Translational implication of Kallmann syndrome-1 gene expression in hepatocellular carcinoma

YURI TANAKA[1], MITSURO KANDA[1], HIROYUKI SUGIMOTO[1], DAI SHIMIZU[1], SATOSHI SUEOKA[1], HIDEKI TAKAMI[1], KAZUHIRO EZAKA[1], RYOJI HASHIMOTO[1], YUKIYASU OKAMURA[2], NAOKI IWATA[1], CHIE TANAKA[1], SUGURU YAMADA[1], TSUTOMU FUJII[1], GORO NAKAYAMA[1], MASAHIKO KOIKE[1], SHUJI NOMOTO[3], MICHITAKA FUJIWARA[1] and YASUHIRO KODERA[1]

[1]Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, Nagoya 466-8550; [2]Department of Hepato-Biliary-Pancreatic Surgery, Shizuoka Cancer Center, Shunto, Shizuoka 411-8777; [3]Department of Surgery, Aichi-Gakuin University School of Dentistry, Chikusa-ku, Nagoya 464-8651, Japan

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Abstract. Accumulation of epigenetic alterations causes inactivation of tumor suppressors and contributes to the initiation and progression of hepatocellular carcinoma (HCC). Identification of methylated genes is necessary to improve our understanding of the pathogenesis of HCC and develop novel biomarkers and therapeutic targets. The Kallmann syndrome-1 (KAL1) gene encodes an extracellular matrix-related protein with diverse oncological functions. However, the function of KAL1 in HCC has not been examined. We investigated the methylation status of the KAL1 promoter region in HCC cell lines, and evaluated KAL1 mRNA levels and those of genes encoding potential interacting cell adhesion factors. KAL1 mRNA expression level was heterogeneous in nine HCC cell lines, and reactivation of KAL1 mRNA expression was observed in cells with promoter hypermethylation of KAL1 gene after demethylation. In addition, KAL1 mRNA levels inversely correlated with those of ezrin in all nine HCC cell lines. KAL1 expression levels in 144 pairs of surgically-resected tissues were determined and correlated to clinicopathological parameters. KAL1 mRNA level was independent of the background liver status, whereas HCC tissues showed significantly lower KAL1 mRNA levels than corresponding noncancerous liver tissues. Downregulation of KAL1 mRNA in HCC was significantly associated with malignant phenotype characteristics, including elevated tumor markers, larger tumor size, vascular invasion, and hypermethylation of KAL1. Patients with downregulation of KAL1 were more likely to have a shorter overall survival than other patients, and multivariate analysis identified downregulation of KAL1 as an independent prognostic factor (hazard ratio 2.04, 95% confidence interval 1.11-3.90, P=0.022). Our results indicated that KAL1 may act as a putative tumor suppressor in HCC and is inactivated by promoter hypermethylation. KAL1 may serve as a biomarker of malignant phenotype of HCC.

Correspondence to: Dr Mitsuro Kanda, Department of Gastroenterological Surgery (Surgery II), Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan
E-mail: m-kanda@med.nagoya-u.ac.jp

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Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and the third most common cause of cancer-related death worldwide (1,2). Development of HCC is considered as a discriminative event because it occurs in chronically damaged tissue due to chronic hepatitis and liver cirrhosis, whereas other common malignancies develop on otherwise healthy tissue (3-5). Because of the accumulated genome instability and numerous epigenetic alterations induced by the microenvironment of the background liver, HCC is a more heterogeneous disease (3).

Aberrant DNA methylation is one of the most common epigenetic alterations in malignancies and is specific to individual organs and diseases (6-8). Furthermore, several studies have shown that aberrant DNA methylation contributes to the initiation and progression of malignant tumors through inactivation of tumor suppressors (9,10). Therefore, identification of novel methylated genes is important for the development of both diagnostic markers and therapeutic targets, such as demethylation agents.

Kallmann syndrome-1 gene (KAL1), also named anosmin-1, encodes an extracellular matrix (ECM) related protein with a role in cellular adhesion. KAL1 contains a WAP domain and three FnIII domains, and promotes the migration of gonadotropin-releasing hormone neurons from the olfactory

placode to the hypothalamus during development (11-13). KAL1 also induces neurite outgrowth and cell migration through fibroblast growth factor receptor 1 (FGFR1) pathways (14,15). Studies have demonstrated that ECM proteins play a vital role in proliferation and invasion of tumor cells (16). However, to date, conflicting results have been reported regarding the oncological role of KAL1. Decreased KAL1 expression is observed in colon, lung, and ovarian cancers compared with corresponding adjacent normal tissues (17). Conversely, KAL1 overexpression promotes brain tumor malignancy through integrin signal pathways and facilitates colon cancer cell migration and anti-apoptotic capacity (15). These studies indicate that KAL1 exhibits diverse functions in cancer initiation and progression. To the best of our knowledge, there have been no studies of expression analysis of KAL1 in HCC. Moreover, although loss-of-function mutations of the KAL1 gene have been known to underlie Kallmann syndrome (18,19), the significance of the methylation status of the KAL1 gene has yet to be determined.

In our previous microarray project exploring HCC-related tumor suppressors, we found that KAL1 was downregulated in HCC tissues (Log2 ratio: -2.1) (16,20-22). Accordingly, we hypothesized that KAL1 might act as a putative tumor suppressor and mediate tumorigenesis of HCC. To systematically address this idea, we examined the expression and methylation status of KAL1 in HCC.

Materials and methods

Sample collection. We purchased nine HCC cell lines from the American Type Culture Collection (Manassas, VA, USA) and cultured cells as previously described (23). Primary HCC and adjacent liver tissues were collected from 144 patients who underwent hepatectomy for HCC at Nagoya University Hospital between January 1998 and January 2012. The ages of the 144 patients ranged from 34 to 84 years (median, 65.5 years), and the male-to-female ratio was 121:23. The median duration of patient follow-up was 40.1 months (range, 2.3-145 months). Thirty-seven were infected with hepatitis B and 80 patients were infected with hepatitis C virus. Ten patients had normal liver, 82 patients had chronic hepatitis, and 52 patients showed cirrhosis. Ninety, 37, and 17 patients were in stages I, II, and III, respectively.

Tissue samples were frozen immediately after resection and stored at -80˚C until use. Genomic DNA and total RNA was extracted from both HCC and adjacent noncancerous tissues approximately 5 mm^2 in diameter, avoiding necrotic areas. Specimens were classified histologically according to the 7th edition of the Union for International Cancer Control (24). Written informed consent for the use of clinical samples and data was obtained from all enrolled patients as required by the Institutional Review Board of Nagoya University, Japan.

Analysis of the KAL1 promoter region. Nucleotide sequencing was used to determine the presence of CpG islands in the KAL1 promoter region, defined as follows: ≥200 bp region with GC content >50% and an observed CpG/expected CpG ratio ≥0.6 (25). CpG Island Searcher software (http://cpgislands.usc.edu/) was used to determine the locations of CpG islands (26).

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR). KAL1 mRNA levels were determined using qRT-PCR. Total RNA (10 μg) was isolated from nine HCC cell lines, 144 primary HCCs, and adjacent noncancerous tissues and used as a template for complementary DNA synthesis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (TaqMan, GAPDH control reagents, Applied Biosystems, Foster City, CA, USA) was quantified in each sample for standardization. Specific primers and annealing temperatures are listed in Table I. qRT-PCR was performed using the SYBR Green PCR Core Reagents kit (Applied Biosystems) as follows: one cycle at 95˚C for 10 min, 40 cycles at 95˚C for 5 sec, and 60˚C for 60 sec. Real-time detection of SYBR Green fluorescence was conducted using an ABI StepOnePlus Real-Time PCR System (Applied Biosystems). All samples were analyzed in triplicate. The expression level of each sample is shown as the value of the KAL1 amplicon divided by that of GAPDH (27).

Methylation-specific PCR (MSP) and bisulfite sequence analysis. Genomic DNA samples from nine HCC cell lines and 144 HCC tissues were subjected to bisulfite treatment. MSP was conducted to determine the presence or absence of promoter hypermethylation of KAL1 gene. Bisulfite DNA from HCC cell lines was sequenced to determine the reliability of MSP results. Primer sequences are shown in Table I.

5-Aza-2'-deoxycytidine (5-aza-dC) treatment. To assess the relation of promoter hypermethylation to KAL1 transcription, HCC cell lines were treated with the DNA methylation inhibitor 5-aza-dC (Sigma-Aldrich, St. Louis, MO, USA) as previously described (10,28).

Expression of genes that encode cell adhesion factors. To identify cell adhesion proteins that may interact with KAL1, expression levels of Ezrin (EZR), focal adhesion kinase (FAK), cellular SRC (SRC) and dihydropyrimidinase-like 3 (DPYSL3) genes were determined by qRT-PCR in HCC cell lines (29,30). Primers specific for EZR, FAK, SRC and DPYSL3 are listed in Table I.

Immunohistochemical (IHC) staining. KAL1 protein localization was determined by IHC using 64 representative formalin-fixed and paraffin-embedded sections of well-preserved HCC tissue using a rabbit polyclonal antibody against KAL1 (ABN486, Millipore, Darmstadt, Germany) diluted 1:150 in antibody diluent (Dako, Glostrup, Denmark) as previously described (7,31). Samples were then washed with phosphate-buffered saline, followed by 10 min incubation with a biotinylated secondary antibody (Histofine SAB-PO(R), Nichirei, Tokyo, Japan). Sections were subsequently developed for 3 min using 3,3'-diaminobenzidine as substrate (Nichirei) and analyzed. To avoid bias, specimens were randomized, coded, and then analyzed by two independent observers who were uninformed of the identities of the samples.

Statistical analysis. The qualitative χ^2 test and quantitative Mann-Whitney test were used to compare two groups. Correlations between mRNA levels of KAL1 and those of EZR,

Table I. Primers and annealing temperatures.

Gene	Experiment	Type	Sequence (5'-3')	Product size (bp)	Annealing temperature (˚C)
KAL1	qRT-PCR	Forward	AACAATGGTTCCCTGGTTTG	110	60
		Reverse	TCACAAAAGCTTTGGCACTG		
	MSP	Forward	GTGCGAACGGGAGAGGC	109	68
		Reverse	GTCAACTACGAACCCGAACG		
	U-MSP	Forward	AAAACCCATAAACCAATCTCA	126	58
		Reverse	TGAATGGGAGAGGTGTTTGT		
	Bisulfite sequencing	Forward	TATTGGGAGGGAGTTTGGGA	411	66
		Reverse	TAC TCC CCA CCC TCA AAC TA		
EZR	qRT-PCR	Forward	GATAGTCGTGTTTTCGGGGA	91	60
		Reverse	CTCTGCATCCATGGTGGTAA		
FAK	qRT-PCR	Forward	GCCAAAAGGATTTCTAAACCAG	110	64
		Reverse	CCTGGTCCACTTGATCAGCTA		
SRC	qRT-PCR	Forward	CTGACCGCATGGACCGT	107	58
		Reverse	AAGCCAACCTGTCACTTGGTA		
DPYSL3	qRT-PCR	Forward	AGAAGAAGGAGGGAGGGAGC	110	60
		Reverse	CTCCCTTGATAAGGAGACGG		
GAPDH	qRT-PCR	Forward	GAAGGTGAAGGTCGGAGTC	226	60
		Probe	CAAGCTTCCCGTTCTCAGCC		
		Reverse	GAAGATGGTGATGGGATTTC		

KAL1, Kallmann syndrome 1 sequence; *EZR*, ezrin; *FAK*, focal adhesion kinase; *SRC*, cellular SRC; *DPYSL3*, Dihydropyrimidinase-like 3; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *qRT-PCR*, quantitative real-time reverse-transcription polymerase chain reaction; *MSP*, methylation specific PCR; *U-MSP*, un-methylation specific PCR.

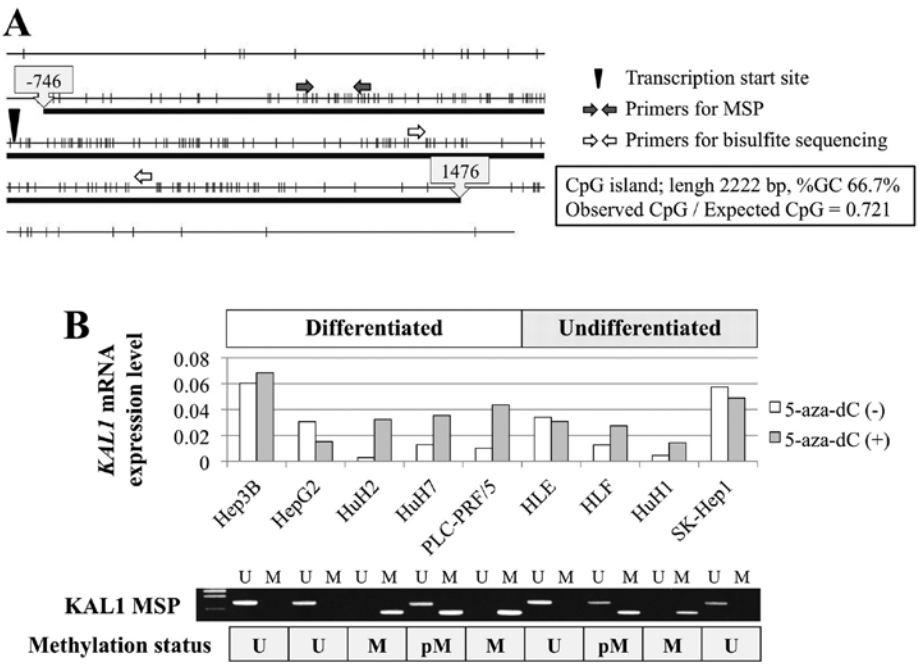


Figure 1. (A) The CpG island (indicated by the underline) is centered on the KAL1 transcription initiation site extending upstream into the promoter region. (B) Top: Bar graphs showing KAL1 mRNA expression levels in nine HCC cell lines before and after 5-aza-dC treatment. Bottom: The methylation status of KAL1 in the nine cell lines was determined by MSP. M, methylated; pM, partially methylated; U, unmethylated.

FAK, SRC, or DPYSL3 as well as tumor size and preoperative serum protein induced by vitamin K antagonists II (PIVKA-II) level were analyzed using the Spearman rank correlation test. Overall and disease-free survival rates were calculated using

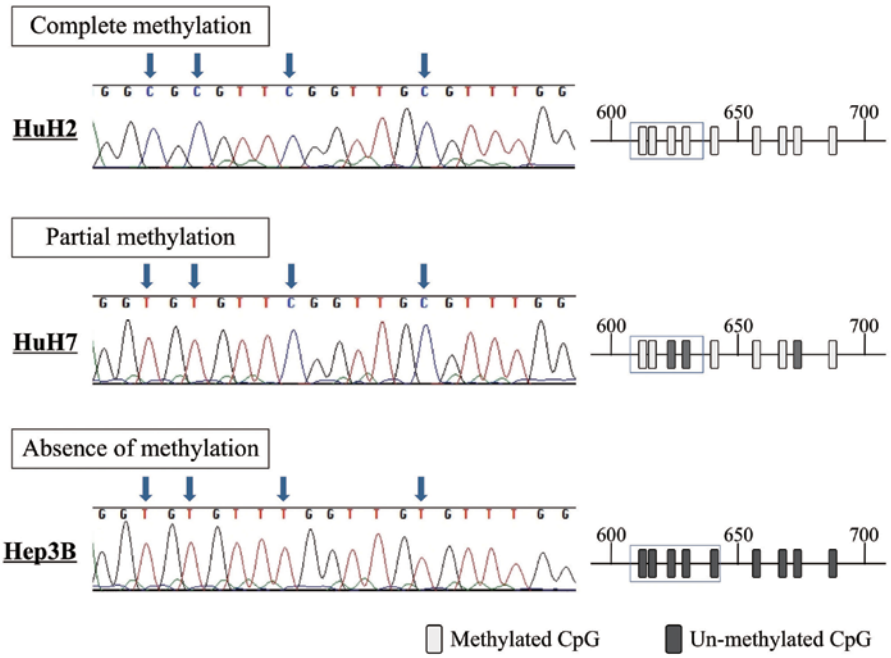


Figure 2. Representative results of bisulfite sequencing analysis. All CpG sites in HuH2 cell were retained as CG, while those of Hep3B were converted to TG. In HuH7, that was identified as a partially methylated cell by MSP, both CG and TG are shown.

the Kaplan-Meier method, and the difference in survival curves was evaluated using the log rank test. A P-value <0.05 was considered statistically significant. All statistical analysis was performed using JMP 10® software (SAS Institute Inc., Cary, NC, USA).

Results

KAL1 mRNA expression and methylation status in HCC cell lines. The KAL1 gene harbors a CpG island around the promoter region (Fig. 1A), suggesting that hypermethylation of the CpG island may regulate KAL1 transcription. KAL1 mRNA expression levels were heterogeneous among nine HCC cell lines, regardless of differentiation (Fig. 1B). MSP revealed methylation in HLF, HuH1, HuH2, HuH7 and PLC/PRF/5 cells. When comparing the levels of KAL1 mRNA in HCC cell lines before and after demethylation by 5-aza-dC treatment, reactivation of KAL1 mRNA expression was observed in cells with promoter hypermethylation of the KAL1 gene (Fig. 1B). Direct sequence analysis revealed that all CpG sites in HuH2 cells (complete methylation) were CG (cytosine and guanine), whereas the corresponding positions in Hep3B cells (absence of methylation) were TG (thymine and guanine) (Fig. 2). These results confirm the accuracy of the MSP results.

Expression analysis of KAL1 and genes encoding putative functional partners in HCC cell lines. We next evaluated the expression levels of genes encoding other cell adhesion factors that could potentially functionally interact with KAL1. The relative expression levels of EZR, FAK, SRC, DPYSL3, and KAL1 mRNAs in HCC cell lines are shown in Fig. 3A. The results showed that KAL1 mRNA levels inversely correlated with those of EZR (correlation coefficient -0.667, P=0.049; Fig. 3B).

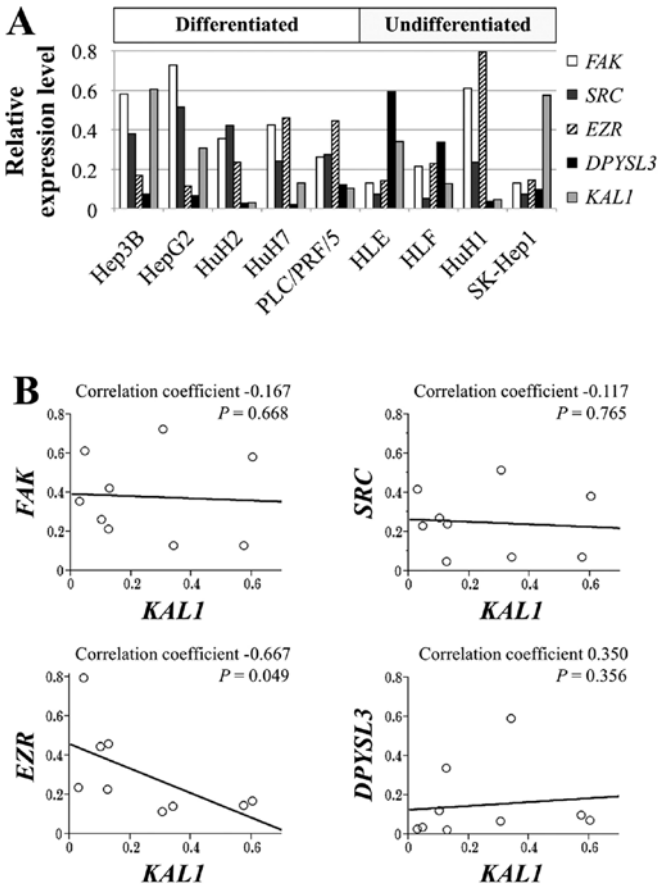


Figure 3. (A) Expression analysis of KAL1, FAK, SRC, EZR and DPYSL3 mRNA levels in nine HCC cell lines. (B) Correlations of mRNA expression levels between KAL1 and the four genes.

KAL1 status in surgically-resected tissues. We next examined KAL1 mRNA levels in 144 HCC tissues compared with the

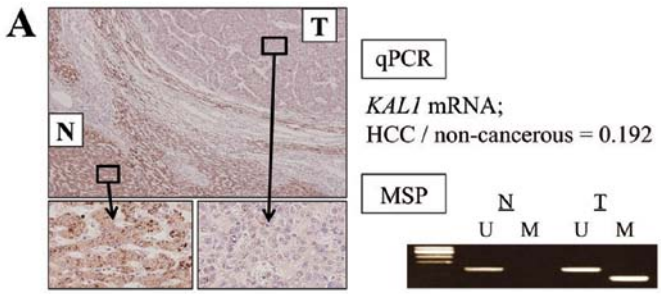
Table II. Association between expression levels of *KAL1* mRNA and clinicopathological parameters in 144 patients with hepatocellular carcinoma (HCC).

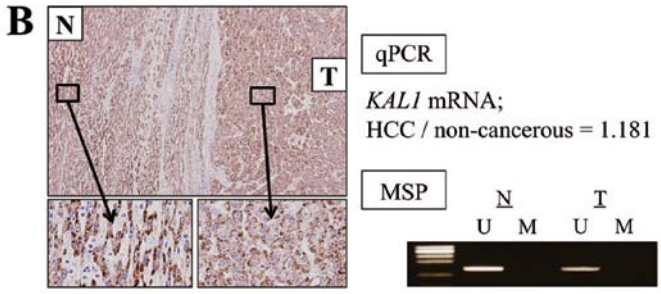
Clinicopathological parameters	Downregulation of *KAL1* mRNA in HCCs (n=62)	Others (n=82)	P-value
Age			0.312
<65 year	25	40	
≥65 year	37	42	
Gender			0.067
Male	56	65	
Female	6	17	
Background liver			0.679
Normal liver	3	7	
Chronic hepatitis	36	46	
Cirrhosis	23	29	
Pugh-Child's classification			0.075
A	55	79	
B	7	3	
Hepatitis virus			0.329
Absent	15	12	
HBV	14	23	
HCV	33	47	
AFP (ng/ml)			0.004[a]
≤20	25	53	
>20	37	29	
PIVKA II (mAU/ml)			0.002[a]
≤40	16	42	
>40	46	40	
Tumor multiplicity			0.928
Solitary	48	64	
Multiple	14	18	
Tumor size			0.004[a]
<3.0 cm	12	34	
≥3.0 cm	50	48	
Differentiation			0.015[a]
Well	9	26	
Moderate to poor	53	56	
Growth type			0.126
Expansive growth	55	65	
Invasive growth	7	17	
Serosal infiltration			0.450
Absent	45	64	
Present	17	18	
Formation of capsule			0.003[a]
Absent	12	35	
Present	50	47	
Infiltration to capsule			0.066
Absent	23	43	
Present	39	39	
Septum formation			0.370
Absent	19	31	
Present	43	51	
Vascular invasion			0.033[a]
Absent	41	67	
Present	21	15	
Hypermethylation of KAL1 in HCCs			0.019[a]
Absent	32	58	
Present	30	24	
UICC pathological stage			0.062
I	33	57	
II	22	15	
III	7	10	

HBV, hepatitis B virus; *HCV*, hepatitis C virus; *AFP*, α-fetoprotein; *PIVKA*, protein induced by vitamin K antagonists; *UICC*, Union for International Cancer Control. [a]P<0.05, statistically significant difference.

corresponding noncancerous liver tissues. Results showed that KAL1 mRNA levels were lower in HCC tissues compared with the corresponding noncancerous liver tissues in 106 (74%) of 144 patients. We next evaluated the association between expression levels of KAL1 mRNA and protein. Results of IHC, qPCR and MSP in representative patients are shown in Fig. 4A and B. One patient with reduced KAL1 mRNA levels showed reduced expression of KAL1 protein in the cytoplasm of HCC cells accompanied with promoter hypermethylation (Fig. 4A). Equivalent expression of KAL1 in cancer and normal cells was detected in a patient without downregulation of KAL1 mRNA and methylation (Fig. 4B). The expression patterns of KAL1 in 64 patients correlated significantly with those of KAL1 mRNA (P=0.023, Fig. 4C).

There were no significant differences in KAL1 mRNA levels between normal liver, chronic hepatitis, and cirrhosis in noncancerous liver tissues. In contrast, HCC tissues showed significantly decreased KAL1 mRNA levels compared with the corresponding noncancerous liver tissues (Fig. 5A). The KAL1 mRNA levels in HCCs correlated inversely with tumor size and preoperative serum PIVKA-II level (Fig. 5B). In 62 patients, KAL1 mRNA expression level in HCC was less than half of that in the corresponding noncancerous liver tissue, and these patients were categorized into the 'downregulation of KAL1' group for the following analyses. Downregulation of KAL1 was significantly associated with α-fetoprotein >20 ng/ml, PIVKA-II >40 mAU/ml, tumor size ≥3.0 cm, moderate to poor differentiation, formation of a capsule, vascular invasion, and hypermethylation of KAL1 (Table II).





C

		qPCR; HCC / normal cancerous		
		>2	0.5 ~ 2	<0.5
Staining intensity	HCC > normal	2	3	0
	Equivalent	2	14	8
	HCC < normal	1	15	19

$P = 0.023$

Figure 4. IHC, qPCR and MSP results of representative patients with reduced (A) and equivalent (B) expression of KAL1 mRNA in HCCs compared with the corresponding noncancerous tissues. (C) The pattern of KAL1 protein expression significantly correlated with that of KAL1 mRNA in HCC tissues.

Impact of KAL1 mRNA expression on patient outcome. Patients with downregulation of KAL1 were more likely to have a shorter overall survival than other patients (5-year survival rates 51% and 78%, respectively, P=0.002) (Fig. 6A). In multivariate analysis, downregulation of KAL1 was identified as an independent prognostic factor (hazard ratio 2.04, 95% confidence interval 1.11-3.90, P=0.022; Table III). Additionally, patients with downregulation of KAL1 tended to have a shorter disease-free survival compared with other patients, although it did not reach statistical significance (3-year survival rates 32% and 50%, respectively, P=0.014) (Fig. 6B).

Discussion

Impaired expression of genes encoding ECM proteins plays an important role in the initiation and progression of HCC (16,32). KAL1, one of the ECM-related proteins, has been reported to have diverse oncological functions (15,17). In the present study, the clinical significance of the expression and methylation status of KAL1 was evaluated in HCC.

Consistent with earlier studies in colon, lung and breast cancer (17), our results showed that expression levels of KAL1 were reduced in HCC tissues compared to adjacent noncancerous liver tissues. Furthermore, KAL1 expression was independent of chronic inflammation or fibrosis of the background liver, suggesting that downregulation of KAL1 is a specific event in hepatocarcinogenesis or at later stages. Loss-of-function mutations in the KAL1 gene are responsible for Kallmann syndrome, a developmental disorder characterized by the association of hypogonadotropic hypogonadism and anosmia (14,18,19). However, no studies have investigated the regulatory mechanisms of KAL1 expression in malignancies. Since a CpG island was found in the promoter region of the KAL1 gene, we focused on aberrant DNA methylation, which is an important mechanism for inactivation of tumor suppressors (33,34). Our results showed that HCC cell lines with profoundly suppressed KAL1 expression also harbored promoter hypermethylation of the KAL1 gene, and expression levels of KAL1 were restored by demethylation. Additionally, there was a significant association between downregulation of KAL1 mRNA and hypermethylation of KAL1 gene in surgically resected HCC tissues. These findings implicated that aberrant methylation is a pivotal regulatory mechanism for KAL1 expression in HCC. Promoter hypermethylation of the KAL1 gene has the potential for becoming a novel biomarker of HCC as well as a therapeutic target for specific demethylation agents (34,35).

We also investigated the levels of other important ECM-related proteins, and found that the expression level of KAL1 had a significant inverse association with EZR expression. EZR is a cytoplasmic peripheral membrane protein that functions as a substrate of protein tyrosine kinases, regulates cellular survival, adhesion, migration, and invasion. Importantly, EZR is also one of the key factors involved in tumor progression and metastasis in HCC (36-39). Our finding supports the notion that KAL1 may function through tumor suppressor mechanisms and led us to speculate that KAL1 may interact with EZR and mediate tumorigenesis of HCC.

The significant correlation between the IHC and qRT-PCR data allowed us to evaluate the prognostic significance of KAL1 mRNA levels in a quantitative manner. Downregulation of KAL1 mRNA in HCC was significantly associated with factors reflecting the malignant potential of HCC and consequently deteriorated patient outcomes after curative hepatectomy. In contrast to the previous study showing that KAL1 overexpression promotes brain tumor malignancy (15), our results instead support a tumor suppressive role for KAL1 in HCC.

KAL1 was first identified through its function in the development of gonadotropin-releasing hormone neurons. Previous studies demonstrated that the expression of KAL1 is modulated by FGFR1 and hypoxia inducible factor-1α (HIF-1α) (11,14). FGFR-1 expression was reported as low in normal liver epithelium and high in human liver cancer epithelium (40,41). FGFR-1 protein may be important in regulating cytoskeletal dynamics and function in cancer cell invasion and metastatic behavior (42). HIF-1α is an important transcription factor in essential adaptive responses to hypoxia, and plays a major role in the development of characteristic tumor phenotypes, including growth rate, angiogenesis, invasiveness, and metastasis, via activation of target genes by binding to hypoxia-responsive elements in the gene regulatory sequences (43-45). The interactions with these major oncogenic pathways might provide a

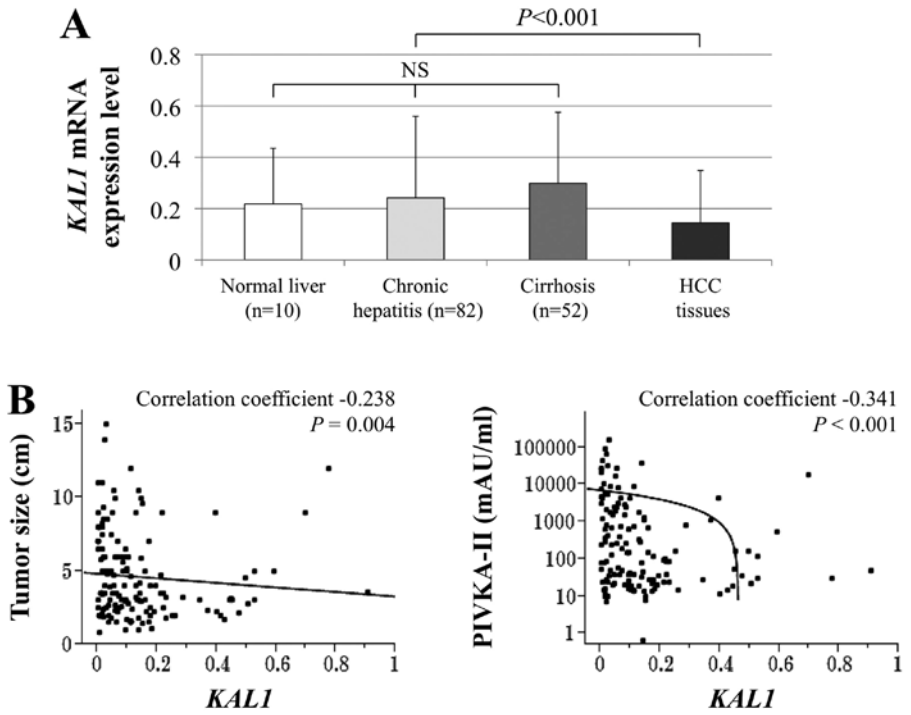


Figure 5. (A) Comparison of KAL1 mRNA levels in normal, chronic hepatitis, cirrhotic, and HCC tissues. (B) Expression levels of KAL1 mRNA were significantly correlated with tumor size and preoperative PIVKA-II levels.

Table III. Prognostic factors of 144 patients with hepatocellular carcinoma (HCC) for overall survival.

		Univariate			Multivariate		
Variable	n	Hazard ratio	95% CI	P-value	Hazard ratio	95% CI	P-value
Age (≥65)	79	1.75	0.96-3.30	0.068			
Gender (male)	121	1.82	0.78-5.29	0.178			
Background liver (cirrhosis)	52	1.53	0.84-2.75	0.161			
Pugh-Child's classification (B)	10	1.68	0.50-4.19	0.360			
AFP (>20 ng/ml)	66	1.96	1.09-3.58	0.024[a]	1.49	0.81-2.78	0.196
PIVKA II (>40 mAU/ml)	86	1.90	1.03-3.71	0.041[a]	1.04	0.50-2.06	0.909
Tumor multiplicity (multiple)	32	1.83	0.94-3.38	0.073			
Tumor size (≥3.0 cm)	98	2.84	1.38-6.64	0.004[a]	1.95	0.88-4.86	0.103
Tumor differentiation (well)	35	0.72	0.34-1.41	0.349			
Growth type (invasive growth)	24	1.71	0.84-3.26	0.136			
Serosal infiltration	35	2.23	1.16-4.11	0.017[a]	1.70	0.87-3.18	0.115
Formation of capsule	97	0.95	0.52-1.81	0.861			
Infiltration to capsule	78	1.24	0.69-2.29	0.478			
Septum formation	94	0.77	0.43-1.43	0.402			
Vascular invasion	36	3.75	2.05-6.78	<0.001[a]	2.48	1.30-4.71	0.006[a]
Hypermethylation of KAL1 in HCCs	54	1.23	0.67-2.33	0.511			
Downregulation of *KAL1* mRNA	62	2.53	1.40-4.73	0.002[a]	2.04	1.11-3.90	0.022[a]

CI, confidence interval; *AFP*, a-fetoprotein; *PIVKA*, protein induced by vitamin K antagonists. Univariate analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model. [a]P<0.05, statistically significant.

mechanism(s) underlying the correlation between KAL1 expression and malignant phenotype of HCC. Future studies, including pathway analysis in hepatocarcinogenesis, hypoxic stress and functional analysis, are required to elucidate the molecular mechanisms that underlie the biological function of KAL1 in HCC.

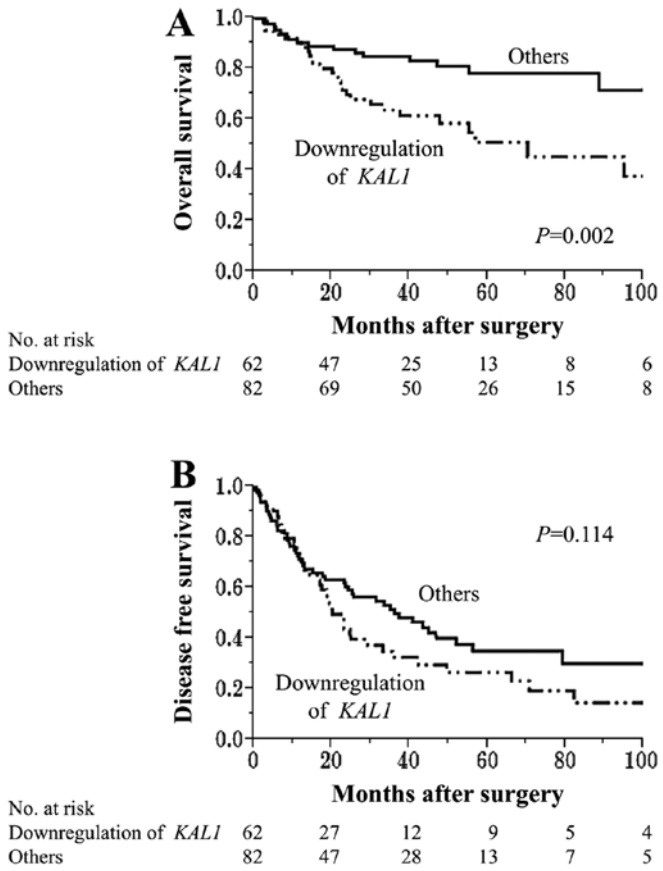


Figure 6. Prognostic impact of downregulation of KAL1. (A) Patients with downregulation of KAL1 mRNA experienced significantly shorter overall survival compared with other patients. (B) Disease-free survival tended to be shorter in patients with downregulation of KAL1 mRNA compared with other patients.

Taken together, our results indicate that KAL1 acts as a putative tumor suppressor in HCC that is inactivated by promoter hypermethylation. Our findings suggest that KAL1 may serve as a promising biomarker of malignant phenotype of HCC.

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