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Cell division cycle 34 is highly expressed in hepatitis C virus-positive hepatocellular carcinoma with favorable phenotypes

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Abstract. Despite tremendous efforts to develop curative agents, there are few effective drugs for the treatment of hepatocellular carcinoma (HCC). This is predominantly due to the variations in individual HCC cases. As numerous HCC cases have no mutations in known tumor-associated genes, identification of novel genes involved in the development and progression of human cancers is considered to be an urgent issue. In the present study, surgical specimens of HCC were analyzed for the expression patterns of ubiquitin-conjugating enzyme, cell division cycle 34 (CDC34), which is hypomethylated in its promoter region and exhibits elevated expression levels in mouse skin tumors. The results of the current study clearly indicated that the elevated CDC34 expression level in cancerous regions was significantly associated with favorable clinicopathological features, such as reduced alanine aminotransferase (ALT) levels and histological grades. Similarly, a higher T/N ratio, which is the ratio of CDC34 expression in HCCs to that in non-tumorous tissues, was significantly associated with favorable features, such as a lower indocyanin green retention rate after 15 min (ICG15R), reduced α-fetoprotein and smaller tumor size. These results indicate that the CDC34 expression level in HCC is a marker for predicting the HCC prognosis and that CDC34 acts as a tumor suppressor.

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Introduction

Hepatocellular carcinoma (HCC) is one of the most common types of cancer in the world and is the second most common cause of cancer-associated mortality (1). More than 70% of HCC cases develop from a background of chronic liver disease, such as chronic hepatitis or liver cirrhosis, caused by viral infection or alcohol abuse (2,3). In Japan, the hepatitis C virus (HCV) is the most common cause of HCC. Although the incidence of non-B non-C-associated HCC is increasing, ~60% of HCC cases are associated with HCV (4).

For early-stage HCC with a limited number of cancerous lesions, smaller size, and without vascular invasion, liver resection is the best treatment; however, the cumulative 5-year recurrence rate remained as high as ~80% following curative hepatectomy (5,6). Chemotherapy is generally applied in advanced cases with multiple cancerous and/or metastatic lesions; however, only a limited number of molecular targeting drugs, such as sorafenib, have demonstrated significant effects during clinical testing (7,8).

It has been reported that the HCV infection induces DNA damage via oxidative stress produced by chronic inflammation. HCV core protein also increases reactive oxygen species by inducing altered mitochondrial function (9,10). The DNA damage generated by these processes is hypothesized to cause mutations in tumor-suppressor genes and oncogenes observed in HCC (11-13). These mutations are thought to be important in the development of HCC; however, there are many HCC cases without mutations in known tumor-associated genes. In addition, a previous study demonstrated that the HCV core protein induces cell transformation directly (14) and this may be mediated by increased c-MYC protooncogene expression (15). This pathogenetic complexity makes it difficult to understand the mechanism of HCC development following HCV infection.

To determine the molecular mechanism underlying HCC development and progression, the current study attempted to identify novel cancer-associated genes in a mouse model of skin cancer. By screening for aberrantly methylated genome regions or genes showing aberrant expression levels in mouse squamous cell carcinoma (SCC) tissues, novel genes involved in

the development and progression of human cancers, including HCC were identified. For example, zygote arrest 1 (Zar1) was aberrantly methylated in mouse SCC genomic DNA and its human ortholog, Zar1, was shown to be aberrantly methylated in the genome of human HCC (16).

Previously, the ubiquitin-conjugating enzyme, CDC34, was found to be highly expressed and hypomethylated in SCC tissue samples (17). In the ubiquitin-proteasome system (UPS), E2 enzymes, including CDC34, conjugate ubiquitin (which is activated by E1 enzymes) onto target molecules in conjunction with E3 enzymes (18). As UPS regulates degradation of various types of protein, it is crucial in a variety of cellular processes. UPS aberrations have been observed in numerous types of human tumor (19,20). However, limited information is available regarding aberrations of CDC34 in HCC. To determine the role of CDC34 in the development or progression of HCC, the current study investigated the expression patterns of CDC34 in human HCC surgical specimens.

Materials and methods

Patients and tissue samples. Fifty-eight HCV-positive HCCs [classified as grade I or II according to the Union for International Cancer Control TNM classification (21)] and paired non-tumorous tissue samples were obtained from the Nihon University Hospital (Tokyo, Japan). These tissues and data were collected from August 1995 to April 2010 after obtaining written informed consent from each subject. The present study complied with the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of Nihon University School of Medicine (approval no. 214-0). All surgical specimens were frozen in liquid nitrogen immediately following resection and stored at -80˚C.

RNA extraction. RNA was extracted from HCC and non-tumorous tissue samples using Invitrogen TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. All samples showed RNA integrity ≥7.0, as determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the tissue samples obtained from the HCV-positive patients. Aliquots of total RNA (500 ng) were reverse-transcribed using iScript (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the generated cDNAs were used for qPCR using SYBR®Premix Ex TaqTM II (Takara Bio, Inc., Otsu, Japan). CDC34 and GAPDH expression levels served as the target and endogenous control, respectively. The amplification conditions were as follows: 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec, annealing at 60˚C for 10 sec and polymerization at 72˚C for 30 sec. To obtain standard curve for each genes, RT-qPCR was done by using a dilution series of cDNA mixture as a template. The experiments were performed in triplicate and the amounts of genes were worked out by extrapolation from the standard curves. The primer sequences were as follows: forward, 5'-CTACGAGGGCGGCTACTTC-3' and reverse, 5'-TCTTGGTCAGGAACCGAAAG-3', for CDC34; and forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGA-3' for GAPDH.

Immunofluorescence staining. Sections (3 μm) were deparaffinized in xylene, dehydrated through a graded alcohol series, washed in phosphate-buffered saline and subjected to hematoxylin-eosin staining or immunofluorescence (IF) staining. Prior to IF staining, sections were heated in 0.05% citrate buffer solution (pH 6.0) at 121˚C for 15 min, for antigen retrieval. To prevent non-specific staining, sections were incubated with 3% H_2O_2 and Protein Block (Dako; Agilent Technologies, Inc.) at room temperature for 10 min. The sections were then incubated with anti-CDC34 rabbit polyclonal primary antibody (dilution, 1:100; cat. no. HPA002382; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 4˚C overnight. For detection of primary antibodies, biotinylated anti-rabbit IgG goat secondary antibody (dilution, 1:2,000; cat. no. E0432, Dako; Agilent Technologies, Inc.) at room temperature for 30 min and streptavidin-conjugated fluorescein isothiocyanate (Dako; Agilent Technologies, Inc.) at room temperature for 10 min. All sections were examined under a fluorescence microscope (DP71 microscope with U-RFL-T fluorescence power supply unit; Olympus Corp., Tokyo, Japan).

Statistical analysis. Continuous data are expressed as the median (range). The χ^2 test was used for categorical variables. Statistical analyses were performed using JMP 10 software (SAS Institute Japan Ltd., Tokyo, Japan) and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinicopathological features of the patients. The 58 patients (48 men and 10 women) had HCV-derived HCC. The median age of the patients was 68.5 years (range 48-78 years). A total of 31 cases (53.4%) had non-liver cirrhosis (LC) background and 27 cases (46.6%) had LC background. The tumor size was ≥3 cm in 24 cases (41.3%), and >2 tumors were found in 6 cases (10.3%). The histological grade was well-differentiated (W/D) in 24 cases (41.3%) and moderately differentiated (M/D) or poorly differentiated (P/D) in 34 cases (58.6%). Details of the pathological parameters are presented in Table I.

Expression in HCV-positive HCC and non-tumorous tissue samples. To examine the role of CDC34 in HCC, the level of CDC34 mRNA expression in 58 HCV-positive HCC and non-tumorous tissue samples were examined by RT-qPCR. The expression patterns of CDC34 in HCC and non-tumorous tissue samples varied between the cases (Fig. 1A). No significant differences were identified in the expression levels of CDC34 between HCC and non-tumorous tissue samples (Fig. 1B). The representative fluorescence staining images using anti-CDC34 antibody are presented in Fig. 2. In case 37 (Fig. 2A), the strength of the staining was similar between cancerous and non-cancerous regions. By contrast, the fluorescence signal indicating CDC34 protein expression was stronger in the cancerous area than the non-cancerous area in case 39 (Fig. 2B). These staining patterns were similar to the expression patterns of CDC34 mRNA in cases 37 and 39, respectively. In the two cases, staining patterns of CDC34 were

Table I. Comparison of clinical backgrounds and expression of CDC34.

Variable	Low tumor expression (n=29)	High tumor expression (n=29)	P-value	T/N ratio <2 (n=47)	T/N ratio ≥2 (n=11)	P-value
Sex			0.16			0.33
M	26	22		40	8	
F	3	7		7	3	
Age (years)[a]	68 (48-78)	70 (50-73)	0.36	67.3 (48-78)	66.5 (50-73)	0.74
ICG15R (%)			0.10			0.03
<15	15	21		26	10	
≥15	14	8		21	1	
Alb (g/dl)			1.00			0.93
<3.5	5	5		8	2	
≥3.5	24	24		39	9	
AST (U/l)			0.37			0.91
<40	6	9		12	3	
≥40	23	20		35	8	
ALT (U/l)			0.03			0.21
< 40	7	15		16	6	
≥40	22	14		31	5	
T.Bil (mg/dl)			0.69			0.14
<1.2	25	26		39	11	
≥1.2	4	3		8	0	
AFP (ng/ml)			0.06			0.03
< 20	9	16		17	8	
≥20	20	30		30	3	
Background			0.45			0.45
non-LC	24	7		24	7	
LC	23	4		23	4	
Vascular invasion			0.60			0.96
(-)	15	17		26	6	
(+)	14	12		21	5	
Tumor size (cm)			0.11			0.02
<3	14	20		24	10	
≥3	15	9		23	1	
Tumor number			0.39			0.34
<1	25	27		43	9	
≥2	4	2		4	2	
Histological grade			0.03			0.10
W/D	8	16		17	7	
M/D, P/D	21	13		30	4	

[a]Data are provided as the medians (range). CDC34, cell division cycle 34; W/D, well-differentiated; M/D, moderately differentiated; P/D, poorly differentiated; ICG15R, indocyanin green retention rate at 15 min; Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T.Bil, total bilirubin; AFP, α-fetoprotein; LC, liver cirrhosis.

inhomogeneous; however, no obvious morphological features or other characteristics were observed in the CDC34-positive cells.

Correlation between CDC34 expression and clinicopathological features. Table I presents the correlations between CDC34 expression in the HCC tissue samples and clinicopathological features. It was clearly demonstrated that reduced ALT levels (<40 U/l) and lower histological grade (W/D) were significantly associated with higher CDC34 expression levels.

The correlations between the T/N ratio, which is the ratio of CDC34 expression in HCCs to that in non-tumorous tissues, and clinicopathological features are presented in Table I.

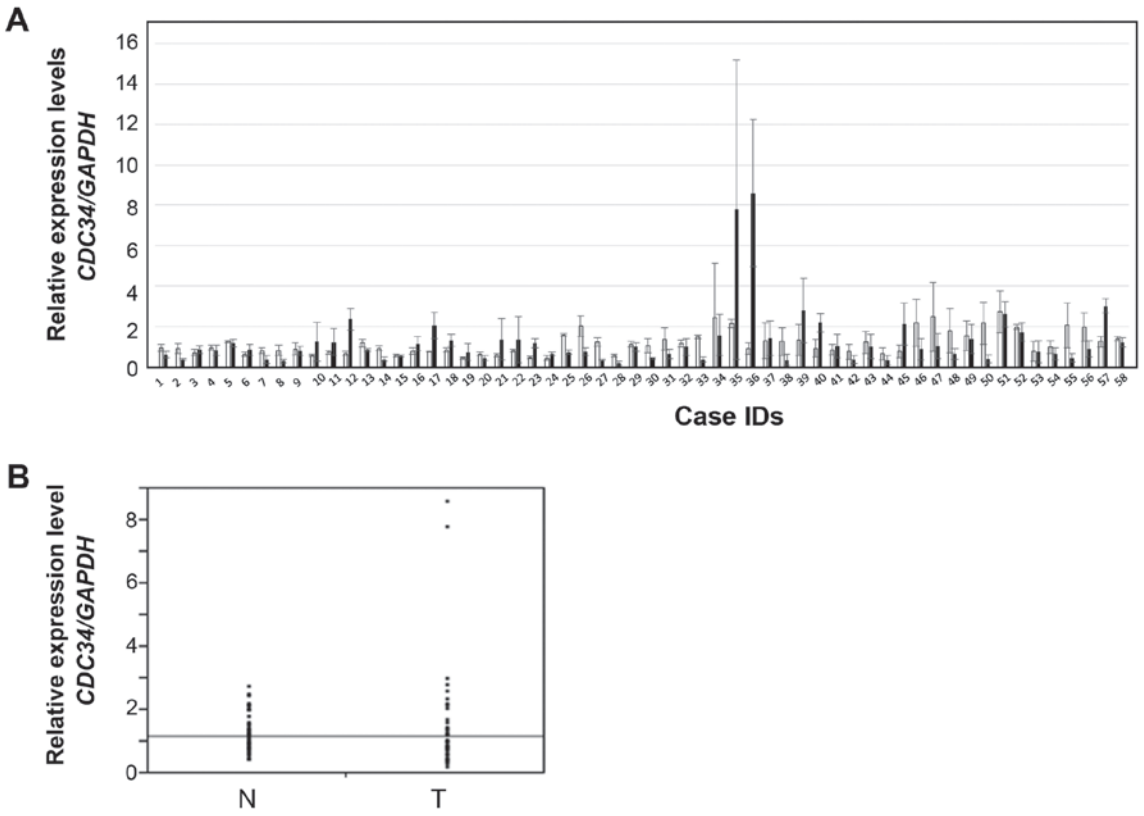

Figure 1. Analysis of CDC34 expression levels in 58 HCC and paired non-tumorous liver tissue samples. (A) Reverse transcription-quantitative polymerase chain reaction was performed to measure CDC34 expression levels in the surgical specimens. The measurements were performed three times. Data are presented as means ± standard deviation. (B) Data presented as scatter plots. No significant differences in CDC34 expression level were observed between the HCC and non-tumorous liver tissue samples. CDC34, cell division cycle 34; HCC, hepatocellular carcinoma; N, non-tumorous; T, tumorous.

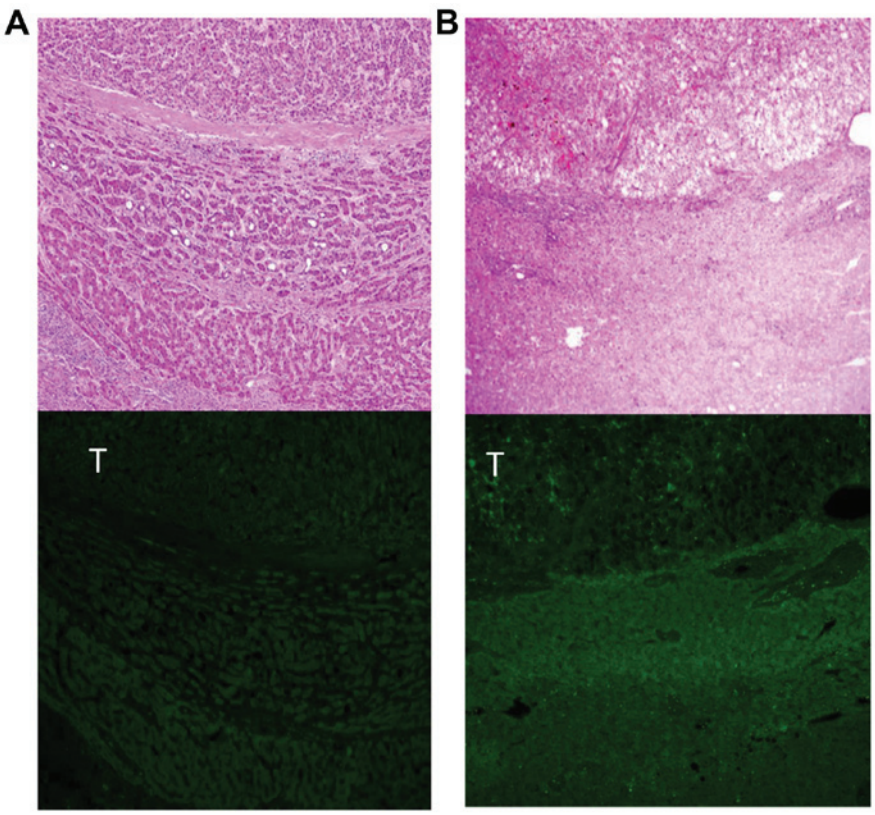

Figure 2. Immunohistochemical analysis of CDC34 in the HCC samples. Hematoxylin and eosin staining (upper) and immunostaining using the anti-CDC34 antibody (lower) were performed. Cases 37 (A) and 39 (B) are presented as representative images (magnification, x200). CDC34, cell division cycle 34; HCC, hepatocellular carcinoma; T, tumorous.

Among the 58 cases, CDC34 expression in HCC was at least 2x higher than that in the non-tumorous tissue samples in 11 cases (19.0%; Table I). In the present study, a smaller tumor size (<3.0 cm) was significantly associated with a higher T/N ratio (T/N ≥2.0; P<0.02). In addition, reduced levels of indocyanine green (ICG; <15%) and α-fetoprotein (AFP; <20 ng/ml) were associated with T/N ≥2.0 (P<0.03).

Discussion

As protein ubiquitination affects protein stability, UPS is crucial for protein catabolism in the cytosol and nucleus. As UPS regulates numerous aspects of cellular function, defects in this system may result in the pathogenesis of various significant human diseases, including tumors (19,20). Ubiquitin may be added to a substrate protein by the concerted action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3) (18).

CDC34, which encodes an E2 enzyme, was originally identified in yeast as a gene essential for G_1/S transition (22). It has been implicated in cell cycle progression by ubiquitination of cyclin-dependent kinase inhibitors, such as $P27^{kip1}$, and the G_2/M checkpoint protein, WEE1 G2 checkpoint kinase (23). In addition, CDC34 was shown to be a critical target of let-7 microRNA, which exerts tumor-suppressive functions (24). These studies indicated that CDC34 performs oncogenic or tumor-promoting functions; however, there have been studies regarding the aberrance of CDC34 in human cancer. To date, upregulated expression levels of CDC34 have been observed in acute lymphoblastic leukemia (25) and HCC (26), in which the quantity of CDC34 mRNA was shown to be significantly higher in surgically resected HCC than in non-tumorous liver tissue samples. In addition, it was demonstrated that the expression level of CDC34 mRNAs was higher in P/D tumors than in W/D and M/D tumors (26). Inconsistent with this previous study (26), the present study indicated that increased CDC34 expression levels in HCC tissue samples were associated with favorable clinicopathological features, including lower ALT levels and histological grade. Furthermore, a higher T/N ratio of CDC34 was associated with favorable features, such as smaller tumor size, lower ICG levels and lower AFP levels. Although not significant, there was a notable trend for W/D histological grade to be associated with an increased T/N ratio of CDC34. ALT is an enzyme produced predominantly by hepatocytes, and a higher blood level of ATL indicates liver damage or disease. ICG retention rate after 15 min is a useful preoperative liver function evaluation factor and serves as an effective postoperative mortality predictor. AFP, which is abundant in fetal plasma, but not in the plasma of adults (27), is a specific marker for HCC. Thus, reduced ALT, ICG and AFP levels are associated with favorable outcome.

The discrepancy between the present results and those reported previously (26) may be attributable to the differences in sample size and viral status of HCC between the two studies. The number of cases in the previous study was limited (26), and the viral status varied among the cases in the previous study. However, all tissue specimens analyzed in the present study were exclusively HCV-positive HCCs.

Numerous studies have indicated that CDC34 exerts oncogenic properties, and there have been studies to develop drugs targeting CDC34 (18); however, the present study indicated the possibility that CDC34 performs tumor-suppressive functions in HCC. This result suggests that CDC34 targets known tumor-suppressive genes, as well as oncogenes.

In conclusion, the present results indicated that increased CDC34 expression levels or T/N ratio in HCC tissue samples are associated with favorable phenotypes of HCC, such as a small tumor size, and reduced levels of ICG and AFP. These findings suggest that there may be genes that perform oncogenic functions among the CDC34 targets in HCC. Therefore, CDC34 knockdown or overexpressing cells may be useful tools to search new cancer related genes. Furthermore, CDC34 expression levels in HCC may serve as an indicator of HCC prognosis in patients.

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