastasis, and poor prognosis (23), it is clinically useful to identify novel prognostic genes which are highly expressed in liver CSCs.

In conclusion, by employing our previously developed databases of cDNA microarray in CSCs, we identified *DKK1* mRNA expression in HCC tissues as representing cancer-specific survival of patients with HCC. Therefore it is expected to serve as a novel prognostic marker in HCC.

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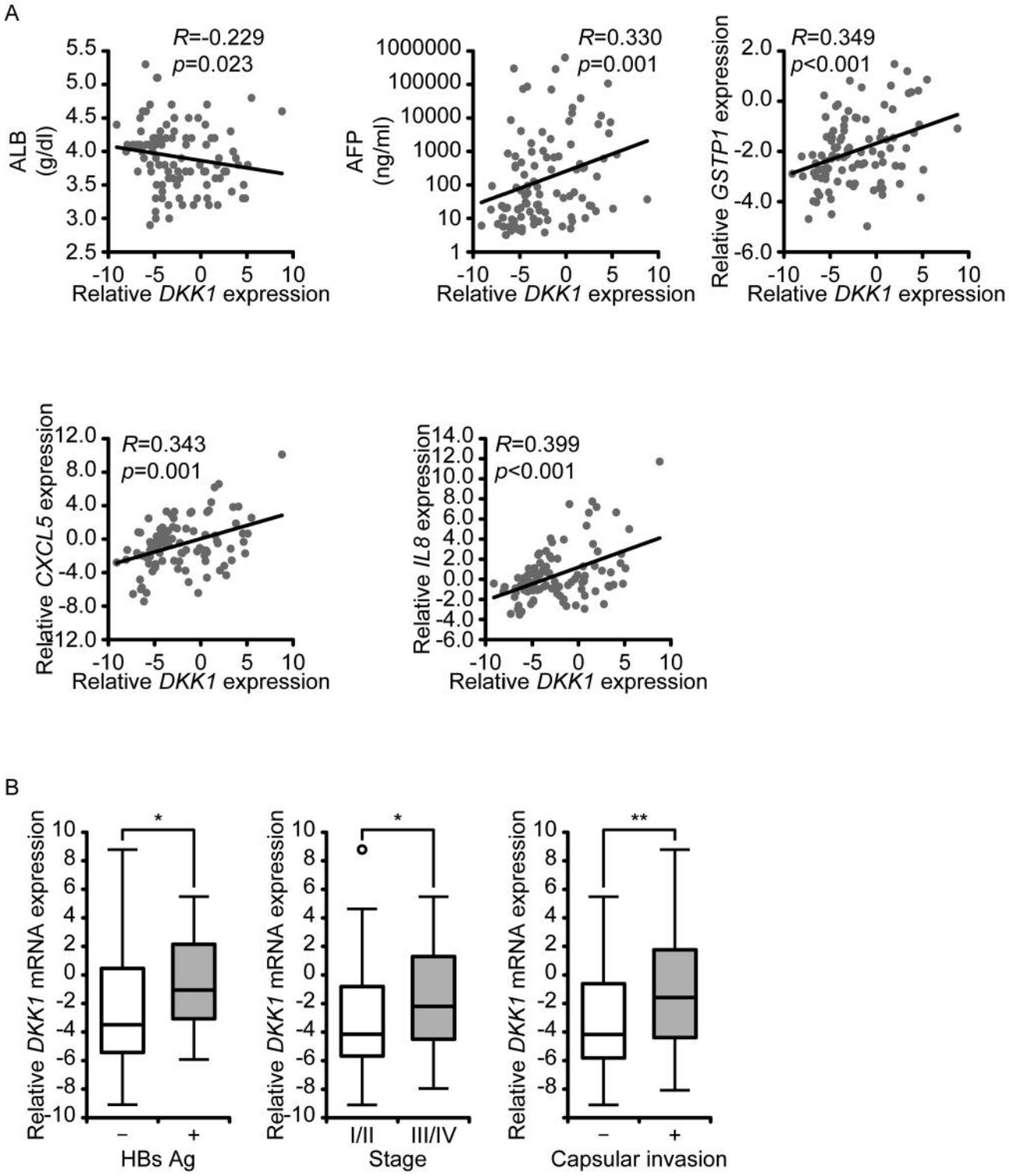

Figure 3. *Comparison of the relationships between dickkopf WNT signaling pathway inhibitor 1 (DKK1) expression and clinicopathological parameters. A: Scatter diagrams showing the results of Spearman's rank correlation analysis between DKK1 expression and albumin (ALB), alpha-fetoprotein (AFP), and mRNA expression of cancer stem cell-related GSTP1, CXCL5 and IL8. mRNA expression levels were normalized by actin beta (ACTB) expression and converted into log2 values. (B) Relative expression level of DKK1 mRNA was analyzed in HCC patients classified into two groups based on their clinicopathological variables. DKK1 expression level was normalized by ACTB expression and converted into log2 values. Mann-Whitney U-test was performed to determine the statistical significance: significantly different at *p<0.05 and **p<0.01.*

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