

# Predictors of hepatocellular carcinoma after hepatitis C virus eradication following direct-acting antiviral treatment: relationship with serum zinc

Itaru Ozeki,\* Tomoaki Nakajima, Hirokazu Suii, Ryoji Tatsumi, Masakatsu Yamaguchi, Tomohiro Arakawa and Yasuaki Kuwata

Department of Hepatology, Sapporo Kosei General Hospital, Kita 3 Higashi 8, Chuo-ku, Sapporo 060-0033, Japan

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The recently approved direct-acting antivirals (DAA) agents are effective in terms of sustained virologic response (SVR) rates and are well tolerated in most hepatitis C virus (HCV) patients. This study aimed to analyze the association between serum zinc levels in patients who developed hepatocellular carcinoma (HCC) following HCV eradication after DAA treatment. The retrospective study included 769 HCV-infected patients who achieved SVR after DAA treatment. We calculated the annual incidence rate of HCC and identified risk factors associated with HCC development. We also assessed serum zinc and clinical factors at both baseline and end of treatment (EOT). During follow-up (median duration 35 months), HCC occurred in 18/769 (2.3%) patients. From the multivariate analysis, serum zinc <60 µg/dl [hazard ratio (HR) 5.936] and AFP ≥6.0 ng/dl (HR 5.862) at baseline, baseline-zinc <60 µg/dl (HR 6.283), EOT-serum zinc <63 µg/dl (HR 6.011), baseline-AFP ≥6.0 ng/dl (HR 8.163), and EOT-M2BPGi ≥2.5 (HR 12.194) at baseline and EOT were independently associated with increased HCC risk. In patients who achieved HCV eradication following DAA treatment, serum zinc levels before and at EOT could be a risk factor for developing HCC.

**Key Words:** zinc, direct-acting antivirals, hepatocellular carcinoma, sustained virologic response, hepatitis C virus

Hepatitis C virus (HCV) infections are an important worldwide health problem with chronic consequences, including cirrhosis and hepatocellular carcinoma (HCC).<sup>(1-3)</sup> The treatment of HCV infections has been revolutionized through the development of direct acting antivirals (DAAs), which are highly effective and well-tolerated.<sup>(4-6)</sup> Nevertheless, the impact of viral eradication after DAA treatment on disease progression, including the development of HCC, has been questioned.<sup>(7-11)</sup>

Zinc is well known to be an active center of or coenzyme for >300 types of enzymes that are involved in numerous biological processes, including DNA synthesis, RNA transcription, cell growth and division, among others. As a result, zinc is considered to be an essential trace element for maintaining life.<sup>(12-15)</sup> In chronic liver disease, a decreased capacity to synthesize albumin and malabsorption of zinc from the intestine cause zinc deficiency.<sup>(16-18)</sup> Long-term zinc supplementation therapy can improve liver pathology and reduced the incidence of HCC in chronic hepatitis C (CHC) patients.<sup>(19,20)</sup> Moreover, hypozincemia has been found to be associated with the development of HCC in HCV-related cirrhosis.<sup>(21)</sup> Therefore, it is important to understand the association between serum zinc and the development of HCC in CHC patients. Here, we examined serum zinc levels at baseline,

end of treatment (EOT) and 24 weeks after EOT (p24w) after DAA treatment.

## Materials and Methods

**Patients and study design.** Of the CHC patients who were treated at Sapporo Kosei General Hospital between February 2012 and December 2018, 769 with a sustained virologic response (SVR) were included in this study. Patients with genotype 1 CHC infections were administered 60 mg daclatasvir once daily plus 100 mg asunaprevir twice daily for 24 weeks ( $n = 258$ ), sofosbuvir/ledipasvir (400/90 mg, combination tablet) once daily for 12 weeks ( $n = 93$ ), ombitasvir/paritaprevir/ritonavir (25/150/100 mg, combination tablet) once daily for 12 weeks ( $n = 53$ ), 50 mg elbasvir once daily plus 100 mg grazoprevir once daily for 12 weeks ( $n = 71$ ), daclatasvir/asunaprevir/beclabuvir (30/200/75 mg, combination tablet) twice daily for 12 weeks ( $n = 18$ ), and glecaprevir/pibrentasvir (300/120 mg, combination tablet) once daily for 8 or 12 weeks ( $n = 46$ ). Patients infected with genotype 2 CHC were administered sofosbuvir 400 mg once daily plus ribavirin (weight-based dosing) for 12 weeks ( $n = 188$ ), ombitasvir/paritaprevir/ritonavir once daily plus ribavirin (weight-based dosing) for 24 weeks ( $n = 4$ ) and glecaprevir/pibrentasvir (300/120 mg, combination tablet) once daily for 8 or 12 weeks ( $n = 38$ ). We collected baseline data at the time of DAA treatment initiation including age, gender, HCV genotype, body mass index (BMI), estimated glomerular filtration rate (eGFR), platelets count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, albumin, Mac-2 binding protein glycosylation isomer (M2BPGi),  $\alpha$ -fetoprotein (AFP), and serum zinc. Moreover, we determined the presence of cirrhosis and diabetes. We also examined laboratory tests after EOT such as platelets count, AST, ALT, total bilirubin, albumin, M2BPGi, AFP, and serum zinc.

We defined SVR as a serum HCV RNA viral load below the lower limit of detection performed at least 24 weeks after EOT. Liver cirrhosis was diagnosed based on liver histology, transient elastography (liver stiffness of  $\geq 14.5$  kPa measured with Fibroscan (Echosens, Paris, France)),<sup>(22)</sup> or the presence of gastroesophageal varices. We defined diabetes in patients with a HbA1c value  $\geq 6.5\%$  or those undergoing treatment with antidiabetic drugs or insulin. Patients with decompensated cirrhosis, chronic kidney disease (CKD) stage  $\geq 4$ , concomitant human immunodeficiency

\*To whom correspondence should be addressed.  
E-mail: i.ozeki@ja-hokkaidoukouseiren.or.jp

virus or hepatitis B virus (HBV) infection, comorbid liver disease associated with autoimmunity, excessive alcohol consumption (daily ethanol consumption was  $\geq 60$  g/day), history of HCC, or HCC detected during the DAA treatment and within 24 weeks after EOT were excluded. We defined the observation period, as the time in weeks since the beginning of treatment. All patients were examined for HCC by ultrasonography (US), dynamic computed tomography (CT), and/or magnetic resonance imaging (MRI) at baseline and every 3–6 months after the beginning of treatment. Patients with cirrhosis underwent CT and/or MRI without US in a similar fashion.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee (registration no. 506) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was a retrospective, single-center, non-randomized, and non-case controlled open trial.

**Clinical and laboratory evaluations.** HCV RNA was determined at a central laboratory using the Roche COBAS TaqMan HCV Auto assay (Roche Diagnostics, Tokyo, Japan; lower limit of quantification, 15 IU/ml). HCV genotype was conducted using a real time polymerase chain reaction assay. Serum zinc levels were measured by atomic absorption spectrometry (Hitachi High-Tech Science Co., Ltd., Tokyo, Japan) until July 2018 and by colorimetry (Jeol Ltd., Tokyo, Japan) from August 2018 onwards.

**Statistical analysis.** The categorical variables are presented as numbers and percentages and the continuous variables are presented as the median (range). Student's *t* test and a Mann-Whitney *U* test were used to analyze continuous data, as appropriate. We calculated the cumulative HCC incidence using the Kaplan-Meier method. Differences among groups were assessed using the log-rank test. Risk factors associated with the development of HCC were determined by the Cox proportional hazard model. The independent variables included age, gender, HCV genotype, cirrhosis, diabetes, BMI, eGFR, platelet count, albumin, AST, ALT, M2BPGi, AFP, and serum zinc at baseline and EOT. Significant predictive factors that contributed to the development of HCC in the univariate analysis were inputted into the multivariate analysis. A stepwise logistic regression model analysis was used to calculate the adjusted hazards ratio (HR) and 95% confidence intervals (CI) for the various factors. Optimal cut-off values were selected from the receiver operating characteristics curve. A *p* value  $< 0.05$  was considered statistically significant. Statistical analyses were performed with R (<http://www.r-project.org/>).

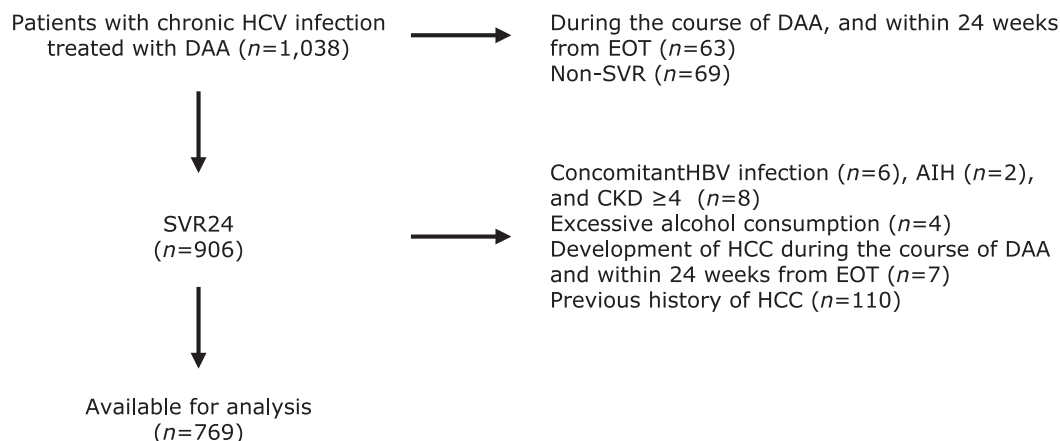
## Results

**Patient flow chart.** Among the 1,038 patients who initiated their antiviral regimen from between February 2012 and December 2018, 906 (87.2%) achieved SVR. After excluding patients with concomitant HBV infection, CKD stage  $\geq 4$ , autoimmune hepatitis, excessive alcohol consumption, previous history of HCC, development of HCC during the course of DAA and within 24 weeks from EOT, 769 patients were included in the analysis (Fig. 1).

**Characteristics of the study population.** The median patient age was 64 years. The patients included 317 men (41.2%) and 539 (70.1%), and 149 (19.4%) patients with genotype 1, and cirrhosis, respectively. Treatment resulted in a significant increase in platelets and albumin, and a significant decrease in AST, ALT, M2BPGi, and AFP (Table 1).

**Changes in serum zinc and albumin levels before and after DAA treatment.** The median serum zinc levels were 69, 68, and 72  $\mu\text{g/dl}$  at baseline, EOT, and p24w, respectively (baseline vs p24w;  $p < 0.001$ ; Fig. 2A). The median serum zinc levels were 70, 68, and 73  $\mu\text{g/dl}$  at baseline, EOT, and p24w in patients without cirrhosis (baseline vs p24w;  $p < 0.001$ ; Fig. 2B). The median serum zinc levels were 60, 64, and 68  $\mu\text{g/dl}$  at baseline, EOT, and p24w in patients with cirrhosis (baseline vs EOT;  $p < 0.01$ , baseline vs p24w;  $p < 0.001$ ; Fig. 2C). The median albumin levels were 4.1, 4.2, and 4.3 g/dl, at baseline, EOT, and p24w, respectively (baseline vs EOT, baseline vs p24w; all  $p < 0.001$ ; Supplemental Fig. 1A\*). The median albumin levels were 4.2, 4.3, and 4.4 g/dl at baseline, EOT, and p24w in patients without cirrhosis (baseline vs p24w;  $p < 0.001$ ; Supplemental Fig. 1B\*). The median albumin levels were 3.7, 4.0, and 4.2 g/dl at baseline, EOT, and p24w in patients with cirrhosis (baseline vs EOT, baseline vs p24w; all  $p < 0.001$ ; Supplemental Fig. 1C\*). Serum zinc levels were positively correlated with albumin levels at baseline ( $r = 0.449$ ,  $p < 0.001$ ; Supplemental Fig. 2A\*). The increase in serum zinc levels from baseline to p24w was significantly associated with the increase in albumin levels from baseline to p24w ( $r = 0.351$ ,  $p < 0.001$ ; Supplemental Fig. 2B\*).

**HCC incidence and predictors.** During follow-up (median duration 35 months, range 8–107 months), HCC occurred in 18/769 (2.3%) patients. The 1, 2, 3, 4, and 5 year overall cumulative rates of HCC were 0.3, 1.7, 2.4, 3.7, and 3.7%, respectively (Fig. 3A). Factors associated with developing HCC at baseline were older age, cirrhosis, higher BMI, lower eGFR, lower platelet count, higher AST, lower albumin, higher M2BPGi, higher AFP, and lower serum zinc (Table 2). A multivariate Cox regression



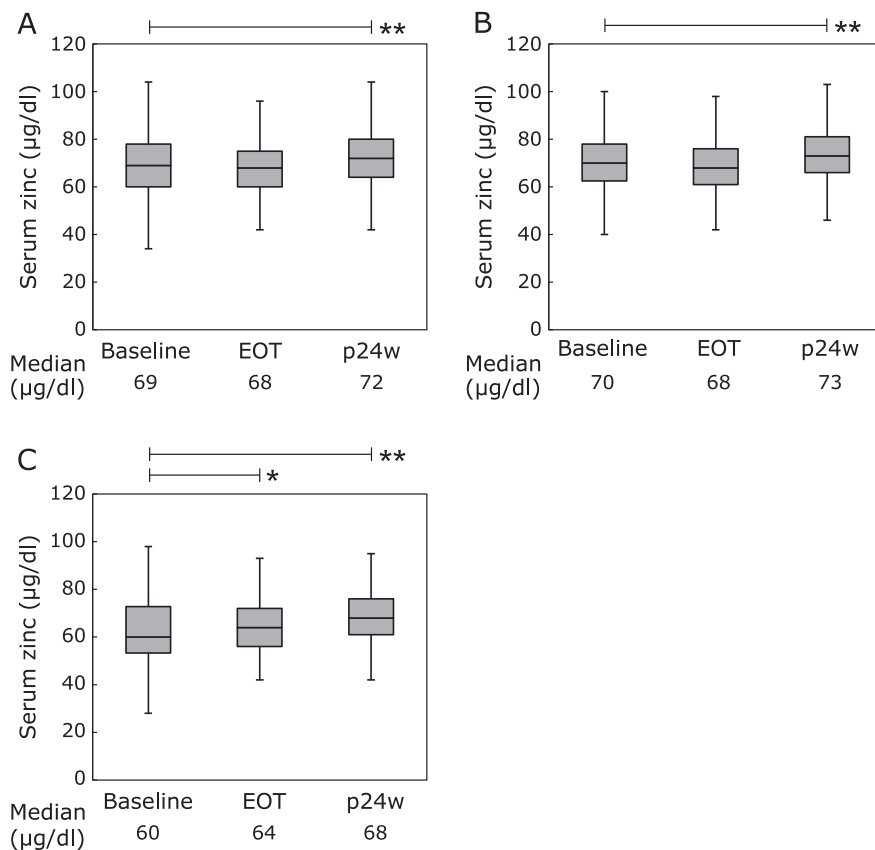
**Fig. 1.** Flowchart for patient selection. AIH, autoimmune hepatitis; HBV, hepatitis B virus; CKD, chronic kidney disease; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; DAA, direct-acting antivirals; EOT, end of treatment; SVR, sustained virologic response.

\*See online. <https://doi.org/10.3164/jcfn.19-98>

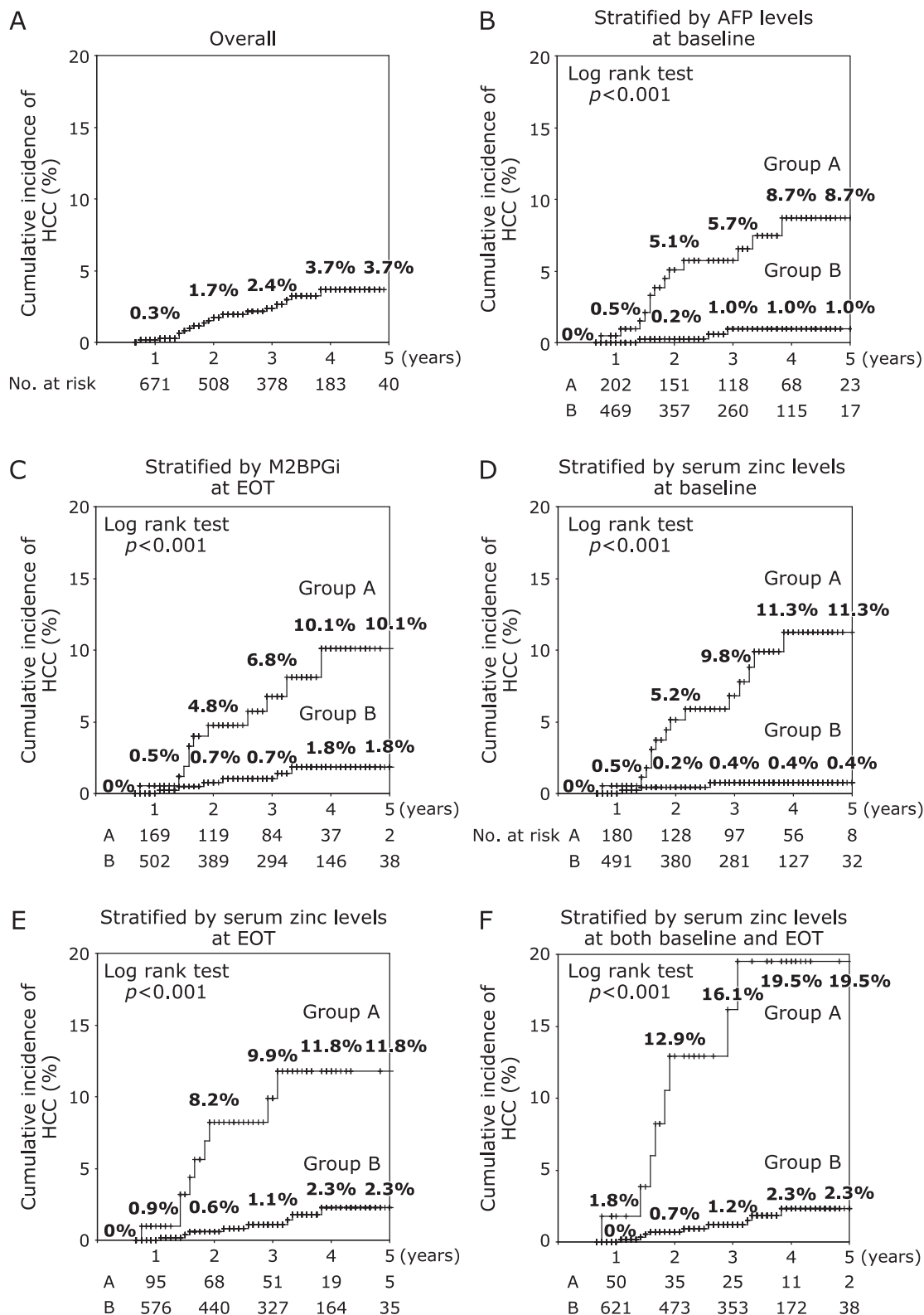
**Table 1.** Patients characteristics at baseline and end of treatment

Characteristics	Baseline	End of treatment	<i>p</i>
Number of patients	769		
Age (years)	64 (18–88)		
Gender, male	317 (41.2)		
Genotype, 1/2	539/230		
Liver cirrhosis	149 (19.4)		
Diabetes	93 (12.1)		
BMI (kg/m <sup>2</sup> )	22.9 (14.4–38.8)		
eGFR (ml/min/1.73 m <sup>2</sup> )	73.6 (30.4–187.8)		
Treatment			
daclatasvir + asunaprevir	258 (33.6)		
sofosbuvir/ledipasvir	93 (12.1)		
ombitasvir/paritaprevir/ritonavir	53 (6.9)		
elbasvir + grazoprevir	71 (9.2)		
daclatasvir/asunaprevir/beclabuvir	18 (2.3)		
glecaprevir/pibrentasvir	84 (10.9)		
sofosbuvir + ribavirin	188 (24.4)		
ombitasvir/paritaprevir/ritonavir + ribavirin	4 (0.5)		
Platelets count (×10 <sup>4</sup> /μl)	15.7 (3.8–22.0)	16.1 (3.8–43.4)	<0.001
AST (U/L)	39 (11–272)	24 (10–99)	<0.001
ALT (U/L)	39 (7–255)	17 (4–106)	<0.001
Total bilirubin (mg/dl)	0.7 (0.2–2.3)	0.7 (0.1–3.0)	0.476
Albumin (g/dl)	4.1 (2.0–5.1)	4.2 (2.7–5.3)	<0.001
M2BPGi	2.0 (0.1–24.2)	1.1 (0.2–11.8)	<0.001
AFP (ng/ml)	4.2 (1.0–634.9)	3.1 (0.9–27.1)	<0.001
Serum zinc (μg/dl)	69 (28–116)	68 (42–142)	0.122

Categorical variables expressed as number (%) and the continuous variables as median (range). AFP,  $\alpha$ -fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; eGFR, estimated glomerular filtration rate; M2BPGi, Mac-2 binding protein glycosylation isomer.



**Fig. 2.** Changes in the serum zinc levels at baseline, EOT, and p24W. (\**p*<0.01, \*\**p*<0.001). EOT, end of treatment; p24w, 24 weeks after EOT. (A) Overall. (B) Non-cirrhotic patients. (C) Cirrhotic patients.



**Fig. 3.** The cumulative rate of HCC by Kaplan-Meier analysis. AFP,  $\alpha$ -fetoprotein; EOT, end of treatment; HCC, hepatocellular carcinoma; M2BPGi, Mac-2 binding protein glycosylation isomer. (A) Overall cumulative incidence of HCC. (B) Cumulative incidence of HCC stratified by AFP at baseline. Group A ( $n = 220$ ); baseline-AFP  $\geq 6.0$  ng/ml, Group B ( $n = 549$ ); baseline-AFP  $< 6.0$  ng/ml (log-rank test  $p < 0.001$ ). (C) Cumulative incidence of HCC stratified by M2BPGi at EOT. Group A ( $n = 194$ ); EOT-M2BPGi  $\geq 2.5$ , Group B ( $n = 575$ ); EOT-M2BPGi  $< 2.5$  (log-rank test  $p < 0.001$ ). (D) Cumulative incidence of HCC stratified by serum zinc levels at baseline. Group A ( $n = 197$ ); baseline-serum zinc levels  $< 60$   $\mu\text{g/dl}$ , Group B ( $n = 572$ ); baseline-serum zinc levels  $\geq 60$   $\mu\text{g/dl}$  (log-rank test  $p < 0.001$ ). (E) Cumulative incidence of HCC stratified by serum zinc at EOT. Group A ( $n = 107$ ); EOT  $< 63$   $\mu\text{g/dl}$ , Group B ( $n = 662$ ); EOT  $\geq 63$   $\mu\text{g/dl}$  (log-rank test  $p < 0.001$ ). (F) Cumulative incidence of HCC stratified by serum zinc at both baseline and EOT. Group A ( $n = 58$ ); baseline-serum zinc levels  $< 60$   $\mu\text{g/dl}$  and EOT-serum zinc levels  $< 63$   $\mu\text{g/dl}$ , Group B ( $n = 711$ ); baseline-serum zinc levels  $\geq 60$   $\mu\text{g/dl}$  and/or EOT-serum zinc levels  $\geq 63$   $\mu\text{g/dl}$  (log-rank test  $p < 0.001$ ).

**Table 2.** Factors associated with developing HCC at baseline

Characteristics	Developing HCC	Non developing HCC	<i>p</i>
Number	18	751	
Age (years)	72 (52–82)	66 (18–88)	0.033
Gender, male/female	11/7	306/445	0.083
Genotype, 1/2	16/2	523/228	0.078
Liver cirrhosis, yes/no	12/6	137/614	<0.001
Diabetes, yes/no	1/17	92/659	0.389
BMI (kg/m <sup>2</sup> )	25.2 (19.4–32.0)	23.0 (14.4–38.8)	0.010
eGFR (ml/min/1.73 m <sup>2</sup> )	64.8 (33.2–105.3)	74.5 (30.4–187.8)	0.026
Platelets count (×10 <sup>4</sup> /μl)	12.5 (3.8–22.0)	16.1 (12.3–20.1)	0.014
AST (U/L)	47 (28–125)	39 (11–272)	0.039
ALT (U/L)	42 (15–101)	39 (7–255)	0.976
Total bilirubin (mg/dl)	0.9 (0.4–2.2)	0.7 (0.2–2.3)	0.083
Albumin (g/dl)	3.6 (2.0–4.7)	4.2 (2.6–5.1)	<0.001
M2BPGi	7.3 (2.2–22.8)	1.9 (0.1–24.2)	<0.001
AFP (ng/ml)	7.3 (1.8–68.5)	4.1 (1.0–634.9)	0.001
Serum zinc (μg/dl)	57 (28–84)	70 (34–116)	<0.001

Categorical variables expressed as number (%) and the continuous variables as median (range). AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; eGFR, estimated glomerular filtration rate; HCC, hepatocellular carcinoma; M2BPGi, Mac-2 binding protein glycosylation isomer.

**Table 3.** Factors associated with developing HCC at baseline on multivariate analysis

Factors	Hazard Ratio	95%CI	<i>p</i>
Serum zinc <60 μg/dl	5.936	1.865–18.886	0.003
AFP ≥6.0 ng/dl	5.862	1.625–21.14	0.007

AFP, α-fetoprotein; CI, confidence interval; HCC, hepatocellular carcinoma.

**Table 4.** Factors associated with developing HCC at end of treatment

Characteristics	Developing HCC	Non developing HCC	<i>p</i>
Platelets count (×10 <sup>4</sup> /μl)	13.1 (4.0–20.1)	16.1 (3.8–43.4)	0.005
AST (U/L)	29 (16–81)	24 (10–99)	0.009
ALT (U/L)	23 (8–51)	17 (4–106)	0.306
Total bilirubin (mg/dl)	0.7 (0.4–1.6)	0.7 (0.1–3.0)	0.343
Albumin (g/dl)	3.8 (2.7–4.6)	4.2 (2.7–5.3)	<0.001
M2BPGi	2.8 (1.0–9.2)	1.1 (0.2–11.8)	<0.001
AFP (ng/ml)	4.6 (2.3–10.7)	3.1 (0.9–27.1)	0.008
Serum zinc (μg/dl)	56 (42–90)	68 (42–142)	<0.001

Continuous variables expressed as median (range). AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; M2BPGi, Mac-2 binding protein glycosylation isomer.

**Table 5.** Factors associated with developing HCC at both baseline and end of treatment on multivariate analysis

Factors	Hazard Ratio	95%CI	<i>p</i>
Baseline-serum zinc <60 μg/dl	6.283	1.655–23.847	0.007
EOT-serum zinc <63 μg/dl	6.011	2.093–17.265	<0.001
Baseline-AFP ≥6.0 ng/dl	8.163	2.188–35.852	0.002
EOT-M2BPGi ≥2.5	12.194	4.184–58.325	<0.001

AFP, α-fetoprotein; CI, confidence interval; HCC, hepatocellular carcinoma; M2BPGi, Mac-2 binding protein glycosylation isomer.

analysis showed that serum zinc <60 μg/dl (HR 5.936, *p* = 0.003) and AFP ≥6.0 ng/dl (HR 5.862, *p* = 0.007) at baseline were independently associated with a higher risk of developing HCC (Table 3). Factors associated with developing HCC at EOT were lower platelet count, higher AST, lower albumin, higher M2BPGi, higher AFP, and lower serum zinc (Table 4). A multivariate Cox regression analysis was carried out including both baseline and EOT factors; accordingly, baseline-serum zinc <60 μg/dl (HR

6.283, *p* = 0.007), EOT-serum zinc <63 μg/dl (HR 6.011, *p* < 0.001), baseline AFP ≥6.0 ng/dl (HR 8.163, *p* = 0.002), and EOT-M2BPGi ≥2.5 (HR 12.194, *p* < 0.001) were identified as independent factors for developing HCC (Table 5).

The analysis of HCC incidence according to AFP levels at baseline is shown in Fig. 3B. Stratified by baseline AFP levels, the cumulative rates of HCC at 1, 3, and 5 years were 0.5%, 5.7%, and 8.7%, respectively, in patients with a baseline AFP ≥6.0 ng/ml

(Group A,  $n = 220$ ) and 0%, 1.0%, and 1.0%, respectively, in patients with a baseline AFP  $<6.0$  ng/ml (Group B,  $n = 549$ ;  $p < 0.001$  by log-rank test). The analysis of HCC incidence according to EOT-M2BPGi levels at EOT is shown in Fig. 3C. Stratified by EOT-M2BPGi levels, the cumulative rates of HCC at 1, 3, and 5 years were 0.5, 6.8, and 10.1%, respectively, in patients with EOT-M2BPGi  $\geq 2.5$  (Group A,  $n = 194$ ) and 0, 0.7, and 1.8%, respectively, in patients with EOT-M2BPGi  $< 2.5$  (Group B,  $n = 575$ ;  $p < 0.001$  by log-rank test). The analysis of HCC incidence according to baseline serum zinc levels at baseline is shown in Fig. 3D. Stratified by baseline serum zinc levels, the cumulative rates of HCC at 1, 3, and 5 years were 0.5, 9.8, and 11.3%, respectively, in patients with baseline serum zinc levels  $< 60$   $\mu\text{g/dl}$  (Group A,  $n = 197$ ) and 0, 0.4, and 0.4%, respectively, in patients with baseline serum zinc levels  $\geq 60$   $\mu\text{g/dl}$  (Group B,  $n = 572$ ;  $p < 0.001$  by log-rank test). The analysis of HCC incidence according to EOT-serum zinc levels is shown in Fig. 3E. Stratified by EOT-serum zinc levels, the cumulative rates of HCC at 1, 3, and 5 years were 0.9, 9.9, and 11.8%, respectively, in patients with EOT-serum zinc levels  $< 63$   $\mu\text{g/dl}$  (Group A,  $n = 107$ ) and 0, 1.1, and 2.3%, respectively, in patients with EOT-serum zinc levels  $\geq 63$   $\mu\text{g/dl}$  (Group B,  $n = 662$ ;  $p < 0.001$  by log-rank test). Stratified by both baseline and EOT-serum zinc levels, the cumulative rates of HCC at 1, 3, and 5 years were 1.8, 16.1, and 19.5%, respectively, in patients with baseline serum zinc levels  $< 60$   $\mu\text{g/dl}$  and EOT-serum zinc levels  $< 63$   $\mu\text{g/dl}$  (Group A,  $n = 58$ ) and 0, 1.2, and 2.3%, respectively, in patients with baseline serum zinc levels  $\geq 60$   $\mu\text{g/dl}$  and/or EOT-serum zinc levels  $\geq 63$   $\mu\text{g/dl}$  (Group B,  $n = 711$ ;  $p < 0.001$  by log-rank test).

## Discussion

Mean serum zinc levels are known to increase to the greatest extent during follow-up after interferon treatment from zinc.<sup>(23)</sup> In two recent studies, serum zinc levels were also found to change in HCV patients before and after DAA treatment.<sup>(24,25)</sup> However, the mechanism underlying these effects has not yet been clarified. It is known that the production of albumin is inhibited by acute phase cytokines, such as interleukin 6 and tumor necrosis factor  $\alpha$ .<sup>(26)</sup> Furthermore, inflammation increases the catabolic rate, resulting in hypoalbuminemia.<sup>(27)</sup> Viral eradication of HCV by DAA treatment suppresses hepatic inflammation and acute phase cytokines.<sup>(28,29)</sup> In the present study, significant increases in serum zinc levels were observed at EOT in cirrhotic patients, and at p24w in all patients. Similarly, significant increases in albumin levels were observed at EOT in all patients, and cirrhotic patients and at p24w in all patients. It has been reported that the baseline albumin levels are lower than the normal limit, and, after achieving SVR, the levels increase, approaching normal levels.<sup>(30)</sup> The same mechanism is likely involved in the increased albumin levels observed, particularly in cirrhotic patients who achieved SVR following DAA, in the present study. We also observed that serum zinc levels were positively correlated with albumin levels at baseline. Furthermore, the increase in serum zinc levels from baseline to p24w was significantly associated with the increase in albumin levels from baseline to p24w. Therefore, as albumin levels increase following DAA, the increase in serum zinc might be a result of the increase in albumin levels.

Numerous studies have demonstrated that interferon-based antiviral treatment reduces the incidence of HCC in patients with HCV-induced SVR.<sup>(31–33)</sup> Similarly, several recent retrospective studies and one recent prospective study have suggested that the risk of HCC decreased after DAA treatment.<sup>(34–39)</sup> Cox regression analyses have highlighted cirrhosis,<sup>(7,8)</sup> low platelet count,<sup>(7,9)</sup> alcohol abuse,<sup>(8,36)</sup> low albumin levels,<sup>(9)</sup> higher AFP levels,<sup>(10)</sup> and older age,<sup>(11,38)</sup> as independent predictors for developing HCC. Therefore, various factors were selected, due to review period, back patient characteristics, and selected independent factors.

Moreover, we excluded patients with a history of HCC and those who reported a high rate of HCC recurrence in advance.<sup>(7,40–44)</sup>

In the present study, a multivariate analysis showed lower serum zinc levels and higher AFP levels at baseline. As several laboratory parameters changed in patients who achieved SVR following DAA, including reductions in AFP and M2BPGi levels, increases in platelet count, albumin, and serum zinc levels,<sup>(28,29,45–47)</sup> we carried out the multivariate analysis at both baseline and EOT. The results showed lower serum zinc levels at baseline and EOT, higher AFP at baseline, and higher M2BPGi at EOT. AFP has previously been identified as a candidate risk factor for the development of HCC.<sup>(10)</sup> Although M2BPGi has been shown to be a noninvasive biomarker for fibrosis, post treatment levels of M2BPGi have been associated with the risk of developing HCC among patients with SVR.<sup>(46)</sup> Serum zinc levels were also lower in patients who developed HCC in the univariate analysis at baseline and EOT. Based on the multivariate analysis, serum zinc was selected as the only common factor associated with HCC development at both baseline and EOT.

Although the long-term administration of zinc to CHC patients with zinc deficiency has been reported to reduce hepatic fibrosis and the risk of developing HCC,<sup>(48)</sup> no consistent views have been reached on whether zinc deficiency increase the risk of developing HCC or not. Many patients with concomitant HCC presumably have advanced hepatic fibrosis. Currently, it is not known whether hypozincemia results from advanced hepatic fibrosis or whether a zinc-deficient state initiates or promotes HCC. Although zinc is an essential nutrient for numerous biological activities, such as suppressing oxidative stress and maintaining the immune system, mitochondrial destruction by HCV severely disrupts zinc homeostasis.<sup>(49)</sup> A recent paper also revealed that hypozincemia is associated with the development of HCC in HCV-related cirrhosis. This study concluded that HCV induces hypozincemia due to a reduction in copper-zinc superoxide dismutase and antioxidative activity, which results in the development HCC.<sup>(21)</sup> However, it did not analyze serum zinc levels in patients who developed HCC following HCV eradication by DAA. Despite HCV eradication, improvements in serum zinc levels were still dependent on the liver fibrosis states.<sup>(21)</sup> Accordingly, we postulate that serum zinc levels increase after DAA treatment and that patients whose serum zinc levels remain unchanged are likely to develop HCC. The association between serum zinc levels and the development of HCC should be further investigated.

This study had several limitations. First, this was a retrospective single-center study. Second, the serum zinc levels show circadian variations, being high in the early morning and decreasing toward the afternoon.<sup>(50,51)</sup> Therefore, blood sample collection should preferably have been done in the early morning when patients were fasting. However, because of the difficulty in collecting samples at the same time from all patients in a clinical setting, this study did not define a fixed sampling time. Lastly, as a major limitation, due to the small number of patients who developed HCC in the present study, the factors, such as serum zinc, identified in the multivariate analysis as contributing to HCC incidence might differ in a larger study. Consequently, larger multi-center studies will be needed to confirm these results.

In summary, the most novel finding of this study was that serum zinc levels were strongly associated with the development of HCC in CHC patients who exhibited eradication of HCV following DAA treatment. Therefore, it is important to pay attention to the serum zinc levels between baseline and EOT following DAA treatment.

## Abbreviations

AFP	$\alpha$ -fetoprotein
ALT	alanine aminotransferase
AST	aspartate aminotransferase

BMI	body mass index
CHC	chronic hepatitis C
CI	confidence intervals
CKD	chronic kidney disease
CT	computed tomography
DAA	direct acting antivirals
eGFR	estimated glomerular filtration rate
EOT	end of treatment
HCC	hepatocellular carcinoma
HBV	hepatitis B virus
HCV	hepatitis C virus
HR	hazards ratio
M2BPGI	Mac-2 binding protein glycosylation isomer
MRI	magnetic resonance imaging
p24w	24 weeks after EOT

SVR	sustained virologic response
US	ultrasonography

## Conflict of Interest

No potential conflicts of interest were disclosed.

## Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee (registration no. 506) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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