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CYLD downregulation is correlated with tumor development in patients with hepatocellular carcinoma

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Abstract. The cylindromatosis (*CYLD*) gene is involved in tumor progression by acting as a negative regulator of nuclear factor-κB (NF-κB). However, the clinical significance of CYLD in patients with hepatocellular carcinoma (HCC) remains unclear. To demonstrate the clinical significance of *CYLD* expression, we analyzed *CYLD* gene expression in 124 paired HCC and non-tumor tissues using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). *CYLD* gene expression was detected in the patients and the cut-off value was determined by the median value of tumor-to-non-tumor (T/N) ratio. qRT-PCR analysis showed that a low *CYLD* expression was associated with a high serum α-fetoprotein (AFP) value. Patients in the low *CYLD* expression group exhibited poorer overall survival compared to those in the high expression group (P=0.0406). Protein expression of CYLD was also investigated in 70 patients with HCC using immunohistochemistry. The findings showed that CYLD protein expression in tumor tissue was associated with *CYLD* gene expression (P=0.031). The findings of the present study suggest that CYLD is clinically associated with tumor development in HCC patients.

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Abbreviations: AFP, α-fetoprotein; *CYLD*, the cylindromatosis gene; HCC, hepatocellular carcinoma; PIVKA-II, protein induced by vitamin K absence or antagonist-II; qRT-PCR, quantitative reverse transcription-polymerase chain reaction

Key words: cylindromatosis gene, hepatocellular carcinoma, quantitative reverse transcription-polymerase chain reaction

Introduction

Hepatocellular carcinoma (HCC) is one of the most common gastrointestinal malignancies and constitutes the leading cause of cancer-related mortality in East Asia and South Africa (1). Currently, the first-line treatment for HCC is liver transplantation or surgical resection (2). However, the overall survival rate after curative therapy is not satisfactory due to the highly chemoresistant nature of this tumor and the frequent intrahepatic recurrence. Identification of the genes responsible for the onset and progression of HCC as well as comprehension of the clinical significance of these genes are critical for the development of successful therapies.

The cylindromatosis (*CYLD*) gene was originally identified as a tumor suppressor, the mutation of which predisposes patients to the development of tumors of hair follicles (cylindromas) (3). It has been reported that CYLD acts as a negative regulator of the nuclear factor-κB (NF-κB) signaling pathway by deubiquitinating NF-κB essential modulator (NEMO), IκB kinase (IKK)-γ, and IKK upstream regulators, including the tumor necrosis factor (TNF), receptor-associated factor 2 (TRAF2), TRAF6, TRAF7 and receptor-interacting protein 1 (RIP1) (4-10). CYLD also regulates transforming growth factor-β (TGF-β) signaling via the deubiquitination of Akt in lung fibrosis (11).

Recent studies have demonstrated that *CYLD* deficiency may promote the development of several types of cancer in addition to skin tumors caused by mutations and loss of the heterozygosity (LOH) of *CYLD*. LOH of chromosome 16q, which includes the *CYLD* gene, has been detected in a large proportion of multiple myeloma cases and has been associated with poor overall survival (12-14). Comparative genomic hybridization (CGH) assays have also suggested potential genetic abnormalities of *CYLD* (reduction in copy number) in HCC, uterine carcinoma and renal cancer (15-17). Moreover, suppressed *CYLD* gene expression may contribute to tumor development in colon cancer, hepatocellular carcinoma and melanoma (18,19).

The aim of this study was to investigate the clinical importance of the *CYLD* gene by analyzing 124 consecutive patients with HCC who were treated with hepatic resection. Distribution of the CYLD protein expression was also examined using immunohistochemistry.

Materials and methods

Clinical tissue samples. Between 2005 and 2010, 124 patients (100 men and 24 women) with HCC were registered at the Department of Gastroenterological Surgery, of the Kumamoto University Hospital (Kumamoto, Japan). Specimens of primary HCC and adjacent normal liver tissues were obtained from the patients after written informed consent was obtained. This study was approved by the Human Ethics Review Committee of the Graduate School of Medical Sciences, Kumamoto University (Kumamoto, Japan).

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was obtained from the frozen tissue samples and cell lines using a mirVana™ miRNA Isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Reverse transcription was performed with 1.0 μg of total RNA as previously described (20). qRT-PCR was performed on a LightCycler 480 II (Roche Diagnostics, Tokyo, Japan) using 2X PCR Master mix (Roche Diagnostics) and Universal ProbeLibrary (Roche Diagnostics). Primers were designed using the Roche website and the Universal ProbeLibrary according to the manufacturer's instructions. The primers used were: CYLD, F: 5'-TCTATGG GGTAATCCGTTGG-3' and R: 5'-CAGCCTGCACACTCAT CTTC-3', and universal probe no. 83; and hypoxanthine phosphoribosyltransferase (HPRT), F: 5'-TGACCTTGATTTA TTTTGCATACC-3' and R: 5'-CGA GCAAGACGTTCAGT CCT-3', and universal probe no. 73. *HPRT*, *18S ribosomal RNA* (rRNA) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) were examined as the internal controls (21). *HPRT* was proved to be the most suitable reference gene. For amplification, an initial denaturation at 95˚C for 10 min was followed by 45 cycles for 15 sec at 95˚C, annealing 15 sec at 60˚C, and extension 13 sec at 72˚C. The experiments were performed twice to confirm reproducibility.

Immunohistochemistry and evaluation of CYLD. Paraffin-embedded tissue sections were dewaxed with xylene and rehydrated using graded concentrations of ethanol. The samples were then stained for CYLD using our previously described technique (22). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. The sections were incubated in 200X diluted primary rabbit anti-CYLD antibody (Sigma, Tokyo, Japan) overnight at 4˚C. A subsequent reaction was performed with a biotin-free horseradish peroxidase enzyme-labeled polymer of the EnVision Plus detection system (Dako Co., Tokyo, Japan). A positive reaction was visualized with a 3,3'-diaminobenzidine (DAB) solution, followed by counterstaining with Mayer's hematoxylin. Each immunohistochemical marker was independently evaluated by two blinded investigators. CYLD expression status in HCC cells was quantified as a percentage of the total number of stained cells detected in ≥5 random high-power fields (magnification, x400) in each section. The positivity of staining cells with 10% was determined as the cut-off value.

Statistical analysis. Statistical analysis was performed using the JMP® 8.0 software (SAS Institute., Cary, NC, USA). Values were presented as the mean ± standard deviation (SD). Differences between groups were calculated using the Wilcoxon test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of CYLD in clinical tissue specimens and their clinicopathological characteristics. We performed qRT-PCR analysis in the primary HCC specimens. CYLD expression was quantified by caluculating the ratio of *CYLD* to *HPRT1* signal. *CYLD* expression was detected in the tumor and non-tumor tissues. CYLD expression of tumor tissue was not markedly different compared to that of non-tumor liver tissue. For the clinicopathological evaluation, patients were allocated into two groups based on the median value of tumor-to-non-tumor (T/N) ratio of *CYLD* expression. Patients with a T/N ratio larger than the median T/N ratio of *CYLD* expression were allocated to the high expression group, while the remaining patients comprised the low expression group. Clinicopathological characteristics associated with the *CYLD* expression status of the 124 patients are summarized in Table I. *CYLD* expression was only correlated with the serum α-fetoprotein (AFP) value ($P=0.0093$).

Correlation between CYLD expression and prognosis. The correlation between each clinicopathological characteristic and prognosis was analyzed by univariate analyses (Table II). The data indicated that poor prognosis in HCC patients correlated with tumor a diameter of >35.5 mm ($P<0.0001$), multiple tumors ($P=0.0048$), positive vascular invasion ($P=0.0021$), the protein induced by vitamin K absence or antagonist (PIVKA)-II >108 ($P=0.0278$), and low *CYLD* expression ($P=0.0406$) (Fig. 1A). In the multivariate analysis, *CYLD* expression was not an independent factor for predicting poor prognosis (data not shown). Although *CYLD* expression was not significantly correlated with disease-free survival ($P=0.1021$) (Fig. 1B), the low *CYLD* expression group had more patients with early recurrence within 2 years (30/37 patients) compared to the high *CYLD* expression group (17/31 patients; $P=0.016$).

Expression of CYLD protein. Among 70 HCC cases, 53 (75.7%) were positive for CYLD expression. CYLD expression was heterogeneously distributed in the tumor tissue and downregulated in tumor cells. In Fig. 2A, a representative case of HCC shows that a number of tumor cells (T1) with a high CYLD expression are well-differentiated and that they demonstrate a trabecular pattern. Conversely, other tumor cells (T2) with low CYLD expression lost their cell polarity and demonstrated dense chromatin in the nucleus. Another case of HCC comprising tumor cells with dense chromatin and a small nucleus that lost CYLD expression, despite being surrounded by CYLD-expressing tumor cells with more cytoplasm and only faint chromatin in the nucleus (Fig. 2B). However, CYLD protein expression was not associated with tumor-related

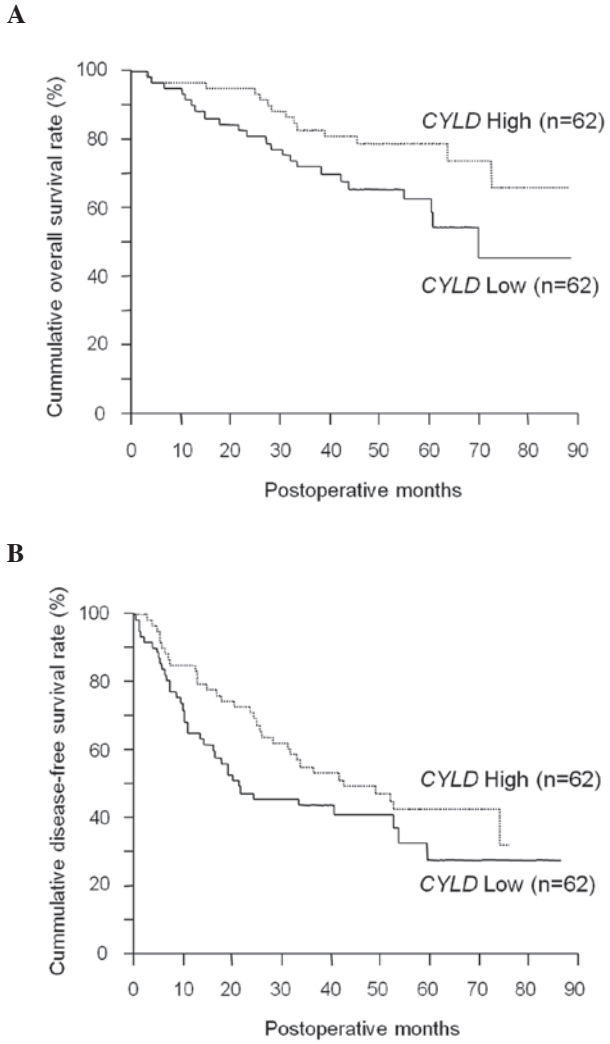

Figure 1. Clinical correlation of *CYLD* expression with HPC prognosis. qRT-PCR analysis of *CYLD* was performed in HCC patients. (A) Overall survival rates of HCC patients based on *CYLD*-mRNA expression status are shown. The overall survival rate was lower in the high compared to the low *CYLD* expression group (P=0.0406). (B) Disease-free survival rates were lower in the high compared to the low *CYLD* expression group (P=0.1021). *CYLD*, cylindromatosis gene; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; HCC, hepatocellular carcinoma.

factors, such as tumor size, tumor diameter, vascular invasion, tumor differentiation and prognosis (data not shown). To confirm the correlation of *CYLD*-mRNA expression with protein expression, *CYLD*-mRNA expression normalized by *HPRT*-mRNA expression in tumor tissue was compared between the high and low-CYLD protein expression groups. This finding showed that the high-CYLD protein expression group demonstrated a markedly higher *CYLD*-mRNA expression compared to the low-CYLD protein expression group (P=0.036) (Fig. 2C).

Discussion

In this study, we showed that reduced *CYLD*-mRNA expression is associated with a poor prognosis in HCC patients, since the incidence of early recurrence (i.e., within 2 years) was higher in the low compared to the high-*CYLD* expression group. The pattern of recurrence was similar between the

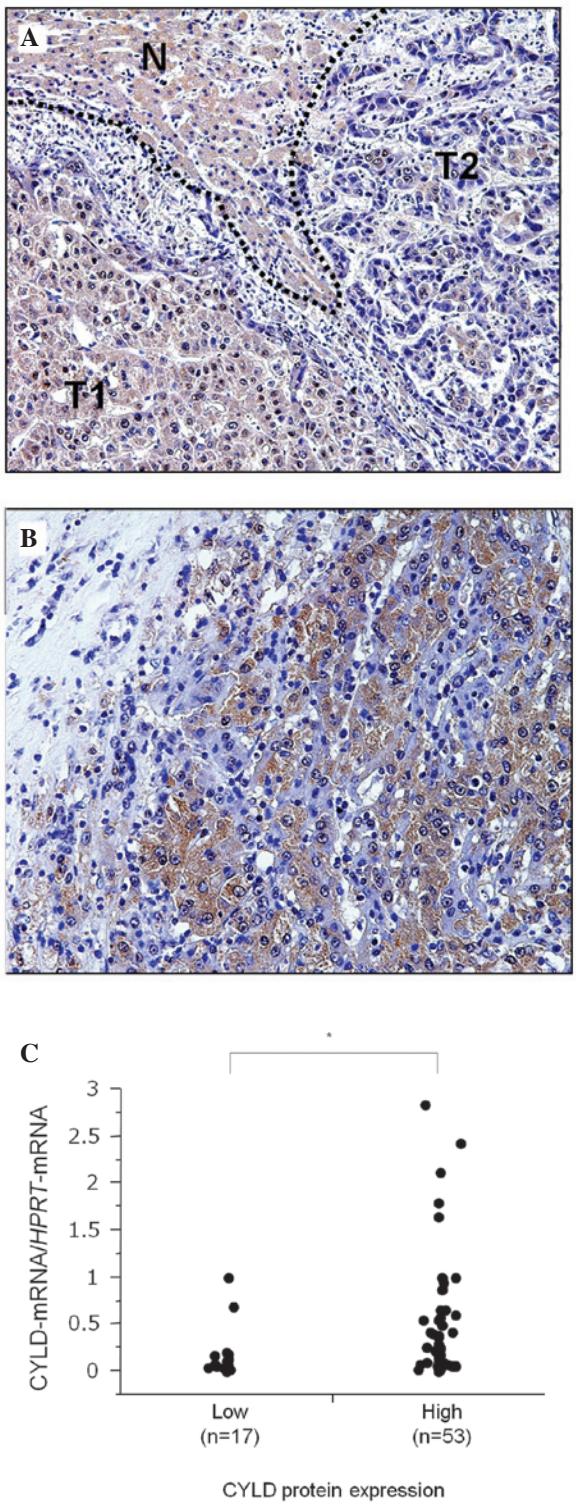

Figure 2. CYLD protein expression in human HCC. (A) Representative CYLD immunohistochemical image of an HCC tumor. Normal hepatocytes (N) and T1 tumor cells demonstrated similar expression levels of CYLD. T2 tumor cells with irregular cell polarity and dense chromatin in the nucleus showed a reduced CYLD expression. The dotted line divides normal hepatocytes and tumor cells. Magnification, x100. (B) Representative CYLD immunohistochemical image of another HCC tumor. Two morphologically distinct types of tumor cells are present that can be distinguished based on the appearance of their nucleus. Cells with high CYLD expression have a faint large nucleus, while others with low CYLD expression have a small dense nucleus. Magnification, x200. (C) The *CYLD*-mRNA level normalized to *HPRT*-mRNA in the low CYLD protein expression group was markedly lower compared to that of the high-CYLD expression group (P=0.036). *P<0.05. CYLD, cylindromatosis; HCC, hepatocellular carcinoma; *HPRT*, hypoxanthine phosphoribosyltransferase.

Table I. *CYLD*-mRNA expression and patient clinicopathological characteristics.

Clinicopathological characteristics	No. of patients	CYLD (T/N ratio)		P-value
		High	Low	
Age[a] (years)				
<66	63	30	33	0.7637
≥66	61	32	29	
Gender				
Male	100	49	51	0.4103
Female	24	13	11	
AFP[b] (U/ml)				
<15.2	68	41	27	0.0093
≥15.2	56	21	35	
PIVKA-II[a] (U/ml)				
<108	61	31	30	0.5000
≥108	69	31	32	
Tumor diameter[a] (mm)				
<35.5	62	32	30	0.4288
≥35.5	62	30	32	
No. of tumors				
Solitary	94	47	47	0.5829
Multiple	30	15	15	
Differentiation				
Well/mod	103	52	51	0.5000
Poor	21	10	11	
Vascular invasion[c]				
Negative	66	36	30	0.1345
Positive	56	24	32	
HCV-Ab				
Negative	70	38	32	0.1826
Positive	54	24	30	
HBs-Ag				
Negative	86	45	41	0.2796
Positive	38	17	21	
Liver cirrhosis[d]				
Negative	87	43	44	0.8444
Positive	37	19	18	

[a]Cut-off value defined as the median value; [b]cut-off value defined as the maximum normal value; [c]pathological vascular invasion; [d]clasified as F4 stage in the Inuyama classification (25). CYLD, cylindromatosis; T/N, tumor to non-tumor; AFP, α-fetoprotein; PIVKA-II, the protein induced by vitamin K absence or antagonist-II; well/mod, well/moderately-differentiated hepatocellular carcinoma; poor, poorly-differentiated hepatocellular carcinoma; HCV-Ab, hepatitis C virus; HBs-Ag, hepatitis B virus.

two groups. Since intrahepatic recurrence within 2 years is considered an intrahepatic metastasis from the primary tumor, this outcome suggests that *CYLD* is associated with metastatic potential and, thus, a poor prognosis. *CYLD*-mRNA expression demonstrated no correlation with tumor-related factors with the exception of serum AFP. AFP production has been strongly associated with specific molecular subtypes of HCC, such as hepatoblastoma (23), while a reduced *CYLD* expression may therefore be associated with a specific molecular phenotype.

A recent *in vivo* study demonstrated that a liver-specific conditional knockout of *CYLD* induced apoptosis in hepatocytes via the chronic activation of TGF-β-activated kinase 1 and c-Jun N-terminal kinase (JNK) in the periportal area.

Table II. Univariate analysis of clinicopathological characteristics for overall survival of patients.

Clinicopathological characteristics	No. of patients	Median survival (months)	P-value
Age[a] (years)			
<66	63	36.0	0.4168
≥66	61	21.6	
Gender			
Male	106	46.7	0.5799
Female	24	41.3	
AFP[b]			
<15.2	68	38.7	0.5008
≥15.2	56	41.1	
PIVKA-II[a]			
<108	61	42.2	0.0278
≥108	63	38.2	
Tumor diameter[a] (mm)			
<35.5	62	45.2	<0.0001
≥35.5	62	33.1	
No. of tumors			
Solitary	94	41.2	0.0048
Multiple	30	36.6	
Differentiation			
Well/mod	103	41.7	0.129
Poor	21	37.3	
Vascular invasion[c]			
Negative	66	42.7	0.0021
Positive	56	38.1	
HCV-Ab			
Negative	72	42.0	0.8255
Positive	58	44.7	
HBs-Ag			
Negative	91	44.8	0.3037
Positive	39	42.0	
Liver cirrhosis[d]			
Negative	87	42.7	0.7831
Positive	37	43.9	
CYLD (T/N ratio)			
Low	62	41.1	0.0406
High	62	37.0	

[a]Cut-off value defined as the median value; [b]cut-off value defined as the maximum normal value; [c]pathological vascular invasion; [d]classified as F4 stage in the Inuyama classification (25). AFP, α-fetoprotein; PIVKA-II, the protein induced by vitamin K absence or antagonist-II; well/mod, well/moderately-differentiated hepatocellular carcinoma; poor, poorly-differentiated hepatocellular carcinoma; HCV-Ab, hepatitis C virus; HBs-Ag, hepatitis B virus; CYLD, cylindromatosis; T/N, tumor to non-tumor.

As a result, this promoted progressive fibrosis and inflammation, resulting in cancer development (24). Although *CYLD* expression was expected to be potentially associated with certain types of carcinogenesis from viral hepatitis or liver cirrhosis due to chronic inflammation, no correlation was observed between *CYLD* expression and non-tumor liver tissue. A previous *in vitro* study demonstrated that HCC cells transfected with the *CYLD* gene showed an increased NF-κB reporter activity (18). The present study supports the clinical and oncological importance of CYLD in HCC progression.

A limited number of clinical studies have investigated the protein expression and distribution of CYLD in solid types of cancer such as HCC. Notably, in this study, immunohistochemical analysis showed that CYLD expression was distributed according to tumor cell morphology within the same tumor, and tumor cells that lost their cell polarity tended to lose CYLD expression. The mechanism underlying staining pattern remains unclear, and further investigation is required to better understand the role of CYLD in dysplastic cell morphology and chromatin structure.

In conclusion, the present study suggests that CYLD is associated with tumor development in HCC patients. This is a preliminary study and, as a result, the functional aspect of CYLD in HCC patients needs to be further investigated. However, the present study is considered to be useful in investigating whether CYLD may be a future molecular target in HCC patients.

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