Original Article

The significance of CDT1 expression in non-cancerous and cancerous liver in cases with hepatocellular carcinoma

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We previously reported that chromatin licensing and DNA replication factor 1 (CDT1) expression was associated with the extent of proliferation of atypical hepatocytes and the time to postoperative recurrence in cases of hepatocellular carcinoma (HCC). This study aimed to clarify the clinical significance or pathogenesis of CDT1 expression in both non-cancerous and cancerous liver in HCC cases, including previously published data. We investigated the association between the expression of CDT1 in non-cancerous or cancerous liver tissues and histologic findings or biochemical examination results in 62 cases. We also examined the dual localization between CDT1 and FbxW7, P57kip2, P53 and c-Myc by confocal laser scanning microscopy. *CDT1* mRNA expression was significantly higher in cancerous liver than in non-cancerous liver (p<0.0001). Elevated CDT1 mRNA expression indicates a significantly degree of inflammatory cell infiltration within lobules, along with elevated serum transaminase levels, and hepatic spare decline. *CDT1* mRNA was highly expressed in a group of poorly differentiated cancer cells. CDT1 co-localized with P57kip2, Fbwx7, P53 and c-Myc in the nucleus or cytoplasm of hepatocytes and cancer cells. We found that *CDT1* mRNA expression could represent the degree of hepatic spare ability and the high carcinogenic state.

***Key Words*: chromatin licensing and DNA replication factor 1, hepatocellular carcinoma, proliferation of atypical hepatocytes, Ki-67, P57 kip2**

The pathogenesis of HCC development remains unclear. On the other hand, we also reported that the degree of irregular regeneration (IR) of hepatocytes were important risk factor for the development of hepatocellular carcinoma (HCC) from chronic hepatitis C (CHC) or liver cirrhosis (LC).[1,2] Furthermore, we have reported the extent of proliferation of atypical hepatocytes (POAH), which is one of the irregular regeneration (IR) of hepatocytes populations as a strong risk factor for the development of HCC from CHC or LC.[3] In addition, we recently examined the pathological findings of non-cancerous liver sections in 356 cases with HCC and reported that the extent of POAH was a significant factor involved in the postoperative recurrence of HCC.[4]

In addition, we examined the pathological findings of non-cancerous liver sections in 356 cases with HCC and reported that the extent of POAH was a significant factor involved in the post-operative recurrence of HCC.[4] It has been reported association with chromatin licensing and DNA replication factor 1 (CDT1) expression and pathogenesis of cancer development in many cancers.[5–9] Yu *et al.*[8] and Karavias *et al.*[9] reported that higher CDT1 expression was significantly associated with reduced overall survival, which supported our results in the previous study. Cai *et al.*[10] CDT1 promoted the proliferation of a cancer cell line *in vitro*. However, these previous reports were based on cancer nodules or cancer cell lines, not on non-cancerous tissues or human non-cancerous cell lines. We consider that the identification of pre-neoplastic cells by focusing on non-cancerous liver of histologic findings is important for future prevention of carcinogenesis and cancer treatment.

In the present study, we focused on CDT1 gene and analyzed the association between the levels of CDT1 expression in non-cancerous or cancerous liver frozen tissues and clinical significance or pathogenesis of HCC. The cases who underwent liver resection used to detect *CDT1* mRNA in the previous study[4] were examined with a more recent extension of the observation period.

In addition, it has recently been reported that the therapeutic efficacy of immune checkpoint inhibitor immunotherapy is differs among different etiologies according to hepatitis B virus (HBV)-associated HCC, hepatitis C virus (HCV)-associated HCC and non-B and non-C HCC.[11] There is no difference in the histologic morphology of POAH in the different etiologies. Therefore, in this study, we also investigated whether CDT1 expression in non-cancerous or cancerous liver tissues are any differences in comparing the clinical status or pathogenesis of HCC postoperative recurrence in according to the different etiology. The etiology shows the following three groups: It has been clarified that hepatitis B virus surface antigen positive cases are HBV (+) and hepatitis C virus antibody positive cases are HCV (+), HBs antigen negative and HCV antibody negative cases. In addition, we hypothesized that POAH has a preneoplastic entity based on our previous study. In this study, we added the following studies to prove whether CDT1 expression is associated with a preneoplastic entity.

Next, CDT1 is normally expressed in the G1 phase and regulates the cell cycle. However, we hypothesized that CDT1, which is expressed in the non-cancerous part of HCC, is also involved in the G0 phase stability, which may be involved in cell cycle abnormalities. Therefore, we investigated the association between P57kip2 and FWXB7, which are mainly expressed in the G0 phase of hepatocytes, and CDT1 expression, which is mainly expressed in the G1 phase, by immunofluorescence (IF)

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using confocal laser scanning microscopy (CLSM). We also investigated whether CDT1 is expressed in POAH, a preneoplastic cell, but also in cells expressing the oncogenes c-Myc or P53 in non-cancerous or cancerous liver sections by IF using CLSM. From these studies, we investigated the significance of the existence of CDT1-expressing cells by comparing the expression morphology of oncogenes and further comparing the expression morphology with genes that control the cell cycle. Based on the above studies, we verified whether the expression of CDT1 indicates the carcinogenic state in the liver.

Subjects and Methods

Study population. The non-cancerous liver tissues of 62 selected cases with HCC who underwent liver resection from February 2011 to February 2012 were examined. These 62 cases were the same cases reported in our previous study,[4] but, these subjects were studied with a more longer observation period. For clinical profiles of these 62 cases, please refer to our previous report.[4] Next, sections from the FFPE block of cancerous and non-cancerous liver tissues of HCC cases who underwent surgical liver resection at our hospital from January to July 2021 were used for IHC or IF study. The breakdown is HCV; 2 cases with F1 stages, which became HCC development after SVR and F4 stages, and NBNC 3 cases with F1, F2, and F3 stages.

For the exclusion criteria and definite diagnosis of postoperative HCC recurrence, please refer to our previous report.[4] All cases were closely monitored for recurrence of HCC at every postoperative outpatient visit until June, 2022.[12] The last observation analyzed in this study was June 29, 2022. The study design conformed to the ethical guidelines of the Declaration of Helsinki.

Blood and biochemical tests. Blood and biochemistry data were used for examination results within one month prior to surgery. Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alanine phosphatase (ALP), γ-glutamyl transpeptidase (γ-GT), total bilirubin (T-Bil), cholinesterase (ChE), blood urea nitrogen (BUN), creatinine (Cr), ammonia (NH_3), HbA1c (hemoglobin A1c), total protein (TP), albumin (Alb), prothrombin time international normalized ratio (PT-INR), and platelet count (PLT). Indocyanine green 15R (ICGR15) levels were also evaluated. Serum concentrations of alpha-fetoprotein (AFP) and des-gamma-carboxy prothrombin (DCP) were determined by enzyme-linked immunosorbent assay as tumor biomarkers.

Evaluation of histologic findings in the liver hematoxylin and eosin (HE)-stained liver sections. The scores of histologic factors in non-cancerous liver HE-stained section were obtained from our previous research data using non-cancerous liver HE-stained sections.[4] For more details on the histologic evaluation of non-cancerous or cancerous liver HE sections and the concept of POAH also please refer to our previous reports.[4] Fig. 1A and B shows an image of POAH. The atypical hepatocyte population is usually detected as dense aggregates of several or more atypical hepatocytes. More details can be found in our previous study.[4]

RNA extraction and RT-qPCR analysis. The levels of *CDT1* mRNA expression data in 62 frozen liver resection of non-cancerous liver tissue in this study used the results of RT-qPCR from previous studies.[4] The detection of *CDT1* mRNA in 62 frozen liver resection of cancerous liver tissue was performed by RT-qPCR. For further details of the RT-qPCR method, please refer to our previous study.[4] The PCR primers used in this study were *CDT1*, 5'-TTC TCC GGG CCA GAA GAT AAA G-3' and 5'-ATG ACG CAA GCT CAG AGA TG-3'; and *β-actin* (ACTB), 5'-ATT CCT ATG TGG GCG ACG AG-3' and 5'-AGG TGT GGT GCC AGA TTT TC-3'. For the methods of RT-qPCR, please refer to our previous report.[4] Quantification was evaluated by delta (Δ) Ct. $\Delta Ct = Ct_{CDT1\ genes\ test} - Ct_{\beta\text{-}actin}$. More details can be found in our previous study.[4]

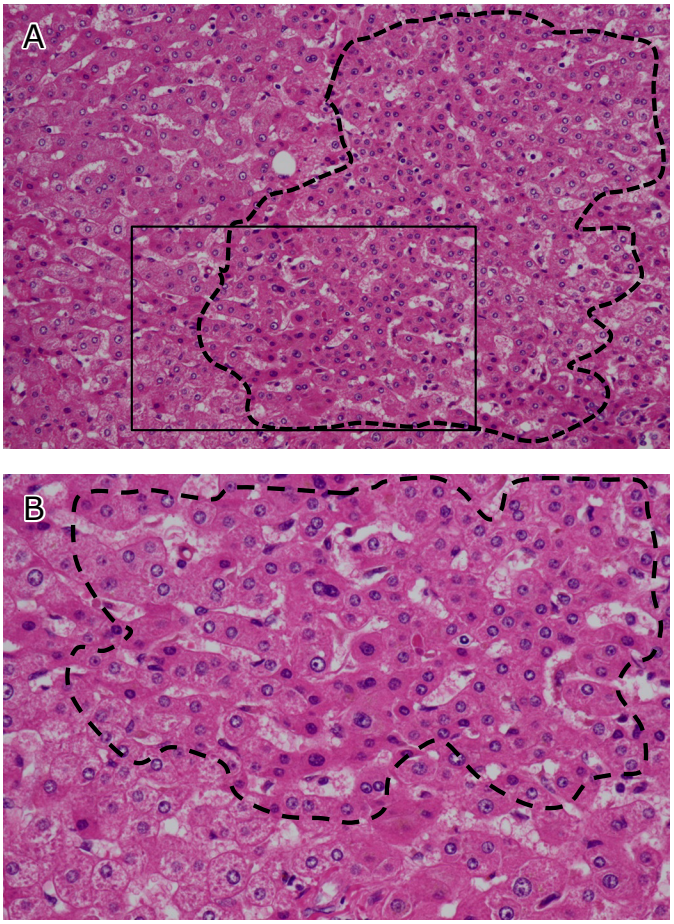

Fig. 1. Representative image of the proliferation of atypical hepatocytes in a non-cancerous liver section. (A) Moderate magnification of proliferation of atypical hepatocyte population. [Hematoxylin and eosin (HE) staining, ×100]. (B) Higher magnification of the dotted line from (A) (HE stain, ×40).

Immunofluorescence study. We examined the association between the localization of CDT1, F-box and WD repeat domain containing 7 (FBXW7), P57-kip2, P53 and c-Myc protein by Immunofluorescence (IF) using rabbit anti-CDT1 polyclonal antibody (14382-1-AP; Proteintech, Rosemont, IL), rabbit anti-Ki-67 polyclonal antibody (27309-1-AP; Proteintech), rabbit anti-Fbxw7 polyclonal antibody (ab109617; Abcam, 330 Cambridge Science Park Milton Road Cambridge, CB4 OFL GBR), rabbit anti-p57 Kip2 monoclonal antibody [(EP2515Y) ab75974; Abcam], rabbit anti-P53 polyclonal antibody (21891-1-AP; Proteintech) and mouse anti-c-Myc monoclonal antibody (sc-40; Santa Cruz, Sallas, TX). Then, we performed double IF staining of CDT1 and Ki-67, CDT1 and P57kip2 or FWbx7, CDT1 and P53 or c-Myc in non-cancerous liver FFPE sections containing cancerous parts. To detect colocalization of CDT1 and P57, Fbwx7, P53, c-Myc, confocal images with IF staining in paraffin sections were performed using a confocal laser scanning microscope (CLSM, TCS SP8; Leica Microsystems, Wetzlar, Germany). For more details on the IF staining and CLSM method, please refer to our previous report.[4,13]

Statistical analysis. Data are expressed as median ± range unless otherwise noted. Categorical variables were compared using the Kruskal–Wallis test, and Steel–Dwass tests were performed for comparisons between multiple groups. Correlation

Table 1. Multivariate Cox proportional regression model analysis of histologic factors in non-cancerous hematoxylin and eosin stained liver sections associated with the postoperative recurrence and recurrence times of hepatocellular carcinoma (n = 62)

Parameter	Risk ratio	95% CI		p
Irregular regeneration of hepatocytes populations				
Anisocytosis of hepatocytes	0.666	0.443	0.992	0.0461
Bulging of hepatocytes	0.871	0.386	1.871	0.7273
Map-like distribution	1.37	0.9	2.115	0.729
Oncocytic change of hepatocytes	0.841	0.535	1.288	1.187
Nodular arrangement of parenchyma	1.63	1.123	2.39	0.0103
Proliferation of atypical hepatocyte	1.924	1.16	3.253	0.0112
Inflammatory cell infiltration				
Periportal	2.524	1.211	5.344	0.0137
Parenchymal	0.843	0.344	2.026	0.7032
Portal	4.898	1.85	13.681	0.0013
F stage	0.641	0.407	0.99	0.0451
Portal lymphoid aggregation	0.249	0.112	0.508	<0.0001
Bile duct damage	1.307	0.609	2.529	0.4658
Portal sclerosis	0.431	0.048	2.62	0.3751
Pre-venular fibrosis	0.523	0.242	1.078	0.0798
Peri-cellular fibrosis	0.887	0.499	1.597	0.685
Steatosis	0.756	0.441	1.277	0.2977
Etiology HCV vs HBV	0.734	0.215	2.626	0.6275
HCV vs NBNC	2.277	0.707	7.864	0.1693
HBV vs NBNC	3.1	0.085	0.855	0.085
CDT1 ΔCt	0.508	0.281	0.849	0.0086

Histologic parameters were evaluated with formalin fixed paraffin imbedded hematoxylin and eosin staining section, and the *chromatin licensing and DNA replication factor 1 (CDT1)* ΔCT were examined by RT-qPCR by frozen liver tissues. Hazard ratios were calculated by Log-rank test using the group in cases with HCC resection. CI, confidence interval; HCV, hepatitis C virus; HBV, hepatitis B virus; NBNC, Non-A Non-B.

analysis between the two groups was performed using Spearman's rank correlation test and robust regression analysis using Huber's M estimation.[14] A p value <0.05 on a two-tailed test was considered significant. Cumulative relapse-free survival (RFS) was estimated using the Kaplan–Meier method, and differences between groups were assessed using the log-rank test. In the analysis of risk factors in non-cancerous and cancerous liver for postoperative HCC recurrence, we tested different etiologies, histologic factors, tumor markers and *CDT1* mRNA (ΔCt) in non-cancerous or cancerous liver tissue obtained at baseline in univariate and multivariate Cox proportional hazards regression analysis. Processing and analysis were performed using JMP software (SAS Inc., Chicago, IL).

Ethics. This study was approved by the Ethics Committee of Nihon University School of Medicine (#131, #241-1, RK-200702-1) and was conducted in accordance with the tenets of the Declaration of Helsinki of 1964 and its subsequent amendments. Written informed consent was obtained in all cases.

Results

Risk factors for HCC postoperative recurrence. Histologically significant risk factors in non-cancerous liver section for postoperative HCC recurrence by multivariate Cox proportional regression model included POAH [hazard ratio (HR) = 1.924; 95% confidence interval (CI), 1.160, 3.253; p = 0.0112], anisocytosis of hepatocytes (HR = 0.666; 95% CI, 0.443–0.992; p = 0.0461), nodular arrangement of parenchyma (HR = 1.630; 95% CI, 1. 123, 2.390; p = 0.0103) and inflammatory cell infiltration in the periportal (HR = 2.524; 95% CI, 1.211, 5.344; p = 0.0137) and portal areas (HR = 4.894; 95% CI, 1.850, 13.681; p = 0.0013), F stage (HR = 0. 641; 95% CI, 0.407, 0.990; p = 0.0451), portal lymphoid aggregation (HR = 0.0.249; 95% CI, 0.112, 0.508; p<0.0001) and CDT1 ΔCt (HR = 0.508; 95% CI, 0.281, 0.849; p = 0.0086; Table 1). Based on the results, we confirmed that *CDT1* ΔCt level was associated with postoperative HCC recurrence. In addition, cases with lymphocytic infiltration in the periportal and portal areas were frequently observed, but a low degree of lymphocytic aggregation in the portal area was identified as a risk factor for recurrence. Interestingly, the risk of postoperative recurrence was low in cases with lymph follicles with germinal center.

Then, histologically significant risk factors in cancerous liver HE stained section for HCC postoperative recurrence by multivariate Cox proportional regression model included sex (M; F, HR = 0.439; 95% CI, 0.204, 1.010; p = 0.0528), AFP (HR = 0.996; 95% CI, 0.9994, 0.9998; p = 0.0016), CA19-9 (HR = 1.048; 95% CI, 1.024, 1.073; p<0.0001), etiology (HCV vs NBNC; HR 6.299, 95% CI, 2.072, 20.543, p = 0.0011) and cancer cell differentiation (poorly vs well; HR 5.943, 95% CI, 2.074, 18.540, p = 0.0008: poorly vs moderately; HR 5.296, 95% CI, 2.165, 13.561, p = 0.0002). The risk factors for recurrence in cancerous liver were extracted by cancer cell differentiation (Poorly vs moderately; r = 5.296, p = 0.0002, Poorly vs well; r = 5.943, p = 0.0008), high AFP level (r = 0.996, p = 0.0016), high carbohydrate antigen 19-9 (CA19-9; r = 1.048, p<0.0001) level, etiology (HCV vs NBNC; r = 6.299, p = 0.0011) and sex (female; r = 0.26, p = 0.0062) (Supplemental Table 1*).

Correlation between *CDT1* ΔCt and fibrosis stage or extent of POAH in non-cancerous liver tissue. Levels of *CDT1* ΔCt was lower in cases with advanced fibrosis (p = 0.014). A similar trend was observed in HCV (+) cases (p = 0.0247) according to the different etiologies (Fig. 2A). However, no significant association was observed in HBV (+) cases (p =

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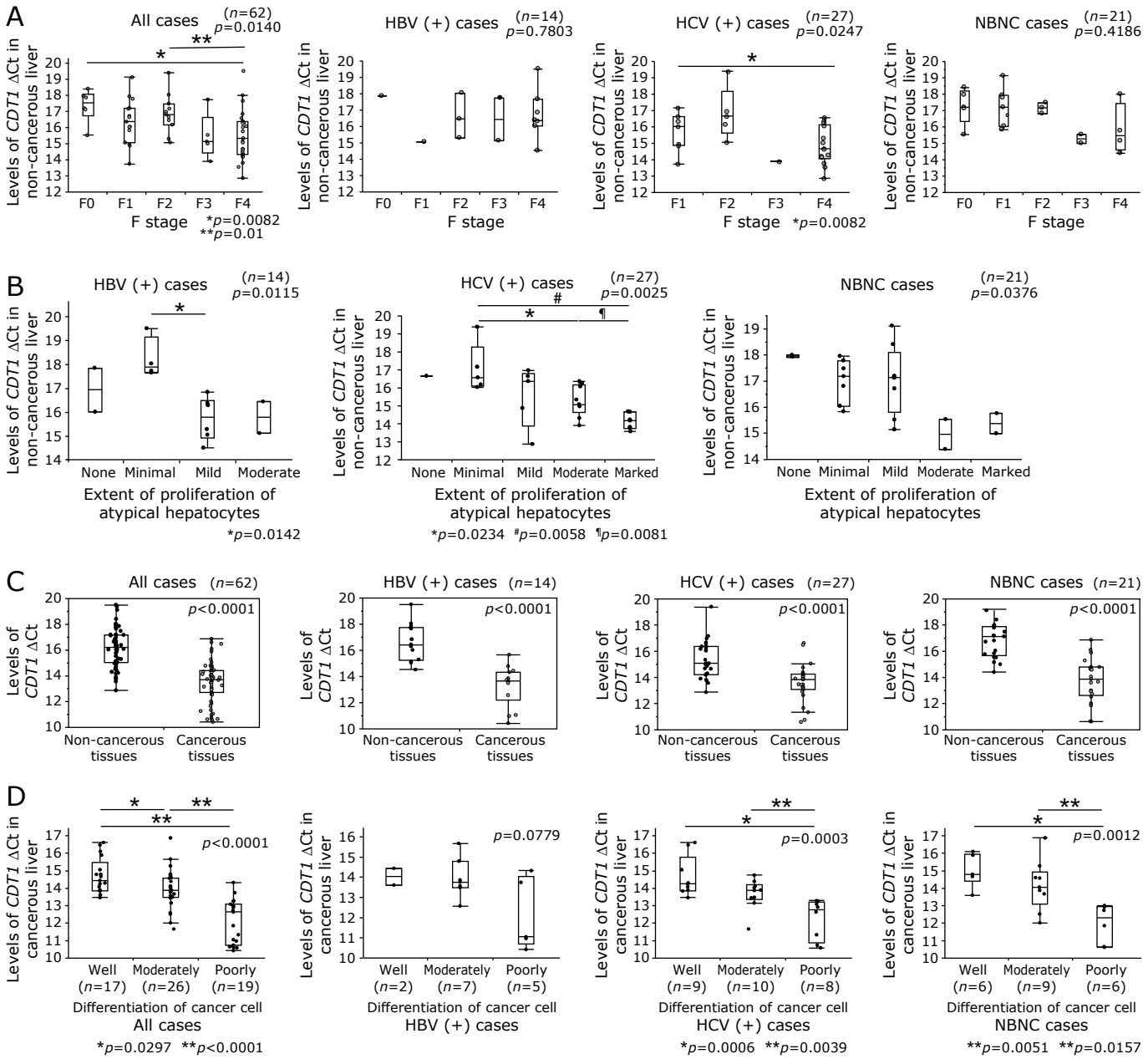

Fig. 2. Comparison between *chromatin licensing and DNA replication factor 1 (CDT1)* mRNA expression levels (ΔCt) and fibrosis stage. (A) All cases ($p = 0.014$, left panel), cases with hepatitis B virus surface antigen-positive (HBV) (+) cases ($p = 0.7803$, middle left panel), cases with hepatitis C virus antibody-positive (HCV) cases (+) ($p = 0.0247$, middle right panel), cases with non-HBV (+) non-HCV (+) cases (NBNC) ($p = 0.4186$, right panel). Data analyzed using Kruskal–Wallis and Steel–Dwass tests. Comparison between *CDT1* ΔCt levels and degree of proliferation of atypical hepatocytes. (B) HBV (+) cases ($p = 0.0115$, left panel), HCV (+) cases ($p = 0.0025$, middle panel), NBNC cases ($p = 0.0376$, right panel). Data analyzed using Kruskal–Wallis and Steel–Dwass tests. Comparisons between *CDT1* ΔCt in cancerous and non-cancerous liver tissue. (C) All cases (left panel, $p<0.0001$), (HBV) (+) cases (middle left panel; $p<0.0001$), middle right panel shows (HCV) (+) cases ($p<0.0001$), right panel shows NBNC cases ($p<0.0001$). Data analyzed by Kruskal–Wallis test. Comparison between *CDT1* ΔCt in cancerous liver tissues and the each well, moderately and poorly differentiation of cancer cells group. (D) All cases (left panel, $p<0.0001$, moderately vs poor; $p<0.0001$, moderately vs well; $p<0.0001$, moderately vs well; $p = 0.0297$, left panel). HBV (+) cases ($p = 0.077$, middle left panel). HCV (+) cases ($p = 0.0003$, moderately vs poorly; $p = 0.0039$, poorly vs well; $p = 0.0006$, middle right). NBNC cases ($p = 0.0012$, moderately vs poorly; $p = 0.0157$, poorly vs well; $p = 0.0051$, right panel). Data analyzed by Kruskal–Wallis and Steel–Dwass tests.

0.7803) and NBNC cases ($p = 0.4186$) cases, which also showed a similar trend to HCV (+) cases. The extent of POAH and *CDT1* ΔCt levels were significantly correlated in all cases ($r = -0.678$, $p<0.0001$) in our previous study.[4] In addition, the extent of POAH and *CDT1* ΔCt levels were significantly correlated in the HBV (+) cases ($p = 0.011$), HCV (+) cases ($p = 0.002$) and NBNC cases ($p = 0.037$, Fig. 2B). From the above results, *CDT1* ΔCt and the extent of POAH were significantly correlated regardless of the etiology. In addition, *CDT1* ΔCt tended to be lower in cases with advanced F stage.

Association between *CDT1* ΔCt levels and the cancerous or non-cancerous liver and differentiation of cancer cells according to the different etiology. When comparing *CDT1* ΔCt levels between cancerous and non-cancerous frozen liver

Table 2. Correlation between blood and biochemical test results and *CDT1* ΔCt levels in non-cancerous liver (*n* = 62)

Parameter	All cases		HBV (+)		HCV (+)		NBNC	
	r	*p*	r	*p*	r	*p*	r	*p*
AST (U/L)	−0.506	<0.0001	−0.293	0.569	−0.368	0.0239	−0.519	0.016
ALT (U/L)	−0.454	0.0002	−0.327	0.4838	−0.354	0.0383	−0.437	0.0474
γ-GT (U/L)	−0.105	0.5931	−0.63	0.3699	−0.105	0.8043	0.168	0.7501
ALP (U/L)	−0.218	0.0883	−0.316	0.4439	−0.111	0.3313	−0.24	0.2945
PLT (×10⁴/μl)	0.393	0.0016	−0.201	0.6385	0.206	0.4095	0.511	0.0179
ICGR15 (%)	−0.41	0.0009	−0.063	0.819	−0.28	0.1092	−0.426	0.0542
Tp (g/dl)	0.13	0.3125	0.479	0.0521	0.154	0.5966	0.288	0.2058
Alb (g/dl)	0.441	0.0003	0.362	0.2238	0.435	0.0138	0.401	0.072
T.bil (mg/dl)	−0.313	0.0131	−0.092	0.3103	0.087	0.5845	−0.665	0.001
ChE (U/L)	0.464	0.0002	0.137	0.9962	0.358	0.0456	0.336	0.136
T chol (mg/dl)	0.163	0.2344	−0.117	0.5553	0.01	0.601	0.252	0.2699
BUN (mg/dl)	0.028	0.8286	0.215	0.7909	−0.009	0.42	0.121	0.6025
Cr (mg/dl)	0.242	0.058	0.018	0.9934	0.273	0.0732	0.012	0.9587
NH_3 (μg/dl)	−0.197	0.1324	−0.577	0.0303	0.034	0.894	−0.371	0.1181
PT-INR (%)	−0.254	0.0465	0.072	0.8698	−0.028	0.905	−0.602	0.0039
AFP (ng/ml)	0.05	0.7016	−0.05	0.8651	0.174	0.3962	−0.098	0.6729
DCP (mAU/ml)	0.051	0.6977	0.503	0.0668	−0.119	0.5721	−0.152	0.5216

Data calculated by Spearman's rank correlation coefficient. AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alanine phosphatase; γ-GT, γ-glutamyl transpeptidase; PLT, platelet count; ICGR15, indocyanine green retention rate 15 min; TP, total protein; T.bil, total bilirubin; Alb, albumin; Che, choline esterase; T chol, total cholesterol; BUN, blood nitro nitrogen; Cr, creatinine; NH3, ammonia; PT-INR, prothrombin time international normalized ratio; AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin.

tissues according to different etiologies, *CDT1* ΔCt levels in cancerous liver tissues were significantly lower than those in non-cancerous liver tissues in HBV (+) ($p<0.0001$), HCV ($p<0.0001$) and NBNC ($p<0.0001$) (Fig. 2C). We then compared the median *CDT1* ΔCt levels in cancerous and non-cancerous liver tissue between different etiologies. Median *CDT1* ΔCt levels according to different etiologies in cancerous liver tissue are all cases; 13.79 (10.42–16.87), HBV (+) cases; 13.67 (10.42–15.66), HCV (+) cases; 13.83 (10.58–16.61), NBNC cases; 13.87 (10.61–16.87). There was no difference ($p = 0.6888$) (Fig. 2C). In non-cancerous liver tissue it was all cases; 16.21 (12.86–19.51), HBV (+) cases; 16.41 (14.52–19.51), HCV (+) cases; 15.07 (12.86–19.39), NBNC cases; 17.12 (14.39–19.12). There was a significantly lower *CDT1* ΔCt between HCV (+) cases and HBV (+) cases ($p = 0.0027$) or NBNC cases ($p = 0.03561$, Fig. 2C). On the other hand, when comparing *CDT1* ΔCt according to the differentiation of cancer cells, *CDT1* ΔCt was significantly lower in poorly differentiated cancer cells than in well differentiated cancer cells ($p<0.0001$) or in moderately differentiated cancer cells ($p<0.0001$), and well differentiated cancer cells is significantly higher than that of moderately differentiated cancer cells in all cases ($p = 0.0297$). In HBV (+) cases, there was no difference in *CDT1* ΔCt according to the differentiation of the cancer cell groups ($p = 0.0779$). In HCV (+) cases, *CDT1* ΔCt was significantly lower in poorly differentiated group than in well ($p = 0.0006$) and moderately ($p = 0.0039$) differentiated group. In NBNC cases, *CDT1* ΔCt was significantly lower in poorly differentiated cancer cells than in well ($p = 0.0051$) and moderately differentiated group ($p = 0.0157$, Fig. 2D). In each group, the levels of CDT1 ΔCt tended to be low in the poorly differentiated group and no difference was observed according to the different etiologies.

Correlation between blood and biochemical test results and *CDT1* ΔCt levels. There was a significant positive correlation between *CDT1* ΔCt levels in non-cancerous frozen liver tissue and levels of serum AST ($r = -0.506$, $p<0.0001$), ALT ($r = -0454$, $p = 0.0002$), total bilirubin ($r = -0.313$, $p = 0.0131$), Che ($r = 0.464$, $p = 0.0002$), albumin ($r = 0.441$, $p = 0.0003$), platelet count ($r = 0.393$, $p = 0.0016$), PT-INR ($r = -0.254$, $p = 0.0465$) and ICG15R ($r = -0.410$, $p = 0.0009$). There were also significant correlations between *CDT1* ΔCt and choline esterase ($r = 0.464$, $p = 0.0002$), albumin concentration ($r = 0.441$, $p = 0.0003$) and platelet count ($r = 0.393$, $p = 0.0016$, Table 2). When comparing the serum tests and *CDT1* ΔCt levels according to the different etiologies, there were significant correlations between NH3 concentration ($r = -0.577$, $p = 0.0303$) and *CDT1* ΔCt levels in the HBV (+) group; albumin ($r = 0.435$, $p = 0.0138$), AST ($r = -0.368$, $p = 0.0239$), ALT ($r = -0. 354$, $p = 0.0383$) and cholinesterase levels ($r = 0.358$, $p = 0.0456$) and *CDT1* ΔCt levels in the HCV (+) group; and AST ($r = -0.519$, $p = 0.0160$), ALT ($r = -0.437$, $p = 0. 0474$), T-Bil (-0.665, $p = 0.0010$), platelet count ($r = 0.511$, $p = 0.0179$) and PT-INR ($r = -0.602$, $p = 0.0039$) and *CDT1* ΔCt levels in the NBNC group (Table 2). In addition, *CDT1* ΔCt levels were not associated with age ($r = 0.0468$, $p = 0.7176$) and sex ($p = 0.9479$).

Using the same parameters, we then investigated the relationship between CDT1 ΔCt levels in cancerous liver tissues. In these results, *CDT1* ΔCt levels in cancerous liver tissues were significantly correlated with BUN level ($r = 0.291$, $p = 0.0226$) in all cases, total cholesterol concentration ($r = 0.581$, $p = 0.0474$) and creatinine concentration ($r = -0.564$, $p = 0.0353$) in HBV (+) cases and choline esterase concentration ($r = 0.4351$, $p = 0.0487$), BUN level ($r = 0.6062$, $p = 0.0036$), PT INR level ($r = 0.5754$, $p = 0.0063$), CA19-9 level ($r = 0.6301$, $p = 0.0029$) in NBNC cases. There was no significant correlation between *CDT1* ΔCt levels in cancerous liver tissue and the same parameters in HCV (+) cases (Supplemental Table 2*).

Correlation between histologic factor scores in HE stained sections and *CDT1* ΔCt levels in frozen liver tissue in non-cancerous liver. A significant correlation was found between *CDT1* ΔCt levels and scores of oncocytic change of hepatocytes ($r = -0.467$, $p = 0.0001$) and scores of POAH ($r = -0.678$, $p<0.0001$) in the non-cancerous liver HE stained sections in all cases. There were also significant correlations between *CDT1*

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Table 3. Correlation between histologic factor scores in non-cancerous hematoxylin and eosin-stained liver sections and *CDT1* ΔCt levels in non-cancerous liver tissue (n = 62)

Parameter	All cases		HBV (+)		HCV (+)		NBNC	
	r	p	r	p	r	p	r	p
Irregular regeneration								
Anisocytosis of hepatocytes	0.021	0.8695	−0.297	0.3026	0.005	0.981	−0.164	0.4784
Bulging of hepatocytes	0.213	0.0967	−0.133	0.6516	−0.017	0.9332	0.403	0.0697
Map like distribution	−0.236	0.0645	−0.008	0.979	−0.09	0.6549	−0.349	0.1212
Oncocytic change of hepatocytes	−0.467	0.0001	−0.396	0.1611	−0.36	0.0651	−0.434	0.0492
Nodular arrangement of hepatocytes	−0.226	0.0775	−0.504	0.066	−0.241	0.2262	−0.012	0.9596
Proliferation of atypical hepatocyte	−0.678	<0.0001	−0.546	0.0433	−0.682	<0.0001	−0.571	0.0068
Inflammatory cell infiltration								
Periportal	−0.48	<0.0001	−0.499	0.0692	−0.524	0.005	−0.13	0.5739
Parenchymal	−0.542	<0.0001	−0.457	0.1004	−0.633	0.0004	−0.361	0.1082
Portal	−0.572	<0.0001	−0.552	0.0409	−0.523	0.0051	−0.289	0.2042
F stage	−0.383	0.0022	0.016	0.9563	−0.381	0.0501	−0.504	0.0199
Lymphoid aggregation in portal area	−0.464	0.0001	−0.261	0.3679	−0.475	0.0461	−0.061	0.7945
Bile duct damage	−0.101	0.4327	−0.078	0.7907	−0.046	0.8207	−0.024	0.9186
Portal sclerosis	0.007	0.9596	−0.124	0.6719	0.174	0.3858	0	1
Peri-venular fibrosis	0.021	0.873	−0.29	0.3141	0.261	0.2073	0.007	0.9746
Peri-cellular	−0.028	0.8315	−0.115	0.6963	−0.038	0.8495	−0.104	0.6523
Steatosis	−0.121	0.3472	−0.4	0.1561	−0.361	0.0646	0.099	0.669

Data calculated by Spearman's rank correlation coefficient.

ΔCt levels and inflammatory cell infiltration scores in the periportal (r = −0.480, p<0.0001), parenchymal (r = −0.542, p<0.0001) and portal areas (r = −0.572, p<0.0001). Lymphoid aggregation scores in the portal area (r = −0.467, p = 0.0002) and fibrosis stage (r = −0.383, p = 0.0022) were also significantly correlated with *CDT1* ΔCt levels (Table 3). According to the different etiologies, significant correlations were found only for portal lymphocytic infiltration (r = −0.562, p = 0.0409) in HBV (+) cases and only for fibrosis (F) stage (r = −0.504, p = 0.0199) in NBNC cases. In HCV (+) cases, *CDT1* ΔCt levels were correlated with lymphocyte infiltration scores in the parenchyma and portal area. There was a significant correlation between *CDT1* ΔCt levels and POAH scores regardless of the different etiologies (All cases; r = −0.678, p = 0.678, p<0.0001, HBV (+) cases; r = −0.546, p = 0.0433, HCV (+) cases; r = −0.682, p<0.0001, NBNC cases; r = −0.571, p = 0.0068, Table 3). There was no significant correlation between *CDT1* ΔCt levels in cancerous liver tissue and the same pathological parameters in non-cancerous liver. There were also no differences in *CDT1* ΔCt levels in non-cancerous liver tissues according to each differentiation of cancer cells (p = 0.2130).

Relationship between *CDT1* ΔCt levels and degree of lymphocyte infiltration in the lobules. Interestingly, *CDT1* ΔCt levels correlated with the degree of lymphocytic infiltration in HCV (+) cases, while HBV (+) cases also showed a weak correlation. However, NBNC cases showed no correlation with the degree of lymphocytic infiltration in the lobules (Table 3). This is consistent with the fact that lymphocyte reactivity in HCC cases with an HCV-associated background differs from that in HCC cases with an HBV-associated or non-viral liver disease background, suggesting that lymphocyte reactivity is weaker in NBNC cases. Furthermore, the degree of portal lymphoid aggregation was significantly inversely correlated with *CDT1* ΔCt levels only in the HCV (+) cases. This suggests that cases with a high *CDT1* ΔCt level tended to have a low degree of portal lymphoid aggregation. This is an interesting finding in relation to the higher RFS in cases with higher *CDT1* ΔCt.

Association between *CDT1* ΔCt levels and clinical features in non-cancerous and cancerous liver. When we compared *CDT1* ΔCt levels in cases who developed recurrence (with recurrence) or cases who did not develop recurrence until January 2022 (without recurrence), *CDT1* ΔCt levels in cases with recurrence were significantly lower than those without recurrence in non-cancerous liver tissue [HBV (+) cases; p = 0.0180, HCV (+) cases; p = 0008 and NBNC cases; p = 0.0179, Fig. 3A], but not in cancerous tissue (Fig. 3B).

Next, we compared *CDT1* ΔCt levels in cancerous or non-cancerous liver tissue between each different etiology in cases with and without recurrence. There were significant correlations in non-cancerous liver in all cases (p = 0.0018, Fig. 3C left panel). Furthermore, there were significant correlations in cases with recurrence in *CDT1* ΔCt levels in non-cancerous liver [HCV (+) cases vs NBNC cases; p = 0.0102, HCV (+) cases vs HBV (+) cases; p = 0.0139, Fig. 3C]. There was no difference between the different etiologies in cases without recurrence (p = 0.1905, Fig. 4D). There was no difference in *CDT1* ΔCt levels between each different etiology in cases with recurrence (p = 0.7331) or without recurrence (p = 0.6315) in cancerous liver (Fig. 3D). When comparing *CDT1* ΔCt in cancerous liver tissues according to the differentiation of cancer cells divided into cases with recurrence and cases without recurrence, there were significant correlations in cases with recurrence (moderately group vs poorly group; p = 0.0219, poorly group vs well group; p = 0.0112, Fig. 3E left panel). However, there was no difference in cancer cell differentiation in cases without recurrence (p = 0.8337, Fig. 3E right panel).

Association between *CDT1* ΔCt levels in non-cancerous or cancerous liver tissue and postoperative recurrence time. When, we divided the groups by each different etiology and compared the time to recurrence in non-cancerous and cancerous frozen liver tissue according to *CDT1* ΔCt levels. Significant correlations were observed for *CDT1* ΔCt levels in non-cancerous liver tissue in all cases (p<0.0001), HBV (+) cases (p = 0.0317), and HCV (+) cases (p = 0.0001), but no significant difference was observed in NBNC cases (p = 0.1752) by robust

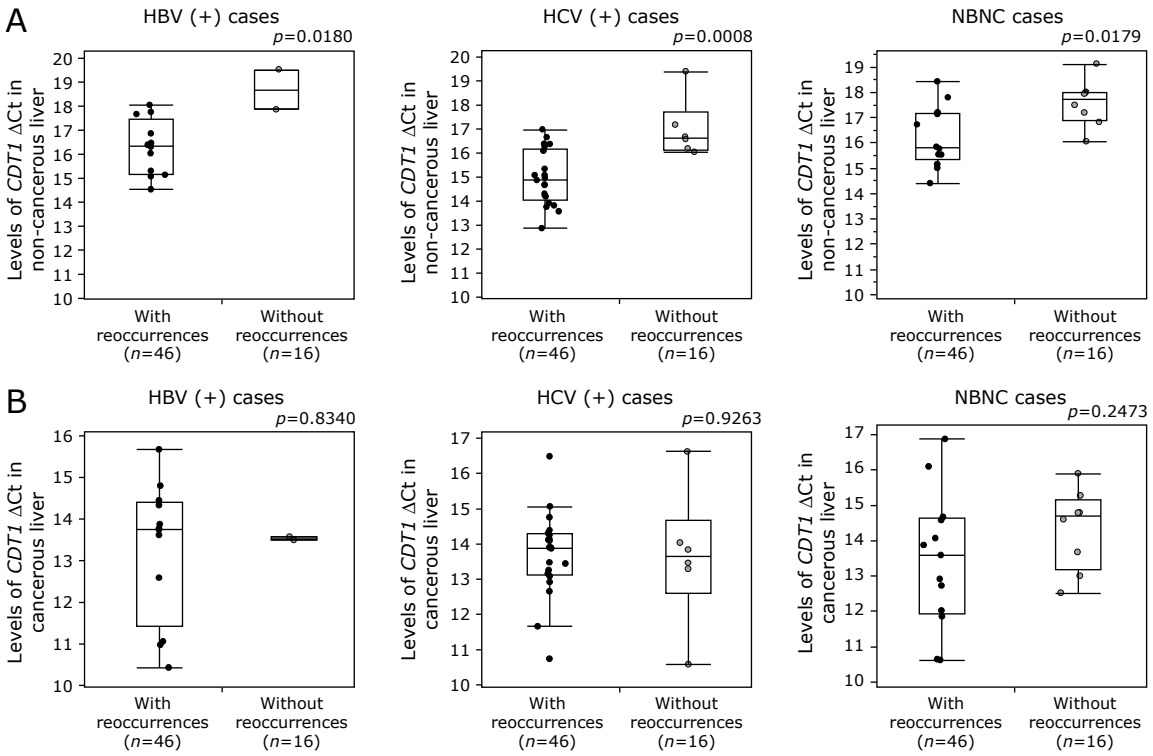

Fig. 3. Comparison between *CDT1* ΔCt levels in cases with recurrence (with recurrence) and cases without recurrence (without recurrence) in non-cancerous liver. (A) HBV (+) cases ($p = 0.133$, left panel), HCV (+) cases ($p = 0.0008$, middle panel), NBNC cases ($p = 0.0529$, left panel). Data analyzed by Kruskal–Wallis test. (B) Comparison between *CDT1* ΔCt levels in cases with recurrence and those without recurrence in cancerous liver. HBV (+) cases ($p = 0.4891$, left panel), HCV (+) cases ($p = 0.4891$, middle panel), NBNC cases ($p = 0.1683$, right panel). Data analyzed by Kruskal–Wallis test. Comparison among *CDT1* ΔCt levels in non-cancerous liver tissues among the each etiology in cases who became recurrence or cases who did not become recurrence. (C) All cases in non-cancerous liver [HCV (+) cases vs NBNC cases; $p = 0.0027$, HCV (+) cases vs HBV (+) cases; $p = 0.0356$, left panel]. Cases who had recurrence [HCV (+) cases vs NBNC cases; $p = 0.0102$, HCV (+) cases vs HBV cases; $p = 0.0139$, middle panel]. Cases who did not have recurrence ($p = 0.1905$, right panel). Data analyzed using Kruskal–Wallis and Steel–Dwass tests. Comparison among *CDT1* ΔCt levels in cancerous liver tissues among the each etiology in cases who had recurrence and cases who did not have recurrence. (D) All cases ($p = 0.6586$, left panel). Cases who had recurrence ($p = 0.7331$, middle panel). Cases who did not have recurrence ($p = 0.6315$, right panel). Data analyzed using Kruskal–Wallis and Steel–Dwass test. (E) The comparison among the *CDT1* ΔCt levels in cancerous liver tissues according to the differentiation of cancer cells in cases who became recurrence or cases who did not become recurrence. Cases who had recurrence (moderately vs poorly groups; $p = 0.0219$, poorly vs well groups; $p = 0.0112$, left panel). Cases who did not have recurrence ($p = 0.8337$, right panel).

regression analysis with Huber M estimation (Fig. 4A). There was no association between *CDT1* ΔCt levels in cancerous liver tissue and the recurrence time in all cases ($p = 0.4581$), HBV (+) cases ($p = 0.8521$), HCV (+) cases ($p = 0.6238$), NBNC cases ($p = 0.1012$, Fig. 4B).

Furthermore, we divided the groups according to the differentiation of the cancer cells and compared the relationship between the time to recurrence and the *CDT1* ΔCt levels in non-cancerous or cancerous liver. The shorter time to recurrence correlated significantly between *CDT1* ΔCt levels in non-cancerous frozen liver with well differentiation ($p<0.0001$), poorly differentiation ($p = 0.0226$) and moderately differentiation groups ($p = 0.0048$) using robust regression analysis using Huber M estimation (Fig. 4C). There was no correlation between time to recurrence and *CDT1* ΔCt levels in cancerous liver and moderately differentiation ($p = 0.6086$) and poorly differentiation groups ($p = 0.2515$). However, the time to recurrence and *CDT1* ΔCt levels in well differentiation group showed an inverse significant correlation ($p = 0.0034$, Fig. 4D). This result may indicate that the duration of recurrence is not related to the differentiation of the cancer cells, but depends on the *CDT1* ΔCt levels of the non-cancerous liver.

Correlation of *CDT1* ΔCt levels between cancerous and non-cancerous frozen livers. We examined the correlation of *CDT1* ΔCt levels between cancerous and non-cancerous frozen liver tissues ($n = 62$). The *CDT1* ΔCt levels between cancerous and non-cancerous liver tissues showed a significant correlation ($p = 0.0009$, Fig. 5A left panel). We then examined the correlation of *CDT1* ΔCt levels between cancerous and non-cancerous liver tissues, dividing the cases with recurrence ($n = 46$) and cases without recurrence ($n = 16$). In these results, *CDT1* ΔCt levels in cases with recurrence ($n = 46$) showed a significant correlation between cancerous and non-cancerous liver tissue ($p = 0.0056$). However, *CDT1* ΔCt levels in cases without recurrence showed no correlation between cancerous and non-cancerous liver tissues ($p = 0.9320$, Fig. 5A).

We further divided the different groups according to etiology and compared the *CDT1* ΔCt levels in non-cancerous and cancerous liver tissue. The *CDT1* ΔCt levels in non-cancerous and cancerous liver tissues were significantly correlated in HBV (+) cases ($n = 14$, $p = 0.0179$) and HCV (+) cases ($n = 27$, $p = 0.0408$), but no significant difference was observed in NBNC cases ($n = 21$, $p = 0.0833$) (Fig. 5B).

We then compared the *CDT1* ΔCt levels in cancerous and non-cancerous tissues of cases with recurrence ($n = 46$) by each different etiology according to each differentiation of cancer cells. In these results, *CDT1* ΔCt levels were significantly correlated in HBV (+) cases ($n = 12$, $p = 0.0212$) and HCV (+) cases ($n = 21$, $p<0.0001$) but not in NBNC cases ($n = 14$, $p = 0.1023$) by robust regression analysis with Huber M estimation (Fig. 5C).

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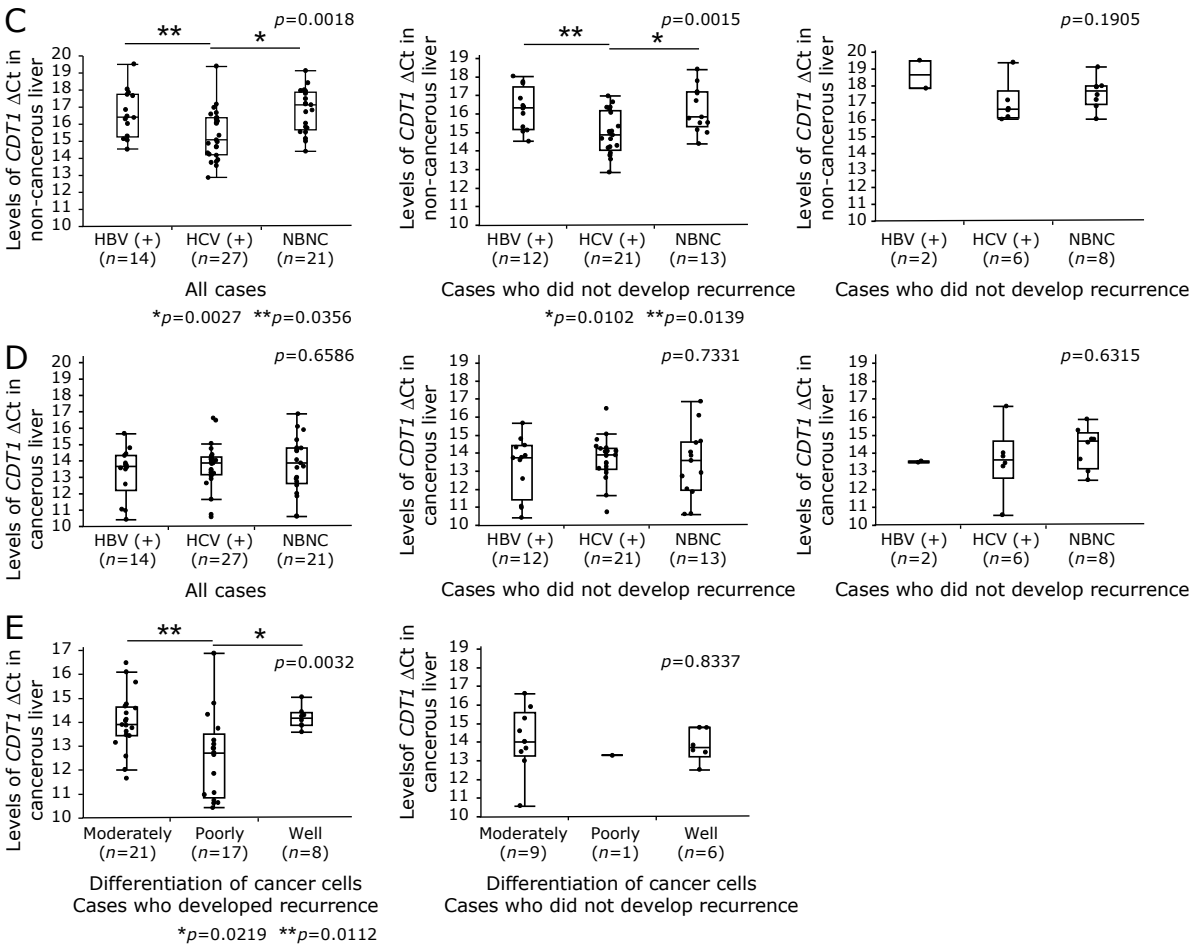

Fig. 3. continued

This indicated that cases with low *CDT1* ΔCt levels in cancerous liver tissue also had low *CDT1* ΔCt levels in non-cancerous liver tissue.

We then compared *CDT1* ΔCt levels between cancerous and non-cancerous liver tissues according to each differentiation of cancer cells in all cases ($n = 62$) or in cases with recurrence ($n = 46$). There was a significant correlation between cancerous and non-cancerous liver tissues in poorly differentiation group ($n = 18$, $p = 0.0006$) and the well differentiation group ($n = 14$, $p = 0.209$), but the correlation was inverted in the well differentiation group in all cases (Fig. 5D). In cases with recurrence ($n = 46$), there was significant correlation between cancerous and non-cancerous liver tissue in poorly differentiation group ($n = 17$. $p = 0.0011$), but not in well differentiation group ($n = 8$, $p = 0.6128$) and moderately differentiation group ($n = 21$, $p = 0.2571$, Fig. 5E).

Immunofluorescence and confocal laser scanning microscopy study. The localization of CDT1 and each gene product in non-cancerous and cancerous liver FFPE sections was confirmed by CLSM. When the CDT1 positive, each gene positive and Hoechst 33342 were merged by CLSM, the positive reaction products were confirmed in the same nucleus and cytoplasm in hepatocytes. The differences in the distribution of CDT1 and P57kip2, FWXB7, p53 and c-Myc staining positive cells in non-cancerous and cancerous sections are shown in Fig. 6. P57 kip2 localized mainly to the nucleus in hepatocytes in the lobules, and P57 kip2-positive cells were also present in the lobules, with moderate to strong localization in each case (Fig. 6A). The merged images showed co-localization with CDT1 and P57kip2 mainly in the nucleus with hepatocytes and cancer cells respectively (Fig. 6A and B). Fbxw7 was also localized to the nucleus and cytoplasm in hepatocytes, and the localization of Fbxw7-positive cells in lobules, which was mild to moderate in localization, was similar to P57kip2 in lobules (Fig. 6C). Fbxw7-positive cells were observed to co-localize with CDT1 mainly in the nucleus and cytoplasm with hepatocytes and cancer cells (Fig. 6C and D). P53 localized to the nucleus and cytoplasm in hepatocytes, and localization of P53-positive cells in lobules was observed which was minimal to mild (Fig. 6E). P53 was observed to co-localize with CDT1 mainly in the nucleus in mainly in the nucleus in hepatocytes and cancer cells (Fig. 6E and F). c-Myc localized at the same as p53 to nucleus and cytoplasm in hepatocytes, and localization of c-Myc positive cells in lobules, the localization degree were scattered to minimal to mild were observed (Fig. 6G). c-Myc observed to co-localize with CDT1 in mainly nucleus with hepatocytes and cancer cell (Fig. 6G and H).

Based on these results, we considered that some of CDT1-positive with atypical hepatocytes has proliferative ability as same as the cancer cells being associated with ki67.[4] Although, CDT1-positive and P57kip2 or FWXB7-positive hepatocytes had co-localization in lobules, the number of CDT1-positive hepatocytes is few numbers than that of P57kip2 or FWXB7 positive cells which present only in G0 phase and has been reported to be involved in carcinogenesis. Therefore, some of the CDT1 was co-localized with the oncogene in non-cancerous or cancerous

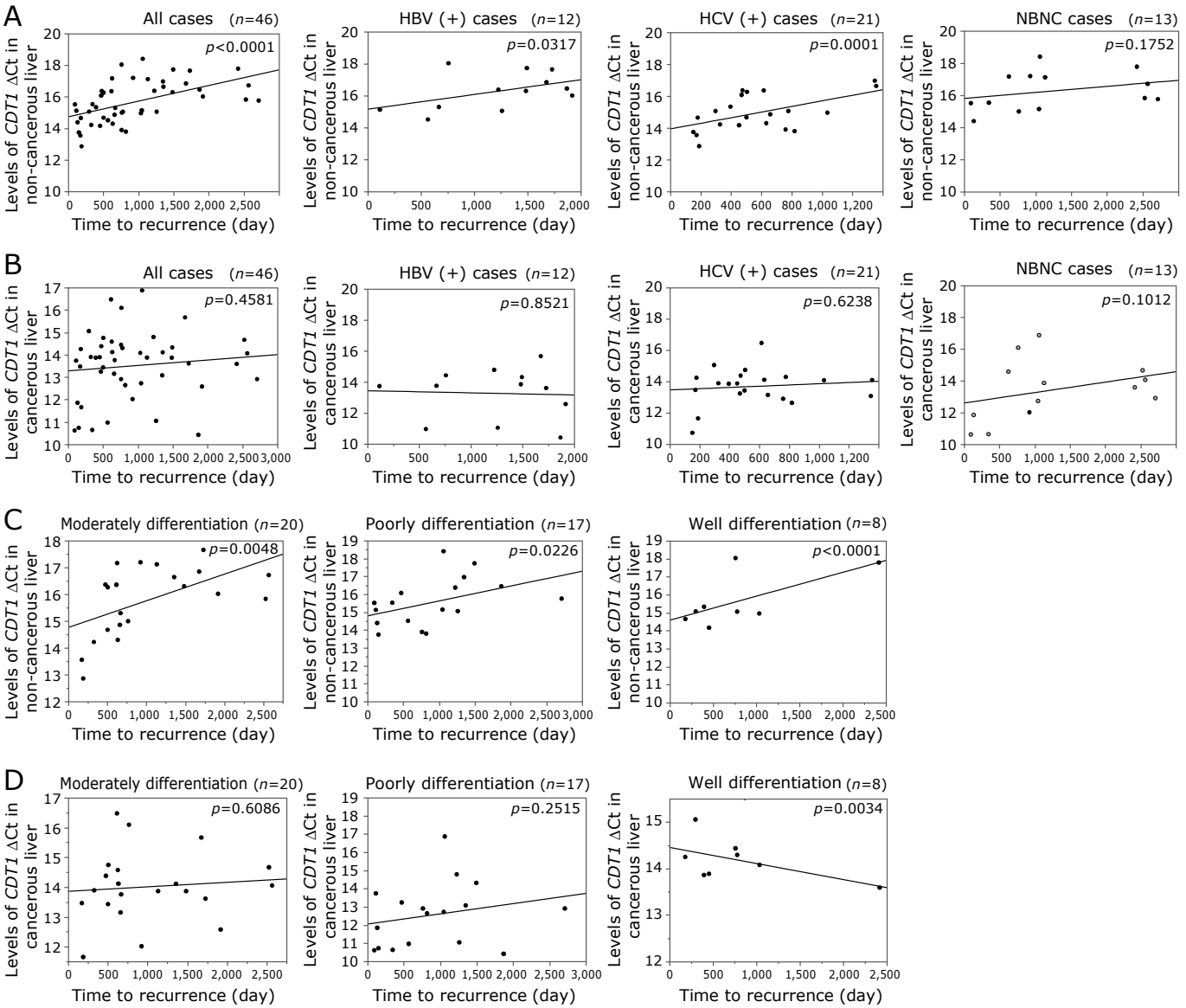

Fig. 4. Association between time to postoperative recurrence and the *CDT1* ΔCt levels in non-cancerous frozen liver tissue. (A) All cases ($p<0.0001$, left panel), HBV (+) cases ($p = 0.0317$. middle left panel), HCV (+) cases ($p = 0.0001$, middle right panel), NBNC cases ($p = 0.1752$, right panel). Data analyzed by robust regression analysis with Huber M estimation. Association between time to recurrence and *CDT1* ΔCt in cancerous liver tissue. (B) All cases ($p = 0.0109$, left panel), HBV (+) cases ($p = 0.8521$, middle left panel), HCV (+) cases ($p = 0.6238$, middle right panel) and NBNC cases ($p = 0.1012$, right panel). Data analyzed by robust regression analysis with Huber M estimation. The association between time to recurrence and *CDT1* ΔCt according to the differentiation group of cancer cells in non-cancerous frozen liver tissue. (C) Moderately differentiation group ($p = 0.0048$, left panel), poorly differentiation group ($p = 0.0026$, middle panel), well differentiation group ($p<0.0001$). The association between time to recurrence and the *CDT1* ΔCt according to the differentiation group of cancer cells in cancerous frozen liver tissue. (D) Moderately differentiation group ($p = 0.6086$, left panel), poorly differentiation group ($p = 0.2515$, middle panel), well differentiation group ($p = 0.0034$). All data were analyzed by robust regression analysis with Huber M-estimation.

cells, suggesting that CDT1 may be associated with carcinogenesis. In addition, we supported our hypothesis that high levels of CDT1 expression indicate a highly carcinogenic state by morphological study.

Discussion

The results of this study showed the following: *CDT1* mRNA expression in cancerous frozen liver tissues and *CDT1* mRNA expression in non-cancerous frozen liver tissues is overwhelmingly higher in cancerous liver (Fig. 2C). However, *CDT1* ΔCt levels in cancerous liver were significantly correlated with *CDT1* ΔCt levels in non-cancerous liver (Fig. 5A). By different etiologies, *CDT1* mRNA expression in cancerous liver tissues was significantly correlated with *CDT1* mRNA expression in non-cancerous liver tissues in cases with HBV (+) and HCV (+), while a similar trend, although not significant, was observed in cases with NBNC (Fig. 5A–C). In other words, cases with high *CDT1* mRNA expression in cancerous liver also showed high *CDT1* mRNA expression in non-cancerous liver. *CDT1* mRNA expression in cancerous liver tissue varied depending on the differentiation of the cancer cells. *CDT1* mRNA expression tended to increase as differentiation worsened (Fig. 2D). Regarding the duration of postoperative recurrence, only *CDT1*

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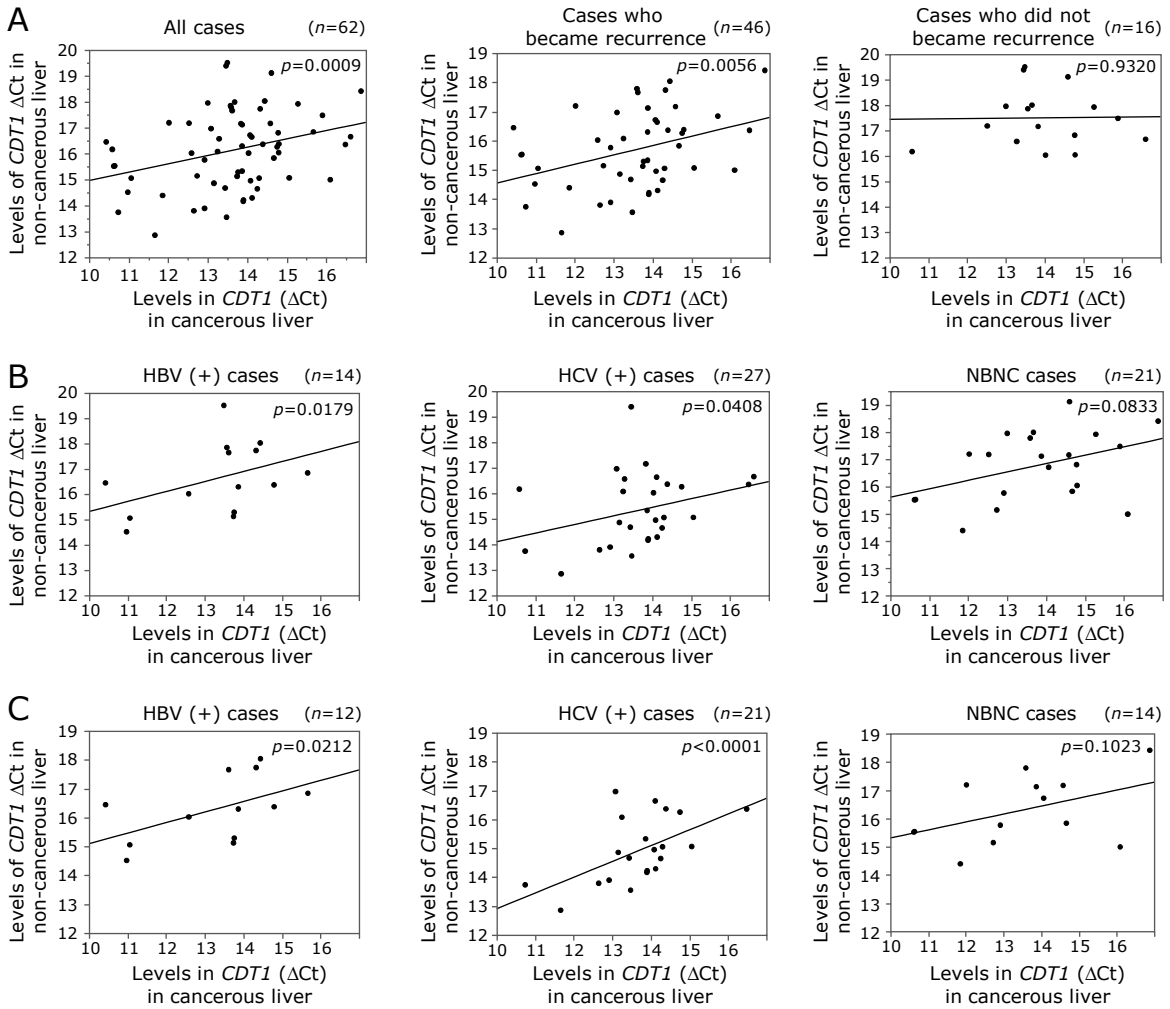

Fig. 5. (A) Comparison of *CDT1* ΔCt levels between cancerous and non-cancerous liver. The relationship of *CDT1* ΔCt levels between cancerous and non-cancerous liver tissues ($n = 62$, $p = 0.0009$, left panel). The relationship of *CDT1* ΔCt levels between cancerous and non-cancerous frozen liver tissues in cases who developed HCC recurrence ($n = 46$, $p = 0.0054$, middle panel) and cases who did not develop HCC recurrence ($n = 16$, $p = 0.9320$, right panel) during the observation periods. Calculated by robust regression analysis with Huber-M estimation. (B) The relationship between the levels of *CDT1* ΔCt in non-cancerous and cancerous liver tissues in cases with HCC recurrence with HBV (+) ($n = 14$, $p = 0.0179$, left panel), with HCV (+) ($n = 27$, $p = 0.0408$, middle panel) and with NBNC ($n = 21$, $p = 0.0833$, right panel). Calculated by robust regression analysis with Huber M estimation. (C) The relationship between the levels of *CDT1* ΔCt in non-cancerous and cancerous liver tissues in cases who did not develop HCC recurrence during the observation periods with HBV (+) ($n = 12$, $p = 0.0212$, left panel), with HCV (+) ($n = 21$, $p<0.0001$, middle panel) and with NBNC ($n = 14$, $p = 0.1023$, right panel). Calculated by robust regression analysis with Huber-M estimation.

mRNA expression in non-cancerous liver tissue was significantly associated, indicating that cases with higher *CDT1* mRNA expression also had shorter recurrence duration (Fig. 4A and B). Furthermore, only *CDT1* mRNA expression in the non-cancerous liver of HBV (+) and HCV (+) cases was found to be associated with the duration of recurrence.

The association between *CDT1* mRNA expression and the duration of recurrence by each differentiation of cancer cells suggests that the duration of recurrence is not related to the amount of *CDT1* mRNA expression in the cancerous area, but to the amount of *CDT1* mRNA expression in the non-cancerous area (Fig. 4C and D). Differences in *CDT1* mRNA expression by etiology were observed only in non-cancerous liver tissue, with NBNC cases tending to have lower *CDT1* mRNA expression than HBV and HCV (+) cases (Fig. 3C). However, there was no difference in *CDT1* mRNA expression by etiology in cancerous liver tissue (Fig. 3D).

The results of the present study would be consistent with the hypothesis that the amount of POAH determines postoperative recurrence and its duration. Intrahepatic lymphocytic infiltration is significantly associated with *CDT1* mRNA expression. This is consistent with the fact that high AST and ALT levels are risk factors for the development of HCC from CHC or LC, although it must be considered that *CDT1* mRNA expression may be due to the abundance of lymphocytes. In other words, the higher the *CDT1* mRNA expression, the greater the degree of intrahepatic inflammatory cell infiltration and the more advanced the state of intrahepatic fibrosis. On the other hand, NBNC cases were not significantly associated with the degree of inflammatory cell infiltration, but were associated with the progression of intrahepatic fibrosis. An important conclusion is that regardless of etiology, cases with a strong degree of POAH also have a high *CDT1* mRNA expression, verifying that the degree of POAH is significantly associated with the degree of *CDT1* mRNA expression.

Furthermore, we were able to confirm that high CDT1 expression, which is associated with the extent of POAH, represents a highly carcinogenic state of the liver, as our previous hypoth-

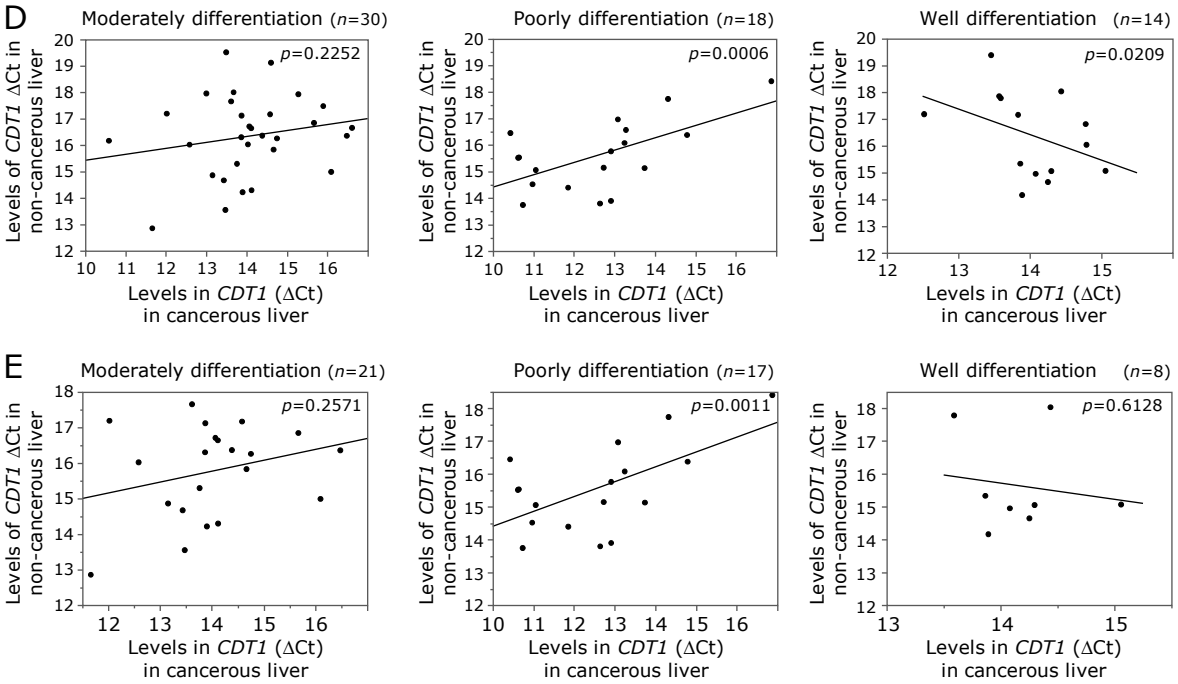

Fig. 5. continued

esis.[4] In this study of clinical laboratory tests and histologic findings in non-cancerous HE stained sections, *CDT1* mRNA expression was higher in cases with higher transaminase levels, in cases with decreased hepatic spare ability, in cases with progression to F stage, and in cases with greater intralobular lymphocyte infiltration in cases with HBV (+) or HCV (+). These factors were significantly correlated with those considered to be important clinical background factors for the development of HCC in cases with chronic liver disease.[1–3] These facts are consistent with the increased risk of carcinogenesis according to the progression of F stage (Fig. 2A). Typically, hepatocyte populations in non-cancerous liver tissue that has already developed HCC are not uniform, and different cell populations compete with each other, as described in our previous reports.[2,4] Therefore, even in hepatocytes that are morphologically close to normal, genetic abnormalities such as high expression of CDT1 have already accumulated in the non-cancerous liver, which is considered to be in a high carcinogenic state. This is also evident from the comparison between the normal and non-atypical hepatocyte populations in the non-cancerous HE stained section in our previous study.[4] These results are consistent with our belief that findings in the non-cancerous liver are important in predicting recurrence.

Multistep carcinogenesis based on an inflammatory response appears to be an important mechanism for HCC development or recurrence, especially in cases with chronic HCV and HBV infection. In addition, *CDT1* mRNA expression varied according to the histologic differentiation of HCC. Poorly differentiated cells had significantly higher *CDT1* mRNA expression than moderately or well differentiated cancer cells. This result supports our hypothesis that CDT1 can be used as a direct marker to reveal the malignant potential of cells. On the other hand, in HCV (+) cases, there was a significant correlation in *CDT1* mRNA expression between the degree of lymphocyte infiltration in the parenchyma and portal area and the degree of lymphoid aggregation in the portal area (Table 3). From these facts, the carcinogenic mechanisms of HCV (+) seemed to be based on the inflammatory reaction or immune responses. However, it is characteristic that NBNC showed no significant correlation between the level of lymphocyte infiltration or lymphoid aggregation in the portal area and the expression of *CDT1* mRNA (Table 3). The mechanism of HCC carcinogenesis can be broadly classified into the necro-inflammatory response caused by hepatitis virus and direct cellular damage caused by other factors, such as chemical carcinogen-induced liver cancer.[15,16] This phenomenon is consistent with our findings in histologic analysis of the relationship between CDT1 expression and inflammatory responses in HCV (+) cases. In other words, the mechanism of carcinogenesis observed in the NBNC case may indicate that CDT1 expression is associated with carcinogenesis not only in cell regeneration based on immune response, but also in direct cytopathic damage.

Even in the same non-cancerous liver tissue, there were cases where the degree of CDT1 staining varied depending on the location of the lobule. That is, in some cases moderate CDT1 expression could be seen in non-atypical hepatocyte populations.[4] These results also show that the non-atypical hepatocyte population is not necessarily a homogeneous or normal cell population, but rather a mixture of cells in which the gene abnormalities are already excited. This fact implies that CDT1 expression is also different in non-atypical hepatocytes in the globules in some cases. These results also suggested that there was heterogeneity in the non-atypical hepatocytes, as they were close to normal hepatocytes or close to atypical hepatocytes as described in our previous study.[4] To summarize the above, the expression of CDT1 in non-cancerous liver depends on the abundance of atypical hepatocytes. However, population of atypical hepatocytes is not homogeneous, and there are a variety of different states of each atypical hepatocytes. The reason why CDT1 expression in non-cancerous liver is correlated with the duration of recurrence is that the abundance of atypical hepatocytes, especially high-grade atypical hepatocytes which is atypical hepatocytes with high CDT1 expression, correlates with the levels of *CDT1* mRNA expression. The expression of *CDT1* mRNA of the non-atypical hepatocytes lesion in non-cancerous section varied depending on the morphological difference of the hepatocytes

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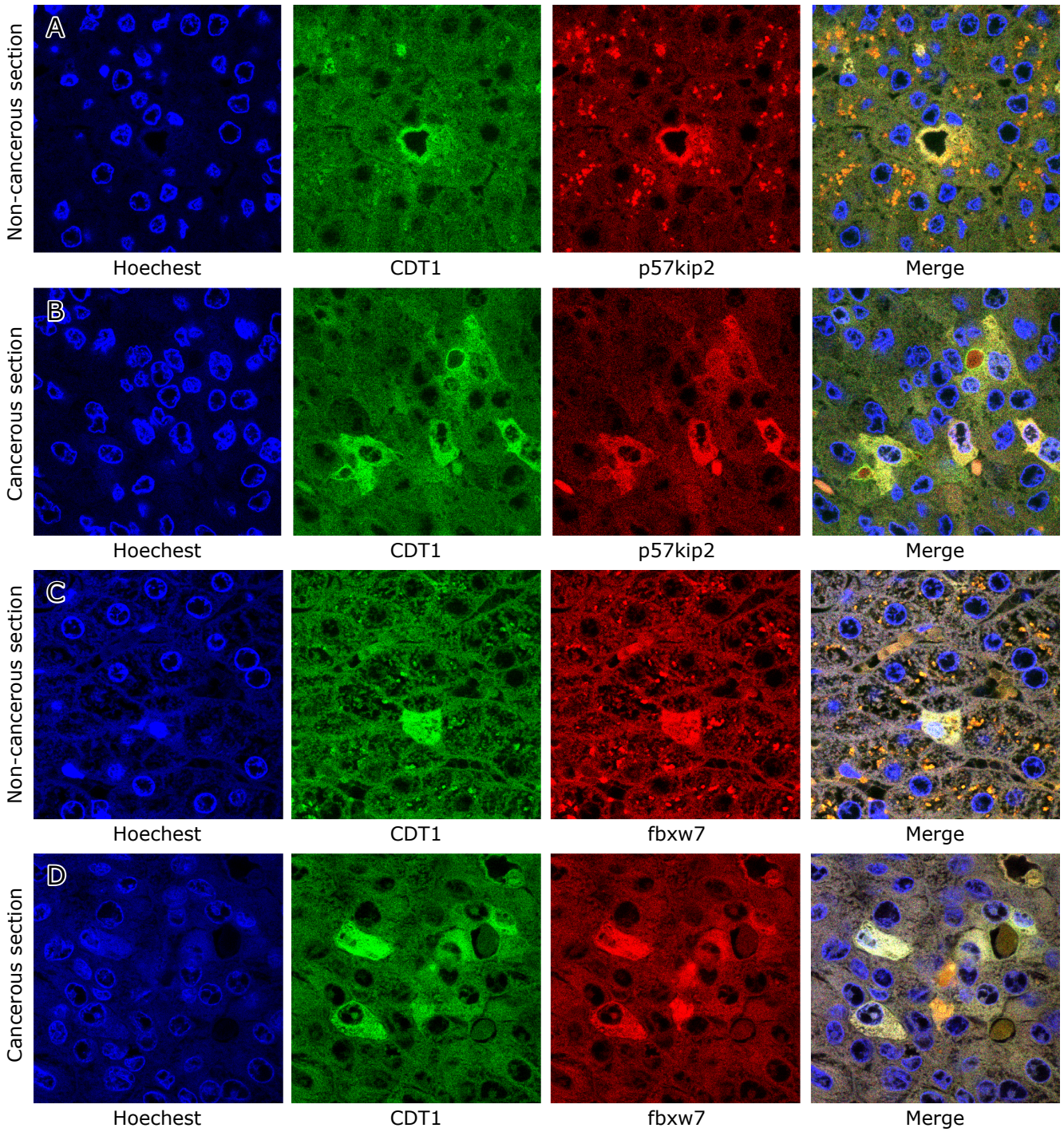

Fig. 6. Representative images of immunofluorescence in non-cancerous liver FFPE section using confocal laser scanning microscopy (CLSM). (A) Fluorescence staining of Hoechst 33342 (blue), P57kip2 (red), and CDT1 (green) and merge images (orange) in non-cancerous section. (B) Fluorescence staining of Hoechst 33342 (blue), P57kip2 (red), and CDT1 (green) and merge images (orange) in cancerous section. (C) Fluorescence staining of Hoechst 33342 (blue), WD repeat domain containing 7 (Fwbx7) (red), and CDT1 (green) and merge images (orange) in non-cancerous section. (D) Fluorescence staining of Hoechst 33342 (blue), Fwbx7 (red), and CDT1 (green) and merge images (orange) in cancerous section. (E) Fluorescence staining of Hoechst 33342 (blue), P53 (red), and CDT1 (green) and merge images (orange) in non-cancerous section. (F) Fluorescence staining of Hoechst 33342 (blue), P53 (red), and CDT1 (green) and merge images (orange) in cancerous section. (G) Fluorescence staining of Hoechst 33342 (blue), c-Myc (red), and CDT1 (green) and merge images (orange) in non-cancerous section. (H) Fluorescence staining of Hoechst 33342 (blue), c-Myc (red), and CDT1 (green) and merge images (orange) in cancerous section.

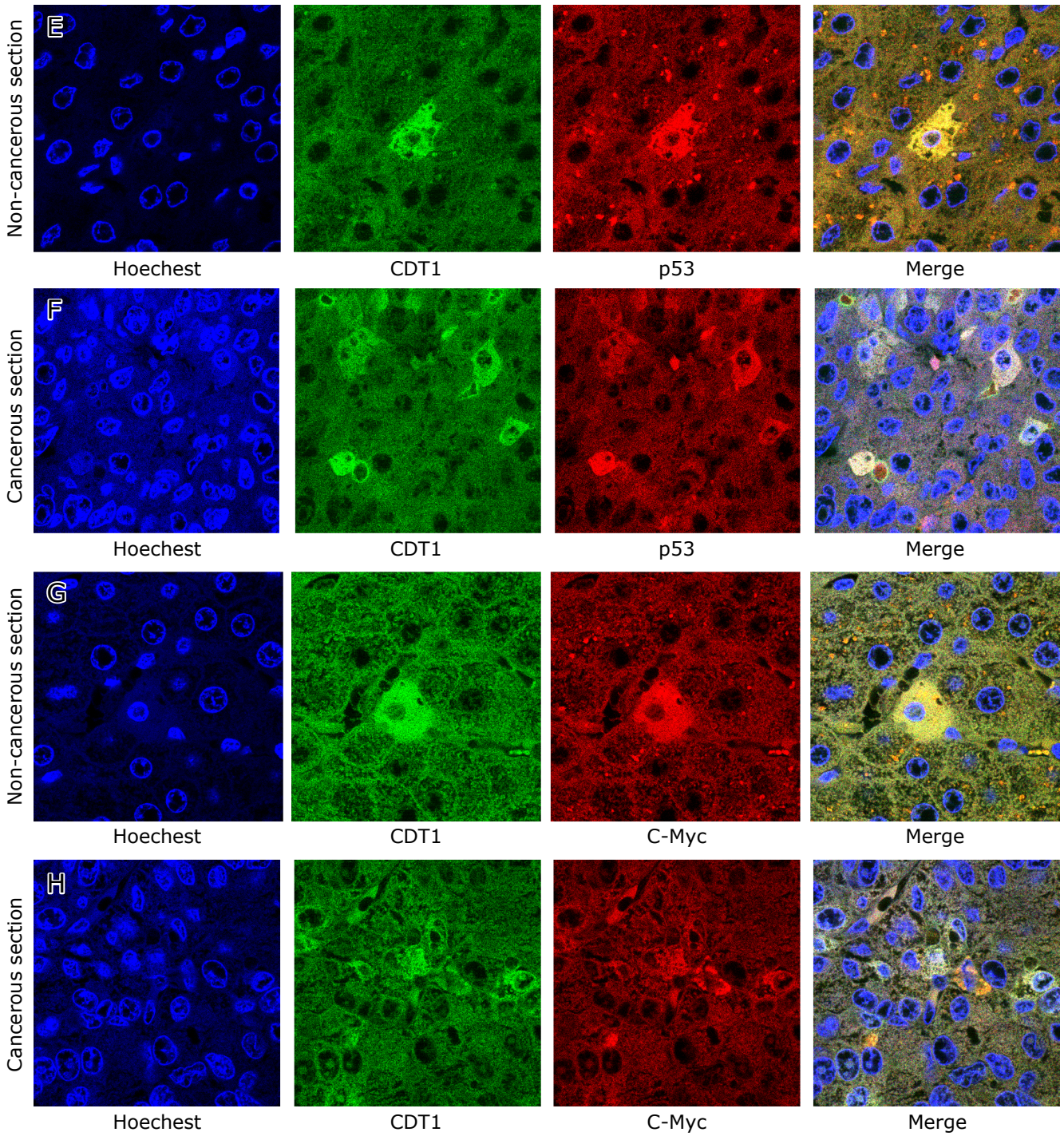

Fig. 6. continued

and was considered to be one of the conclusive pieces of evidence for multi-step carcinogenesis by described in our previous study.[4]

CDT1 is an important cell cycle control factor that is mainly expressed in the G1 phase and is degraded by the action of geminin.[17] Therefore, it is suggested that the reason why *CDT1* mRNA is highly expressed is that cell rotation from the G1 phase continues to be stimulated because it is not degraded. This overexpression of CDT1 is not an oncogene, but it is consistent as one of the pathogenic mechanisms of cancer development. In the study by co-author Midorikawa *et al.*,[4,18,19] no abnormalities of the CDT1 gene were found in HCC or its non-cancerous liver tissue. Therefore, we consider that the cause of CDT1 overexpression is an abnormality in the upstream promoter region or other regions.

We then investigated morphologically whether CDT1 overexpression has carcinogenic potential. We examined the co-localization of CDT1, which is thought to be expressed in POAH, with oncogenes and genes thought to be involved in cell cycle regulation and hepato-carcinogenesis. Ki-67 is used as a

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marker of cell proliferation because it is expressed in all cell nuclei except the G0 phase.[4,20] CDT1 staining patterns in non-cancerous liver sections were scattered and more widespread than staining patterns in Ki-67-positive cells in our previous study.[4] These facts indicate that excessive cell proliferation was not observed in the region of POAH, but is considered to be indicative of cell cycle progression.[4] p57 kip2 is expressed in post-mitotic cells of organs such as the liver and causes cell cycle arrest in G1.[21,22] Fbxw7 is the type of SCF ubiquitin ligase complex that targets positive regulators of the cell cycle, including cyclin E, c-Myc, Notch and c-Jun, for ubiquitination and subsequent degradation by the 26S proteasome to promote cell cycle exit.[23,24] The localization of CDT1-positive cells, which are considered identical to POAH, is consistent with P57 kip2 and Fbxw7-positive cells. Since P57 kip2 and Fbxw7 gene expression has been reported to be involved in carcinogenesis.[21–24] We considered whether excessive expression of CDT1 in POAH is also associated with hepato-carcinogenesis. Furthermore, the expression of P53- and c-Myc-positive hepatocytes were almost the same as the degree of CDT1-positive hepatocytes in lobules, but the number of positive cells was more than CDT1-positive cancer cell in cancer nodule. Therefore, we assume that CDT1-positive cells have malignant potential, because the localization of CDT1 in cancer cells is co-localized in the nucleus with genes involved in oncogenes, and CDT1 co-localization with oncogenes is also observed in POAH even in non-cancerous areas. It is also possible that hepatocytes overexpressing CDT1 already have a genetic abnormality as a cancer cell, and there is no contradiction as a preneoplastic cell. We consider that these results also suggest that CDT1 may be associated with oncogene addiction.

In fact, it has been reported that high expression of CDT1 in other cancers worsens the long-term prognosis of cases. It has been reported in prostate adenocarcinoma, breast cancer, HCC, ovarian cancer, etc. and our results also support to these results.[6–9] It has also been reported that CDT1 and Geminin are involved in the state of malignant transformation or preneoplastic entity by exciting DNA replication abnormalities and genomic instability.[25]

Also in this study, it was shown that CDT1 expression clinically represents a highly carcinogenic state of the liver, so we consider it is important to confirm the expression of CDT1 in non-cancerous tissues in the future in order to predict the long-term prognosis of cases. Therefore, we believe that the results of our study will greatly contribute to the clinical practice of HCC in terms of the selection of initial treatment methods or the need for additional postoperative therapy and long-term prognosis.

The first limitation of this study is that the sample size is small. Therefore, we expect to study a larger number of cases in the future. In particular, *CDT1* mRNA detection using FFPE sections, by adding a larger number example, there is likely to be stared significant difference in statistically. In addition, the *CDT1* mRNA level should be considered to be influenced by the abundance of hematopoietic cells such as lymphocytes. These points need to be further investigated.

In summary, the expression of CDT1 in non-cancerous liver depends on the abundance of POAH. The reason why CDT1 expression in non-cancerous liver is correlated with the duration of recurrence is that the abundance of POAH, especially high-grade POAH with high CDT1 expression, is associated with the level of *CDT1* mRNA expression. We found that CDT1 was the gene most responsible for the extent of POAH and the recurrence times, and the detection of expression of CDT1 could be a parameter of high carcinogenic state in cases with HCC regardless of the different etiology. In other words, high expression of CDT1 indicates that the liver is in a high carcinogenic state and the heterogeneity of CDT1 expression in different hepatocyte populations suggested multi-step carcinogenesis in the liver. These results suggest that hepatocytes overexpressing CDT1 with abnormal morphological features may be involved in the induction or differentiation of cancer cells.

Author Contributions

MO, YM, and MM contributed to data collection, statistical analyses, interpretation of data, the writing of the manuscript and was the main author of the manuscript. TT and HNakayama contributed to data and sample collection, MM were contributed for study concept and design. MM, HNakamura, and MS were contributed for histologic analysis. KKanemaru, TM, and MM were contributed for IF or confocal laser scanning microscope study analysis. MM, MO, YM, KKuroda, and TS were responsible or analysis and interpretation of data. MO and MM were responsible for drafting of the manuscript. MM and KKuroda were responsible for statistical analysis. MM, TT, and MS were responsible for study supervision.

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Consent for Publication

Not applicable.

Abbreviations

CDT1	chromatin licensing and DNA replication factor 1
CHC	chronic hepatitis C
CLSM	confocal laser scanning microscope
ΔCt	$Ct_{\ CDT1\ \text{genes test}} - Ct_{\ \beta\text{-actin}}$
FBXW7	F-box and WD repeat domain containing 7
FFPE	formalin-fixed paraffin-embedded
HE	hematoxylin and eosin
HBV (+)	hepatitis B virus surface antigen-positive
HCC	hepatocellular carcinoma
HCV (+)	hepatitis C virus antibody-positive
IHC	immunohistochemistry
IF	Immunofluorescence study
IR	irregular regeneration
LC	liver cirrhosis
POAH	proliferation of atypical hepatocytes
RFS	relapse free survival
RT-qPCR	real-time quantitative PCR

Conflict of Interest

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

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