Original Article

Clinical significance of *CDT1* mRNA expression in chronic hepatitis C or liver cirrhosis

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We have previously reported that *chromatin licensing and DNA replication factor 1* (*CDT1*) is associated with the postoperative recurrence of hepatocellular carcinoma (HCC). Based on this fact, we verified whether *CDT1* mRNA expression is also associated with HCC development from chronic hepatitis C (CHC) and liver cirrhosis (LC). There were 142 cases with CHC or LC who underwent liver biopsy. Detection of *CDT1* mRNA in liver was performed by RT-qPCR using frozen liver biopsy tissues. We examined the association between the *CDT1* mRNA expression and clinical conditions and long-term outcome. We then examined the association between serum cytokine/chemokine levels and *CDT1* mRNA expression in 58 cases. The cumulative incidence rates of HCC development in cases with *CDT1* mRNA in the low expression group showed significantly lower than those in the high expression group ($p = 0.0391$). A significant correlation was found between *CDT1* mRNA expression and the extent of proliferation of atypical hepatocytes in hematoxylin and eosin-stained sections ($p<0.0001$). *CDT1* mRNA expression has been associated with cytokines involved in tumorigenesis in experimental and human cancers. We found that cases with high *CDT1* mRNA expression were at risk for developing HCC, even if they were CHC or LC.

***Key Words*: proliferation of atypical hepatocytes, hepatocellular carcinoma, chronic hepatitis C, *chromatin licensing and DNA replication factor* (*CDT1*), serum cytokines/chemokine concentration**

The long-term prognosis in cases of hepatocellular carcinoma (HCC) show a 10-year survival rate of approximately 10% regardless of their different etiologies, such as viral-associated and non-viral associated HCC.[1] Therefore, new methods to predict and prevent the occurrence of HCC are important. We consider that the identification of preneoplastic lesions by detailed histological examination of noncancerous liver tissue in cases with HCC is useful for the development of liquid biopsies to prevent hepatocarcinogenesis. In addition, we consider that the identification of the genes that promote the development of preneoplastic lesions will play an important role in drug discovery to prevent liver carcinogenesis, and we are continuing our research.

From this perspective, we have been analyzed histological findings in noncancerous liver tissue and liver biopsy tissue to investigate their association with recurrence or occurrence of HCC.[2–7] From these results, we have reported the importance of the degree of proliferation of atypical hepatocytes (POAH) as a background factor in the development of HCC from chronic hepatitis. Furthermore, in our recent study, *chromatin licensing and DNA replication factor 1* (*CDT1*) was verified as one of the gene associated with the extent of POAH.[2] In fact, it has also been reported that the time to postoperative recurrence is short in cases whose *CDT1* mRNA expression in non-cancerous liver tissue is high. Based on these studies, we found that the extent of POAH is consistent with a histological risk of HCC recurrence. Therefore, based on our previously reported work, we propose that CDT1 is also associated with the induction of POAH, which histologically represents a high-risk lesion for hepatocellular carcinoma. Furthermore, we propose that *CDT1* mRNA expression is a marker of high carcinogenicity in the liver.

CDT1 is one of the licensing control factors of the cell cycle and ensures that chromosome replication occurs exactly once in the cell cycle.[8,9] Recently, CDT1 has been reported to play an important role in human carcinogenesis. It has also been reported that its expression is associated with the prognosis of not only HCC but also other somatic cell carcinomas in humans.[10–17] However, all of these reports are the results of studies using tissue from cancerous nodules or carcinoma cell lines. Thus, there are no studies using tissues from chronic hepatitis/cirrhosis or human liver cell lines.

Based on our previous results and the above reports, we hypothesized that *CDT1* mRNA expression represents a highly carcinogenic state in the development of HCC and that it has carcinogenic potential even in the state of chronic hepatitis C (CHC) or liver cirrhosis (LC). We then tested this hypothesis clinically by examining the association between *CDT1* mRNA expression and histologic findings or clinical status of cases with CHC or LC and the association between *CDT1* mRNA expression and the occurrence of HCC. In addition, we investigated whether *CDT1* mRNA expression was associated with serum cytokine and chemokine levels in 58 cases using a Bioplex Suspension Array System (Bio-Rad Laboratories, Hercules, CA). The numerical values used in this study were basesd on the results measured in the previous study.[17]

Based on the above studies, we investigated whether the detection of CDT1 expression could be an indicator of pathophysiology and long-term prognosis in cases with CHC or LC.

Subjects and Methods

Cases that underwent liver biopsy. A total of 142 cases with CHC or LC visited the Department of Gastroenterology and Hepatology, Nihon University Itabashi Hospital from January 1996 to March 2008. The number of cases who underwent initial liver biopsy in our institute during this period was 384. Of these, 142 cases agreed to participate in this study and had usable

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Table 1. Clinical profiles in cases with chronic hepatitis C and liver cirrhosis whose liver tissue was analyzed by real-time quantitative PCR (n = 142)

	Chronic hepatitis (F0 to F2 stage)	Liver cirrhosis (F3 to F4 stage)	p
Number	104	38	
Observation periods (days)	4,250.5 (118–9,042)	6,740.5 (335–8,266)	0.4733
Age (years)	53.1 (23.5–70.3)	58.0 (44.8–74.1)	0.0004
Gender (males)	55 (52.9%)	18 (47.4%)	0.5604
AST (U/L)	46.0 ± 25.0	78.5 ± 47.6	<0.0001
ALT (U/L)	59.5 ± 44.3	95.3 ± 64.0	0.0003
γ-GT (U/L)	55.2 ± 58.0	70.0 ± 58.5	0.1835
ALP (U/L)	228.4 ± 101.9	337.1 ± 215.1	<0.0001
Total Bilirubin (mg/dl)	0.6 ± 0.3	0.7 ± 0.2	0.4676
Platelet counts (×10^4)	18.8 ± 5.6	12.9 ± 3.6	<0.0001
Total protein (g/dl)	7.2 ± 0.6	7.4 ± 0.6	0.0813
Albumin (g/dl)	4.2 ± 0.5	4.0 ± 0.7	0.1408
Prothrombin time (%)	95.4 ± 6.6	90.5 ± 8.0	0.0005
Ammonia (μg/dl)	42.7 ± 16.5	42.4 ± 18.6	0.9526
ICGR15 (%)	8.1 ± 5.2	14.1 ± 12.4	0.0009
Zinc concentration (μg/dl)	78.1 ± 12.6	71.0 ± 16.0	0.0165
Ferritin (μg/dl)	113.7 ± 118.2	206.1 ± 194.1	0.0718
Alpha fetoprotein (ng/dl)	10.1 ± 36.5	23.8 ± 38.3	0.0751
F stages			<0.0001
F0	3 (2.9%)	—	
F1	61 (58.6%)	—	
F2	40 (38.5%)	—	
F3	—	15 (39.5%)	
F4	—	23 (60.5%)	
HCV RNA			0.9158
High	80 (79.2%)	29 (78.4%)	
Low	21 (20.8%)	8 (21.6%)	
Serotype			0.3334
1	72 (69.2%)	23 (60.5%)	
2	32 (30.8%)	15 (39.5%)	

P value was calculated by Kruskal–Wallis test. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; γ-GT, γ-glutamyltransferase; PT-INR, prothrombin time international normalized ratio; AFP, alpha-fetoprotein; HCV RNA high, ≥10^6 copies/ml; HCV RNA low, <10^5 copies/ml; HCC, hepatocellular carcinoma.

frozen liver biopsy tissue. Therefore, these 142 cases were included in this study. We investigated the mRNA expression of *CDT1* in cryopreserved liver tissue obtained at the time of liver biopsy. The clinical background factors of the 142 cases at the time of initial liver biopsy are shown in Table 1. These cases were cases of CHC or LC that were hepatitis C virus (HCV) RNA positive. They were either cases with interferon (IFN) treatment or cases without IFN treatment (untreated), and all cases agreed to this study of long-term prognosis. Of these cases, 53 of 142 achieved a sustained virologic response (SVR), 63 relapsed after treatment with IFN (non-SVR), and 26 were untreated with IFN (UT). The UT cases are cases in which informed consent for IFN therapy was not obtained or IFN treatment was not covered by Japanese insurance at the time.

We then measured serum cytokine/chemokine concentrations in 58 cases. Of these, 26 cases were F1 stage, 15 were F2 stage, 7 were F3 stage, and 10 were F4 stage. Serum cytokine/chemokine concentrations were measured using a Bioplex Suspension Array System (Bio-Rad Laboratories) as described in our previous study.[18] These measurements were available for 58 cases. Enrolled cases were diagnosed with F stage classification based on the new INUYAMA and Desmet classification.[19–21] Cases were diagnosed with CHC if they were F0 to F3 stage and LC if they were F4 stage. SVR is a case with HCV RNA negativity that persists for more than 6 months after IFN treatment. Non-SVR is a case in which HCV RNA remains positive after treatment.

HCV RNA examination. All cases were positive for serum HCV RNA. Serum HCV RNA levels were determined using the Amplicor HCV Monitor (Roche Diagnostic K.K., Tokyo, Japan) or TaqMan PCR methods (COBAS TaqMan HCV [auto] ver. 2.0; Roche Diagnostic K.K.). The serum HCV RNA level of each patient was classified as high (≥100 kilo copies/ml or 5.0 logU/ml) or low (<100 kilo copies/ml or 5.0 logU/ml). HCV serotype was determined using an EIA kit (Imucheck F-HCV Gr1 and Gr2 reagent, International Reagent Corporation, Tokyo, Japan) according to the manufacturer's instructions.

Exclusion criteria. Cases were excluded if they were less than 18 years of age, habitual alcoholics (ethanol intake of more than 30 g per day), hepatitis B surface antigen [enzyme-linked immunosorbent assay (EIA); Abbott Tokyo, Japan], anti-smooth muscle antibody (fluorescent antibody method; FA), anti-mitochondrial M2 antibody (EIA), and current intravenous drug users.

Definite diagnosis of HCC. Definitive diagnosis of HCC was made by histological diagnosis or by abdominal angiography, which was performed when HCC nodule was suspected by contrast-enhanced abdominal ultrasonography (CEUS) or computed tomography (CT).[2,5] Cases visited the outpatient clinic

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every 1 to 3 months and underwent CT or AUS every 6 months after IFN treatment, depending on the degree of F stage.

Histologic analysis of liver biopsy specimen. The histologic findings of liver biopsy were mainly decided by MM and HN at the histologic conference of our department. Liver biopsy has been performed in our department since 1992, and the histological findings of each parameter have been registered in the database until now. The data of these histologic parameters were used in the present study. For histologic evaluation, the score of each parameter and the histologic findings related to the development of HCC were examined according to previous reports.[2–6] Liver biopsy tissues were obtained from CHC and LC by percutaneous needle biopsy (Tru-Cut Soft Tissue Biopsy Needle, 14 G; Baxter, Deerfield, IL; or BARD MONOPTY Tru Guid, 14 G; Medicon, Tokyo, Japan). Liver tissues obtained by needle biopsy were fixed in 10 to 20% buffered formalin and embedded in paraffin. Each paraffin-embedded specimen was cut into 3- to 4-micrometer sections and stained with hematoxylin and eosin (HE). Each liver biopsy tissue was more than 10 mm in length and contained more than 2 portal areas.

Liver biopsy sections were analyzed semiquantitatively by assigning a score to each of the following features: (1) the degree of inflammatory cell infiltration (0 for none, 1 for minimal, 2 for mild, 3 for moderate, and 4 for severe) in the periportal, parenchymal, and portal areas; (2) the severity of F-stage fibrosis (0 for F0, 1 for F1, 2 for F2, 3 for F3, and 4 for F4); (3) the degree of lymphoid aggregates in the portal area (0 for none, 1 for mild, 2 for scattered, 3 for clusters, 4 for lymphoid follicles without germinal center, and 5 for lymphoid follicles with germinal center); (4) the severity of each of portal sclerotic changes, pericellular fibrosis, and steatosis (on a scale of 0–4 with 0 for none to 4 for severe); (5) severity of bile duct damage (on a scale of 0–4 with 0 for none to 4 for disappearance); (6) severity of bridging necrosis (0 for none, 1 for presence); (7) severity of irregular regeneration of hepatocytes (IR) (0 for none; 1 for <33% of hepatocytes in the sample affected by anisocytosis and pleiomorphism of hepatocytes, bulging of regenerated hepatocytes, nodular arrangement of parenchyma, map-like distribution, proliferation of atypical hepatocytes, and oncocytic change of hepatocytes; 2 for 33–50% of hepatocytes so affected; 3 for 50–75% of hepatocytes so affected; 4 for all hepatocytes diffusely affected) as described in our previous reports.[2–6] The histologic scores were interpreted at a conference centered on MM and HN without knowledge of the cases' characteristics. MS finalized the interpretation when the two graders had different scores.

Reverse transcription quantitative real-time PCR (RT-qPCR). Detection of CDT1 mRNA by RT-qPCR methods was performed according to our previous report.[2] CDT1 gene expression was quantified by ΔCt. $\Delta Ct = Ct_{CDT1\ gene} - Ct_{\beta\text{-actin}}$. RT-qPCR was performed in triplicate. Total RNA was extracted from frozen liver tissues that were embedded in optimal cutting temperature (OCT) compound using TRIzol (Thermo Fisher Scientific, Waltham, MA), and cDNA synthesis was synthesized using the SuperScript™ III First-Strand Synthesis System (Thermo Fisher Scientific). cDNA was used to examine gene expression by RT-qPCR. The cDNA samples were then subjected to PCR using the following primer pairs: CDT1, 5'-TTC TCC GGG CCA GAA GAT AAA G-3' and 5'-ATG ACG CAA GCT CAG AGA TG-3'; β-actin (ACTB), 5'-ATT CCT ATG TGG GCG ACG AG-3' and 5'-AGG TGT GGT GCC AGA TTT TC-3'.[2]

Real-time PCR was performed with THUNDERBIRD®SYBR® qPCR Mix (TOYOBO, Osaka, Japan) using an ABI7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Tokyo, Japan). For details, please refer to our previous report.[2]

Blood and biochemical tests. Blood and biochemical tests were performed on samples from CHC and LC cases obtained at the time of the liver biopsy. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alanine phosphatase (ALP), γ-glutamyl transpeptidase (γ-GT), total bilirubin, total protein (T.P), albumin (Alb), prothrombin time international normalized ratio (PT-INR), platelet count (PLT), alpha-fetoprotein (AFP), and indocyanine green retention rate 15 min (ICGR15) were measured.

Measurement of serum cytokine and chemokine levels. S Serum cytokine and chemokine levels in 58 subjects were measured using a Bioplex Suspension Array System (Bio-Rad Laboratories) according to the manufacturer's instructions. The numerical values used in this study were based on the results measured in the previous study.[17] The cytokines and chemokines measured were measured as follows Cutaneous T-cell-attracting chemokine (CTACK), growth-regulated alpha protein (GROa), interleukin (IL)-1α, IL-2 receptor α (Rα), IL-3, IL-12p40, IL-16, IL-18, leukemia Inhibitory Factor (LIF), monocyte-specific chemokine 3 (MCP-3), macrophage colony stimulating factor (M-CSF), macrophage migration inhibitory factor (MIF), Hu migration inducing gene (MIG), b-nerve growth factor (NGF), c-kit receptor present on mast cells and stem cell factor (SCF), stem cell growth factor β (SCGF)-β, stromal cell-derived factor 1α (SDF-1α), tumor necrosis factor (TNF)-β, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), hepatocyte growth factor (HGF), Hu interferon α2 (IFN-α2), platelet-derived growth factor receptor (PDGF)-ββ, IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, FGF basic, granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), interferon gamma-induced protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1) (MCAF), macrophage inflammatory protein 1 (MIP-1α), MIP-1β, regulated on activation, normal T-cell expressed and secreted (RANTES), TNF-α, and vascular endothelial growth factor (VEGF).

Statistical analysis. Data are presented as mean ± SD or median (range). Categorical variables were compared using the Mann–Whitney *U* test (nonparametric). The Kruskal–Wallis test and the Steel–Dwass test were used for comparisons between multiple groups. For correlation analysis between the two groups, Spearman's rank correlation coefficient test was used. A p value <0.05 on a two-tailed test was considered significant. The cumulative incidence of HCC occurrence was estimated using the Kaplan–Meier method, and the differences between groups were assessed by using the log-rank test.

Ethics. Only cases that agreed to participate in these studies were included in this study. This study was approved by the Ethics Committee of Nihon University School of Medicine (#RK100910-15, #RK-100910-14) and was conducted in accordance with the tenets of the Declaration of Helsinki of 1964 and its subsequent amendments. Only cases who agreed to participate in this study were enrolled; they were asked to cooperate in the study at the time of liver biopsy and at future follow-up visits. Written informed consent was obtained from these cases.

Results

Development of HCC from CHC and LC. Among the 142 cases, 16 cases (11.2%) developed HCC. Among them, 4 cases (7.5%) of the 53 SVR cases, 5 cases (7.9%) of the 63 non-SVR cases, and 7 cases (26.9%) of the 26 untreated cases had HCC occurrence. The 5-year incidence rate of occurrence of HCC was 2.11% for SVR cases, 3.69% for non-SVR cases, and 14.71% for UT cases. The incidence rate of HCC was significantly lower in SVR cases than in non-SVR cases (p<0.0001) and in untreated cases (p = 0.0001). Furthermore, this rate was also significantly lower in non-SVR cases (p<0.0001) than in untreated cases (p = 0.0018).

Relationship between *CDT1* mRNA expression (*CDT1* ΔCt) and liver histologic scores. There was a significant correlation only between the extent of POAH and *CDT1* ΔCt ($p<0.0001$; Fig. 1A). However, there was no difference according to the development of F stages ($p = 0.1413$; Fig. 1B). When comparing the *CDT1* ΔCt at the initial liver biopsy between the SVR, non-SVR, and untreated groups, no difference in the *CDT1* expression was observed ($p = 0.4091$; Fig. 1C). Furthermore, when the pretreatment *CDT1* ΔCt was compared between the cases with HCC development and the cases that did not become HCC development, there was no significant difference in the pretreatment levels between all cases and the SVR, non-SVR, and untreated groups (Fig. 1D–G). The *CDT1* ΔCt in cases who developed HCC was generally between 9.0 and 14.0 in ΔCt levels. The cases who developed HCC was not detected over 14.0 in ΔCt levels.

Correlation between the blood and biochemical test results, histological scores and *CDT1* ΔCt levels. Then, there was only a significant weak correlation between *CDT1* ΔCt and ALP levels in all cases ($r = 0.167$, $p = 0.0499$), but there were no differences between *CDT1* ΔCt and blood and biochemical examination results in SVR, non-SVR and untreated cases (Table 2). Furthermore, there was no significant correlation between *CDT1* ΔCt and the blood and biochemical examination results in cases that developed HCC or cases that did not develop HCC during the observation periods (Table 2). Histologic findings showed significant inverse correlations between *CDT1* ΔCt levels and POAH ($r = -0.437$, $p<0.0001$). In addition, there were no significant correlations between *CDT1* ΔCt and the degree of inflammatory cell infiltration, lymphoid aggregation or F stage. However, there was a significant weak correlation between *CDT1* ΔCt and the characteristic findings of the degree of portal sclerosis ($r = 0.274$, $p = 0.0010$), peri-venular fibrosis ($r = 0.360$, $p<0.0001$), and peri-cellular fibrosis ($r = 0.238$, $p = 0.0044$) (Table 2). Among them, there were significant inverse correlations between *CDT1* ΔCt and the degree of nodular arrangement of parenchyma ($r = -0.301$, $p = 0.0288$), extent of POAH ($r = -0.447$, $p = 0.0008$), and the degree of perivascular fibrosis ($r = 0.331$, $p = 0.0154$) in SVR cases. In the non-SVR group, there was only a significant inverse correlation between *CDT1* ΔCt and the extent of POAH ($r = -0.426$, $p = 0.00005$), portal sclerosis ($r = 0.334$, $p = 0.0075$), and peri-venular fibrosis ($r = 0.373$, $p = 0.0026$). In the untreated group, there were significant correlations between *CDT1* ΔCt and the extent of POAH ($r = -0.446$, $p = 0.0225$), portal sclerosis ($r = 0.479$, $p = 0.0134$), peri-venular fibrosis ($r = 0.395$, $p = 0.0134$), peri-cellular fibrosis ($r = 0.400$, $p = 0.0428$), and steatosis ($r = 0.421$, $p = 0.0322$) (Table 2). Characteristically, *CDT1* ΔCt levels tended to be associated with histologic findings of fibrosis or steatosis in the liver.

Furthermore, there were significant correlations between the *CDT1* ΔCt levels and the extent of POAH in cases that developed HCC ($r = -0.498$, $p = 0.0493$) and cases that did not develop HCC during observation periods ($r = -0.438$, $p<0.0001$), but there were no differences between *CDT1* ΔCt and the blood and biochemical examination results in both groups (Table 2). When comparing the histologic parameters and *CDT1* ΔCt levels, there was a significant correlation between *CDT1* ΔCt and the extent of POAH ($r = -0.443$, $p<0.0001$), the degree of portal sclerosis ($r = 0.230$, $p = 0.0052$) and peri-venular fibrosis ($r = 0.279$, $p = 0.0006$) in cases that developed HCC. Characteristically, there was no correlation between the *CDT1* ΔCt levels and the degree of portal sclerosis ($r = 0.180$, $p = 0.5954$), the peri-venular fibrosis ($r = 0.424$, $p = 0.1935$) and peri-cellular fibrosis ($r = 0.404$, $p = 0.2180$) in cases that developed HCC (Table 2).

Relationship between *CDT1* ΔCt and long-term outcomes. Furthermore, the cumulative incidence rates of HCC development were compared by simply dividing the CDT1 ΔCt into 2 groups in the high group and the low group, the same numerical numbers (Fig. 2A–D). The cumulative 5-year incidence rates of all cases ($n = 142$) were 10.2% and 0% between the high ΔCt ($n = 71$) and low ΔCt ($n = 71$) groups, respectively. The cumulative incidence rates of low ΔCt in all cases were risk ratio; 3.71, 95% CI 0.512–68.361, $p = 0.0351$ (Fig. 2A). The 5-year cumulative incidence rates of the SVR group ($n = 53$) were 0% and 3.7% between the low ΔCt and high ΔCt groups, respectively. The cumulative incident rates of low ΔCt in the SVR group were hazard ratio; 4.24, 95% CI 0.542–85.95, $p = 0.1728$. (Fig. 2B). The 5-year cumulative incidence rates of the non-SVR group ($n = 63$) were 3.2% and 3.2% between the low ΔCt and high ΔCt groups, respectively. The cumulative incident rates of low ΔCt in the non-SVR group were hazard ratio; 3.487, 95% CI 0.512–68.36, $p = 0.2162$ (Fig. 2C). The cumulative 5-year incidence rates of the untreated group were 10% and 10% between the low ΔCt and high ΔCt groups, respectively. The cumulative incidence rates of low ΔCt in the untreated group were hazard ratio; 2.54, 95% CI 0.546–17.78, $p = 0.2409$ (Fig. 2D). The cumulative incidence rates of HCC development between high and low groups, the high ΔCt groups tended to have a lower incidence. Furthermore, the cumulative incidence rates of HCC development were compared by simply classifying *CDT1* ΔCt into 3 groups, the high ΔCt group, the medium ΔCt group and the low ΔCt group, the same numerical numbers (Fig. 2E). The 5-year incidence rates in all cases were 10.25%, 5.26%, and 0% in the low, medium, and high *CDT1* ΔCt groups, respectively. The cumulative incidence rates of HCC development were significantly different among these 3 groups (low ΔCt vs medium ΔCt; risk ratio; 0.727, 95% CI 0.239–2.091, $p = 0.5536$, low ΔCt vs high ΔCt; risk ratio; 3.24, 95% CI 0.746–2.22.13, $p = 0.1203$, medium ΔCt vs low ΔCt; risk ratio; 4.458, 95% CI 1. 0.116–29.55, $p = 0.0385$). The cumulative incidence rate of HCC development was significantly higher in the low *CDT1* ΔCt group. In addition, the cumulative incidence rates of HCC development in high, medium and low CT groups in non-SVR ($p = 0.2037$, Fig. 2F), SVR ($p = 0.3951$, Fig. 2G) and untreated cases ($p = 0.2054$, Fig. 2H) did not observe significant. Significantly, among the three groups of *CDT1* ΔCt of SVR cases, no HCC incident was observed in the high *CDT1* ΔCt group. We consider that the development of HCC can be predicted up to 10 years after a liver biopsy specimen, but not after that from above results. More specifically, 5 years from the time of specimen collection may be a reasonable prediction period for HCC development.

Association between the serum cytokine/chemokine levels and *CDT1* ΔCt levels. We investigated the relationship between serum cytokine/chemokine levels and *CDT1* ΔCt in 58 cases in which cryopreserved serum collected at the time of liver biopsy was available. No significant correlation was observed between *CDT1* ΔCt levels in liver tissue and serum cytokine/chemokine levels (Table 3). There were no significant correlations between *CDT1* ΔCt levels and serum cytokine/chemokine levels. However, there was a trend towards an association between *CDT1* ΔCt levels and macrophage migration inhibitory factor (MIF) levels ($r = 0.2485$, $p = 0.0673$).

Association between *CDT1* ΔCt and time to HCC development from initial biopsy. The association between time to HCC development and *CDT1* ΔCt levels at initial liver biopsy was investigated. As a result, the time to HCC development and *CDT1* ΔCt levels were significantly related in all cases ($p = 0.0140$, Fig. 3A). The significant correlation was observed in the SVR group ($p = 0.0023$, Fig. 3B), non-SVR group ($p = 0.0066$, Fig. 3C) and the untreated group ($p = 0.0053$, Fig. 3D). The time to development of HCC tended to be shorter in the cases with a low ΔCt level of *CDT1*.

Discussion

The time to HCC development from the initial liver biopsy in

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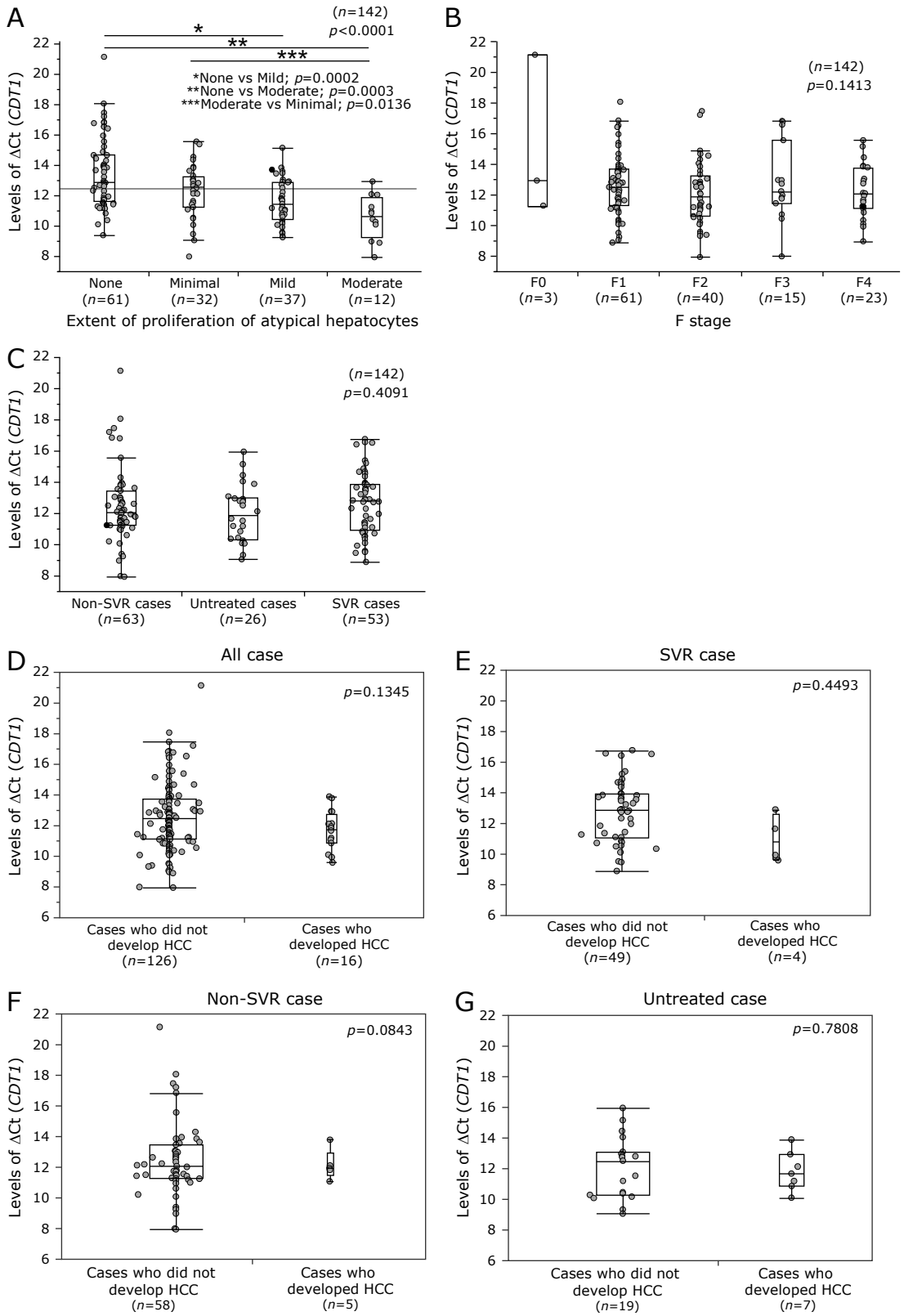

Fig. 1. Comparison between the mRNA expression of *chromatin licensing and DNA replication factor 1* (*CDT1*) (ΔCt) and the extent of proliferation of atypical hepatocytes (POAH), according to F stages and each efficacy of interferon therapy in cases with chronic hepatitis C and liver cirrhosis at initial liver biopsy section. (A) Comparison of the levels of *CDT1* ΔCt and the extent of POAH. (B) Comparison of *CDT1* ΔCt levels and F stages ($p = 0.1413$). (C) Comparison of *CDT1* ΔCt levels with sustained virologic response (SVR), non-SVR and untreated groups ($p = 0.409$). Data in (A) through (C) were analyzed using Kruskal–Wallis and Steel–Dwass tests. Comparison of *CDT1* ΔCt at the liver biopsy in cases who developed hepatocellular carcinoma (HCC) and in cases who did not developed HCC. (D) The levels of *CDT1* ΔCt in all cases ($p = 0.1345$). (E) SVR group ($p = 0.4493$). (F) Non-SVR group ($p = 0.0843$). (G) Untreated group ($p = 0.7808$). Data in (D) to (G) were analyzed by Kruskal–Wallis test.

Table 2. Correlation between the scores of histological parameters or biochemical examination results and expression of *CDT1* mRNA (*CDT1* ΔCt) in liver, and correlation between the cases who became HCC development or who did not become HCC development and *CDT1* ΔCt levels in liver (*n* = 142)

Parameter	All cases (*n* = 142)		SVR cases (*n* = 53)		Non-SVR cases (*n* = 63)		Untreated cases (*n* = 26)		HCC development cases (*n* = 16)		Without HCC Development cases (*n* = 126)	
	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*
Blood and biochemical examinations												
AST (IU/L)	−0.054	0.5235	−0.146	0.2989	−0.075	0.5584	0.127	0.5336	0.009	0.9809	−0.075	0.6211
ALT (IU/L)	−0.03	0.7201	0.007	0.9564	−0.069	0.5905	0.026	0.8997	−0.031	0.9366	−0.078	0.607
γ-GT (IU/L)	−0.04	0.5858	−0.082	0.5596	0.014	0.9078	0.202	0.3205	0.073	0.8515	0.007	0.9628
ALP (IU/L)	0.167	0.0499	0.129	0.3667	0.1157	0.3707	0.328	0.1008	−0.018	0.7031	0.036	0.8129
Total bilirubin (mg/dl)	0.089	0.2958	0.07	0.6255	0.0548	0.6696	0.245	0.2259	−0.493	0.1778	0.071	0.6377
Platelet count (×10³)	0.015	0.854	0.11	0.4354	0.0039	0.9757	−0.29	0.1502	−0.251	0.514	−0.032	0.8346
Total protein (g/dl)	0.117	1784	0.088	0.5442	0.128	0.3317	0.199	0.3287	0.294	0.4432	0.167	0.2662
Albumin (g/dl)	−0.168	0.1081	−0.122	0.5442	−0.118	0.4328	−0.336	0.1596	−0.019	0.9622	−0.099	0.5119
Prothrombin time (INR)	−0.123	0.1479	−0.171	0.26	−0.147	0.2577	−0.153	0.4533	0.129	0.7402	−0.241	0.1066
α-fetoprotein (ng/ml)	−0.099	0.2961	−0.14	0.2617	−0.151	0.3033	0.029	0.9063	0.16	0.6813	−0.09	0.5532
ICGR15	0.002	0.9739	−0.177	0.3636	0.072	0.6431	−0.057	0.8328	0.178	0.5864	−0.011	0.4891
Histological examinations												
Irregular regeneration of hepatocyte groups												
Anisocytosis of hepatocytes	−0.039	0.6392	−0.071	0.6155	−0.083	0.5156	0.004	0.9846	0.344	0.3009	0.014	0.8948
Bulging of heopatocytes	−0.047	0.5754	−0.222	0.1104	0.034	0.7894	−0.253	0.2118	0.138	0.685	−0.139	0.186
Map-like distribution	0.142	0.8675	−0.254	0.0662	0.111	0.3859	0.319	0.1124	0.135	0.6915	−0.019	0.8611
Oncocytic change of hepatitis	0.014	0.8655	−0.013	0.9248	−0.005	0.9708	0.188	0.3566	0.354	0.2849	0.009	0.9316
Nodular arrangement of parenchyma	−0.163	0.0526	−0.301	0.0288	−0.116	0.3675	−0.155	0.4509	−0.409	0.2121	−0.193	0.0659
Proliferation of atypical hepatocyte	−0.437	<0.0001	−0.447	0.0008	−0.426	0.0005	−0.446	0.0225	−0.498	0.0493	−0.438	<0.0001
Inflammatory cell infiltration												
Periportal	−0.012	0.8789	−0.114	0.4172	0.008	0.9478	0.116	0.5731	−0.246	0.4651	0.047	0.6583
Parenchymal	0.025	0.7908	−0.04	0.7749	0.074	0.5654	0.007	0.9743	−0.126	0.713	0.091	0.3869
Portal	0.099	0.2393	0.064	0.6474	0.166	0.1948	0.056	0.7878	−0.307	0.3591	0.19	0.0699
Fibrosis (F) stage	−0.071	0.4005	−0.22	0.114	0.029	0.8237	0.181	0.3762	−0.115	0.737	−0.016	0.878
Lymphocytes aggregation in portal area	0.05	0.5523	−0.084	0.5524	0.254	0.0444	−0.116	0.5717	−0.29	0.387	0.133	0.2072
Bile duct damage	0.104	0.2194	−0.026	0.852	0.197	0.1219	0.081	0.695	−0.012	0.9719	0.059	0.5767
Portal sclerosis	0.274	0.001	0.005	0.9727	0.334	0.0075	0.479	0.0134	0.18	0.5954	0.243	0.0197
Peri-venular fibrosis	0.36	<0.0001	0.331	0.0154	0.373	0.0026	0.395	0.0457	0.424	0.1935	0.278	0.0073
Peri-cellular fibrosis	0.238	0.0044	0.188	0.1767	0.218	0.0862	0.4	0.0428	0.404	0.218	0.179	0.0881
Bridging necrosis	0.068	4190	ND	ND	0.142	0.2669	0.075	0.7129	−0.082	0.7627	0.117	0.1906
Steatosis	−0.005	0.9512	−0.14	0.3167	−0.07	0.5852	0.421	0.0322	0.326	0.3286	−0.014	0.8974

Data analyzed by Spearman's rank correlation coefficient test. ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; γ-GT, γ-glutamyltransferase; ICGR15, indocyanine green retention rate 15 min; ND, not detected.

cases that developed HCC and *CDT1* ΔCt levels were generally correlated. This fact probably reflects the fact that cases who developed HCC already had a high carcinogenic state of the liver at the state of CHC and LC. In other words, we consider that it is possible to predict the development of HCC, even from a small number of samples, in cases where the time to the onset of HCC is short, i.e., in cases where the potential for carcinogenesis has already increased. In addition, the group with low *CDT1* ΔCt levels was also suggested to be a high-risk group for HCC development. These results indicate that there are cases that already represent a high carcinogenic state in CHC or LC.

Regarding liver histology, significant correlations were observed with the extent of POAH and histologic findings related to liver fibrosis. Thus, even in cases with chronic hepatitis or cirrhosis who did not develop HCC, extent of POAH showed a significant inverse correlation with *CDT1* ΔCt levels, confirming that *CDT1* mRNA expression is also higher in cases with a strong extent of POAH. In addition, histologic findings related to liver fibrosis suggested that cases with a progression of fibrosis also had low *CDT1* ΔCt levels and a strong tendency for *CDT1* mRNA expression. Furthermore, in UT cases, *CDT1* mRNA expression tended to be low in cases with a high degree of steatosis. The authors suggest that these events are probably due to the number of hepatocyte populations within the lobule.

Next, when the cumulative incidence of HCC in the first 5 years after IFN treatment was compared among the high, medium, and low *CDT1* ΔCt groups, the incidence of HCC in the high *CDT1* ΔCt group was 0%. The incidence of HCC development in the low *CDT1* mRNA expression group was significantly better than that in the medium and high *CDT1* mRNA expression groups. Interestingly, the pre-treatment *CDT1* ΔCt levels in cases who developed HCC were detected within a certain range without any difference among the three groups. In addition, the cases who developed HCC tended to have high *CDT1* mRNA expression at the initial liver biopsy. Thus, there was a clear difference in the incidence of HCC up to 5 years after IFN treatment depending on *CDT1* mRNA expression. This fact also supports our previous report that high CDT1 expression in a noncancerous liver is a high risk factor for HCC recurrence.

Although no significant associations were found between *CDT1* mRNA expression and anti-inflammatory cytokines, blood cell-related cytokines, and cytokines that promote cell proliferation, *CDT1* ΔCt levels and MIF levels tended to be related. Cytokines, such as MIF, are known to promote or inhibit tumorigenesis in experimental and human cancers. In particular, MIF is considered to be a major cytokine in many cancers, and it has been reported that this cytokine is produced by both malignant cells and infiltrating leukocytes.[22]

It is estimated that in the advanced stages of CHC or LC, oxidative stress of liver cells is increased due to various factors.

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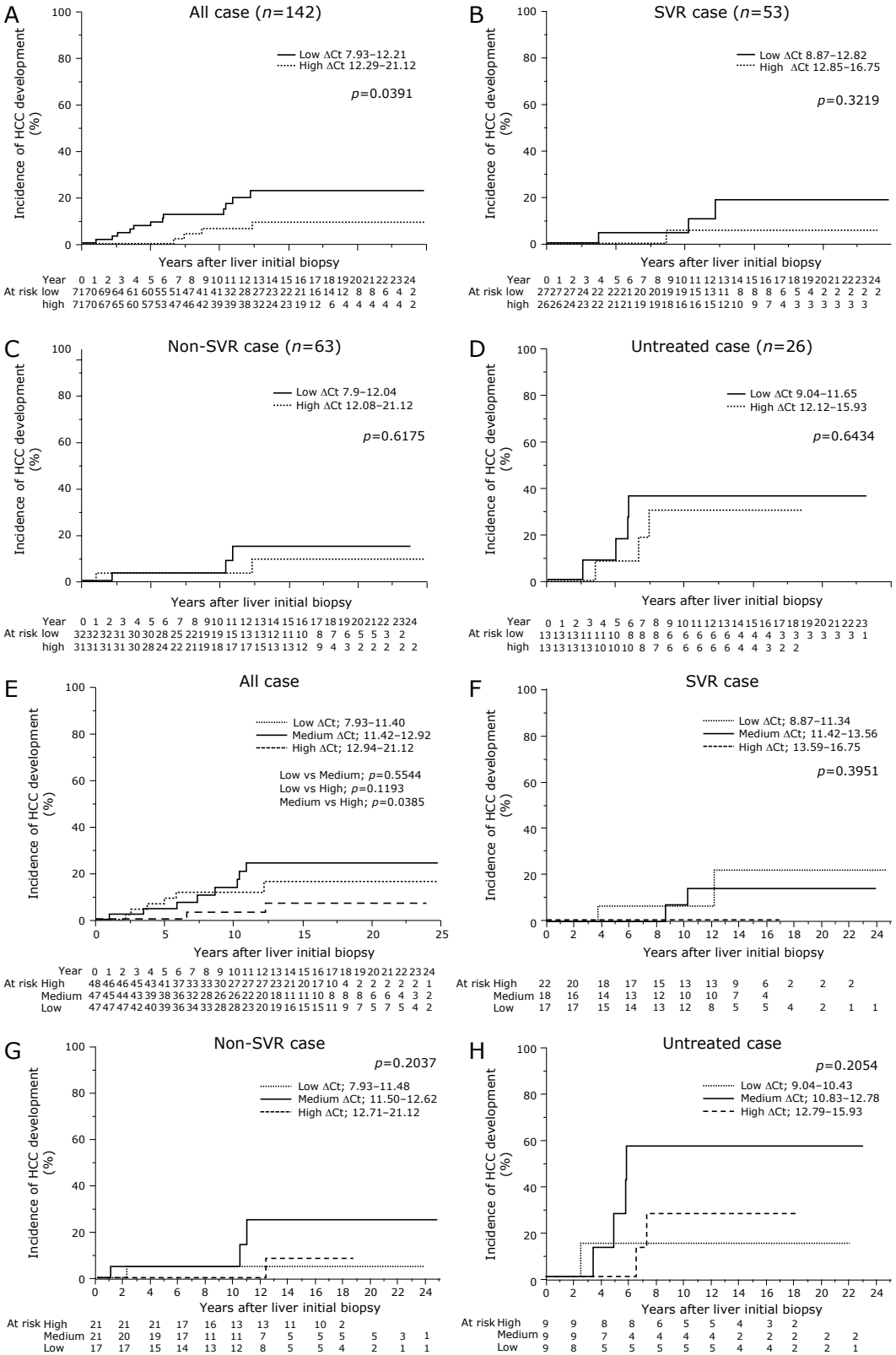

Fig. 2. Comparison of cumulative incidence rates of hepatocellular carcinoma (HCC) development by simple classification of *CDT1* mRNA expression levels (ΔCt) between the high-level group and the low-level group, which were evenly distributed based on the upper numerical levels. (A) All cases ($p = 0.0391$). (B) Sustained virologic response (SVR) cases ($p = 0.3219$). (C) Non-SVR cases ($p = 0.6175$). (D) Untreated cases ($p = 0.6434$). The cumulative incidence rates of HCC development were compared by simply classifying into 3 groups according to the levels of *CDT1* ΔCt. (E) The cumulative incidence rates of HCC development in the high, medium, and low (ΔCt) groups were evenly distributed across the upper numerical levels (low vs medium; $p = 0.0338$, low vs high; $p = 0.1193$, medium vs high; $p = 0.5544$). Data in (A) through (E) were estimated using the Kaplan–Meier method, and then differences between groups were assessed using the log-rank test.

Table 3. Association between *chromatin licensing and DNA replication factor 1* (*CDT1*) mRNA expression (ΔCt) in liver biopsy tissue and serum cytokine and chemokine levels using serum obtained at the time of liver biopsy (*n* = 58)

Cytokine and chemokine	*CDT1* mRNA expression	
	r	*p*
Hu CTACK	0.078	0.5727
Hu GROa	0.054	0.6956
Hu IL-1alpha	–0.083	0.5489
Hu IL-2Ra	–0.172	0.2125
Hu IL-3	0.04	0.7727
Hu IL-12p40	–0.082	0.5519
Hu IL-16	–0.001	0.9941
Hu IL-18	0.038	0.7833
Hu LIF	0.07	0.6129
Hu MCP-3	–0.032	0.8139
Hu M-CSF	0.113	0.4149
Hu MIF	0.248	0.0673
Hu MIG	–0.083	0.549
Hu b-NGF	0.158	0.2519
Hu SCF	–0.155	0.261
Hu SCGF-b	0.045	0.7445
Hu SDF-1a	–0.019	0.8894
Hu TNF-beta	–0.026	0.8498
Hu TRAIL	–0.007	0.9585
Hu HGF	0.115	0.4076
Hu IFN-a2	–0.028	0.8404
Hu platelet-derived growth factor-bb	–0.117	0.3992
Hu IL-1b	–0.018	0.897
Hu IL-1ra	0.019	0.8862
Hu IL-2	0.041	0.7657
Hu IL-4	0.0089	0.949
Hu IL-5	–0.047	0.7314
Hu IL-6	–0.061	0.6607
Hu IL-7	–0.19	0.1673
Hu IL-8	–0.063	0.6496
Hu IL-9	–0.099	0.4753
Hu IL-10	–0.187	0.1746
Hu IL-12 (p70)	–0.039	0.7785
Hu IL-13	–0.119	0.3905
Hu IL-15	0.028	0.8389
Hu IL-17	0.017	0.9022
Hu Eotaxin	–0.094	0.4964
Hu FGF basic	0.207	0.1319
Hu G-CSF	–0.054	0.6972
Hu GM-CSF	–0.057	0.6779
Hu IFN-gamma	0.009	0.9487
Hu IP-10	–0.146	0.2903
Hu MCP-1(MCAF)	–0.068	0.6215
Hu MIP-1a	0.07	0.6124
Hu MIP-1b	–0.151	0.2751
Hu RANTES	–0.149	0.2811
Hu TNF-alpha	0.022	0.8705
Hu VEGF	0.008	0.954

Cutaneous T-cell-attracting chemokine (CTACK), growth-regulated alpha protein (GROa), interleukin (IL)-1α, IL-2 receptor α (Rα), IL-3, IL-12p40, IL-16, IL-18, leukemia inhibitory factor (LIF), monocyte-specific chemokine 3 (MCP-3), macrophage colony-stimulating factor (M-CSF), macrophage migration inhibitory factor (MIF), Hu migration inducing gene (MIG), b-nerve growth factor (NGF), c-kit receptor present on mast cells and stem cell factor (SCF), stem cell growth factor β (SCGF)-β, stromal cell-derived factor 1 α (SDF-1α), tumor necrosis factor (TNF)-β, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), hepatocyte growth factor (HGF), Hu interferon α2 (IFN-α2), platelet-derived growth factor receptor (PDGF)-ββ, IL-1b, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, eotaxin, FGF basic, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage-colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), interferon gamma-induced protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1) (MCAF), macrophage inflammatory protein-1 (MIP-1α), MIP-1β, regulated on activation, normal T-cell expressed and secreted (RANTES), TNF-α, and vascular endothelial growth factor (VEGF).

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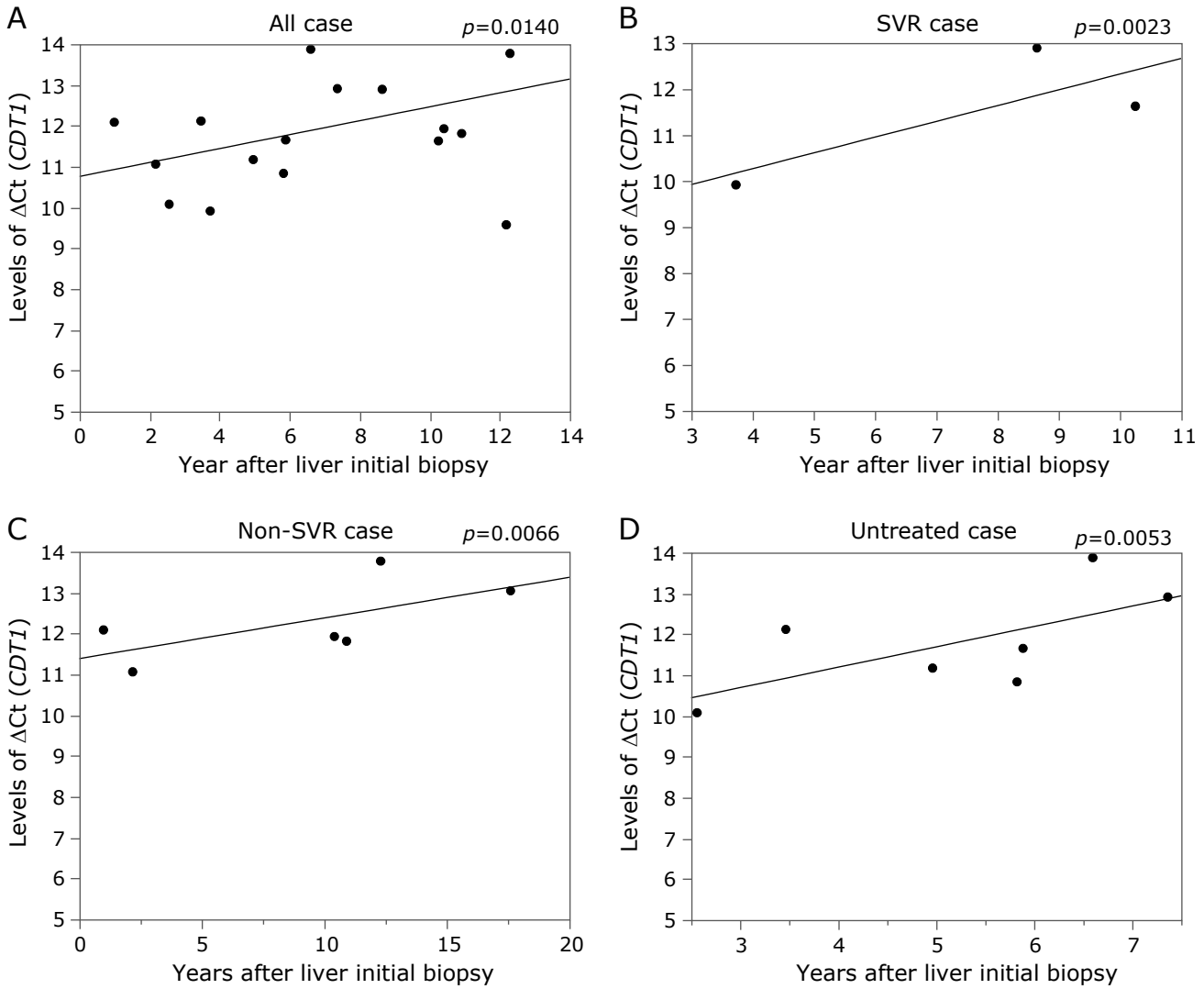

Fig. 3. Association between *CDT1* ΔCt and time to HCC development from initial liver biopsy. (A) All cases ($p = 0.0140$). (B) Non-SVR cases ($p = 0.0066$). (C) SVR cases ($p = 0.0023$). (D) Untreated groups ($p = 0.0053$). Data analyzed by Robust regression analysis by Huber M estimation.

We have previously reported a reduction in urinary 8OH-dG concentration by the use of glutathione administration in patients with CHC or LC (our unpublished data). In addition, an increase in intracellular taurine has been reported to be associated with antioxidant defense mechanisms.[23] In such situations with increased oxidative stress, it is considered that there may be disturbances in the DNA replication process of liver cells. The functional association between the *CDT1* gene and antioxidant activity has not been reported until recently. Therefore, *CDT1* is primarily a protein involved in DNA replication, and direct involvement in antioxidant activity is not expected. However, it is possible that cellular oxidative stress may interfere with DNA replication. Reduction of oxidative stress by antioxidant activity may enhance support for accurate DNA replication in liver cells. *CDT1* is a protein involved in the initiation of DNA replication and is necessary for proper cell cycle progression. When oxidative stress increases in the cell, it can cause damage to DNA and proteins, potentially affecting the expression and function of *CDT1*. Such effects can interfere with DNA replication, thereby disrupting the precise functioning of the cell. Therefore, understanding the relationship between oxidative stress and *CDT1* is critical to the progression and treatment of liver disease. Future research is expected to provide a more detailed elucidation of how oxidative stress influences *CDT1* expression and how antioxidant activity may affect DNA replication in liver cells. This knowledge may lead to the development of novel therapeutic and preventive approaches for liver diseases.

The development of a therapeutic method that can directly improve the abundance of proliferation of atypical hepatocytes is considered a future task. As one strategy, we would like to focus on the *CDT1* gene or genes related to *CDT1* that could lead to the prevention and prediction of HCC by drug discovery and the construction of biomarkers. Even if the *CDT1* ΔCt level is high at the time of measurement, it is assumed that the necroinflammatory reaction in the liver may become more severe or the lymphocytic infiltration may become more severe in the long term. In such examples, the long-term prognosis was not consistent with the *CDT1* ΔCt level at the time of measurement. Therefore, the *CDT1* ΔCt level indicates the highly carcinogenic state of the liver at the time of sampling. Since we consider that the predictable time may be approximately 5 years, hepatic *CDT1* mRNA expression should be continuously monitored.

This study had limitations. If the sample volume is adequate, as in the case of non-cancerous tissue from HCC, the atypical hepatocyte population should be included. However, it should be considered that a small amount of liver biopsy tissue sample may

not reflect the actual presence of atypical hepatocytes in individual cases.

In summary, we found that *CDT1* was overexpressed in some cases of CHC and LC. In other words, in some cases of CHC and LC, we found that *CDT1* was already highly expressed, and high carcinogenesis from the state of CHC and LC. Thus, detection of *CDT1* mRNA can identify individuals who are at high risk of developing HCC, even in CHC and LC. Our results suggest that *CDT1* mRNA expression in the liver before interferon therapy is also associated with carcinogenesis in cases with SVR. Based on this study, our report can greatly contribute to the future medical treatment of chronic liver disease and treatment of HCC by targeting HCC prevention.

Author Contributions

MO and MM contributed to data collection, statistical analyses, interpretation of data, the writing of the manuscript and was the main author of the manuscript. HN, MM, MS, and MO contributed to data and sample collection; MM and MO were contributed for study concept and design; MM, HN, MO, and MS were contributed for histological analysis; MM, KK, TS, and MO were responsible or analysis and interpretation of data; MM, MO, and KK were responsible for drafting of the manuscript; MO and MM were responsible for statistical analysis.

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Conflict of Interest

MM; Towa Pharmaceutical Co., Ltd. The other authors have no conflict of interest to declare.

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