Gastro Hep Advances 2023;2:83–95

ORIGINAL RESEARCH—CLINICAL

Protein Kinase C Delta Is a Novel Biomarker for Hepatocellular Carcinoma

Tsunekazu Oikawa,[1,*] Kohji Yamada,[2,*] Akihito Tsubota,[3,*] Chisato Saeki,[1] Naoko Tago,[2] Chika Nakagawa,[1] Kaoru Ueda,[1] Hiroshi Kamioka,[1,2] Tomohiko Taniai,[4] Koichiro Haruki,[4] Masanori Nakano,[1] Yuichi Torisu,[1] Toru Ikegami,[4] Kiyotsugu Yoshida,[2] and Masayuki Saruta[1]

[1]Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, Tokyo, Japan; [2]Department of Biochemistry, The Jikei University School of Medicine, Tokyo, Japan; [3]Core Research Facilities, Research Center for Medical Science, The Jikei University School of Medicine, Tokyo, Japan; and [4]Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, The Jikei University School of Medicine, Tokyo, Japan

BACKGROUNDS AND AIMS: Hepatocellular carcinoma (HCC) is the most common cancer with a poor prognosis. Identification of an alternative biomarker that can detect early-stage and conventional tumor marker-negative HCC is urgently needed. We found that protein kinase C delta (PKCδ) is specifically secreted from HCC cell lines into extracellular space and contributes to tumor development and that its serum levels were elevated in HCC patients. This study aimed to assess the practical usefulness of serum PKCδ for detecting HCC in chronic liver disease (CLD) patients. **METHODS:** Serum PKCδ levels in 313 CLD patients with and without HCC (n = 187 and 126, respectively) were measured using a sandwich enzyme-linked immunosorbent assay. The diagnostic performance of PKCδ for HCC was evaluated using the receiver operating characteristic curve analysis and was compared with that of conventional markers, α-fetoprotein (AFP), and des-γ-carboxy prothrombin (DCP). **RESULTS:** Serum PKCδ levels in HCC patients were significantly higher than those in CLD patients without HCC. PKCδ distinguished HCC patients from CLD patients without HCC, with high sensitivity and specificity. Subgroup analyses revealed that the diagnostic performance of PKCδ for HCC was comparable to that of AFP and DCP, and that approximately 40% of AFP/DCP double-negative HCC patients were positive for PKCδ. PKCδ yielded better diagnostic performance for detecting solitary small-sized (ie, very early stage) HCC than AFP and DCP. There was no significant correlation between serum PKCδ and AFP/DCP levels. **CONCLUSION:** Serum PKCδ is a novel HCC biomarker, which is independent of and complementary to conventional markers. Specifically, PKCδ may be useful for detecting very early-stage or AFP/DCP double-negative HCC.

Keywords: HCC; PKCδ; Biomarker; Tumor Marker; Early Detection

See editorial on page 158

Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and is the fourth leading cause of cancer-related mortality worldwide.[1] The only curative treatments for patients with early-stage HCC are surgical resection and liver transplantation. However, most patients are diagnosed with advanced-stage HCC when these therapies are not recommended. Alternatively, transcatheter arterial chemoembolization (TACE) and systemic chemotherapy, including molecular-targeted agents, have been performed in patients with intermediate- to advanced-stage HCC.[2] The advent of immune checkpoint inhibitors has substantially improved the treatment outcome in combination with molecular-targeted agents for such patients.[3] However, the number of patients who benefit from innovative treatment is limited due to its limited effectiveness. Therefore, early detection of HCC is urgently required to eradicate this aggressive cancer.

α-Fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP), also known as protein induced by vitamin K absence or antagonist-II (PIVKA-II), have been commonly used as conventional biomarkers for HCC in clinical practice.[4,5] Although numerous studies on HCC have shown their usefulness for diagnosis, surveillance, monitoring progression and recurrence, and the evaluation of treatment response,[6–9] several problems remain. Specifically, the sensitivity and specificity for HCC diagnosis, especially at the early stage, are not fully satisfactory. Only 40%–60% of

***Contributed equally to this work with Tsunekazu Oikawa, Kohji Yamada, and Akihito Tsubota.**

Abbreviations used in this paper: **AFP, α-fetoprotein; ALT, alanine aminotransferase; AUC, area under the receiver operating characteristic curve; BCLC, Barcelona Clinic Liver Cancer; CH, chronic hepatitis; CLD, chronic liver disease; CT, computed tomography; DCP, Des-γ-carboxy prothrombin; ELISA, enzyme-linked immunosorbent assay; Gd-EOB-DTPA, gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid; HCC, hepatocellular carcinoma; LC, liver cirrhosis; MRI, magnetic resonance imaging; NPV, negative predictive values; PKCδ, protein kinase C delta; Plt, platelet; PPV, positive predictive values; TACE, transcatheter arterial chemoembolization.**

Most current article

2772-5723
https://doi.org/10.1016/j.gastha.2022.07.020

HCC patients are positive for these markers, and the positive rate further decreases to around 30% in early-stage patients although it increases along with progression toward the late stage.[10,11] AFP levels are elevated even in acute or chronic liver damage caused by various etiologies and other cancers, resulting in reduced specificity. Furthermore, it should be noted that elevated DCP levels are found in patients with vitamin K deficiency associated with jaundice and when antiangiogenic agents or antibiotics that inhibit the vitamin K cycle are administered.[12] Therefore, it is necessary to identify an alternative biomarker that can identify HCC patients, especially AFP/DCP double-negative or false-positive patients.

Protein kinase C delta (PKCδ) has been identified as an intracellular serine/threonine kinase, and its activation is found in various cancers, including HCC, and is associated with cell survival and invasion.[13–15] Recently, we reported for the first time that PKCδ is unconventionally secreted into the extracellular space in HCC cells, but not in gastrointestinal cancer cells or normal hepatocytes.[16] Extracellularly secreted PKCδ behaves like growth factors; that is, it stimulates the IGF1R and EGFR signaling and subsequently enhances the ERK1/2 and STAT3, leading to the progression of HCC.[16,17] Moreover, we demonstrated that serum PKCδ levels in HCC patients were significantly higher than those in patients with chronic liver disease (CLD) and healthy individuals, suggesting that serum PKCδ could be a potential biomarker for screening or detecting HCC.

This study aimed to evaluate the usefulness of serum PKCδ as a novel biomarker for HCC diagnosis in patients with CLD by comparing conventional tumor markers.

Patients and Methods

Study Design

This preliminary study assessed the usefulness of serum PKCδ as a novel biomarker for HCC using serum samples from CLD patients with and without HCC and healthy individuals. All participants were older than 20 years and recruited at the Jikei University School of Medicine. They all voluntarily provided written informed consent. Serum samples from HCC patients were collected before treatment (surgical resection, ablation, TACE, and/or systemic chemotherapy) between 2018 and 2022. Aside from the etiology, CLD was diagnosed using biochemistry, imaging (ultrasonography, dynamic computed tomography [CT], and/or magnetic resonance imaging [MRI]), and/or histologic analysis.[18] HCC, including solitary small-sized HCC (≤20 mm in diameter), was diagnosed based on contrast-enhanced imaging findings (perflubutane [Sonazoid; Daiichi Sankyo, Tokyo, Japan]-enhanced ultrasonography, dynamic iodinated contrast medium-enhanced CT, and/or gadolinium ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced MRI [Gd-EOB-DTPA-enhanced MRI]) and/or tumor biopsy according to the American Association for the Study of Liver Diseases guidelines.[19,20] HCC conditions were staged according to the eighth edition of the tumor, node, metastasis classification system released by the American Joint Committee on Cancer/Union for International Cancer Control[21] and the Barcelona Clinic Liver Cancer (BCLC) staging systems.[22] Patients with the following conditions were excluded: (1) presence of double cancers (HCC with another extrahepatic cancer); (2) presence of obstructive jaundice and severe hepatic failure; (3) pregnancy; and (4) treatment with antibiotics or antiangiogenic drugs. This study was conducted in accordance with the Declaration of Helsinki and ethical guidelines issued by administrative departments and was approved by the Local Ethics Committee of the Jikei University School of Medicine (approval no. 29–135 [8751]).

Serum PKCδ, AFP, and DCP Measurements

Serum PKCδ levels were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) kit using only 1 μL of 100-fold diluted serum, according to the manufacturer's instructions (MyBioSource, San Diego, CA). Serum AFP and DCP levels were measured using a chemiluminescence enzyme immunoassay (Tosoh bioscience, Brisbane, CA).

Statistical Analysis

Fisher's exact test, χ^2 test, Student's t-test, Mann-Whitney U test, and McNemar's test were used to compare 2 groups, as appropriate. Multiple comparisons of continuous variables among 3 groups were performed using the Kruskal-Wallis test, followed by the Steel-Dwass post-hoc test. The association between a variable with 2 categories and a variable with multiple categories was analyzed using the Cochran-Armitage trend test. Spearman's correlation was used to evaluate the correlation between serum PKCδ and conventional markers (AFP and DCP). The diagnostic performance of serum PKCδ for HCC was evaluated in terms of sensitivity, specificity, positive and negative predictive values (PPV and NPV, respectively), and the area under the receiver operating characteristic curve (AUC). The optimal cutoff value for diagnosing HCC was determined using Youden J statistics.[23] Propensity score matching involving one-to-one pairing of patients was performed with propensity scores matched at 2 decimal places. Propensity score matching was conducted based on age, gender, aspartate aminotransferase, and presence of cirrhosis for the matched cohort 1; and age, aspartate aminotransferase, platelet count, and presence of cirrhosis for the matched cohort 2 with calibration of 0.2. All P values were 2-tailed, and a value of $<.05$ was considered statistically significant. All statistical analyses were performed using R version 4.0.3 (The R Foundation for Statistical Computing, http://www.R-project.org) and IBM SPSS version 23.0 (IBM Japan, Tokyo, Japan).

Results

Characteristics of Patients

More recently, we reported that 19 CLD patients with HCC had significantly higher serum PKCδ levels than 16 CLD patients without HCC and 8 healthy subjects.[16] In this study, we added 278 CLD patients (168 with and 110 without HCC) and 1 healthy subject to the previous cohort. Accordingly, a total of 313 CLD patients with and without HCC were included in this analysis. These patients were divided into 2 groups according to the time of sample collection (2018–2020 and 2021–2022): cohort A (CLD with HCC ["HCC"], n = 108; and CLD without HCC ["non-HCC"],

Table 1. Characteristics of Patients in Cohort A and B

Patient characteristics	Cohort A (n = 182)	Cohort B (n = 131)	*P* value
Age (y)	70 (61–77)	68 (57–74)	.165
Gender			
Male	129 (70.9%)	101 (77.1%)	.271
Female	53 (29.1%)	30 (22.9%)	
Etiology			
Viruses	87 (47.8%)	43 (32.8%)	.011
Others	95 (52.2%)	88 (67.2%)	
Liver damage			
CH	48 (26.4%)	42 (32.1%)	.332
LC	134 (73.6%)	89 (67.9%)	
Child-Pugh classification			.778
A	146 (80.2%)	101 (77.1%)	
B	33 (18.1%)	27 (20.6%)	
C	3 (1.6%)	3 (2.3%)	
Biochemistry			
AST (U/L)	33 (24–50)	35 (24–57)	.535
ALT (U/L)	23 (17–41)	27 (19–46)	.397
Plt ($10^4/\mu$L)	14.9 (9.9–20.0)	15.1 (10.5–20.1)	.860
Tumor markers			
PKCδ (ng/mL)	41.9 (32.9–56.6)	45.0 (35.7–58.2)	.148
AFP (ng/mL)	5.0 (3.0–11.0)	5.0 (3.0–12.0)	.556
DCP (mAU/mL)	24.0 (16.0–81.0)	27.0 (18.0–128.0)	.340
HCC			
Presence/absence	108/74	79/52	
UICC Stage			.389
I	40 (37.0%)	39 (49.4%)	
II	40 (37.0%)	22 (27.8%)	
III	18 (16.7%)	11 (13.9%)	
V	10 (9.3%)	7 (8.9%)	
BCLC stage			.491
0	20 (18.5%)	21 (26.6%)	
A	42 (38.9%)	29 (36.7%)	
B	30 (27.8%)	19 (24.1%)	
C	16 (14.8%)	10 (12.7%)	

Data are shown as median (interquartile range) or number (percentage).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; CH, chronic hepatitis; CLD, chronic liver disease; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; LC, liver cirrhosis; PKCδ, protein kinase C delta; Plt, platelet; UICC, Union for International Cancer Control.

n = 74) and cohort B (HCC, n = 79; and non-HCC, n = 52) (Table 1). Furthermore, matched cohort 1 for patients with BCLC all stages (HCC, n = 63; and non-HCC, n = 63) and the matched cohort 2 for those with BCLC stage 0 (HCC, n = 23; and non-HCC, n = 23) were created by one-to-one matching based on their propensity scores (Table A1). A flow diagram of this study is shown in Figure 1A.

Serum PKCδ Levels in HCC Patients

In cohort A, serum PKCδ levels significantly differed between healthy subjects, non-HCC patients, and HCC patients ($P < .001$; Figure 1B). Of note, they significantly increased from healthy subjects to HCC patients. The median levels in healthy subjects, non-HCC patients, and HCC patients were 27.0, 37.9, and 46.9 ng/mL, respectively. Thus, serum PKCδ levels in HCC patients were the highest among the 3 groups (vs non-HCC patients and vs healthy subjects, $P < .001$ for both; Figure 1B). In contrast, serum PKCδ levels were extremely low in healthy subjects (vs non-HCC patients, $P = .003$). These results suggest that PKCδ may be a useful novel marker for HCC.

Diagnostic Performance of Serum PKCδ for HCC

The diagnostic performance of serum PKCδ for HCC was evaluated using the receiver operating characteristic curve analysis in cohort A. PKCδ clearly distinguished between HCC patients and healthy subjects (AUC, 0.968; sensitivity, 88.9%; specificity, 100.0%; Table A2). PKCδ also discriminated HCC patients from non-HCC patients (including those with chronic hepatitis [CH] and liver

cirrhosis [LC]) and from those with LC alone. The AUC and cutoff values of PKCδ for HCC diagnosis were 0.686 (vs non-HCC patients with CH and LC) and 0.548 (vs non-HCC patients with LC alone) and 57.7 ng/mL for both (Table A2). When PKCδ of >57.7 ng/mL was set as abnormal and considered positive, PPV for PKCδ (95.3%)

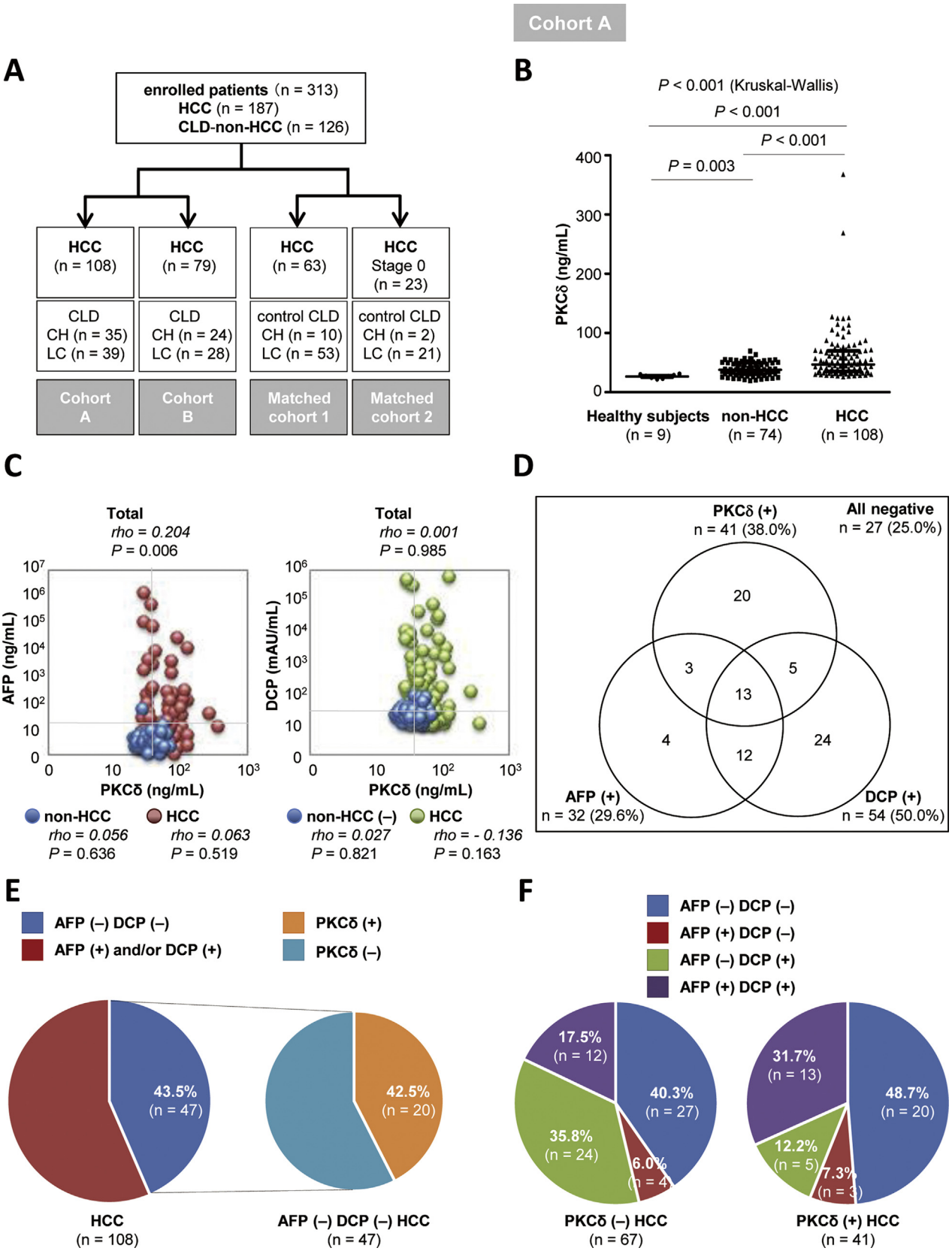


Table 2. Diagnostic Performance of PKCδ, AFP, and DCP for HCC

Cohort A	Sensitivity	Specificity	PPV	NPV	Accuracy
Single marker					
AFP	29.6	98.6	97.0	49.0	57.7
DCP	50.0	93.2	91.5	56.1	66.7
PKCδ	38.0	97.3	95.3	51.8	62.1
Double markers					
AFP/DCP	56.5	91.9	91.0	59.1	70.9
PKCδ/AFP	52.8	95.9	95.0	58.2	70.3
PKCδ/DCP	71.3	90.5	91.7	68.4	79.1
Triple markers					
PKCδ/AFP/DCP	75.0	89.2	91.0	71.0	80.8
Cohort B	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
Single marker					
AFP	32.9	94.2	89.7	48.0	57.3
DCP	55.7	86.5	86.3	56.2	67.9
PKCδ	38.0	92.3	88.2	49.5	59.5
Double markers					
AFP/DCP	67.1	80.8	84.1	61.8	72.5
PKCδ/AFP	54.4	86.5	86.0	55.6	67.2
PKCδ/DCP	75.9	82.7	87.0	69.4	78.6
Triple markers					
PKCδ/AFP/DCP	78.5	76.9	83.8	70.2	77.9
Matched cohort 1	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
Single marker					
AFP	33.3	100.0	100.0	60.0	66.7
DCP	44.4	87.3	77.8	61.1	65.9
PKCδ	42.9	92.1	84.4	61.7	67.5
Double markers					
AFP/DCP	55.6	87.3	81.4	66.3	71.4
PKCδ/AFP	55.6	92.1	87.5	67.4	73.8
PKCδ/DCP	69.8	82.5	80.0	73.2	76.2
Triple markers					
PKCδ/AFP/DCP	73.0	82.5	80.7	75.4	77.8

AFP, α-fetoprotein; CH, chronic hepatitis; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; LC, liver cirrhosis; NPV, negative predictive values; PKCδ, protein kinase C delta; PPV, positive predictive values.

was not inferior or comparable to that of AFP (>20.0 ng/mL; 97.0%) or DCP (>40.0 mAU/mL; 91.5%) (Tables 2 and A2; vs AFP, $P = .212$; and vs DCP, $P = .118$). There were no significant differences in sensitivity or specificity between PKCδ and conventional markers.

These results suggest that a high level of serum PKCδ is indicative of the presence of HCC and that the diagnostic

Figure 1. (A) A flow diagram of this study. (B) Serum PKCδ levels in HCC patients, non-HCC patients, and healthy subjects in cohort A. There were significant differences in serum PKCδ levels among the 3 groups ($P < .001$ by the Kruskal-Wallis test). Serum PKCδ levels in HCC patients were significantly higher than those in non-HCC patients and healthy subjects ($P < .001$ for both by the Steel-Dwass test). The longest horizontal line through the middle of each plot represents the median. The median serum PKCδ levels in healthy subjects, CLD patients, and HCC patients were 27.0, 37.9, and 46.9 ng/mL, respectively. (C) Correlation between serum PKCδ and conventional markers (AFP and DCP) in HCC and non-HCC patients in cohort A. (D) The numbers of PKCδ-, AFP-, and DCP-positive HCC patients in cohort A. (E) The proportion of AFP/DCP double-negative and AFP- and/or DCP-positive HCC patients in cohort A (left). The proportion of PKCδ-positive and PKCδ-negative patients in the AFP/DCP double-negative group (right). (F) The percentages of AFP-positive/AFP-negative and/or DCP-positive/DCP-negative patients in the PKCδ-negative and PKCδ-positive HCC groups in cohort A. The cutoff values of PKCδ, AFP, and DCP were 57.7 ng/mL, 20.0 ng/mL, and 40.0 mAU/mL, respectively. AFP, α-fetoprotein; CH, chronic hepatitis; CLD, chronic liver disease; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; LC, liver cirrhosis; PKCδ, protein kinase C delta.

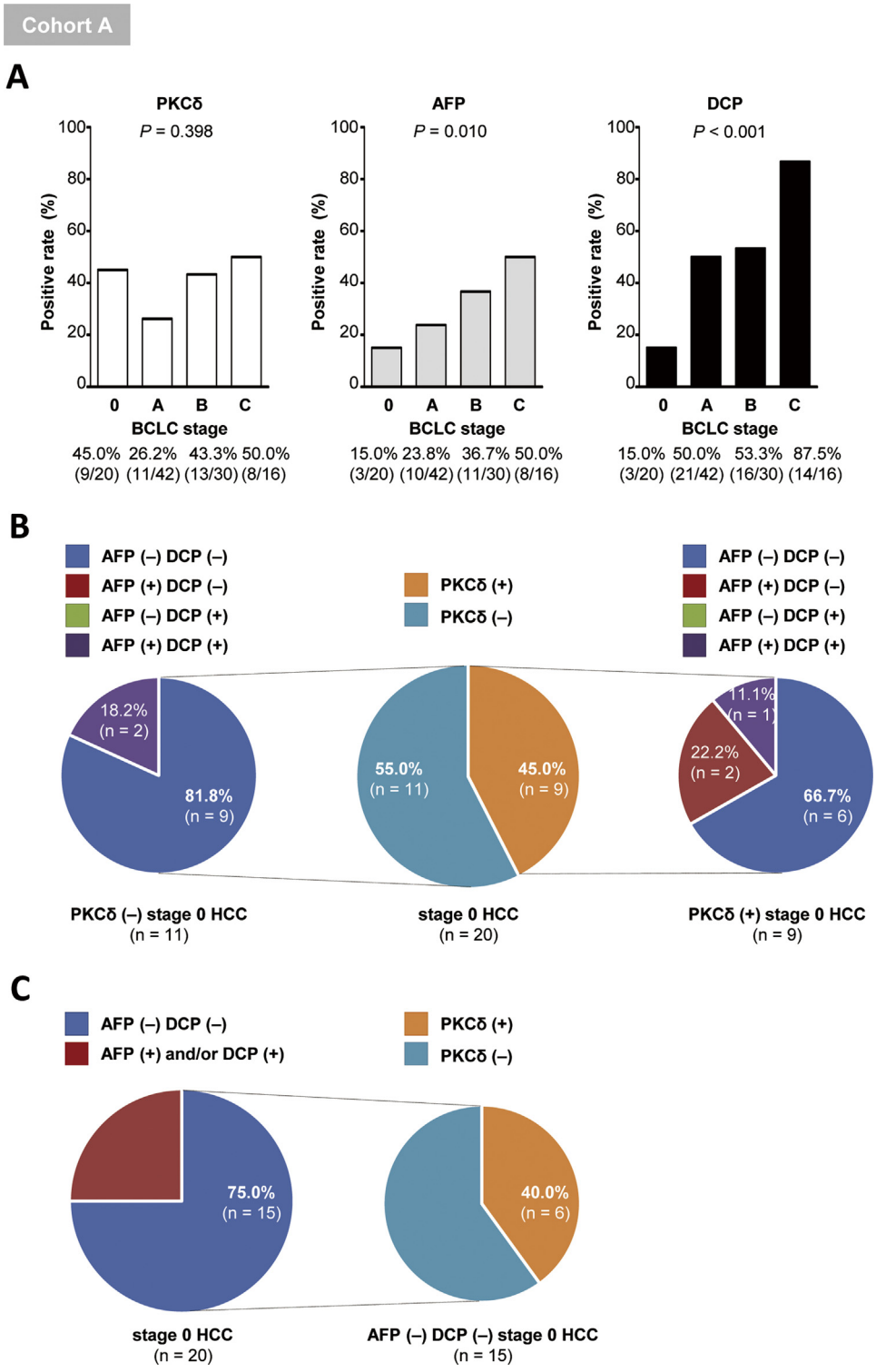


Figure 2. (A) The positive rates of PKCδ, AFP, and DCP according to the BCLC stages in cohort A. The PKCδ-positive rates were similar across all stages ($P = .398$), whereas the AFP- and DCP-positive rates were significantly increased stepwise along with advanced HCC stages ($P = .010$ and <0.001 for AFP and DCP, respectively, by the Cochran-Armitage test). (B) The proportion of PKCδ-positive and PKCδ-negative patients with BCLC stage 0 HCC in cohort A (middle). The percentages of AFP-positive/AFP-negative and/or DCP-positive/DCP-negative patients in the PKCδ-negative (left) and PKCδ-positive (right) groups. (C) The proportion of AFP/DCP double-negative and AFP- and/or DCP-positive patients with BCLC stage 0 HCC in cohort A (left). The proportion of PKCδ-positive and PKCδ-negative patients in the AFP/DCP double-negative group (right). AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; PKCδ, protein kinase C delta.

performance of PKCδ for HCC is not inferior or comparable to that of conventional tumor markers.

Correlation Between Serum PKCδ and Conventional Tumor Markers

The correlation between serum PKCδ and conventional markers was analyzed in cohort A (Figure 1C). A very weak correlation with AFP was noted ($rho = 0.204$), while no correlation with DCP was observed ($rho = 0.001$). PKCδ had no correlation with AFP and DCP in HCC patients ($rho = 0.063$ and -0.136, respectively) and non-HCC patients. The AFP- and DCP-positive rates did not significantly differ between PKCδ-positive and PKCδ-negative HCC patients ($P = .128$ and .428, respectively; Figure A1).

The numbers of PKCδ-, AFP-, and DCP-positive HCC patients are shown in Figure 1D. Of the 108 HCC patients, 41

(38.0%), 32 (29.6%), and 54 (50.0%) were positive for PKCδ (>57.7 ng/mL), AFP (>20.0 ng/mL), and DCP (>40.0 mAU/mL), respectively. Thirteen (12.0%) patients were positive for all 3 markers, whereas 27 (25.0%) were negative for them.

Of the 108 HCC patients, 47 (43.5%) were negative for both AFP and DCP (Figure 1E, left). Notably, of these 47 AFP/DCP double-negative patients, 20 (42.5%) were positive for PKCδ (Figure 1E, right), suggesting that PKCδ may be useful for detecting HCC in AFP/DCP double-negative patients. When the 108 patients were divided according to PKCδ-positive or PKCδ-negative HCC (n = 41 and 67, respectively), the positive rates of AFP and DCP were examined respectively (Figure 1F). Of the 67 PKCδ-negative patients, 4 (6.0%), 24 (35.8%), and 12 (17.9%) were positive for AFP alone, DCP alone, and both AFP/DCP, respectively (Figure 1F, left). Meanwhile, of the 41 PKCδ-positive HCC patients, 20 (48.7%) were negative for both AFP and DCP (Figure 1F, right). The use of triple markers (combination of PKCδ, AFP, and DCP) enhanced sensitivity, NPV, and accuracy to the highest levels in single markers and double/triple combinations (Table 2).

These results suggest that PKCδ, AFP, and DCP are independent of each other and that PKCδ is complementary to conventional markers, AFP and DCP, for HCC screening, especially in AFP/DCP double-negative individuals.

PKCδ for Detecting Very Early-Stage HCC

The positive rates of PKCδ and conventional markers were investigated in HCC patients with BCLC stages 0–C in cohort A. The PKCδ-positive rates were 45.0% (9/20), 26.2% (11/42), 43.3% (13/30), and 50.0% (8/16) for stages 0, A, B, and C, respectively (Figure 2A). Accordingly, they were similar across all stages. Meanwhile, the AFP- and DCP-positive rates significantly increased stepwise as the disease stage progressed, consistent with previous reports.[10,11] It is noteworthy that PKCδ, unlike AFP and DCP, was positive at a high rate at BCLC stage 0 (ie, very early stage). This led us to analyze whether PKCδ is useful for detecting solitary small-sized HCC (≤20 mm in diameter), which corresponds to BCLC stage 0.[22]

Of the 20 stage 0 patients, 9 (45.0%) were positive for PKCδ (Figure 2B, middle). Of these 9 PKCδ-positive patients, 6 (66.7%) were AFP/DCP double-negative (Figure 2B, right). Thus, 6 (30%) of the 20 stage 0 patients were positive only for PKCδ. Meanwhile, 11 (55.0%) of the 20 stage 0 patients were negative for PKCδ (Figure 2B, middle). Of these 11 PKCδ-negative patients, 9 (81.8%) were also negative for both AFP and DCP, while 2 (18.2%) were positive for both AFP and DCP (Figure 2B, left). Thus, 9 (45%) of the 20 stage 0 patients were PKCδ/AFP/DCP triple-negative. Only 2 (10%) of the patients were AFP/DCP double-positive/PKCδ-negative. From the viewpoint of AFP/DCP, 15 (75.0%) of the 20 stage 0 patients were AFP/DCP double-negative (Figure 2C). Of these 15 AFP/DCP double-negative patients, 6 (40.0%) were positive for PKCδ.

The diagnostic performances of PKCδ, AFP, and DCP for detecting stage 0 HCC are summarized in Table 3. In cohort A, PKCδ yielded the highest sensitivity (45.0%) with high specificity, PPV, NPV, and accuracy (97.3%, 81.8%, 86.7%, and 86.2%, respectively) compared with AFP and DCP. In contrast, AFP and DCP had low sensitivity (only 15.0% for both). The combination of AFP and DCP did not exceed the diagnostic performance of PKCδ. Moreover, PKCδ had the highest AUC among the 3 markers (0.762, 0.710, and 0.562 for PKCδ, AFP, and DCP, respectively).

These results suggest that serum PKCδ can be more useful than conventional markers in detecting very early-stage HCC (ie, solitary small-sized HCC).

Verification of Diagnostic Performance of Serum PKCδ for HCC in Cohort B and Propensity-Matched Cohorts

We verified the diagnostic performance of serum PKCδ for HCC in cohort B. Similar to the results in cohort A, serum PKCδ levels in HCC patients were higher than those in non-HCC patients ($P = .002$; Figure 3A). PKCδ distinguished between HCC patients and non-HCC patients with CH and LC: AUC, 0.651; sensitivity, 38.0%; specificity, 92.3%; PPV, 88.2%; NPV, 49.5%; and accuracy, 59.5%. These characteristics were not inferior or comparable to those of AFP or DCP (Tables 2 and A2). Of the 79 HCC patients, 26 (32.9%) were AFP/DCP double-negative (Figure 3B, left). Of these 26 patients, 9 (34.6%) were positive for PKCδ (Figure 3B, right), indicating that there is a certain proportion of PKCδ-positive patients in AFP/DCP double-negative HCC patients. The correlations between PKCδ and conventional markers, the numbers of PKCδ-, AFP-, and DCP-positive HCC patients, and the PKCδ-positive rate in AFP/DCP double-negative patients with BCLC stage 0 HCC are shown in Figure 3C–E. These results in cohort B were similar to those in cohort A, indicating that PKCδ is independent of and complementary to conventional markers in detecting HCC.

Furthermore, we also verified the diagnostic performance of PKCδ for HCC in the propensity score-matched cohort 1 and 2 (Table 2, 3, and A2). The matched cohort 1 and 2 mainly matched cirrhotic conditions between HCC and non-HCC patients and predominantly included patients with LC (Table A1). The PKCδ-positive rates for stages 0–C in the matched cohort 1 were similar to those in cohort A; that is, the PKCδ-positive rate was high even at BCLC stage 0, unlike conventional markers, whose positive rates increased with disease-stage progression (Figure 4A). The diagnostic performance of PKCδ for HCC in the matched cohort 1 was comparable to that of conventional markers (Table A2). In the matched cohort 2, PKCδ yielded the highest diagnostic performance values for stage 0 HCC among the 3 markers (Tables 3 and A2). Additionally, PKCδ improved the diagnostic performance in combination with AFP/DCP in both the matched cohort 1 and 2 (Tables 2 and 3). Similar to the results in cohort A and B, the PKCδ-positive rates in AFP/DCP double-

Table 3. Diagnostic Performance of PKCδ, AFP, and DCP for BCLC Stage 0 HCC

Cohort A	Sensitivity	Specificity	PPV	NPV	Accuracy
Single marker					
AFP	15.0	98.6	75.0	81.1	80.9
DCP	15.0	93.2	37.5	80.2	76.6
PKCδ	45.0	97.3	81.8	86.7	86.2
Double markers					
AFP/DCP	25.0	91.9	45.5	81.9	77.7
PKCδ/AFP	45.0	95.9	75.0	86.6	85.1
PKCδ/DCP	55.0	90.5	61.1	88.2	83.0
Triple markers					
PKCδ/AFP/DCP	55.0	89.2	57.9	88.0	81.0
Cohort B	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
Single marker					
AFP	0	94.2	0	70.0	67.1
DCP	42.9	86.5	56.2	78.9	74.0
PKCδ	52.4	92.3	73.3	82.8	80.8
Double markers					
AFP/DCP	66.7	76.9	53.8	85.1	74.0
PKCδ/AFP	52.4	86.5	61.1	81.8	76.7
PKCδ/DCP	66.7	82.7	60.9	86.0	78.1
Triple markers					
PKCδ/AFP/DCP	66.7	76.9	53.8	85.1	74.0
Matched cohort 2	**Sensitivity**	**Specificity**	**PPV**	**NPV**	**Accuracy**
Single marker					
AFP	4.3	100	100	51.1	52.2
DCP	34.8	87.0	72.7	57.1	60.9
PKCδ	39.1	91.3	81.8	60.0	65.2
Double markers					
AFP/DCP	34.8	87.0	72.7	57.1	60.9
PKCδ/AFP	39.1	91.3	81.8	60.0	65.2
PKCδ/DCP	60.9	82.6	77.8	67.9	71.7
Triple markers					
PKCδ/AFP/DCP	60.9	82.6	77.8	67.9	71.7

AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; CH, chronic hepatitis; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; LC, liver cirrhosis; NPV, negative predictive values; PKCδ, protein kinase C delta; PPV, positive predictive values.

negative patients were 39.3% and 40% in the matched cohort 1 and 2, respectively, (Figure 4B–C).

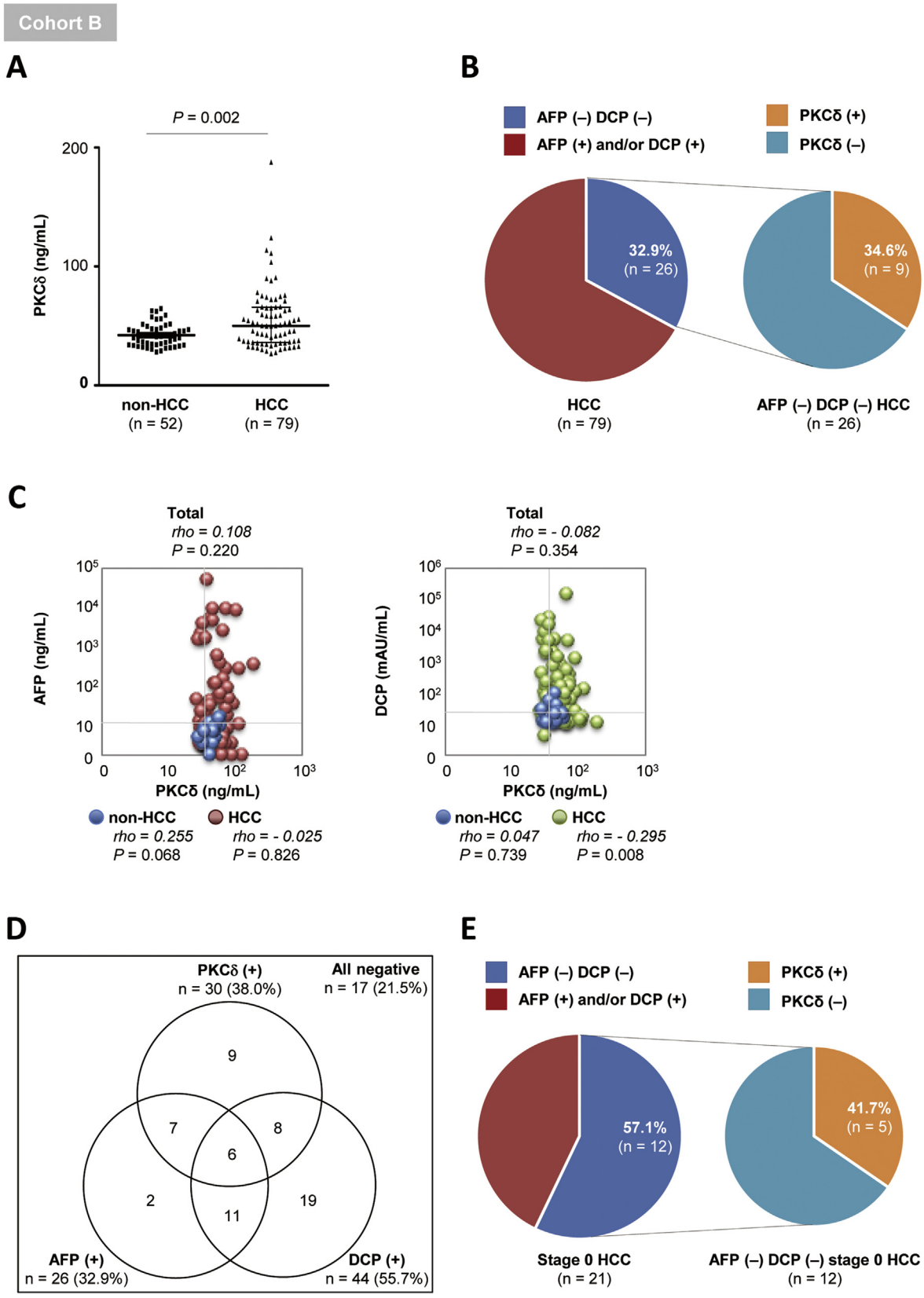
Taken together, these results in cohort B and matched cohort 1 and 2 verified that the diagnostic performance of serum PKCδ is not inferior or comparable to that of conventional markers and that PKCδ is independent of and complementary to conventional markers in the detection of HCC. Specifically, PKCδ may be a useful marker for detecting very early-stage and AFP/DCP-double-negative HCC.

Discussion

The main causes of death in CLD patients are HCC and liver failure. The American Association for the Study of Liver Disease, European Association for the Study of the Liver, and Japanese Society of Hepatology have proposed the guidelines for the surveillance of HCC in CLD patients.[7–9] Regular radiological examinations by dynamic CT and/or Gd-EOB-DTPA-enhanced MRI every 3–6 months are recommended, especially in patients with LC at a high risk of HCC. However, a typical imaging finding (ie, early arterial enhancement and subsequent washout of contrast medium) is usually lacking in small-sized, well-differentiated HCC, thereby making it difficult to detect early-stage HCC on images.[24]

AFP and DCP are commonly used as conventional biomarkers for HCC, and their serum levels are elevated along with advanced HCC stages. However, serum AFP levels can be elevated in other conditions, such as liver injury, cirrhosis, pregnancy, and other malignant tumors, including gastric and gynecological cancers.[10,11] DCP is a nonfunctional coagulation protein arising from the lack of vitamin K-dependent carboxylation of the amino-terminal glutamic acid residues. Obstructive jaundice and intrahepatic cholestasis that impair absorption of vitamin K from the intestinal tract and ingestion of drugs such as warfarin that inhibit vitamin K-related enzymes and antibiotics that

suppress vitamin K-synthesizing enterobacteria can lead to vitamin K deficiency and consequently elevate serum DCP levels.[12] Accordingly, these tumor markers are less specific for HCC, and their measurements are not recommended for the definitive diagnosis of HCC in the aforementioned guidelines.[7–9] Alternatively, they are useful for screening for



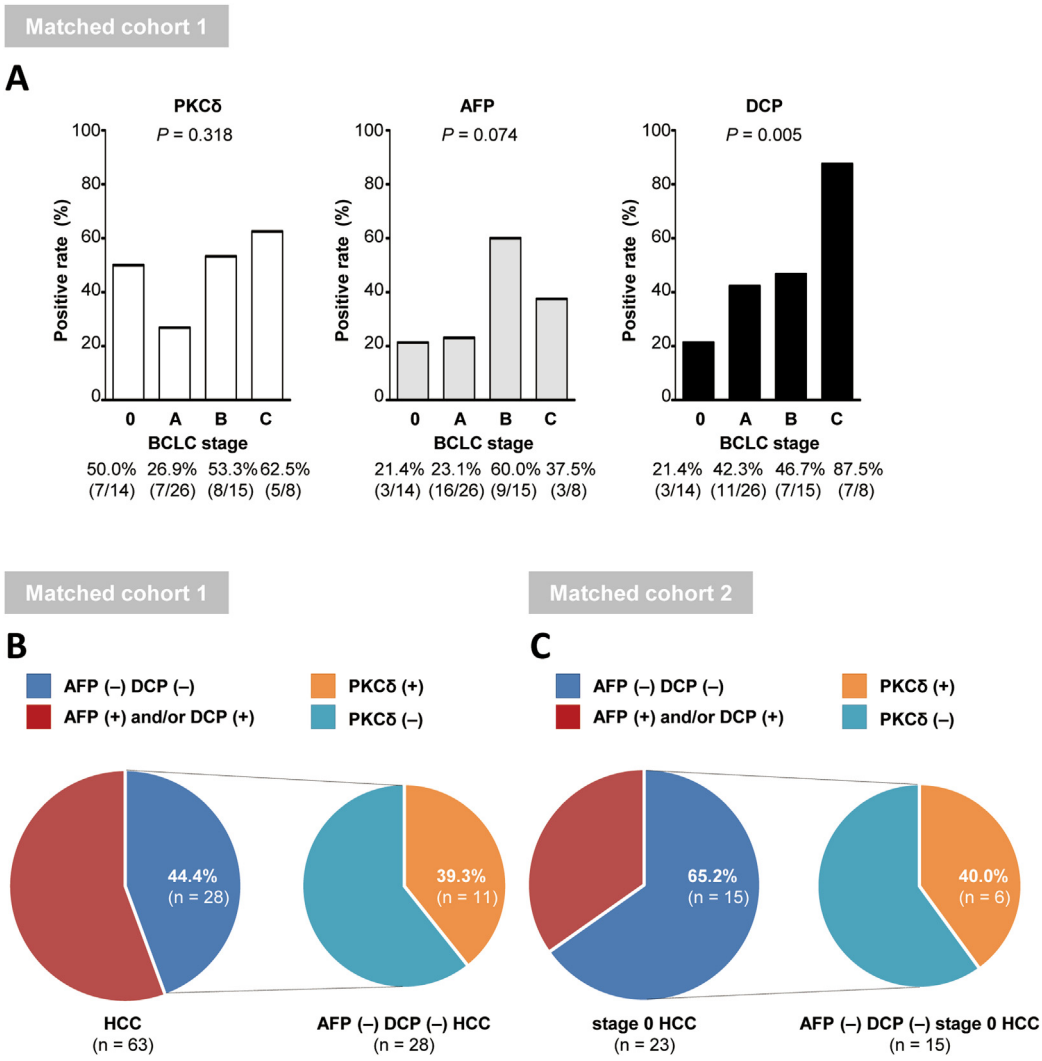


Figure 4. (A) The positive rates of PKCδ, AFP, and DCP according to the BCLC stages in the matched cohort 1. The PKCδ-positive rates were similar across all stages ($P = .318$), whereas the AFP- and DCP-positive rates were marginally or significantly increased along with advanced HCC stages ($P = .074$ and .005 for AFP and DCP, respectively, by the Cochran-Armitage test). (B) The proportion of AFP/DCP double-negative and AFP- and/or DCP-positive HCC patients in the matched cohort 1 (left). The proportion of PKCδ-positive and PKCδ-negative patients in the AFP/DCP double-negative group (right). (C) The proportion of AFP/DCP double-negative and AFP- and/or DCP-positive patients with BCLC stage 0 HCC in the matched cohort 2 (left). The proportion of PKCδ-positive and PKCδ-negative patients in the AFP/DCP double-negative group (right). AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; PKCδ, protein kinase C delta.

HCC in clinical practice. However, as shown in this study, nearly half or one-third of the HCC patients and three-quarters of those with solitary small-sized HCC were AFP/DCP double-negative. Thus, an alternative or complementary biomarker to AFP/DCP is required to identify such HCC patients.

PKC, a serine/threonine kinase, is mainly localized in the cytoplasm of cells and plays an essential role in

Figure 3. (A) Serum PKCδ levels in HCC and non-HCC patients in cohort B. (B) The proportion of AFP/DCP double-negative and AFP- and/or DCP-positive HCC patients in cohort B (left). The proportion of PKCδ-positive and PKCδ-negative patients in the AFP/DCP double-negative group (right). (C) Correlation between serum PKCδ and conventional markers (AFP and DCP) in non-HCC and HCC patients in cohort B. (D) The numbers of PKCδ-, AFP-, and DCP-positive HCC patients in cohort B. (E) The proportion of AFP/DCP double-negative and AFP- and/or DCP-positive patients with BCLC stage 0 HCC in cohort B (left). The proportion of PKCδ-positive and PKCδ-negative patients in the AFP/DCP double-negative group (right). AFP, α-fetoprotein; DCP, des-γ-carboxy prothrombin; HCC, hepatocellular carcinoma; PKCδ, protein kinase C delta.

phosphorylation to activate several signaling pathways. Ten PKC isoforms have been identified in humans as pivotal molecules involved in cell proliferation, survival, and apoptosis.[13–15] We have recently revealed that HCC cells, unlike other solid cancer cells and normal hepatocytes, aberrantly secreted PKCδ from the cytoplasm into the extracellular space and that the secreted PKCδ extracellularly contributed to tumor development and the serum levels were increased in HCC patients.[16] These new findings suggest that serum PKCδ could be a useful biomarker for HCC. This clinical study demonstrated that serum PKCδ distinguished HCC patients from CLD patients without HCC and healthy individuals with high sensitivity and specificity. The diagnostic performance of PKCδ for HCC was comparable to or not inferior to that of conventional tumor markers. This is the first report of serum PKCδ as a novel biomarker for HCC, independent of conventional tumor markers (AFP and DCP).

It has been reported that the combined measurement of at least 2 markers improved the sensitivity while minimizing a decrease in specificity for the tumor detection, which is conceivable given the molecular tumor heterogeneity. Accumulating evidence has demonstrated that the combined use of AFP and DCP enhances diagnostic performance because these markers are independent and do not correlate with each other.[10,11] This study revealed that there was no or very weak correlation between PKCδ and AFP/DCP and that PKCδ was an HCC biomarker independent of AFP/DCP. Notably, nearly half or one-third of the HCC patients were double-negative for AFP/DCP, and nearly half or one-third of them were positive for PKCδ alone. When PKCδ and AFP/DCP were combined for HCC diagnosis, their performance was enhanced. These findings indicate that PKCδ is a complementary biomarker to AFP/DCP for assessing the risk of HCC development.

Despite recent advances in radiological imaging and therapy, the 5-year survival rate of HCC patients is extremely poor (approximately 20%).[25,26] Although it was once thought that there was no intrahepatic metastasis in early-stage HCC, vascular invasion and intrahepatic metastasis, which are related to poor prognosis, were found even in small-sized HCC.[27,28] Accordingly, a novel examination, including a tumor marker, is required to detect early-stage HCC and introduce therapeutic intervention. To date, several useful biomarkers for HCC have been reported, such as glypican-3 (also known as phosphatidylinositol proteoglycan), insulin-like growth factor-II, osteopontin, and dickkopf-1.[12,29–33] However, none have surpassed or replaced conventional biomarkers even more than 50 years after the discovery of AFP. Therefore, a novel biomarker that can identify HCC patients, especially those who are AFP/DCP double-negative and, therefore, lose the opportunity to undergo radiological imaging, is required.

Regardless of the recent advances in molecular biomarkers (so-called "liquid biopsy", such as cell-free DNA, circulating tumor cells, cell-free noncoding RNA [eg, microRNAs, long noncoding RNAs] and extracellular vesicles [eg, exosomes]), there still remain many issues (eg, high cost, low sensitivity and reproducibility, technical difficulty and complexity of handling with samples, time-consuming process) to be overcome before it can be applied in clinical use.[34] Considering these issues, measurements of serum PKCδ can be easily and reproducibly performed using a sandwich ELISA without complicated processing. In addition, PKCδ can be measured by diluting only 1 μL of serum 100-fold, and its detection is possible on the order of ng/mL.

This study has some limitations. First, the sample size was too small owing to a single-center preliminary study to determine the clinical features of PKCδ as a biomarker for HCC. Second, the relationship between serum PKCδ levels and tumor characteristics (tumor burden and malignant potential, such as gene signatures and cancer stem cell markers[35,36]) remains unclear. Third, it is necessary to clarify whether any factors or conditions influencing PKCδ measurements are present or absent, such as elevated AFP during pregnancy or abrupt liver damage and elevated DCP during antibiotic or antiangiogenetic use. Currently, we are planning to conduct a large-scale, multicenter study to resolve these issues in real-world clinical practice.

In conclusion, serum PKCδ can be a novel biomarker for HCC and is complementary to conventional HCC markers, AFP and DCP. Specifically, PKCδ is useful for detecting very early-stage or AFP/DCP double-negative HCC.

Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2022.07.020.

References

1. Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2018;68:394–424.
2. Llovet JM, Kelley RK, Villanueva A, et al. Hepatocellular carcinoma. Nat Rev Dis Primers 2021;7:6.
3. Finn RS, Qin S, Ikeda M, et al. Atezolizumab plus bevacizumab in unresectable hepatocellular carcinoma. N Engl J Med 2020;382:1894–1905.
4. Liebman HA, Furie BC, Tong MJ, et al. Des-gamma-carboxy (abnormal) prothrombin as a serum marker of primary hepatocellular carcinoma. N Engl J Med 1984;310:1427–1431.
5. Taketa K. Alpha-fetoprotein: reevaluation in hepatology. Hepatology 1990;12:1420–1432.
6. Bolondi L, Sofia S, Siringo S, et al. Surveillance programme of cirrhotic patients for early diagnosis and treatment of hepatocellular carcinoma: a cost effectiveness analysis. Gut 2001;48:251–259.
7. Heimbach JK, Kulik LM, Finn RS, et al. AASLD guidelines for the treatment of hepatocellular carcinoma. Hepatology 2018;67:358–380.

8. European Association for the Study of the Liver. EASL clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol 2018;69:182–236.
9. Kokudo N, Takemura N, Hasegawa K, et al. Clinical practice guidelines for hepatocellular carcinoma: the Japan society of hepatology 2017 (4th JSH-HCC guidelines) 2019 update. Hepatol Res 2019; 49:1109–1113.
10. Toyoda H, Kumada T, Tada T, et al. Tumor markers for hepatocellular carcinoma: simple and significant predictors of outcome in patients with HCC. Liver Cancer 2015;4:126–136.
11. Berhane S, Toyoda H, Tada T, et al. Role of the GALAD and BALAD-2 serologic models in diagnosis of hepatocellular carcinoma and prediction of survival in patients. Clin Gastroenterol Hepatol 2016;14:875–886.e6.
12. Bertino G, Ardiri A, Malaguarnera M, et al. Hepatocellualar carcinoma serum markers. Semin Oncol 2012;39:410–433.
13. Tsai JH, Hsieh YS, Kuo SJ, et al. Alteration in the expression of protein kinase C isoforms in human hepatocellular carcinoma. Cancer Lett 2000;161: 171–175.
14. Wu TT, Hsieh YH, Wu CC, et al. Overexpression of protein kinase C alpha mRNA in human hepatocellular carcinoma: a potential marker of disease prognosis. Clin Chim Acta 2007;382:54–58.
15. Yoon CH, Kim MJ, Park MJ, et al. Claudin-1 acts through c-Abl-protein kinase Cdelta (PKCdelta) signaling and has a causal role in the acquisition of invasive capacity in human liver cells. J Biol Chem 2010;285:226–233.
16. Yamada K, Oikawa T, Kizawa R, et al. Unconventional secretion of PKCdelta exerts tumorigenic function via stimulation of ERK1/2 signaling in liver cancer. Cancer Res 2021;81:414–425.
17. Yamada K, Kizawa R, Yoshida A, et al. Extracellular PKCdelta signals to epidermal growth factor receptor for tumor proliferation in liver cancer cells. Cancer Sci 2022; 113:2378–2385.
18. Heidelbaugh JJ, Bruderly M. Cirrhosis and chronic liver failure: part I. Diagnosis and evaluation. Am Fam Physician 2006;74:756–762.
19. Bruix J, Sherman M, American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. Hepatology 2011;53:1020–1022.
20. Marrero JA, Kulik LM, Sirlin CB, et al. Diagnosis, staging, and management of hepatocellular carcinoma: 2018 practice guidance by the American Association for the Study of Liver Diseases. Hepatology 2018; 68:723–750.
21. Brierley DJ, Gospodarowicz KM, Wittekind C. TNM classification of malignant tumours. 8th ed. Hoboken, NJ: Wiley-Blackwell in affiliation with the Union for International Cancer Control (UICC), 2017:90–93.
22. Reig M, Forner A, Rimola J, et al. BCLC strategy for prognosis prediction and treatment recommendation: the 2022 update. J Hepatol 2022;76:681–693.
23. Youden WJ. Index for rating diagnostic tests. Cancer 1950;3:32–35.
24. Simmons O, Fetzer DT, Yokoo T, et al. Predictors of adequate ultrasound quality for hepatocellular carcinoma surveillance in patients with cirrhosis. Aliment Pharmacol Ther 2017;45:169–177.
25. Kawano Y, Sasaki A, Kai S, et al. Short- and long-term outcomes after hepatic resection for hepatocellular carcinoma with concomitant esophageal varices in patients with cirrhosis. Ann Surg Oncol 2008; 15:1670–1676.
26. Stefaniuk P, Cianciara J, Wiercinska-Drapalo A. Present and future possibilities for early diagnosis of hepatocellular carcinoma. World J Gastroenterol 2010; 16:418–424.
27. Nakashima O, Sugihara S, Kage M, et al. Pathomorphologic characteristics of small hepatocellular carcinoma: a special reference to small hepatocellular carcinoma with indistinct margins. Hepatology 1995; 22:101–105.
28. Takayama T, Makuuchi M, Hirohashi S, et al. Early hepatocellular carcinoma as an entity with a high rate of surgical cure. Hepatology 1998;28:1241–1246.
29. Nakatsura T, Yoshitake Y, Senju S, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 2003;306:16–25.
30. Hippo Y, Watanabe K, Watanabe A, et al. Identification of soluble NH2-terminal fragment of glypican-3 as a serological marker for early-stage hepatocellular carcinoma. Cancer Res 2004;64:2418–2423.
31. Tsai JF, Jeng JE, Chuang LY, et al. Serum insulin-like growth factor-II as a serologic marker of small hepatocellular carcinoma. Scand J Gastroenterol 2005; 40:68–75.
32. Kim J, Ki SS, Lee SD, et al. Elevated plasma osteopontin levels in patients with hepatocellular carcinoma. Am J Gastroenterol 2006;101:2051–2059.
33. Shen Q, Fan J, Yang XR, et al. Serum DKK1 as a protein biomarker for the diagnosis of hepatocellular carcinoma: a large-scale, multicentre study. Lancet Oncol 2012; 13:817–826.
34. Pelizzaro F, Cardin R, Penzo B, et al. Liquid biopsy in hepatocellular carcinoma: where are we now? Cancers (Basel) 2021;13:2274.
35. Hoshida Y, Moeini A, Alsinet C, et al. Gene signatures in the management of hepatocellular carcinoma. Semin Oncol 2012;39:473–485.
36. Oikawa T. Cancer stem cells and their cellular origins in primary liver and biliary tract cancers. Hepatology 2016; 64:645–651.

Received March 9, 2022. Accepted July 25, 2022.

Correspondence:
Address correspondence to: Tsunekazu Oikawa, MD, PhD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, The Jikei University School of Medicine, 3-25-8, Nishi-shimbashi, Minato, Tokyo 105-8461, Japan. e-mail: oitsune@jikei.ac.jp.

Acknowledgment:
The authors thank Ms. K. Katagiri for the technical assistance with sandwich ELISA and Ms. Y. Numata and all medical doctors who were involved in the collection of data.

Authors' Contributions:
The project was originally conceived and designed by Tsunekazu Oikawa, K. Yamada, and K. Yoshida. Acquisition, analyses, and interpretation of data were done by Tsunekazu Oikawa, K. Yamada, Akihito Tsubota, Chisato Saeki, Naoko Tago, Chika Nakagawa, Kaoru Ueda, and Hiroshi Kamioka Samples

were obtained by Masanori Nakano, Yuichi Torisu, Tomohiko Taniai, Koichiro Haruki, and Toru Ikegami Statistical analysis was done by Tsunekazu Oikawa The article was drafted and edited by Tsunekazu Oikawa and Akihito Tsubota Study supervision was done by Akihito Tsubota, K. Yoshida, and Masayuki Saruta All the authors have read and approved of the final manuscript.

Conflicts of Interest:
The authors disclose no conflicts.

Funding:
This work was supported by grants from AMED under grant number B326TS in part by the Japan Society for the Promotion of Science and JP21ck0106712 to K. Yamada; the Japan Society for the Promotion of Science (KAKENHI Grant Numbers JP22K08063 to T. Oikawa.; JP18K15253 and JP20K07621 to K. Yamada.; JP18K19484 and JP20H03519 to K. Yoshida); the Jikei University Graduate Research Fund to K. Yamada and T. Oikawa.; Takeda Science Foundation to K. Yamada.; and The Science Research promotion fund to K. Yoshida.

Ethical Statement:
The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

Data Transparency Statement:
Data, analytic methods, and study materials are not available for public access; however, this information could be procured directly from the corresponding author upon reasonable request.