

# Impact of *hepcidin* mRNA expression in the liver of patients with chronic hepatitis C or liver cirrhosis

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## Abstract

**Background:** We aimed to investigate the correlation between *hepcidin* mRNA expression and the pathophysiology of the liver or the long-term prognosis in patients with chronic hepatitis C (CHC) or liver cirrhosis (LC).

**Methods:** A total of 82 patients with CHC or LC who underwent liver biopsy were included in this study. *Hepcidin* mRNA expression was detected in the frozen liver tissues obtained from patients with CHC or LC who underwent liver biopsy. *Hepcidin* mRNA expression in frozen liver tissues was assessed using real-time quantitative polymerase chain reaction. We previously reported that the extent of proliferation of atypical hepatocytes (POAH) within the six-cell lesion of irregular regeneration (IR) of hepatocytes is a significant histological finding associated with the development of hepatocellular carcinoma (HCC) from CHC or LC. Therefore, we investigated the association between *hepcidin* mRNA expression and disease severity, pathological findings such as the extent of POAH, and the incidence of HCC development from CHC and LC. Furthermore, we compared *hepcidin* mRNA expression with serum cytokine and chemokine levels using a Bio-plex suspension array system (Bio-Rad Laboratories, CA, USA).

**Results:** *Hepcidin* mRNA expression was associated with serum ferritin concentration ( $p=0.009$ ) and unsaturated iron-binding capacity ( $P=0.0003$ ) in the serological parameters, but no association was found with histological parameters. The cumulative incidence of HCC development was significantly higher in the low *hepcidin* mRNA expression group than in the high *hepcidin* mRNA expression group ( $p=0.003$ ). Additionally, patients with lower *hepcidin* mRNA levels tended to exhibit more extensive POAH ( $p=0.0089$ ). *Hepcidin* mRNA expression was also significantly associated with apoptosis-related and anti-inflammatory cytokines/chemokines.

**Conclusions:** Low *hepcidin* mRNA expression may be a risk factor for carcinogenesis in patients with CHC or LC.

## Keywords:

hepcidin, Real-time quantitative RNA (RT-qPCR), hepatocellular carcinoma (HCC), chronic hepatitis C (CHC), liver cirrhosis (LC), Bio-plex suspension array, proliferation of atypical hepatocytes (POAH)

## Competing interests:

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

## Introduction

Although iron is an essential element for the human body,

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Received: November 15, 2023

Accepted: January 22, 2024

Released online: February 15, 2024



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©2024 Moriyama M. et al. [DOI https://doi.org/10.11299/metallomicsresearch.MR202310](https://doi.org/10.11299/metallomicsresearch.MR202310)

excessive amounts of free iron ( $\text{Fe}^{2+}$ ) can be toxic. Excess iron can catalyze the formation of free radicals, which may lead to various cellular disorders. Therefore, the human body tightly regulates intracellular iron levels and the peptide hormone hepcidin, plays a crucial role by binding to the iron export transporter ferroportin (FPN) [1]. Elevated serum iron levels results in rapid inhibition of iron transport [2] and the degradation of cell-surface FPN [3-6], which in turn inhibits FPN-mediated dietary iron absorption and iron release from intracellular iron stores. As a result, dysregulation of hepcidin or FPN expression can lead to iron disorders in humans.

Hepcidin expression in the liver is a complex process that is regulated by various factors including iron overload and inflammation which increase hepcidin expression, whereas hypoxia and erythropoiesis decrease expression [7-9]. In patients with chronic viral liver disease, hepcidin expression has also been reported to influence hepatic morbidity and pathophysiology [10-12]. We hypothesized that hepatic hepcidin expression is associated with liver morbidity in nonalcoholic steatohepatitis (NASH). Therefore, we previously investigated hepatic hepcidin expression using a rat model of NASH development [13], and found that *hepcidin* mRNA expression decreases with the progression of NASH, which led us to speculate that hepcidin may be linked to the pathogenesis of NASH.

According to reports, hepcidin is involved in the progression of steatohepatitis, including NASH and metabolic dysfunction-associated steatotic liver disease [14-16]. Impaired iron translocation from the hepatocytes and Kupffer cells is the primary cause of iron accumulation in nonalcoholic fatty liver disease. Impaired iron transport is associated with inflammation and metabolic abnormalities that affect iron regulators, such as hepcidin, ferroportin, transferrin receptors, ferritin, and copper [17,18].

Furthermore, our previous research revealed that phlebotomy in patients with chronic hepatitis C (CHC) or liver cirrhosis (LC) can reduce transaminase levels and the cumulative incidence of hepatocellular carcinoma (HCC) development in patients with CHC and LC [19]. Therefore, we hypothesized iron overload is associated with the progression of CHC or LC and the development of HCC from CHC or LC. We have also reported that intrahepatic iron concentration affects the pathogenesis of chronic hepatitis [20]. Herein, we hypothesized that liver iron overload and *hepcidin* mRNA expression in the liver are linked to the severity of liver disease and the development of HCC.

Regarding histological factors that are associated with carcinogenesis in liver sections, the degree of irregular regeneration (IR) of hepatocytes was reported by Uchida et. al. [21-23]. The theory of the IR of hepatocytes was proposed by Peters [24] and Uchida [21-23]. The IR of hepatocytes in lobules is a general term encompassing anisocytosis of hepatocytes, map-like distribution, nodular arrangement of parenchyma, oncocytic change of hepatocytes, proliferation of atypical hepatocytes, and bulging of hepatocytes.

Recently, we defined the grade according to the IR of each group of hepatocytes, and examined the correlation between the IR grade in hepatocytes and the occurrence of HCC in chronic hepatitis C or cirrhosis [25]. The analysis revealed that the extent of proliferation of atypical hepatocytes (POAH) was a strong histological risk factor for HCC development. Atypical hepatocytes are generally characterized by smaller cells with a higher nucleoplasmic (N/C) ratio, nuclei that show mild size disparity, cytoplasm with enhanced eosinophilia, a clear distinction between atypical and normal hepatocytes, and the absence of fat droplets [26,27]. It has been reported that the degree of proliferation of atypical hepatocytes (POAH) within the six-cell lesion of IR of hepatocytes is a significant histological finding associated with the development of HCC from CHC or LC [19,23,25]. Therefore, we hypothesized that the level of expression of *hepcidin* mRNA in chronic hepatitis represents a highly carcinogenic state of the liver. Hence, we investigated the relationship between liver histology, including the extent of POAH and *hepcidin* mRNA expression.

We then examined the cytokines/chemokines that regulate *hepcidin* mRNA expression using a multiplex cytokine assay to detect serum cytokine/chemokine concentrations. Based on these studies, we investigated whether *hepcidin* mRNA expression levels are associated with the progression of liver disease and the development of HCC from CHC and LC. The expression of *hepcidin* mRNA was quantified using the formula:  $\Delta Ct = Ct_{\text{for hepcidin gene test}} - Ct_{\text{glyceraldehyde-3-phosphate dehydrogenase (GAPDH) test}}$ .

## Materials and methods

### Participants

The study population comprised 82 hepatitis C virus (HCV) RNA-positive patients who underwent liver biopsy at Nihon University Itabashi Hospital between January 1, 2001 and December 25, 2008. These patients were selected based on the feasibility of using cryopreserved liver biopsy specimens. The participants were HCV RNA-positive patients with CHC or LC

who consented to long-term prognostic evaluation and participation in the study. The clinical characteristics of the patients are presented in **Table 1**. The enrolled patients were assigned an F stage according to the INUYAMA and Demet classification, with F0 to F3 designated as CHC, and F4 as LC [28,29].

Of the 82 patients, 49 whose cytokine and chemokine levels were previously measured [30] were analyzed for correlations between *hepcidin* mRNA expression and cytokine and chemokine concentrations. These patients comprised 27 males and 22 females and were classified into four F stages as follows: F1 (n=21), F2 (n=13), F3 (n=6), and F4 (n=9).

**Table 1.** | Clinical profiles of participants (n=82)

	Chronic hepatitis (F0 to F3)	Liver cirrhosis (F4)
number	63	19
observation periods (yrs)	8.8 (0.1-14.1)	6.3 (0.4-12.4)
age (yrs)	54.0 (23.5-73.7)	59.8 (44.8-74.1)
gender (males)	35 (54.7%)	9 (47.4%)
AST (U/L)	44.5 (15-183)	74.0 (40-129)
ALT (U/L)	55.5 (10-328)	79.0 (45-138)
r-GT (U/L)	39.0 (10-303)	50.0 (24-128)
ALP (U/L)	246.0 (111-624)	331.0 (163-1370)
total bilirubin (mg/dl)	0.60 (0.25-1.47)	0.70 (0.40-1.38)
platelet counts(x10 <sup>4</sup> )	18.2 (7.3-38.1)	11.9 (9.6-22.8)
total protein (g/dl)	7.2 (6.1-8.6)	7.3 (6.7-8.6)
albumin (g/dl)	4.0 (3.4-4.7)	3.6 (3.1-6.9)
prothrombin time (%)	96 (69-100)	92 (79-100)
BTR	5.94 (4.06-12.20)	5.29 (2.82-8.00)
BCAA	489 (352-677)	477 (259-825)
tyrosine	83 (40-126)	92 (63-151)
ammonia (µg/dl)		
ICGR15 (%)	7.0 (1.4-21.2)	14.0 (2.1-71.1)
zinc concentration (µg/dl)	74 (47-101)	64 (48-100)
F stages		
F0	0	
F1	32 (50.8%)	
F2	21 (33.3%)	
F3	9 (14.3%)	
F4		19
HCV RNA		
high	52 (82.5%)	17 (89.5%)
low	11 (17.5%)	2 (10.5%)
Serotype		
1	41 (49.4%)	12 (14.5%)
2	22 (26.6%)	7 (8.4%)

*p* was obtained using the Mann-Whitney U test,

Abbreviations: BCAA, branched chain amino acids; BTR, BCAA to tyrosine molar ratio; CH, chronic hepatitis; LC, liver cirrhosis; AST, aspartate amino transferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; r-GT, γ-glutamyltransferase; ICGR15, the retention rate of indocyanine green at 15 min, HCV RNA high, ≥10<sup>6</sup>copy/mL; HCV RNA low, <10<sup>5</sup>copy/mL

## Clinical and laboratory evaluation of patients with CHC and LC

Serum and liver biopsy specimens were collected at the time of liver biopsy and stored at  $-80^{\circ}\text{C}$  until analysis. Samples were only collected from the patients who provided informed consent for their serum samples to be stored for subsequent laboratory analysis. The exclusion criteria for CHC and LC were as follows: age  $< 18$  years, habitual alcohol consumption ( $> 30$  g ethanol/day), presence of hepatitis B surface antigen (enzyme-linked immunosorbent assay (ELISA); Abbott Tokyo, Japan), presence of anti-smooth muscle antibody (fluorescent antibody method; FA), presence of anti-mitochondrial M2 antibody (enzyme immunoassay; EIA), and current intravenous drug use. All patients were positive for serum HCV RNA and were followed up for  $> 1$  year. A definitive diagnosis of HCC was obtained using abdominal angiography or liver tumor biopsy. HCC nodules were evaluated every 3–12 months if detected using contrast-enhanced abdominal ultrasonography or enhanced CT. All patients were closely followed up for HCC development at our outpatient clinic until May 31, 2020.

## Hematological and biochemical examinations

The serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alanine phosphatase (ALP),  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT), total bilirubin, total protein (TP), albumin (Alb), branched-chain amino acid-to-tyrosine molar ratio (BTR), prothrombin time (PT), platelet count, and zinc were evaluated. In addition, the serum concentration of alpha-fetoprotein (AFP) was measured as a tumor marker, and the indocyanine green retention rate at 15 min (ICGR15) was also assessed. Serum zinc concentrations were determined using conventional atomic absorption spectrophotometry with a Zeeman polarized atomic absorption spectrophotometer (Z-6100; Hitachi, Tokyo, Japan). Serum ferritin levels were assessed using chemiluminescent ELISA or EIA (SRL, Tokyo, Japan). Serum levels of HCV RNA were determined using the Amplicor HCV Monitor (Roche Diagnostics KK, Tokyo, Japan) or TaqMan PCR methods (Cobas TaqMan HCV [auto] v2.0; Roche Diagnostics KK, Tokyo, Japan). Each patient's serum HCV RNA level was classified as high ( $\geq 100$  kilo copies/mL or  $5.0 \log \text{U/mL}$ ) or low ( $< 100$  kilo copies/mL or  $5.0 \log \text{U/mL}$ ). The HCV serotype was determined using an EIA kit (Imucheck F-HCV Gr1 and Gr2 reagents; International Reagent Corporation, Tokyo, Japan) according to the manufacturer's instructions.

## Histological analysis of hematoxylin-eosin-stained sections from liver biopsy

Liver biopsy specimens were obtained from patients with CHC or LC through percutaneous needle biopsy (Tru-Cut soft tissue biopsy needles, 14 G, Baxter, Deerfield, IL, USA) or hard Monopty needles (14 G, Medicon, Tokyo, Japan). These sections were fixed in 10%–20% buffered formalin and embedded in paraffin. The paraffin-embedded specimens were cut into 3- to 4- $\mu\text{m}$  sections and stained with hematoxylin and eosin (HE). Each liver biopsy specimen was analyzed semi-quantitatively, with a score assigned to each parameter, as previously reported [25,27,30]. The HE-stained sections obtained from the liver biopsies performed at our department were evaluated by the first author and co-authors at a conference without knowledge of the patients' characteristics. The data were entered into a database and subsequently relevant patient data were retrieved from the database and analyzed.

## Real-time quantitative PCR analysis

*Hepcidin* mRNA expression was evaluated using real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from the liver using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, U.S.A.) [27]. Complementary DNA was synthesized from 4  $\mu\text{g}$  of total RNA using the SuperScript<sup>TM</sup> III First-Strand Synthesis System (Thermo Fisher Scientific). qPCR was performed using a THUNDERBIRD<sup>®</sup> SYBR qPCR Mix (TOYOBO, Osaka, Japan) and a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). Gene expression was normalized to GAPDH expression. The qPCR conditions included  $95^{\circ}\text{C}$  for 1 min, followed by 40 cycles of  $95^{\circ}\text{C}$  for 15 s, and  $60^{\circ}\text{C}$  for 60 s. The RT-qPCR data were analyzed using a standard curve. The correlation coefficient of the standard curve was  $> 0.90$  for all patients. The PCR primer sequences used for detection were hepcidin F (5'-CAC AAC AGA CGG GAC AAC TT-3'; 20-mer), hepcidin R (5'-CGC AGC AGA AAA TGC AGA TG-3'; 20-mer), GAPDH F (5'-TGA Superscript CAT CAA GAA GGT Superscript GAA G-3'; 25-mer), and GAPDH R (5'-TCC TTG GAG GCC ATG TGG GCC AT-3'; 23-mer). The expression of *hepcidin* mRNA was quantified using the formula:  $\Delta Ct = Ct_{\text{for hepcidin gene test}} - Ct_{\text{GAPDH test}}$ . The RT-qPCR experiments were performed in triplicate.

## Long-term patient outcomes

In order to assess long-term patient outcomes, we compared the cumulative probability of HCC based on the classification of *hepcidin* mRNA expression ( $\Delta$ Ct) as high or low. The high  $\Delta$ Ct group included patients with *hepcidin*  $\Delta$ Ct levels exceeding the middle value for all patients, while the low  $\Delta$ Ct group included those with *hepcidin*  $\Delta$ Ct levels below the middle level.

## Measurement of serum cytokine and chemokine concentrations

Cytokine and chemokine levels in the serum of 49 patients were measured using a Bio-plex Suspension Array System (Bio-Rad Laboratories, HERCULES, CA, USA) following concentrations measured in our previous study [30]. The cytokines and chemokines measured were cutaneous T-cell-attracting chemokine (CTACK), growth-regulated alpha protein (GRO $\alpha$ ), interleukin (IL)-1 $\alpha$ , IL-2 receptor  $\alpha$ (R $\alpha$ ), 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  $\beta$ (SCGF)- $\beta$ , stromal cell-derived factor 1  $\alpha$  (SDF-1 $\alpha$ ), tumor necrosis factor (TNF)- $\beta$ , tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), hepatocyte growth factor (HGF), Hu interferon  $\alpha$ 2 (IFN- $\alpha$ 2), platelet-derived growth factor receptor (PDGF)- $\beta$  $\beta$ , 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- $\gamma$ ), interferon gamma-induced protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1)(MCAF), macrophage inflammatory protein 1 (MIP-1 $\alpha$ ), MIP-1 $\beta$ , regulated on activation normal T-cell expressed and secreted (RANTES), TNF- $\alpha$ , and vascular endothelial growth factor (VEGF).

## Statistical analysis

Statistical analysis was performed using JMP 12 software (SAS Institute Inc., Tokyo, Japan). Data are expressed as the mean  $\pm$  SD or median (range). Categorical variables were compared using the Mann–Whitney U test (non-parametric). The Kruskal–Wallis and Steel–Dwass tests were used for multiple-group comparisons. The Spearman's rank correlation coefficient test was used for correlation analysis between the two groups.  $p < 0.05$  was considered statistically significant. The cumulative incidence of HCC development was estimated using the Kaplan–Meier method, and differences between groups were assessed using the log-rank test. Statistical analyses was performed using JMP 12 software (SAS Institute Inc., Tokyo, Japan).

## Ethical Approval

This study was approved by the Ethics Committee of Nihon University School of Medicine (RK-100611-13, # RK100910-15) and conducted in accordance with the tenets of the Declaration of Helsinki of 1964 and its subsequent amendments. Written informed consent was obtained from all participants. The enrolled patients agreed to cooperate with the study procedures and for publication of the results.

## Results

### *Hepcidin* mRNA expression ( $\Delta$ Ct) levels of liver biopsy specimens obtained from patients with CHC or LC

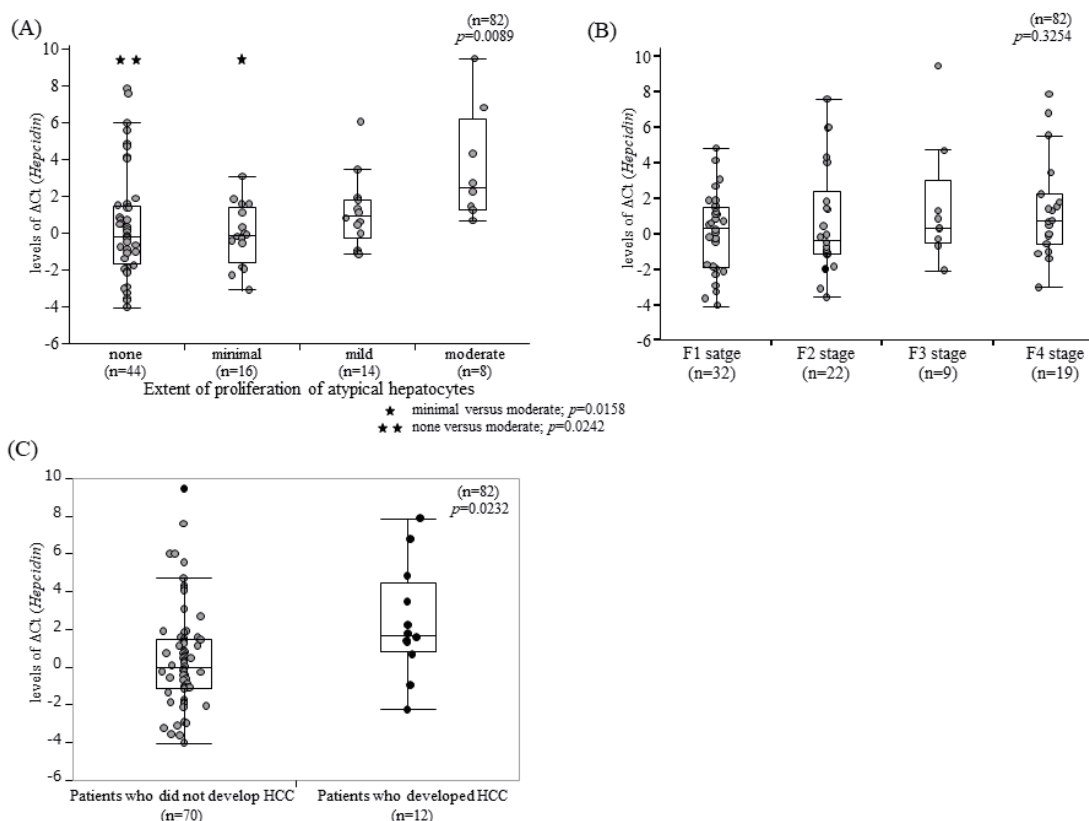
The median levels of *hepcidin* ( $\Delta$ Ct) did not show any significant differences between the sexes or between different age groups. Additionally, no significant differences were observed in the median *hepcidin*  $\Delta$ Ct levels between patients with different stages of chronic liver disease (F0 to F4) or between different HCV serotypes or serum HCV RNA levels (Table 2).

### Correlation between *hepcidin* $\Delta$ Ct levels and blood, biochemical, and histological parameters

*Hepcidin*  $\Delta$ Ct levels did not exhibit significant correlations with most biochemical parameters, except for serum ferritin concentration ( $r = -0.357$ ;  $p = 0.0161$ ) and unsaturated iron binding capacity ( $p = 0.0003$ ; Table 3). Interestingly, *hepcidin*  $\Delta$ Ct levels did not show any significant associations with the scores of histological factors, although a significant correlation was observed between the extent of POAH and *hepcidin*  $\Delta$ Ct levels ( $r = 0.288$ ,  $p = 0.0086$ ). Patients with higher *hepcidin*  $\Delta$ Ct levels were more likely to exhibit a greater degree of POAH (minimal versus moderate,  $p = 0.0158$ ; none versus moderate,  $p = 0.0242$ , Figure 1A). Notably, no significant association was observed between *hepcidin*  $\Delta$ Ct levels and the F-stages ( $p = 0.3254$ ; Figure 1B).

**Table 2.** | Associations between *hepcidin* mRNA expression ( $\Delta Ct$ ) and clinical characteristics of patients (n=82).

Parameter	number	r	p
Gender			0.5223
male	44	0.0744	
female	38	0.4817	
Age			0.0669
<65 yrs	66	0.2139	
≥65 yrs	16	1.3036	
Genotype			0.9200
1a	54	0.3267	
2a	15	0.5013	
2b	12	-0.1300	
HCV RNA			0.3351
High	67	0.5012	
Low	12	-0.2411	



**Figure 1.** (A) *Hepcidin* mRNA expression was quantified using the formula:  $\Delta Ct = Ct_{\text{for hepcidin gene test}} - Ct_{\text{glyceraldehyde-3-phosphate dehydrogenase (GAPDH) test}}$ . The degree of proliferation of atypical hepatocytes (POAH) and *hepcidin*  $\Delta Ct$  levels were significantly associated with the degree or extent of POAH ( $p=0.0089$ ) based on robust regression analysis using Huber M estimation. Significant differences in *hepcidin*  $\Delta Ct$  levels were observed between moderate and minimal ( $p=0.0158$ ) and none and moderate ( $p=0.0242$ ) POAH, with *hepcidin*  $\Delta Ct$  levels being significantly higher in moderate cases ( $p=0.0267$ ) based on Kruskal–Wallis and Steel–Dwass tests. (B) Comparison between *hepcidin*  $\Delta Ct$  levels and F1 to F4 stages in patients with hepatitis C virus (HCV) RNA-positive chronic hepatitis. No significant correlation was observed between *hepcidin*  $\Delta Ct$  level and F stage ( $p=0.3254$ ). Data were analyzed using Kruskal–Wallis and Steel–Dwass tests. (C) Comparison between *hepcidin*  $\Delta Ct$  levels and the development of hepatocellular carcinoma in patients. Patients who did not develop HCC exhibited significantly lower *hepcidin*  $\Delta Ct$  levels than those who developed HCC ( $p=0.0232$ ). Data were analyzed using ANOVA.

**Table 3.** Associations between *hepcidin* mRNA expression levels ( $\Delta$ Ct), blood and biochemical examination results or histological parameters of patients (n=82)

Parameter	number	r	p
<b>Blood and Serological Examination.</b>			
aspartate aminotransferase (U/L)	82	0.100	0.367
alanine aminotransferase (U/L)	82	0.017	0.875
$\gamma$ -glutamyl transpeptidase (U/L)	82	-0.114	0.306
alkaline phosphatase (U/L)	82	-0.099	0.306
total Bilirubin (mg/dl)	82	-0.176	0.112
platelet counts ( $\times 10^4$ )	82	-0.020	0.852
total protein (g/dl)	74	0.023	0.837
albumin (g/dl)	74	-0.151	0.210
prothrombin time (%)	74	0.031	0.779
BTR	62	-0.135	0.315
BCAA	62	0.089	0.508
tyrosine	62	0.191	0.153
indocyanine green retention rate 15min	62	0.043	0.700
alpha-feto protein (ng/ml)	70	0.029	0.798
zinc concentration ( $\mu$ g/ml)	62	-0.013	0.914
ferritin concentration (ng/ml)	62	-0.382	0.009
FIB-4	82	0.178	0.123
APRI	82	0.141	0.221
unsaturated iron binding capacity	62	0.582	0.0003
<b>Histology of liver biopsy section (score)</b>			
Irregular regeneration of hepatocytes	82		
anisocytosis of hepatocytes		0.084	0.448
bulging of hepatocytes		0.106	0.341
map-like distribution		0.123	0.269
oncocytic change of hepatocytes		0.089	0.424
nodular arrangement of hepatocytes		0.136	0.052
proliferation of atypical hepatocytes		0.288	0.0086
Inflammatory cell infiltration			
peri-portal		0.101	0.366
parenchyma		0.138	0.215
portal		-0.044	0.692
F stage		0.144	0.196
lymphoid aggregation of portal tract		-0.020	0.858
portal sclerotic change		-0.068	0.563
bile duct damage		-0.124	0.266
peri-venular fibrosis		-0.150	0.177
peri-cellular fibrosis		0.005	0.958
bridging necrosis		0.002	0.858
steatosis		0.173	0.118

Data were analyzed using Spearman's rank correlation coefficients. Abbreviations: BCAA, branched-chain amino acids; BTR, BCAA-to-tyrosine molar ratio, FIB-4; fibrosis-4; APRI, AST-to-platelet ratio index.

### Correlations between *hepcidin* $\Delta$ Ct levels and HCC development in patients

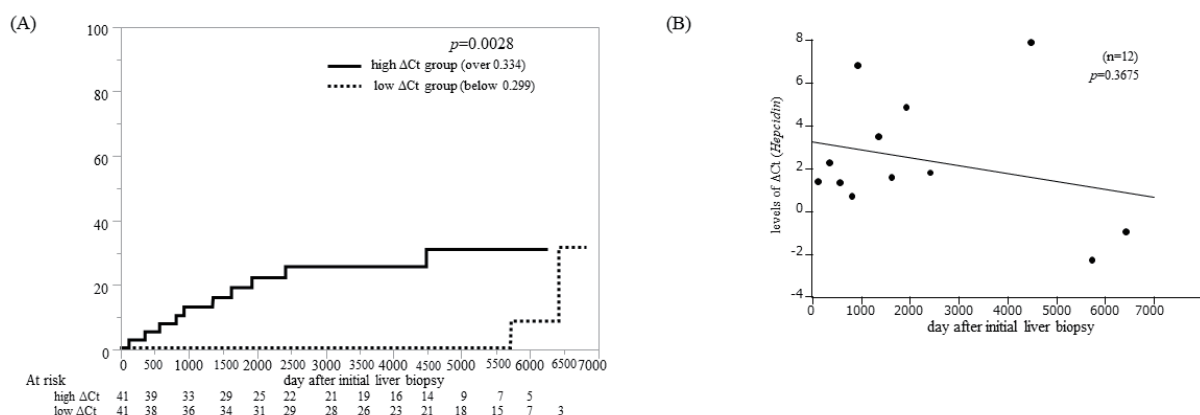
The relationship between *hepcidin*  $\Delta$ Ct levels and the development of HCC was analyzed in patients, and revealed that the levels of *hepcidin*  $\Delta$ Ct in the liver biopsy specimens of patients who developed HCC were significantly higher than those of patients who did not develop HCC ( $p=0.0232$ ; **Figure 1C**).

### Relationship between *hepcidin* $\Delta$ Ct levels and cumulative incidence of HCC development

The cumulative incidence of HCC development was analyzed in the cohort of 82 patients. These patients were divided into low ( $<0.299$ ) and high ( $\geq 0.334$ ) groups based on their *hepcidin*  $\Delta$ Ct levels. The low  $\Delta$ Ct group consisted of patients with *hepcidin*  $\Delta$ Ct expression below the middle value, whereas the high  $\Delta$ Ct group comprised patients with *hepcidin*  $\Delta$ Ct expression exceeding the middle level. The cumulative incidence of HCC in the low  $\Delta$ Ct group, after a median follow-up of 12.06 (range: 0.11–18.71) years, was significantly lower than that in the high  $\Delta$ Ct group, with a median follow-up of 8.13 (range: 0.32–17.18) years ( $p=0.0028$ ; **Figure 2A**). Moreover, the 5-year incidence rates were 0% and 19.2% in the low and high  $\Delta$ Ct groups, respectively. The association between *hepcidin*  $\Delta$ Ct levels in patients who developed HCC and the time to HCC development after the first liver biopsy was then examined. Although no statistically significant difference was observed, there was a tendency for a shorter time to HCC development in patients with low *hepcidin*  $\Delta$ Ct levels ( $p=0.3675$ ; **Figure 2B**). These findings suggest that higher *hepcidin*  $\Delta$ Ct levels are associated with an increased risk of HCC development.

### Correlations between serum cytokine/chemokine concentrations and *hepcidin* $\Delta$ Ct levels

Significant correlations were observed between *hepcidin*  $\Delta$ Ct levels and the concentrations of certain cytokines (**Table 4**). The *hepcidin*  $\Delta$ Ct levels was positively correlated with the concentrations of IL-1 $\alpha$  ( $r=0.3323$ ;  $p=0.0197$ ), IL-5 ( $r=0.3771$ ;  $p=0.0076$ ), IL-8 ( $r=0.2823$ ;  $p=0.0493$ ), IL-10 ( $r=0.3395$ ;  $p=0.0170$ ), IL-12 (p70) ( $r=0.4243$ ;  $p=0.0024$ ), IL-13 ( $r=0.3110$ ;  $p=0.0024$ ), G-CSF ( $r=0.3294$ ;  $p=0.0208$ ), GM-CSF ( $r=0.3208$ ;  $p=0.0246$ ), IFN- $\gamma$  ( $r=0.3063$ ;  $p=0.0323$ ), MIP-1b ( $r=0.2840$ ;  $p=0.0480$ ). Conversely, a negative correlation was observed between *hepcidin*  $\Delta$ Ct levels and the concentration of TRAIL ( $r=-0.2933$ ,  $p=0.0408$ ).



**Figure 2.** (A) The cumulative incidence of HCC development in the high *hepcidin*  $\Delta$ Ct group ( $\geq 0.334$ , straight line) was significantly higher than that in the low *hepcidin*  $\Delta$ Ct group ( $<0.299$ , dashed line) ( $p=0.0028$ ). Data were analyzed using the Kaplan–Meier method, and between-group differences were assessed using the log-rank test. (B) Comparison between days after initial biopsy during HCC development and *hepcidin*  $\Delta$ Ct levels ( $p=0.3675$ ). Data were analyzed using Spearman's rank correlation test.



**Table 4.** Associations between *hepcidin* levels ( $\Delta Ct$ ) and serum cytokine and chemokine concentrations (n=49). The expression of each gene mRNA was quantified using the formula:  $\Delta Ct = Ct_{\text{for each gene test}} - Ct_{\beta\text{-actin}}$ . RT-qPCR experiments were performed in triplicate.

	r	p		r	p
Hu CTACK	-0.0407	0.7812	Hu IL-2	0.2238	0.1222
Hu GROa	0.1041	0.4766	Hu IL-4	0.0254	0.8624
Hu IL-1 $\alpha$	0.3323	0.0197	Hu IL-5	0.3771	0.0076
Hu IL-2Ra	0.2457	0.0888	Hu IL-6	0.2791	0.0521
Hu IL-3	0.1984	0.1717	Hu IL-7	0.2149	0.1381
Hu IL-12p40	0.0416	0.7766	Hu IL-8	0.2823	0.0493
Hu IL-16	0.0280	0.8485	Hu IL-9	0.0889	0.5436
Hu IL-18	0.0272	0.8526	Hu IL-10	0.3395	0.0170
Hu LIF	-0.0367	0.8024	Hu IL-12(p70)	0.4243	0.0024
Hu MCP-3	0.0958	0.5126	Hu IL-13	0.3110	0.0296
Hu M-CSF	0.1273	0.3834	Hu IL-15	0.1814	0.2122
Hu MIF	0.0861	0.5565	Hu IL-17	0.1021	0.4851
Hu MIG	0.2319	0.1088	Hu Eotaxin	0.0131	0.9290
Hu b-NGF	0.1598	0.2726	Hu FGF basic	-0.1754	0.2280
Hu SCF	-0.0669	0.6477	Hu G-CSF	0.3294	0.0208
Hu SCGF-b	0.0456	0.7556	Hu GM-CSF	0.3208	0.0246
Hu SDF-1a	0.1161	0.4270	Hu IFN- $\gamma$	0.3063	0.0323
Hu TNF- $\beta$	0.2257	0.8692	Hu IP-10	0.0033	0.9820
Hu TRAIL	-0.2933	0.0408	Hu MCP-1(MCAF)	0.1930	0.1841
Hu HGF	0.0583	0.6909	Hu MIP-1a	-0.1451	0.3200
Hu IFN-a2	0.1822	0.2103	Hu MIP-1b	0.2840	0.0480
Hu PDGF-bb	0.0384	0.7935	Hu RANTES	0.1317	0.3669
Hu IL-1b	0.2537	0.0797	Hu TNF-alpha	0.2411	0.0952
Hu IL-1ra	-0.0337	0.8183	Hu VEGF	0.1174	0.4216

The expression of each gene mRNA was quantified using the formula:  $\Delta Ct = Ct_{\text{for each gene test}} - Ct_{\beta\text{-actin}}$ . RT-qPCR experiments were performed in triplicate. The following cytokines and chemokines were measured: cutaneous T-cell-attracting chemokine (CTACK), growth-regulated alpha protein (GROa), Interleukin (IL)-1 $\alpha$ , IL-2 receptor  $\alpha$ (Ra), IL-3, IL-12p40, IL-16, IL-18, leukemia Inhibitory Factor (LIF), monocyte-specific chemokine 3 (MCP-3), macrophage colonystimulating 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  $\beta$ (SCGF)- $\beta$ , stromal cell-derived factor 1  $\alpha$  (SDF-1 $\alpha$ ), tumor necrosis factor (TNF)- $\beta$ , tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), hepatocyte growth factor (HGF), Hu interferon  $\alpha$ 2 (IFN- $\alpha$ 2), platelet-derived growth factor receptor (PDGF)-  $\beta\beta$ , 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- $\gamma$ ), interferon gamma-induced protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1)(MCAF), macrophage inflammatory protein 1 (MIP-1 $\alpha$ ), MIP-1 $\beta$ , regulated on activation, normal T-cell expressed and secreted (RANTES), TNF- $\alpha$ , and vascular endothelial growth factor (VEGF).

## Discussion

The results of our study suggest that patients with low *hepcidin* mRNA expression in liver biopsy tissues are at higher risk of developing HCC. This is supported that the majority of patients who developed HCC exhibited high *hepcidin*  $\Delta$ Ct levels. Previous studies have reported a reduction in *hepcidin* mRNA expression in HCC tissues compared to that in non-cancerous liver tissues [31,32].

Based on the premise that low *hepcidin* mRNA expression (high *hepcidin*  $\Delta$ Ct level) is a risk factor for HCC development, we propose a hypothesis regarding the underlying mechanism. We hypothesize that within the lobules of patients with chronic hepatitis or cirrhosis, some of the atypical regenerating hepatocytes are more similar to HCC cells than to normal hepatocytes. Consequently, these hepatocytes may possess a diminished capacity to produce *hepcidin* mRNA. This may explain the observed decrease in *hepcidin* mRNA expression under these conditions. Based on our hypothesis, we speculate that livers with low *hepcidin* mRNA expression may have atypical hepatocytes that exhibit similar characteristics to those of HCC cells to some extent, when compared to normal hepatocytes.

Although the frequency of patients with advanced POAH was low, those with mild or higher POAH exhibited significantly lower *hepcidin* mRNA expression (Figure 1A). To date, we have reported the extent of POAH as a risk factor for the development of HCC from chronic hepatitis C and as a risk factor for HCC recurrence [33,34]. This suggests that hepcidin may play a direct role in the development of POAH. Further investigation is required for more detailed exploration of this hypothesis, which is beyond the scope of the present study. Our results support the finding that low hepcidin expression in cancerous areas is associated with HCC progression and poor prognosis [34,35].

While low *hepcidin* mRNA expression in liver tissue is a significant predictor of HCC development, the exact role of hepcidin in inhibiting or promoting HCC development remains unclear. Further studies are required to elucidate the precise mechanisms and determine the specific impact of hepcidin on HCC development. Furthermore, *hepcidin* mRNA expression showed an inverse positive correlation with blood ferritin concentration and a positive correlation with unsaturated iron-binding capacity levels, although only a few patients were analyzed. Based on this finding, it appears plausible that blood ferritin concentration reflects *hepcidin* mRNA expression in liver biopsy tissues. However, it is important to note that blood ferritin levels may be elevated under carcinogenic conditions or in patients with high levels of inflammation. Therefore, caution should be exercised when interpreting the blood ferritin level as a direct indicator of *hepcidin* mRNA expression in the liver. In contrast, *hepcidin* mRNA expression was not associated with the degree of inflammatory lymphocytic infiltration in liver lobules. This finding may suggest that *hepcidin* mRNA expression is unrelated to the mechanism of hepatocellular injury recognized by immunocompetent cells. In general, low *hepcidin* mRNA expression in hepatocytes indicates that ferroportin expression is not suppressed, thereby resulting in an increased export of iron ions from the cells, leading to a reduction in the intracellular iron ion levels. Hence, the reduction of iron ions can be seen as a mechanism that aids in suppressing the generation of radicals that can cause cellular damage.

We further investigated the relationship between serum cytokine and chemokine concentrations and *hepcidin* mRNA expression levels in the liver biopsy tissues. We found that *hepcidin* mRNA expression positively correlated with TRAIL only. On the other hand, *hepcidin* mRNA expression exhibited inverse correlations with IL-1 $\alpha$ , IL-5, IL-8, IL-10, IL-12 (P40), IL-13, G-CSF, GM-CSF, IFN- $\gamma$ , and MIP-1b. These findings suggest a complex interplay between *hepcidin* mRNA expression and various cytokines and chemokines in the liver microenvironment. The lifespan of neutrophils is normally 2–3 days at most to induce apoptosis, and the inverse correlation between G-CSF and GM-CSF suggests that low *hepcidin* mRNA expression may induce apoptosis and respond to infection by increasing the production of the granulocyte lineage and monocytes. Further, TRAIL is an antitumor cytokine that transduces apoptosis-inducing signals into cells by binding to specific receptors on the cell membrane [35]. Therefore, the positive correlation between *hepcidin* mRNA expression and TRAIL may indicate a pro-apoptotic effect of hepcidin. Zerafa et al. reported that TRAIL-knockout mice exhibit increased rates of carcinogenesis [36], and this positive correlation also suggests that hepcidin may exert an inhibitory effect on hepatocarcinogenesis. Thus, *hepcidin* mRNA levels positively correlated with TRAIL and negatively correlated with IL-13 and G-CSF, suggesting that hepcidin regulates apoptosis in a hyperactivating direction. Next, *hepcidin* mRNA expression was inversely correlated with MIP-1 $\beta$  (CCL4). Although MIP-1 $\alpha$ / $\beta$  display similar responses, MIP-1 selectively induces migration of CD8+ lymphocytes and MIP-1 $\beta$  of CD4+ lymphocytes [37]. Additionally, MIP-1 (CCL4) promotes tumor development and progression by recruiting regulatory T cells and pro-tumorigenic macrophages to enhance their pro-tumorigenic capacity [38]. IL-10 acts on immune cells such as

T cells and macrophages, directly suppressing cell activation and weakening the antigen-presenting capacity of macrophages, thereby suppressing the immune response [39]. IL-12 (p70) is a cytokine essential for the induction of Th1 responses through the production of IFN- $\gamma$ , which is important for protective immunity against infection and the induction of tumor immunity [40]. IL-13 has been observed to be a mediator of cytotoxicity *in vitro* and it has been reported that IL-13 induces cell death when applied to the colon cancer epithelial cell line HT29 [41,42]. It is believed that the disruption of the cell structure by IL-13 causes unstable cells to undergo apoptosis. Taken together, these results suggest that low *hepcidin* mRNA expression facilitates the enhancement of cell apoptosis and may also modulate the inflammatory response in an inhibitory manner. Comparison of cytokine/chemokine concentrations requires the detection of *hepcidin* mRNA expression and cytokine/chemokine levels in the liver tissue. To further elucidate the function of hepcidin and the mechanisms regulating *hepcidin* mRNA expression, we compared the differences in hepcidin expression in cell lines in which hepcidin was highly expressed in cultured cells or knocked down using small interfering RNA, and performed RNA-Seq to confirm the differences in gene expression. This study was based on a small number of liver biopsy tissues. We would like to recruit more patients and perform further studies using more liver tissue samples.

In conclusion, this study suggests that low *hepcidin* mRNA expression in patients with HCV-associated liver disease is associated with a high risk of HCC development. Thus, low *hepcidin* mRNA expression appears to indicate a hypercarcinogenic liver state. Furthermore, measuring *hepcidin* mRNA expression can be clinically useful for predicting the long-term prognosis of patients and for guiding treatment decisions.

### Acknowledgments

We greatly thank for Motomi Yamazaki, PhD. And Ms. Kayo Iwaguchi, and Ms. Shinobu Arai for technical assistance.

### Author Contributions:

M.O. and M.M., contributed to data collection, statistical analyses, interpretation of data, the writing of the manuscript and was the main author of the manuscript.

M.O., H.N. and M.M., contributed to data and sample collection,

M.O., M.M., and K.K. were contributed for study concept and design;

H.N. and M.M. were contributed for histological analysis;

M.M., T.S. and K.K. were responsible for analysis and interpretation of data;

M.M., and K.K. were responsible for statistical analysis;

### Ethics approval and consent to participate:

This study was approved by the Ethics Committee of Nihon University School of Medicine (RK-100611-13, # RK100910-15). The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

**Consent for participate:** Written informed consent was obtained from each patient.

**Availability of data and material:** Not applicable.

**Funding:** Not applicable.

**Consent for publication:** Not applicable.

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