

Zinc Transporter 1 Expression in Hepatocellular Carcinoma Correlates with Prognosis: A Single-Center Retrospective Study

Naruyasu Kakita^a, MD, PhD; Kazuhiro Katayama^a, MD, PhD; Toshimitsu Yasui^a, MD; Shin Satake^a, MD; Kenji Aoi^a, MD; Hisashi Jo^a, MD; Yong Kook Kim^b, MD; Masaru Yamazaki^c, MD, PhD; Ayako Hashimoto^d, PhD; and Taiho Kambe^e, PhD

^aDepartment of Gastroenterology and Hepatology, Kaizuka City Hospital, Kaizuka city, Osaka, Japan

^bDepartment of Surgery, Kaizuka City Hospital, Kaizuka city, Osaka, Japan

^cDepartment of Pathology, Kaizuka City Hospital, Kaizuka city, Osaka, Japan

^dDepartment of Food and Nutrition, Faculty of Home Economics, Kyoto Women's University, Kyoto city, Kyoto, Japan

^eGraduate School of Biostudies, Kyoto University, Kyoto city, Kyoto, Japan

Naruyasu kakita, n.kakita@hosp.kaizuka.osaka.jp

Kazuhiro Katayama, d.ktym-afro-8233@live.jp

Toshimitsu Yausi, can19dy@hotmail.co.jp

Shin Satake, s.satake@hosp.kaizuka.osaka.jp

Kenji Aoi, k.aoi@hosp.kaizuka.osaka.jp

Hisahi Jo, h.joh@hosp.kaizuka.osaka.jp

Yong Kook Kim, y-kim@hosp.kaizuka.osaka.jp

Masaru Yamazaki, ma.yamazaki@hosp.kaizuka.osaka.jp

Ayako Hashimoto, hashimoa@kyoto-wu.ac.jp

Taiho Kambe, kambe.taiho.7z@kyoto-u.ac.jp

Corresponding author:

Dr. Naruyasu Kakita

Department of Gastroenterology and Hepatology

Kaizuka City Hospital, 3-10-20 Hori, Kaizuka city, Osaka 5970015, Japan

Tel.: +81-724-22-5865

Fax: +81-724-39-6061

E-mail: naruta1974@yahoo.co.jp¹

¹ ZIP, zrt-, irt-related protein; ZNT, zinc transporter¹

Abstract

Objectives

Zinc is crucial in the pathogenesis of hepatocellular carcinoma; however, no reports have examined its association with clinical parameters and zinc transporter 1 (ZNT1) expression intensity. We assessed the association between ZNT1 expression and prognosis in hepatocellular carcinoma patients.

Methods

This retrospective study included 65 patients who underwent surgical hepatocellular carcinoma resection at a single center between January 2011 and June 2015. ZNT1 expression on hepatocellular carcinoma cells from specimens was assessed using immunohistochemistry, and the relationship between its intensity and various clinical indexes was examined with univariate and multivariable analyses and the Mann–Whitney U, Kruskal–Wallis, Bonferroni, and log-rank tests.

Results

ZNT1 expression on the hepatocellular carcinoma cell membrane was negative in 31 and positive in 34 patients, including nine with strongly positive expression. Patients with and without ZNT1 expression had similar blood zinc concentrations, α -fetoprotein levels, protein induced by vitamin K absence-antagonist-II levels, gross classification, maximal tumor diameters, and background liver disease. Patients with strongly positive ZNT1 expression (57.0 ± 22.1) had significantly lower blood zinc concentrations than those with positive (71.1 ± 14.2 ; $P=0.015$) or no ZNT1 expression (72.9 ± 14.1 ; $P=0.043$). Overall survival was significantly shorter in ZNT1-expressing than in non-expressing patients (log-rank test, $P=0.024$). Multivariable analysis using the Cox proportional hazards model identified maximal tumor diameter (hazard ratio, 1.018; 95% confidence interval,

1.002–1.034; $P=0.026$) and ZNT1 expression status (hazard ratio, 2.082; 95% confidence interval, 1.196–3.621; $P=0.010$) as prognostic contributing factors.

Conclusions

ZNT1 expression on the hepatocellular carcinoma cell membrane and its intensity are useful predictors of postoperative survival.

Keywords

Hepatocellular carcinoma, surgical resection, prognostic factors, zinc metabolism, Immunohistochemistry,

Introduction

Hepatocellular carcinoma is the seventh most common cancer worldwide and the second leading cause of cancer-related death, making its pathogenesis and clinical management an important health issue. Currently, the worldwide incidence rates of this cancer type are the highest in Asia and Africa, with the rates increasing in many regions [1,2].

The risk of carcinogenesis that leads to hepatocellular carcinoma increases with fibrosis progression due to chronic inflammation. Viral hepatitis, alcohol consumption, and fatty liver disease are the main causes of chronic hepatic inflammation. While the prevalence of viral hepatitis diseases has decreased recently, the frequency of non-viral hepatitis diseases is increasing [1]. Hepatic carcinogenesis might be caused by the accumulation of genetic mutations due to chronic inflammation and the resulting abnormal cellular functions, including the acquisition of cell proliferative capacity and impaired tumor immune function. However, the detailed mechanism remains unknown [3,4].

Zinc is an essential trace metal, required by many living organisms for enzymatic activity and protein-structure maintenance. It plays crucial roles in many biological phenomena, such as human growth, wound healing, immune response, and antioxidant and anti-inflammatory activities. It is also involved in cell proliferation and differentiation by playing important roles in DNA synthesis, repair, maintenance, and cell division [5].

Chronic liver diseases, especially cirrhosis, have been frequently associated with hypozincemia [6]. Several studies have shown that patients with hypozincemia had a higher rate of subsequent hepatic carcinogenesis and a poorer prognosis after treatment for hepatocellular carcinoma than did those without [7-10]. These epidemiological data suggest that hypozincemia in chronic liver diseases plays a facilitative role in

hepatocarcinogenesis and cancer progression. Oxidative stress and chronic inflammation are pro-carcinogenic, and zinc antioxidant and anti-inflammatory effects might not work well when zinc is deficient. Zinc also plays an important role in the immune response, and zinc deficiency might compromise tumor immunity [11].

Zinc status in hepatocarcinoma cells should be analyzed to further elucidate its role in hepatocarcinogenesis and cancer progression. Low intracellular zinc content has previously been implicated in cellular carcinogenesis and cancer progression [12]. Intracellular zinc homeostasis is maintained by zinc transporters, including the zrt-, irt-related protein (ZIP) and zinc transporter (ZNT), as well as metallothionein. Therefore, the expression kinetics of these transporters in cancer cells might be involved in hepatocarcinogenesis and maintenance of cancer grade [13]. Franklin et al. compared the expression kinetics of ZIP1, 2, 3, and 14 in hepatocarcinoma and noncancerous tissues. They found a decrease in ZIP14 expression and zinc concentration in cancer tissues, suggesting that this transporter was involved in hepatocarcinogenesis and cancer progression [13]. In contrast, some reports found that the expression of ZIP4, ZIP14, and ZNT9 increased in hepatocarcinoma tissues [14]. The association between the expression of these transporters and cancer pathology has been noted in other cancers as well; for example, ZIP10 promoted lymph node metastasis, whereas ZIP6 expression was correlated with a favorable prognosis in breast cancer patients [15].

The ZIP family transfers extracellular or intra-organelle zinc into the cytoplasm, whereas the ZNT family transfers zinc in the opposite direction to reduce intracytoplasmic zinc. ZNT1 is the only transporter on the plasma membrane that translocates zinc to the extracellular space [16]; however, it has rarely been studied in hepatocellular carcinoma

and has not been associated with any clinical outcomes. We report the expression dynamics of ZNT1 at the protein level in patients with hepatocellular carcinoma and investigate its association with clinical outcomes.

Materials and Methods

Participants

This retrospective study included 65 patients (median age, 72 years; 52 men) who underwent surgical resection and were pathologically diagnosed with hepatocellular carcinoma at Kaizuka City Hospital between January 2010 and December 2017.

Background diseases included type B cirrhosis in 11 patients, type C cirrhosis in 28, alcoholic cirrhosis in 15, and non-alcoholic fatty liver disease in 11 patients. The gross classification, based on the General Rules for the Clinical and Pathological Study of Primary Liver Cancer, 6th Edition, Revised Version, of the Liver Cancer Study Group of Japan [17], was as follows: small nodular type with indistinct margins in 15 patients, simple nodular type in 28, simple nodular type with extra-nodular proliferation in 9, and confluent multinodular type in 13. The patient baseline characteristics are summarized in Table 1.

Immunohistochemical staining for ZNT1 expression

Tissue sections were de-paraffined with xylene and rehydrated through an ethanol series and phosphate-buffered saline. Antigen retrieval was performed by microwave treatment with citrate buffer (pH 6.0). Endogenous peroxidase was blocked with 0.3% H₂O₂ in methanol for 30 min, followed by incubation with G-block (Genostaff Co., Ltd., Tokyo, Japan) and Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA, USA). The

sections were incubated with 5 µg/mL of mouse anti-human ZNT1 monoclonal antibody (mAb) at 4 °C overnight [18,19]. Subsequently, the sections were incubated with biotin-conjugated anti-mouse IgG (Vector Laboratories) for 30 min at room temperature (approximately 15–25 °C), followed by the addition of peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan) for 5 min. Peroxidase activity was visualized using diaminobenzidine. The sections were counterstained with Mayer's hematoxylin (Muto Pure Chemicals, Co., Ltd., Tokyo, Japan) and mounted with malinol (Muto Pure Chemicals). The microscope used was a NanoZoomerS210 (C13239-01, <https://www.hamamatsu.com/jp/ja/product/life-science-and-medical-systems/digital-slide-scanner/C13239-01.html>) with a 20× NA 0.75 objective; scanning was performed in the 40-image mode. The software used for image processing after data acquisition was NDP.view2 (Hamamatsu Photonics KK, Shizuoka, Japan).

Preparation of mouse anti-human ZNT1 monoclonal antibody (mAb).

The procedure to generating anti-human ZNT1 mAb was briefly described previously (①), and is described in more detail here. Fused proteins consisting of the cytosolic C-terminal portion of human ZNT1 (167 residues from Leu341 to stop codon) and maltose-binding protein were used as antigens. The hybridoma that produces the anti-human ZNT1 mAb was screened using glutathione-S-transferase protein fused with the same portion of human ZNT1. The hybridoma was subcloned by the limiting dilution method, and a clone stably producing the mAb was established. A ascites was generated by injection of 1×10^7 hybridoma cells into pristine-primed mice, and the anti-human ZNT1 mAb was purified from ascites using the MabTrap Kit (Cytiva, Marlborough, MA, USA) according to the manufacture's instruction. The purified mAb was the IgG1 subclass,

which was determined using IsoStrip Kit (Merck, Darmstadt, Germany). Its specifically to detect human ZNT1 was confirmed in several experiments in previous studies (①, ②, ③), and the specificity of it used in immunohistochemical staining was confirmed using human cells lacking ZNT1 (Supplemental Figure 1).

①, Nishito Y, Tsuji N, Fujishiro H, Takeda T, Yamazaki T, Teranishi F, Okazaki F, Matsunaga A, Tuschl K, Rao R, Kono S, Miyajima H, Narita H, Himeno S and Kambe T “Direct Comparison of Manganese Detoxification/Efflux Proteins and Molecular Characterization of ZnT10 Protein as a Manganese Transporter” - *J. Biol. Chem.*, 291, 14773-14787, 2016

②, Nishito Y, and Kambe T

“Zinc transporter 1 (ZNT1) expression on the cell surface is elaborately controlled by cellular zinc levels”- *J. Biol. Chem.*, 294, 15686-15697, 2019

③, Nagamatsu S, Nishito Y, Yuasa H, Yamamoto N, Komori T, Suzuki T, Yasui H, Kambe T

“Sophisticated expression responses of ZNT1 and MT in response to changes in the expression of ZIPs”- *Sci. Rep.*, 12, 7334, 2022

Statistical analysis

All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R version 2.13.0 (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add the statistical functions frequently used in biostatistics. Univariate analysis was performed using a Cox proportional hazards model to determine which of the following 12 factors contributed to overall survival: α -

fetoprotein, Alb, NH3, protein induced by vitamin K absence-antagonist-II (PIVKAI), platelet count, prothrombin time (PT), total bilirubin, zinc, maximal tumor diameter of hepatocellular carcinoma, gross pathological classification, differentiation, and the expression of ZNT1. Factors with $P < 0.05$ were selected for multivariable analysis. The proportional hazards property was verified by visual inspection of the survival curves and by checking the parallelism of the stratified log-log plots. Significant factors with a P -value < 0.05 were included in the multivariable analysis. The Mann–Whitney U or chi-square tests were performed for two-group comparisons of background variables. The Kruskal–Wallis test was performed for multi-group comparisons and the Bonferroni test for pairwise comparisons. The cumulative overall survival rate in the study groups was determined by the Kaplan–Meier method and compared using the log-rank test.

Results

Correlation between ZNT1 expression and clinical parameters

The hepatocellular carcinoma cell membrane of 31 patients did not express ZNT1, whereas positive expression was noted in 34 patients: 9 strong positives and 25 positives according to their expression intensity. Two gastroenterologists determined whether a sample tested negative, positive, or strongly positive (Figure 1).

The negative and positive (including strong positive) groups were similar in blood zinc level (72.9 ± 14.1 vs. 66.7 ± 18.0 $\mu\text{g/dL}$, $P = 0.150$), α -fetoprotein level (52.2 ± 137.5 vs. $961.1 \pm 3,709.0$ ng/mL , $P = 0.731$), PIVKAI ($14,881.0 \pm 74,420.0$ vs. $6,325.0 \pm 25,255.0$ mAU/mL , $P = 0.737$), gross classification (small nodular type with indistinct margin/simple nodular type/simple nodular type with extra-nodular growth/confluent

multinodular type; 8/15/6/5 vs. 9/13/3/6, $P = 0.622$; Table 2), maximal tumor diameter ($P = 0.218$), and background liver disease ($P = 0.415$). When ZNT1-positive cases were divided into strong positive and positive, the blood zinc level in the strong positive group ($57.0 \pm 22.1 \mu\text{g/dL}$) was significantly lower than that in the positive ($71.1 \pm 14.2 \mu\text{g/dL}$, $P = 0.015$) and negative ($72.9 \pm 14.1 \mu\text{g/dL}$, $P = 0.043$) groups. The three groups were similar in terms of all other clinical parameters (Table 2).

ZNT1 expression and survival

Figure 2 shows the results of the Kaplan–Meier analysis of cumulative overall survival rates according to ZNT1 expression. The overall survival of patients with ZNT1 expression was significantly shorter than that of patients without ZNT1 expression (log-rank test, $P = 0.024$).

Univariate and multivariable analyses of factors contributing to overall survival

The univariate and multivariable analysis results on factors associated with survival are summarized in Table 3. The univariate analysis revealed a significant association with serum PIVKAI level, maximal hepatocellular carcinoma tumor diameter, and ZNT1 expression. The multivariable analysis showed that the maximal tumor diameter (hazard ratio [HR], 1.018; 95% confidence interval [CI], 1.002–1.034, $P = 0.026$) and ZNT1 expression (HR, 2.082; 95% CI, 1.196–3.621, $P = 0.010$) were significant prognostic factors.

Discussion and Conclusion

This study showed that the expression of ZNT1 on the hepatocellular carcinoma cell

membrane and its intensity were useful prognostic predictors of postoperative survival and that ZNT1 expression could be correlated with cancer grade. Bitirim [12] also reported immunohistochemistry results of ZNT1 expression in hepatocellular carcinoma, showing that histological differentiation correlated with ZNT1 expression; cases with ZNT1 expression were less differentiated, and therefore, had more malignant characteristics, supporting our results. However, while Bitirim [12] found an association between ZNT1 expression and the pathological grade, the clinical outcomes were not examined, making our report the first to show such an association between ZNT1 expression and the clinical outcomes after hepatocellular carcinoma resection.

ZNT1 is a transporter that moves intracytoplasmic zinc out of the cytoplasm. When its expression increases, the intracytoplasmic zinc concentration decreases. It has been noted in several studies that zinc concentration in hepatocellular carcinoma tissues [20,21] and cell lines [12] is lower than that in noncancerous tissues.

Zinc is thought to play an important role in carcinogenesis and cancer progression due to its multiple physiological functions, particularly since it is deficient in hepatocellular carcinoma tissues [12,22]; however, the detailed underlying mechanism remains unknown. Since zinc transporters play a key role in maintaining intracellular zinc homeostasis, the expression kinetics of these transporters can lead to abnormalities in intracellular zinc concentration. Consequently, their expression kinetics have been investigated in several cancers, including breast, prostate, and gallbladder cancer, and differences between cancer and non-cancer cells have been found [23,25]. A review published in 2014 by Costello et al. showed that the ZIP14 gene and protein expression levels in hepatocellular carcinoma cells were lower than those in noncancerous cells, indicating that low intracellular zinc levels might promote the carcinogenic process [22].

They also noted that other transporters that take up zinc (ZIP family) had been examined but with less clear results and that while the zinc efflux transporter ZNT1 had not been examined in human samples at the time, its expression was enhanced in the HepG2 cell line [22].

Later, in 2021, Bitirim reported the expression kinetics of several zinc transporters in hepatocellular carcinoma at the gene and protein levels [12]. In that report, it was shown that ZIP14 and ZNT1 protein expression differed significantly between cancerous and noncancerous tissues. The report also showed that the expression of these two transporters is reciprocal; ZIP14 expression decreases and ZNT1 expression increases as the cancer cell differentiation level decreases. Furthermore, the relationship between these two transporters and intracellular zinc concentration has been examined in two hepatocellular carcinoma cell lines with different degrees of differentiation [12]. The researchers showed that zinc concentration and ZIP14 expression were lower, and ZNT1 expression was higher in the less differentiated cell line. These studies showed that the zinc transporters were expressed on the plasma membrane.

Gartmann et al. showed in 2018 that the expression of ZIP4, ZIP14, and ZNT1 was enhanced in hepatocellular carcinoma, but they were localized to the cytoplasm [14], contrasting the reports mentioned above. Since transporters essentially function on the plasma membrane, it is possible that those detected by Gartmann et al. were not functioning as they should. Lehvy et al. examined the association between ZNT1 gene expression and the post-treatment prognosis in several carcinomas (hepatocellular carcinoma not included) [16]. They found that ZNT1 expression was upregulated in several cancer types and that while some carcinomas had a poorer prognosis with upregulated expression, others showed no difference in prognosis.

These results suggested that ZNT1 expression was enhanced and ZIP14 expression decreased in hepatocellular carcinoma, resulting in decreased intracytoplasmic zinc concentration, a significant impact on carcinogenesis and malignancy, and a worse prognosis after treatment. Although the detailed mechanisms by which intracytoplasmic zinc levels are involved in carcinogenesis and malignant transformation are unknown, it is possible that the low intracellular zinc concentration causes DNA damage, interferes with DNA damage repair, and reduces p53 binding [12]. Zinc has also been shown to suppress cancer by inducing A-20 [15,26], a negative regulator of intracellular nuclear factor kappa B, which is thought to act in a pro-cancer manner. Zinc is also involved in the urea cycle in hepatocytes, and intrahepatic zinc deficiency causes abnormal ammonia metabolism owing to impaired urea cycle function. Consequently, the abnormal ammonia metabolism causes abnormal protein metabolism due to branched-chain amino acid deficiency [27], which contributes to hepatocarcinogenesis in cirrhosis [28]. Zinc is involved in many functions. The mechanism associating the enhanced ZNT1 expression in hepatocellular carcinoma with worse clinical prognosis, as revealed in this study, remains to be clarified. These findings could direct clinicians in the identification of their patients at an elevated risk to optimize treatment strategies.

Limitations of this study include the rather small number of cases ($n = 65$), the single-center and retrospective nature of the study, as well as the limited conditions leading to tumor resection. For future research, we are considering conducting a large multicenter study with further follow-up data.

Lastly, discussion from a different perspective should be provided; Specifically, a moonlighting function of ZNT1. ZNT1 is known to be functional as a binding partner for several proteins through its cytosolic carboxy terminal portion, including the regulatory

β -subunit of the L-type calcium channel and RAF-1 (④, ⑤). Among them, interaction with RAF1 is extremely intriguing, because the interaction activates RAF1, leading to downstream activation of ERK (⑤, ⑥). Since the ERK signaling is known to be associated with cancer and ERK signaling pathway closely link with hepatocellular carcinoma, ZNT1 involved in hepatocellular carcinoma might not be related to zinc transport but rather to the effect of ZNT1 on the ERK signal transduction pathway. This hypothesis might be a possible mechanism and needs further investigation in future study.

④. Molecular basis for zinc transporter 1 action as an endogenous inhibitor of L-type calcium channels

Levy S, Beharier O, Etzion Y, Mor M, Buzaglo L, Shaltiel L, Gheber LA, Kahn J, Muslin AJ, Katz A, Gitler D, Moran A.- J Biol Chem, 2009 284, 32434-43.

⑤. Cation diffusion facilitator proteins modulate Raf-1 activity

T Jirakulaporn, AJ Muslin - Journal of Biological Chemistry, 2004, 27807–27815

⑥ . Zinc ions and cation diffusion facilitator proteins regulate Ras-mediated signaling, J Bruinsma, T Jirakulaporn, AJ Muslin, K Kornfeld
Developmental cell, 2002, 567–578

In conclusion, ZNT1 expression in resected hepatocellular carcinoma tissue samples was significantly associated with poor prognosis. To the best of our knowledge, this study is the first to demonstrate this association. Further studies are needed to elucidate the mechanism in more detail.

Statements and Declarations

Funding

Dr. Taiho Kambe has received research support from a Grant-in-Aid for Scientific Research on Innovative Areas “Integrated Bio-metal Science” (MEXT KAKENHI Grant Number JP19H05768) from the Ministry of Education, Culture, Sports, Science, and Technology.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Naruyasu Kakita, Kazuhiro Katayama, Ayako Hasimoto and Taiho Kambe. The first draft of the manuscript was written by Naruyasu Kakita and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors are grateful to Ms. Nakanishi, Ms. Takamure, and Mr. Ono of the Clinical Laboratory Department of Kaizuka City Hospital for their support in processing

hepatocellular carcinoma specimens, Ms. Ishihara and Ms. Tanaka of Clarks Medical, Kaizuka City Hospital, for their support in data collection, and Ms. Asami and Ms. Yura of the Nursing Bureau, Kaizuka City Hospital, for secretarial support. All acknowledged individuals have consented to be named in the manuscript.

(2) **Informed consent:** All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Approval was obtained from the ethics committees of Kaizuka City Hospital (No. 225) and Kyoto University Graduate School and Faculty of Medicine (No. R2546), and the requirement for written informed consent was waived; the study was presented on the institutional website (hosp.kaizuka.osaka.jp/hospital/rinsyo-kenkyu/optout.html, kyoto-u.ac.jp/ja/research/rule/opt-out), and the patients were given the opportunity to refuse participation by opting out.

References

- [1] P.K. To PK, M.H. Do, J.H. Cho, C. Jung, Growth modulatory role of zinc in prostate cancer and application to cancer therapeutics, *Int. J. Mol. Sci.* 21(2020)2991. <https://doi.org/10.3390/ijms21082991>.
- [2] European Association for the Study of the Liver, EASL Clinical Practice Guidelines: management of hepatocellular carcinoma, *J. Hepatol.* 69(2018)182–236. <https://doi.org/10.1016/j.jhep.2018.03.019>.
- [3] K.A. McGlynn, J.L. Petrick, H.B. El-Serag, Epidemiology of hepatocellular carcinoma, *Hepatology* 73(2021)4–13. <https://doi.org/10.1002%2Fhep.31288>.
- [4] J. Zucman-Rossi, A. Villanueva, J.C. Nault, J.M. Llovet, Genetic landscape and biomarkers of hepatocellular carcinoma, *Gastroenterology* 149(2015)1226–1239.e4. <https://doi.org/10.1053/j.gastro.2015.05.061>.
- [5] G. Khemlina, S. Ikeda, R. Kurzrock, The biology of hepatocellular carcinoma: implications for genomic and immune therapies, *Mol Cancer.* 16(2017)149. <https://doi.org/10.1186/s12943-017-0712-x>.
- [6] K. Katayama, T. Kawaguchi, K. Shiraishi, T. Ito, K. Suzuki, C. Koreeda, T. Ohtake, M. Iwasa, Y. Tokumoto, R. Endo, N. Kawamura, The prevalence and implication of zinc deficiency in patients with chronic liver disease, *J. Clin. Med. Res.* 10(2018)437–444. <https://doi.org/10.14740/jocmr3374w>.
- [7] A. Hosui, E. Kimura, S. Abe, T. Tanimoto, K. Onishi, Y. Kusumoto, Y. Sueyoshi, K. Matsumoto, M. Hirao, T. Yamada, N. Hiramatsu, Long-term zinc supplementation improves liver function and decreases the risk of developing hepatocellular carcinoma, *Nutrients* 10(2018)1955. <https://doi.org/10.3390/nu10121955>.
- [8] R. Shigefuku, M. Iwasa, K. Katayama, A. Eguchi, T. Kawaguchi, K. Shiraishi, T. Ito,

- K. Suzuki, C. Koreeda, T. Ohtake, Y. Tokumoto, R. Endo, N. Kawamura, M. Shiraki, D. Habu, H. Sakai, A. Kato, S. Nishiguchi, H. Moriwaki, K. Suzuki, Y. Takei, Hypozincemia is associated with human hepatocarcinogenesis in hepatitis C virus-related liver cirrhosis, *Hepatol. Res.* 49(2019)1127–1135. <https://doi.org/10.1111/hepr.13388>.
- [9] Y. Tamai, M. Iwasa, A. Eguchi, R. Shigefuku, K. Sugimoto, H. Hasegawa, Y. Takei, Serum copper, zinc and metallothionein serve as potential biomarkers for hepatocellular carcinoma, *PLoS One.* 15(2020)e0237370. <https://doi.org/10.1371/journal.pone.0237370>.
- [10] K. Imai, T. Beppu, T. Yamao, H. Okabe, H. Hayashi, H. Nitta, D. Hashimoto, K. Mima, S. Nakagawa, K. Sakamoto, A. Chikamoto, T. Ishiko, H. Baba, Clinicopathological and prognostic significance of preoperative serum zinc status in patients with hepatocellular carcinoma after initial hepatectomy. *Ann. Surg. Oncol.* 21(2014)3817–3826. <https://doi.org/10.1245/s10434-014-3786-3>.
- [11] I. Stamoulis, G. Kouraklis, S. Theocharis, Zinc and the liver: an active interaction, *Dig. Dis. Sci.* 52(2007)1595–1612. <https://doi.org/10.1007/s10620-006-9462-0>.
- [12] C.V. Bitirim, The role of zinc transporter proteins as predictive and prognostic biomarkers of hepatocellular cancer, *PeerJ.* 9(2021)e12314. <https://doi.org/10.7717/peerj.12314>.
- [13] R.B. Franklin, B.A. Levy, J. Zou, N. Hanna, M.M. Desouki, O. Bagasra, L.A. Johnson, L.C. Costello, ZIP14 zinc transporter downregulation and zinc depletion in the development and progression of hepatocellular cancer, *J. Gastrointest. Cancer.* 43(2012)249–257. <https://doi.org/10.1007/s12029-011-9269-x>.
- [14] L. Gartmann, T. Wex, K. Grüngreiff, D. Reinhold, T. Kalinski, P. Malfertheiner, K. Schütte, Expression of zinc transporters ZIP4, ZIP14 and ZnT9 in hepatic

- carcinogenesis—an immunohistochemical study. *J. Trace. Elem. Med. Biol.* 49(2018)35–42. <https://doi.org/10.1016/j.jtemb.2018.04.034>.
- [15] D. Skrajnowska, B. Bobrowska-Korczak, Role of zinc in immune system and anti-cancer defense mechanisms. *Nutrients* 11(2019)2273. <https://doi.org/10.3390/nu11102273>.
- [16] A.I. Lehy, G. Horev, Y. Golan, F. Glaser, Y. Shammai, Y.G. Assaraf, Alterations in ZnT1 expression and function lead to impaired intracellular zinc homeostasis in cancer, *Cell. Death. Discov.* 5(2019)144. <https://doi.org/10.1038/s41420-019-0224-0>.
- [17] M. Kudo, M. Kitano, T. Sakurai, N. Nishida, General rules for the clinical and pathological study of primary liver cancer, nationwide follow-up survey and clinical practice guidelines: the Outstanding Achievements of the Liver Cancer Study Group of Japan, *Dig. Dis.* 33(2015)765–770. <https://doi.org/10.1159/000439101>.
- [18] Y. Nishito, T. Kambe, Zinc transporter 1 (ZNT1) expression on the cell surface is elaborately controlled by cellular zinc levels, *J. Biol. Chem.* 294(2019)15686–15697. <https://doi.org/10.1074/jbc.ra119.010227>.
- [19] S. Nagamatsu, Y. Nishito, H. Yuasa, N. Yamamoto, T. Komori, T. Suzuki, H. Yasui, T. Kambe, Sophisticated expression responses of ZNT1 and MT in response to changes in the expression of ZIPs, *Sci. Rep.* 12(2022)7334. <https://doi.org/10.1038/s41598-022-10925-2>.
- [20] M. Ebara, H. Fukuda, R. Hatano, H. Saisho, Y. Nagato, K. Suzuki, K. Nakajima, M. Yukawa, F. Kondo, A. Nakayama, H. Sakurai, Relationship between copper, zinc and metallothionein in hepatocellular carcinoma and its surrounding liver parenchyma, *J. Hepatol.* 33(2000)415–422. [https://doi.org/10.1016/s0168-8278\(00\)80277-9](https://doi.org/10.1016/s0168-8278(00)80277-9).
- [21] K.Y. Liaw, P.H. Lee, F. C. Wu, J. S. Tsai, S.Y. Lin-Shiau, Zinc, copper, and

superoxide dismutase in hepatocellular carcinoma, *Am. J. Gastroenterol.* 92(1997)2260–2263.

[22] L.C. Costello, R.B. Franklin, The status of zinc in the development of hepatocellular cancer: an important, but neglected, clinically established relationship, *Cancer. Biol. Ther.* 15(2014)353–360. <https://doi.org/10.4161/cbt.27633>.

[23] T. Takatani-Nakase, Zinc transporters and the progression of breast cancers, *Biol. Pharm. Bull.* 41(2018)1517–1522. <https://doi.org/10.1248/bpb.b18-00086>.

[24] Z. Pan, S. Choi, H. Ouadid-Ahidouch, J.M. Yang, J.H. Beattie, I. Korichneva, Zinc transporters and dysregulated channels in cancers, *Front. Biosci. (Landmark Ed)* 22(2017)623–643. <https://doi.org/10.2741/4507>.

[25] C. Hogstrand, P. Kille, R.I. Nicholson, K.M. Taylor, Zinc transporters and cancer: a potential role for ZIP7 as a hub for tyrosine kinase activation, *Trends. Mol. Med.* 15(2009)101–111. <https://doi.org/10.1016/j.molmed.2009.01.004>

[26] A.S. Prasad, F.W.J. Beck, D.C. Snell, O. Kucuk, Zinc in cancer prevention, *Nutr. Cancer.* 61(2009)879–887. <https://doi.org/10.1080/01635580903285122>.

[27] K. Katayama. Zinc and protein metabolism in chronic liver diseases, *Nutr. Res.* 74(2020)1–9. <https://doi.org/10.1080/01635580903285122>.

[28] T. Kawaguchi, K. Shiraishi, T. Ito, K. Suzuki, C. Koreeda, T. Ohtake, M. Iwasa, Y. Tokumoto, R. Endo, N.H. Kawamura, M. Shiraki, D. Habu, S. Tsuruta, Y. Miwa, A. Kawaguchi, T. Kakuma, H. Sakai, N. Kawada, T. Hanai, Shin-ichi Takahashi, A. Kato, M. Onji, Y. Takei, Y. Kohgo, T. Seki, M. Tamano, K. Katayama, T. Mine, M. Sata, H. Moriwaki, K. Suzuki, Branched-chain amino acids prevent hepatocarcinogenesis and prolong survival of patients with cirrhosis, *Clin. Gastroenterol. Hepatol.* 12(2014)1012–1018.e1. <https://doi.org/10.1016/j.cgh.2013.08.050>.

Tables

Table 1. Patient characteristics

Characteristic	Value
Age (years)	69.7±9.8
Sex (male/female)	51/14
Etiology: HBV/HCV/alcohol/NAFLD/others	11/28/15/10/1
Albumin (g/dL)	4.1±0.4
Total bilirubin (mg/dL)	0.7±0.3
PT (%)	88.4±14.3
Platelet count (10 ⁴ /μL)	16.4±6.6
AFP (ng/mL)	514.0±2724.0
PIVKAI (mAU/mL)	10107.0±53453
Zn (μg/dL)	70.5±15.7
NH ₃ (μg/dL)	44.0±23.5
Child–Pugh classification A/B	63/2
Expression of ZNT1: negative/positive/strongly positive	31/25/9
Gross classification (#1/#2/#3/#4)	15/28/9/13
Maximal tumor diameter (mm)	28.5±20.1

Data are presented as the number of patients or mean ± standard deviation, as appropriate.

The #1, #2, #3, and #4 in the gross classification represent small nodular type with indistinct margins, simple nodular type, simple nodular type with extra-nodular proliferation, and confluent multinodular type, respectively.

Abbreviations: HBV, hepatitis B; HCV, hepatitis C; NAFLD, non-alcoholic fatty liver

disease; PT, prothrombin time; AFP, α -fetoprotein; PIVK_{II}, protein induced by vitamin K absence/antagonist-II; ZNT1, zinc transporter-1.

Table 2. Comparison of clinical indicators by ZNT1 expression intensity

	Expression of ZNT1		
	Negative (-)	Positive (+, 2+)	P-value
Zn (μg/dL)	72.9±14.1	66.7±18.0	0.150
AFP (ng/mL)	52.2±137.5	961.1±3709.0	0.731
PIVKaII (mAU/mL)	14881.0±74420.0	6325.0±25255.0	0.737
Gross classification (#1/#2/#3/#4)	9/13/3/6	8/15/6/5	0.622

	Expression of ZNT1			
	Negative (-)	Positive (+)	Strongly positive (2+)	P-value
Zn (μg/dL)	72.9±14.1	71.1±14.2	57.0±22.1	*0.043
		* **		**0.015
AFP (ng/mL)	52.2±137.5	1246.0±4305.0	170.3±443.0	0.480
PIVKaII (mAU/mL)	14881.0±74420.0	6788.0±28695.0	5038.0±12580.0	0.372
Gross classification (#1/#2/#3/#4)	9/13/3/6	7/11/4/3	1/4/2/2	0.794

The #1, #2, #3, and #4 denotations in the gross classification represent small nodular type with indistinct margin, simple nodular type, simple nodular type with extra-nodular proliferation, and confluent multinodular type, respectively. There were no differences in zinc, AFP, PIVKAI, or gross classification between the negative and positive ZNT1 groups by Mann–Whitney U test; however, when comparing the negative, positive, and strongly positive groups, the strongly positive group had significantly lower serum zinc levels than the negative and positive groups. The Kruskal–Wallis test was used for comparison among the three groups, and the Bonferroni test was used for pairwise comparison.

Abbreviations: ZNT1, Zn transporter-1; AFP, α -fetoprotein; PIVKAI, protein induced by vitamin K absence/antagonist-II.

Table 3. Univariate and multivariable analyses of the investigated factors associated with survival

	Univariate analysis			Multivariable analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
AFP (ng/mL) <10, >10	2.058	0.935-4.527	0.072			
Alb (g/dL)	0.584	0.194-1.758	0.339			
NH3 (μg/dL)	1.016	0.997-1.036	0.100			
PIVKAlI (mAU/mL) <75, >75	2.364	1.045-5.352	0.038	1.702	0.661-4.385	0.271
Platelet count (10 ⁴ /μL)	1.022	0.963-1.084	0.470			
PT (%)	0.991	0.962-1.020	0.519			
Total bilirubin (mg/dL)	0.269	0.059-1.227	0.089			
Zn (μg/dL)	0.994	0.967-1.023	0.695			
Maximal tumor diameter (mm)	1.021	1.007-1.035	0.004	1.018	1.002-1.034	0.026

Gross pathological classification, four categories	1.332	0.923-1.923	0.125			
Differentiation high, poor	1.376	0.708-2.672	0.346			
Expression of ZNT1 positive, negative	2.557	1.094-5.976	0.030	2.082	1.196-3.621	0.010

The multivariable analysis of factors contributing to prognosis has identified maximal tumor diameter and ZNT1 expression as significant factors.

Abbreviations: HR, hazard ratio; CI, confidence interval; AFP, α -fetoprotein; PIVKAI, protein induced by vitamin K absence/antagonist-II; PT, prothrombin time; ZNT1, Zn transporter-1.

Figure Captions

Fig. 1 Immunohistochemical staining of cell membranes for ZNT1

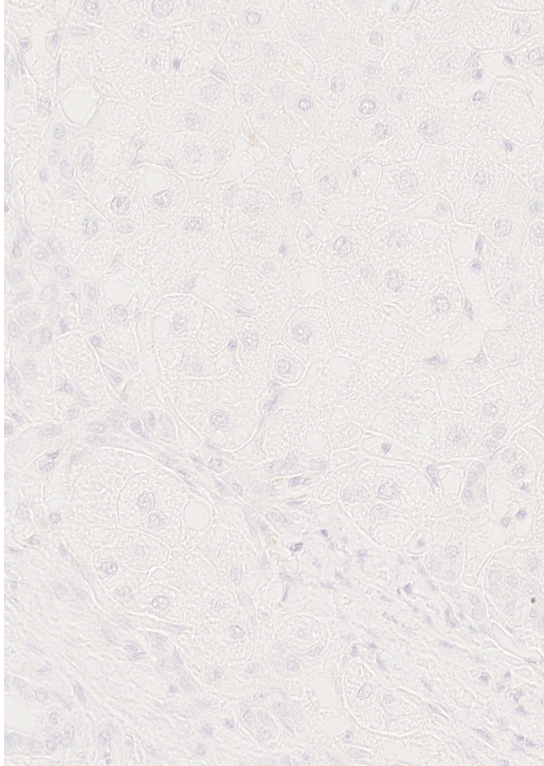
(a) ZNT1 negative; (b) ZNT1 positive; (c) ZNT1 strongly positive.

Two gastroenterologists determined the ZNT1 expression intensity. ZNT1, zinc transporter 1

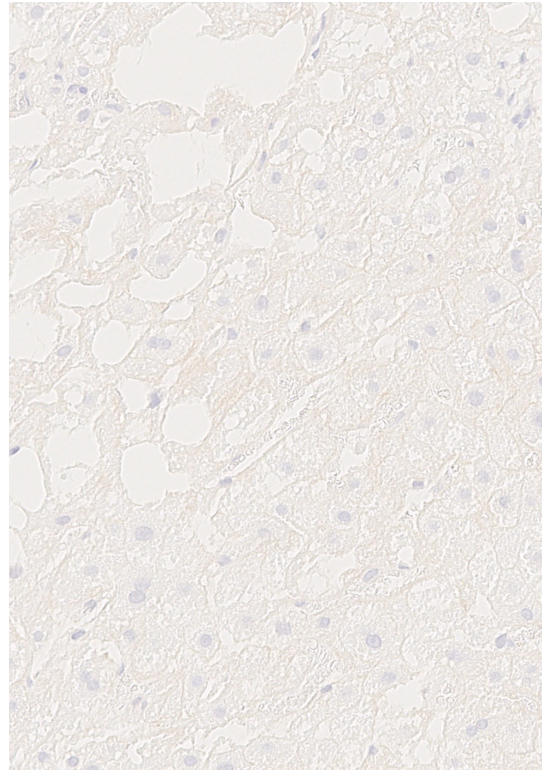
Fig. 2 Cumulative overall survival according to the Kaplan–Meier method

The group with no ZNT1 expression had a significantly better prognosis than the group with positive expression, with median overall survival over 120 and 58 months, respectively (log-rank test, $P = 0.024$)

A) Negative



B) Positive



C) Strongly Positive

