Title: CD133 and epithelial cell adhesion molecule expressions in cholangiocarcinoma component are prognostic factors for combined hepatocellular-cholangiocarcinoma

Running title: Cancer ctem cell markers in cHCC-CCA

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Abstract

Background: A new classification of combined hepatocellular-cholangiocarcinoma (CHC) was recently reported. Cancer stem cells have been associated with CHC carcinogenesis. This study examined the association of cancer stem cell marker expression and prognosis in CHC classified using the new classification.

Methods: We enrolled 26 CHC patients and classified them according to the new classification. We evaluated the expression of cancer stem cell markers (CD56, CD133 and epithelial cell adhesion molecule (EpCAM)) by immunohistochemical staining in each component. We analyzed the association between expressions and prognosis.

Results: Seven cases were hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) (cHCC-CCA), 12 were HCC and intermediate cell carcinoma (HCC-INT) and 7 were intermediate cell carcinoma (INT). The CD133 positive rate tended to be higher in the CCA (42.9%) and INT component (50.0%) than the HCC component (14.3%) in cHCC-CCA. In HCC-INT, the CD133 positive rate in the INT component (83.3%) was significantly higher than the HCC component (8.3%) (P=0.001). For EpCAM, the positive rate in the CCA component (71.4%) and INT component (50.0%) tended to be higher than the HCC component (14.3%) in cHCC-CCA. Overall survival and disease-

free survival were significantly worse in cases with CD133-positive (P=0.048 and P=0.048, respectively) or EpCAM-positive (P=0.041 and P=0.041, respectively) CCA component in cHCC-CCA.

Conclusions: INT and CCA components showed higher expression rates of cancer stem cell markers than the HCC component. CD133 or EpCAM expression in the CCA component was associated with poor prognosis in cHCC-CCA.

Key words: cancer stem cells, CD133, combined hepatocellular-cholangiocarcinoma, EpCAM

Introduction

Combined hepatocellular-cholangiocarcinoma (CHC) is a rare type of primary liver cancer that contains components of hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (iCCA). Its frequency is 1.0%–4.7% of all primary liver cancers.[1-6] The definition of CHC has changed with the elucidation of its pathology. CHC was classified into double cancer (type A), combined type (type B), and mixed type (type C) by Allen and Lisa in 1949.[7] Goodman et al. modified it by classifying CHC into collision (type I), transitional (type II), and fibrolamellar tumors (type III) in 1985.[8] In 2010,Two types of CHC were defined by the fourth edition of the World Health Organization (WHO) classification: the classical type and the subtypes with stem cell features, the latter of which were subdivided into typical, intermediate-cell, and cholangiolocellular subtypes.[9] However, CHC often contains various components, and it is difficult to clearly classify CHC clinically. Therefore, in the consensus paper published in 2018 and the new WHO classification published in 2019, the subtypes were abolished and the current guidelines indicate that primary liver carcinomas with both hepatocellular and cholangiocytic differentiation within the same tumor is CHC.[10, 11] Thus, stem cell phenotypes/features may be present in CHC, but it is not sufficient for diagnosis of CHC. In this new proposal, CHC can be classified

according to the presence of various components, including an HCC component, cholangiocarcinoma (CCA) component, intermediate cell carcinoma (INT) component and cholangiolocarcinoma (CLC) component. Tumors including the HCC component and CCA component are defined as combined hepatocellular-cholangiocarcinoma (cHCC-CCA) in the new classification. INT alone is a classification of CHC. CLC alone and the combination of CCA and CLC are not classifications of CHC because they are included in the iCCA spectrum (Table 1). [10] The definition of CHC was revised based on better understanding of the relationship between the carcinogenesis of CHC and cancer stem cells or hepatic progenitor cells. Several reports have reported the relationship between CHC and cancer stem cells, but few reports have examined the relation between the presence of cancer stem cells and prognosis in CHC. In this study, we classified a panel of hepatectomy cases diagnosed as CHC in our institution using the new classification and analyzed the relationship between stem cell markers and prognosis.

## Methods

### Patients and tissue samples

We retrospectively screened 28 CHC patients who underwent hepatic resection at our institution between January 1999 and December 2016. All tissue samples were obtained from CHC patients who underwent segmentectomy or sectionectomy with or without lymph node dissection. From the 28 patients, 26 tissues were available. We performed lymph node dissection for CHC or iCCA. This study included cases diagnosed as HCC preoperatively, and 11 of 26 case were performed lymph node dissection. The diagnosis of CHC was performed based on the criteria that were most common at that time. Double cancer and collision cancer were not included. Diagnosis of CHC was made by one or more pathologist.

Patients were followed-up postoperatively on an outpatient basis by monitoring of tumor markers and imaging studies every 3 months. Recurrence was diagnosed by combined examination of tumor markers and imaging studies.

We reviewed the medical records of all patients for clinicopathological information, including sex, age, viral markers, serum alpha-fetoprotein (AFP), protein induced by vitamin K absence or antagonists-II (PIVKA-II), carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9), surgical procedure, and pathological

reports. Pathological data included tumor number, tumor size, lymph node metastasis, vascular invasion and findings of non-cancerous liver. Tumor node metastasis (TNM) stage was determined according to the criteria by the Liver Cancer Study Group of Japan (6th edition).[12] This study was performed in compliance with the Helsinki Declaration and approved by the institutional review board of our institution (016-0237).

Classification of CHC

Classification of CHC was performed based on the consensus paper published in 2018.[10] Diagnosis was based on routine histopathology with hematoxylin and eosin. The cHCC-CCA type is primary liver carcinoma with both hepatocellular and cholangiocytic differentiation within the same tumor. CLC and INT are other types of primary liver carcinoma, but both may coexist with an HCC, iCCA, or cHCC-CCA component. According to this new proposal, CHC can be classified into 8 groups, as shown in Table 1. The 26 cases were evaluated and grouped using this new classification. CLC alone and the combination of CCA and CLC are not classifications of CHC because they are included in the iCCA spectrum, and thus these were not included in this study. One pathologist diagnosed components of CHC.

Immunohistochemical staining

We selected CD56, CD133 and epithelial cell adhesion molecule (EpCAM) as stem cell markers.[13] Immunohistochemical staining was performed on thin sections (4 μm) of formalin-fixed and paraffin-embedded specimens. The samples were deparaffinized with xylene and ethanol, and antigen retrieval was performed with target retrieval solution pH 9.0 (415211, Nichirei Biosciences Inc., Tokyo, Japan) for 30 min at 95°C. Sections were incubated with Block Ace (UKB80, KAC Co., Ltd. Kyoto, Japan) for 5 min to block nonspecific antibody binding sites and then incubated with the following primary antibodies: CD56 (NCL-CD56-1B6, diluted 1:200, 1 h at room temperature; Leica Biosystems Nussloch GmbH, Nussloch, Germany), CD133 (130-090-422, diluted 1:20, overnight at 4°C; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and EpCAM (sc-25308, diluted 1:100, 1 h at room temperature; Santa Cruz Biotechnology, Dallas, TX, USA). Sections were then incubated in Histofine Simple Stain MAX PO (MULTI) (724152, Nichirei Biosciences Inc., Tokyo, Japan) for 30 min at room temperature. Staining was visualized using 3,3′ diaminobenzidine, and samples were counterstained with hematoxylin.

Immunoreactivity was scored according to the percentage of positive cells regardless of staining intensity: 0, no staining; 1, 1%–10% positive cells; 2, 11%–50% positive cells; 3, 51%–90% positive cells; and 4, 91%–100% positive cells. Samples were divided into the negative expression group (score 0) and positive expression group (score 1–4). Each component of CHC (the HCC, CCA and INT components) was evaluated separately.

## Statistical analysis

Statistical analyses were performed using EZR version 1.35 (Saitama Medical Center, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).[14] Clinicopathological variables and the expression of stem cell markers were examined by Fisher's exact test and one-way analysis of variance. Overall survival curves and disease-free survival curves were plotted using the Kaplan-Meier method, and curves were compared using the log-rank test. A $P$ value of $<0.05$ was considered statistically significant.

## Results

### Classification of CHC and clinicopathological characteristics

The 26 cases were classified into the following subtypes according to the latest criteria: 7 were cHCC-CCA, 12 were HCC-INT and 7 were INT (Table 1). In the cHCC-CCA group, 3 of the 7 cases contained an INT component.

The clinicopathological characteristics of the 26 patients are summarized in Table 2. There was a tendency for a high frequency of males in all groups, but there were no overall significant differences between groups. The frequency of positive AFP level was higher in the cHCC-CCA group and HCC-INT group compared with the INT group. Other tumor markers showed no significant differences between groups. There was a tendency for frequent multiple tumors in the cHCC-CCA group (85.7%), but there was no significant differences between groups. Tumor size tended to be larger in INT patients at 9.1±5.5 cm, but the difference was not significant. Vascular invasion, lymph node metastasis and stage showed no differences between groups. All patients in the cHCC-CCA and INT groups had no cirrhosis, but 41.7% of HCC-INT patients had cirrhosis.

### Survival analyses

The 5-year OS rates in the cHCC-CCA, HCC-INT and INT groups were 28.6%, 25.0% and 0%, respectively (Figure 1A). There were no significant differences between the groups (P=0.723). The 5-year DFS rates were 14.3%, 16.7%, 0%, respectively (Figure 1B). There were no significant differences between groups (P=0.851). We also compared the prognoses of cHCC-CCA patients with the presence or absence of INT component, but there were no significant differences in OS and DFS between groups (P=0.997, P=0.673, respectively) (Figure 2).

Cancer stem cell marker expression in tumor components

We next evaluated the expression of cancer stem cell markers in the individual components in tumor specimens. Representative immunohistochemical staining of cancer stem cell markers are shown in Figure 3, and immunohistochemical staining results are summarized in Table 3, Table 4 and Table 5. CD56 was negative in all components of cHCC-CCA and the HCC component of HCC-INT. In contrast, the INT component of HCC-INT and INT were positive for CD56 in 25.0% and 42.9% of cases, respectively. Regarding CD133, in cHCC-CCA, the positive rate tended to be higher in the CCA component (42.9%) and INT component (50.0%) than the HCC component (14.3%). In HCC-INT, the CD133 positive rate in the INT component (83.3%) was

significantly higher than in the HCC component (8.3%) (P=0.001). In addition, INT showed a high CD133 positive rate of 85.6%. For EpCAM, the trends were similar to CD133; the positive rate in the CCA component (71.4%) and INT component (50.0%) tended to be higher than in the HCC component (14.3%). Similarly, in HCC-INT, the EpCAM positive rate in the INT component (83.3%) tended to be higher than in the HCC component (58.3%). All INT cases were positive for EpCAM.

Cancer stem cell marker expression and prognosis

We next analyzed the relationship between stem cell marker expression and survival or recurrence. We only performed analyses with groups with more than 2 samples; we thus examined the INT component of HCC-INT and INT for CD56, the CCA component of cHCC-CCA and INT component of HCC-INT for CD133, and the CCA component of cHCC-CCA, HCC component of HCC-INT and INT component of HCC-INT for EpCAM. The INT component of cHCC-CCA was excluded. No relationship between CD56 expression and prognosis was found (Fig. S1). For CD133 expression, both OS and DFS were significantly worse in the cHCC-CCA cases with CD133 positive expression in the CCA component than those with negative expression (P=0.048 and P=0.048, respectively) (Figure 4). However, the prognosis of HCC-INT

cases was not different in the subgroups with negative and positive CD133 expression in the INT component (Fig. S2). Regarding EpCAM expression, OS and DFS were significantly worse in cHCC-CCA cases with EpCAM positive expression in the CCA component compared with the negative group (P=0.041 and P=0.041, respectively) (Figure 5). The prognosis of HCC-INT was not different between subgroups with positive and negative EpCAM expression in both components (Fig. S3).

## Discussion

CHC is a rare type of primary liver cancer that shows various histological appearance, and thus the classification of CHC has been a clinical and pathological problem. The classification of CHC has changed over time; the latest version was suggested in the consensus paper published in 2018 and the WHO classification has been revised in 2019.[10,11] There is no report of CHC classified according to this new classification at the time of writing, and thus this paper represents the first report using the new proposal. Several papers reported the features of CHC and comparisons with other primary liver cancers, and similar to other reports,[15-18] we found that the prognosis of CHC is worse than that of HCC and iCCA. This finding indicates that CHC is a high grade malignancy cancer. There are reports that cancer stem cells or hepatic progenitor cells are associated with the carcinogenesis of CHC, but the origin of CHC cells remains unclear. [19-21] On the other hand, cancer stem cells are also reported to be associated with recurrence, metastasis and therapy resistance of liver cancer.[22-24] Based on these findings, this study was conducted to investigate the relationship of cancer stem cells in CHC with patient prognosis.

Because there have been no reports that verified new proposal, we first classified our CHC cases according to the new proposal. Tumors with a CLC component

were not included because they were diagnosed independently from CHC. We histopathologically investigated CLC cases from our institution, and they did not contain other components and therefore were excluded. We observed a tendency for higher frequencies of males in all groups as with HCC. However, no significant difference was detected between groups in most characteristics, possibly due to the small number of cases in our study. High AFP level and a relation with liver cirrhosis were observed in the subtype with HCC component, and these observations may reflect the properties of HCC.

Previous studies showed that cancer stem cells are associated with prognosis in liver cancer. CD133 is expressed in 22.1% of primary liver cancers and is a risk factor for survival in HCC,[25] and CD133 is expressed in 48.3% in iCCA and is a prognostic indicator.[26] Only few reports are available on the relationship between cancer stem cell markers and prognosis in CHC. In this study, INT or the INT component showed a high frequency of expression of stem cell markers, especially CD133 and EpCAM. This suggests that cells in the INT component are more associated with stem cells than those in the HCC or CCA component. These results could suggest that the prognosis of INT would be worse than the other types, although we found no statistically significant differences in prognoses among groups. Future studies should

be performed using a larger number of cases to clarify the potential relationship with prognosis.

Our results showed that the expression of cancer stem cell markers is different between components in the same tumor. In the cHCC-CCA type, stem cell markers were expressed more frequently in the CCA component than in the HCC component. This trend is similar to a previous study that analyzed cancer stem cell markers according to the WHO classification of 2010.[13] Few reports are available on cancer stem cells and prognosis in CHC. One report showed that CD44 expression was associated with poor prognosis in CHC.[27] Our study demonstrated that CD133 or EpCAM expression in the CCA component is associated with overall survival and recurrence in cHCC-CCA type.

This study had some limitations. First, this study was a single-center study and the sample size was small. Further high volume, multicenter studies are necessary to validate our results. Second, only a few cancer stem cell markers were selected in this study and therefore future studies should be performed using more stem cell markers.

In conclusion, we analyzed expression of stem cell markers in CHC and found that the INT component and CCA component showed a higher expression rate of cancer

stem cell markers than the HCC component. CD133 or EpCAM expression in the CCA component was associated with overall survival and recurrence in the cHCC-CCA type. CHC has a poor prognosis, and in particular, CHC with stem cell marker positive is found to have a poorer prognosis. Treatment targeting stem cells is expected to improve prognosis of CHC in the future.

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Figure legends

Figure 1

Overall survival (OS) (A) and disease-free survival (DFS) (B) of patients with combined hepatocellular-cholangiocarcinoma (cHCC-CCA), hepatocellular carcinoma and intermediate cell carcinoma (HCC-INT) and intermediate cell carcinoma (INT). The 5-year OS rates in cHCC-CCA, HCC-INT and INT were 28.6%, 25.0% and 0%, respectively. The 5-year DFS rates were 14.3%, 16.7%, and 0%, respectively. There were no significant differences in prognosis between these groups.

Figure 2

Overall survival (OS) (A) and disease-free survival (DFS) (B) of combined hepatocellular-cholangiocarcinoma (cHCC-CCA) with or without intermediate cell carcinoma (INT) component. The 5-year OS rates in cHCC-CCA without INT and cHCC-CCA with INT were 25.0% and 33.3%, respectively. The 5-year DFS rates were 25.0% and 0%, respectively. There were no significant differences between the groups.

Figure 3

Representative hematoxylin and eosin (HE) staining and immunohistochemical staining of CD56, CD133 and epithelial cell adhesion molecule (EpCAM) in combined hepatocellular-cholangiocarcinoma (cHCC-CCA), hepatocellular carcinoma and intermediate cell carcinoma (HCC-INT) and intermediate cell carcinoma (INT). cHCC-CCA showed the hepatocellular carcinoma (HCC) component (A, left) and cholangiocarcinoma (CCA) component (A, right). CD56 was negative in both components (B), CD133 was positive in the CCA component (C), and EpCAM was positive in both components (D). HCC-INT showed the HCC component (E, left) and INT component (E, right). CD56 and CD133 were positive in the INT component (F and G) and EpCAM was positive in both components (H). INT showed the intermediate appearance of HCC and CCA (I). CD56, CD133 and EpCAM were positive in this section (J, K and L).

Figure 4

Overall survival (OS) (A) and disease-free survival (DFS) (B) of combined hepatocellular-cholangiocarcinoma (cHCC-CCA) with or without CD133 expression in the cholangiocarcinoma component (cca). The 5-year OS rates in the CD133 negative and CD133 positive groups were 50.0% and 0%, respectively. The 5-year DFS rates

were 50.0% and 0%, respectively. OS and DFS were significantly worse in the CD133 positive patients than in the CD133 negative patients in cHCC-CCA (P=0.048 and P=0.048, respectively).

Figure 5

Overall survival (OS) (A) and disease-free survival (DFS) (B) of combined hepatocellular-cholangiocarcinoma (cHCC-CCA) with or without epithelial cell adhesion molecule (EpCAM) expression in the cholangiocarcinoma component (cca). The 5-year OS rates in the EpCAM negative and EpCAM positive groups were 100.0% and 0%, respectively. The 5-year DFS rates were 50.0% and 0%, respectively. OS and DFS were significantly worse in EpCAM positive patients than EpCAM negative patients in cHCC-CCA (P=0.041 and P=0.041, respectively).

**Table1** The new classification of CHC according to combinations of components and the CHC patients in this study

| Subtype (combinations of components) | Number of patients |
|---|---|
| cHCC-CCA | 7 |
| HCC-CCA | 4 |
| HCC-CCA-INT | 3 |
| HCC-CCA-CLC | 0 |
| HCC-CCA-INT-CLC | 0 |
| HCC-INT | 12 |
| HCC-CLC | 0 |
| HCC-INT-CLC | 0 |
| CCA-INT | 0 |
| CCA-INT-CLC | 0 |
| INT | 7 |
| INT-CLC | 0 |
| CCA-CLC | Not included in CHC |
| CLC | Not included in CHC |

CHC, combined hepatocellular-cholangiocarcinoma; cHCC-CCA, combined hepatocellular-cholangiocarcinoma type; HCC, hepatocellular carcinoma; CCA, cholangiocarcinoma; INT, intermediate cell carcinoma; CLC, cholangiolocarcinoma.

**Table 2** Classification and clinicopathological characteristics of the CHC patients in this study (n, %)

| Characteristics | cHCC-CCA (n=7) | HCC-INT (n=12) | INT (n=7) | *P* value |
|---|---|---|---|---|
| Sex | | | | |
| Male | 5 (71.4) | 11 (91.7) | 6 (85.7) | 0.784 |
| Female | 2 (28.6) | 1 (8.3) | 1 (14.3) | |
| Age (yr) | 64.7±10.4 | 61.8±13.9 | 58.7±10.1 | 0.657 |
| Viral infection | | | | |
| HBV | 2 (28.6) | 5 (41.7) | 4 (57.1) | 0.542 |
| HCV | 1 (14.3) | 2 (16.7) | 0 (0.0) | 0.774 |
| Child-Pugh classification | | | | |
| A | 7 (100.0) | 12 (100.0) | 7 (100.0) | 1.000 |
| B | 0 (0.0) | 0 (0.0) | 0 (0.0) | |
| AFP (ng/ml) | | | | |
| <10 | 2 (28.6) | 2 (16.7) | 5 (71.4) | 0.071 |
| ≥10 | 5 (71.4) | 10 (83.3) | 2 (28.6) | |
| PIVKA-II (mAU/ml) | | | | |
| <40 | 2 (28.6) | 6 (50.0) | 4 (57.1) | 0.682 |
| ≥40 | 5 (71.4) | 6 (50.0) | 3 (42.9) | |
| CEA (ng/ml) | | | | |
| <6.5 | 5 (71.4) | 8 (66.7) | 6 (85.7) | 0.853 |
| ≥6.5 | 2 (28.6) | 4 (33.3) | 1 (14.3) | |
| CA199 (U/ml) | | | | |
| <37 | 3 (42.9) | 5 (41.7) | 3 (42.9) | 1.000 |
| ≥37 | 4 (57.1) | 5 (41.7) | 4 (57.1) | |
| Surgical procedure | | | | |
| Anatomical resection | 7 (100.0) | 9 (75.0) | 6 (85.7) | 0.548 |
| Non-anatomical resection | 0 (0.0) | 3 (25.0) | 1 (14.3) | |
| Tumor number | | | | |
| Solitary | 1 (14.3) | 5 (41.7) | 5 (71.4) | 0.104 |
| Multiple | 6 (85.7) | 7 (58.3) | 2 (28.6) | |
| Tumor size (cm) | 4.5±2.7 | 5.3±3.3 | 9.1±5.5 | 0.067 |
| Vascular invasion | 4 (57.1) | 9 (75.0) | 5 (71.4) | 0.860 |
| Portal vein invasion | 4 (57.1) | 8 (66.7) | 5 (71.4) | 1.000 |
| Hepatic vein invasion | 0 (0.0) | 1 (8.3) | 1 (14.3) | 1.000 |
| Hepatic artery invasion | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1.000 |
| Bile duct invasion | 0 (0.0) | 2 (16.7) | 0 (0.0) | 0.483 |
| Lymph node metastasis | 1 (14.3) | 2 (16.7) | 3 (42.9) | 0.487 |
| pStage† | | | | |
| I | 1 (14.3) | 0 (0.0) | 1 (14.3) | 0.463 |

| | | | | |
|---|---|---|---|---|
| II | 0 (0.0) | 2 (16.7) | 1 (14.3) | |
| III | 2 (28.6) | 6 (50.0) | 1 (14.3) | |
| IVa | 4 (57.1) | 4 (33.3) | 3 (42.9) | |
| IVb | 0 (0.0) | 0 (0.0) | 1 (14.3) | |
| Non-cancerous liver | | | | |
| Cirrhosis | 0 (0.0) | 5 (41.7) | 0 (0.0) | 0.055 |
| Non-cirrhosis | 7 (100.0) | 7 (58.3) | 7 (100.0) | |

† Liver Cancer Study Group of Japan, 6th edition.

CHC, combined hepatocellular-chorangiocarcinoma; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; HCC-INT, hepatocellular carcinoma and intermediate cell carcinoma; INT, intermediate cell carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9.

**Table 3** Immunohistochemical staining of CD56

| CD56 | IHC score | | | | | Judgement | | Positive rate (%) |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | negative | positive | |
| cHCC-CCA | | | | | | | | |
| hcc | 7 | 0 | 0 | 0 | 0 | 7 | 0 | 0.0 |
| cca | 7 | 0 | 0 | 0 | 0 | 7 | 0 | 0.0 |
| int | 4 | 0 | 0 | 0 | 0 | 4 | 0 | 0.0 |
| HCC-INT | | | | | | | | |
| hcc | 12 | 0 | 0 | 0 | 0 | 12 | 0 | 0.0 |
| int | 9 | 3 | 0 | 0 | 0 | 9 | 3 | 25.0 |
| INT | 4 | 2 | 1 | 0 | 0 | 4 | 3 | 42.9 |

IHC, immunohistochemical; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; HCC-INT, hepatocellular carcinoma and intermediate cell carcinoma; INT, intermediate cell carcinoma; hcc, hepatocellular carcinoma component; cca, cholangiocarcinoma component; int, intermediate cell carcinoma component.

**Table 4** Immunohistochemical staining of CD133

| CD133 | IHC score | | | | | Judgement | | Positive rate (%) |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | negative | positive | |
| cHCC-CCA | | | | | | | | |
| hcc | 6 | 0 | 1 | 0 | 0 | 6 | 1 | 14.3 |
| cca | 4 | 2 | 0 | 1 | 0 | 4 | 3 | 42.9 |
| int | 2 | 1 | 0 | 1 | 0 | 2 | 2 | 50.0 |
| HCC-INT | | | | | | | | |
| hcc | 11 | 0 | 1 | 0 | 0 | 11 | 1 | 8.3* |
| int | 2 | 2 | 4 | 4 | 0 | 2 | 10 | 83.3* |
| INT | 1 | 1 | 2 | 3 | 0 | 1 | 6 | 85.6 |

*P<0.05

IHC, immunohistochemical; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; HCC-INT, hepatocellular carcinoma and intermediate cell carcinoma; INT, intermediate cell carcinoma; hcc, hepatocellular carcinoma component; cca, cholangiocarcinoma component; int, intermediate cell carcinoma component.

**Table 5** Immunohistochemical staining of EpCAM

| EpCAM | IHC score | | | | | Judgement | | Positive rate (%) |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | negative | positive | |
| cHCC-CCA | | | | | | | | |
| hcc | 6 | 0 | 1 | 0 | 0 | 6 | 1 | 14.3 |
| cca | 2 | 0 | 1 | 1 | 3 | 2 | 5 | 71.4 |
| int | 2 | 0 | 1 | 0 | 1 | 2 | 2 | 50.0 |
| HCC-INT | | | | | | | | |
| hcc | 5 | 0 | 2 | 3 | 2 | 5 | 7 | 58.3 |
| int | 2 | 1 | 3 | 3 | 3 | 2 | 10 | 83.3 |
| INT | 0 | 0 | 1 | 2 | 4 | 0 | 7 | 100.0 |

EpCAM, epithelial cell adhesion molecule; IHC, immunohistochemical; cHCC-CCA, combined hepatocellular-cholangiocarcinoma; HCC-INT, hepatocellular carcinoma and intermediate cell carcinoma; INT, intermediate cell carcinoma; hcc, hepatocellular carcinoma component; cca, cholangiocarcinoma component; int, intermediate cell carcinoma component.

Figure 1

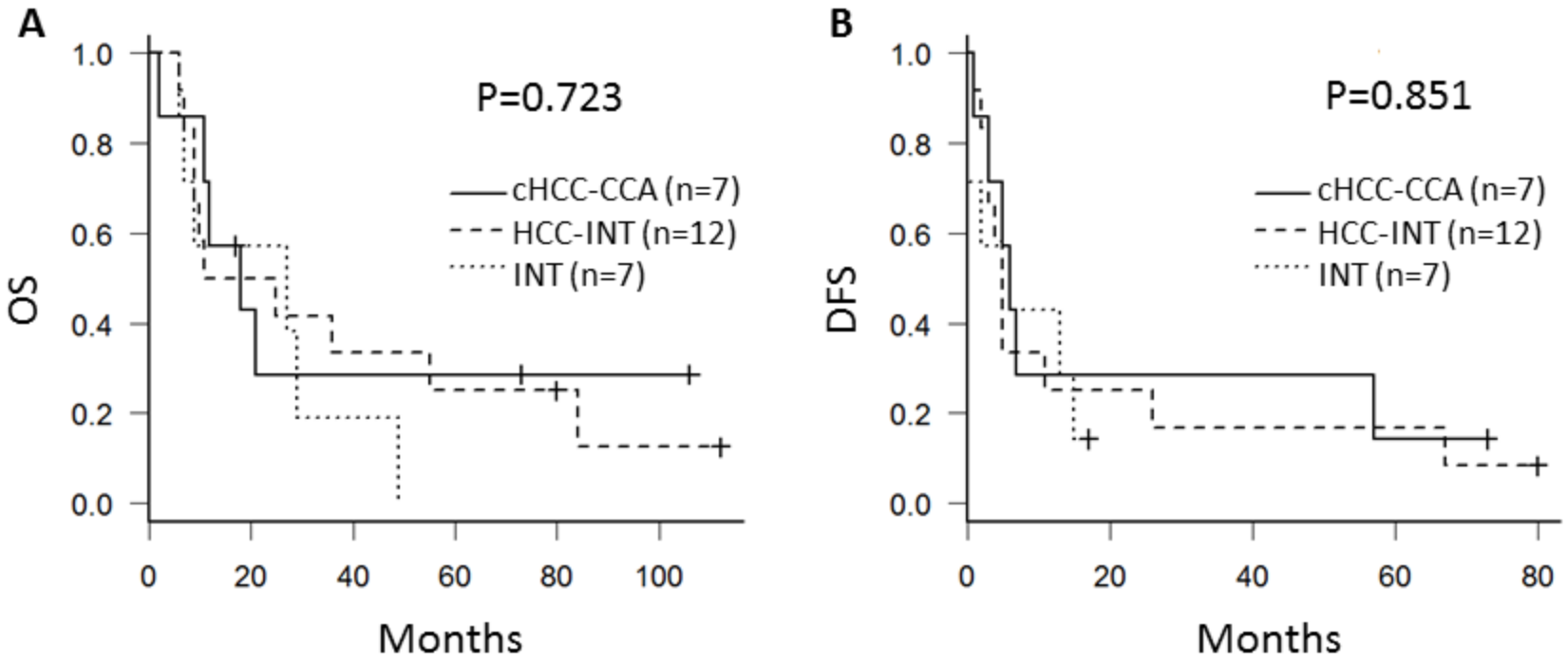

A
OS
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Months
P=0.723
cHCC-CCA (n=7)
HCC-INT (n=12)
INT (n=7)
B
DFS
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Months
P=0.851
cHCC-CCA (n=7)
HCC-INT (n=12)
INT (n=7)

Figure 2

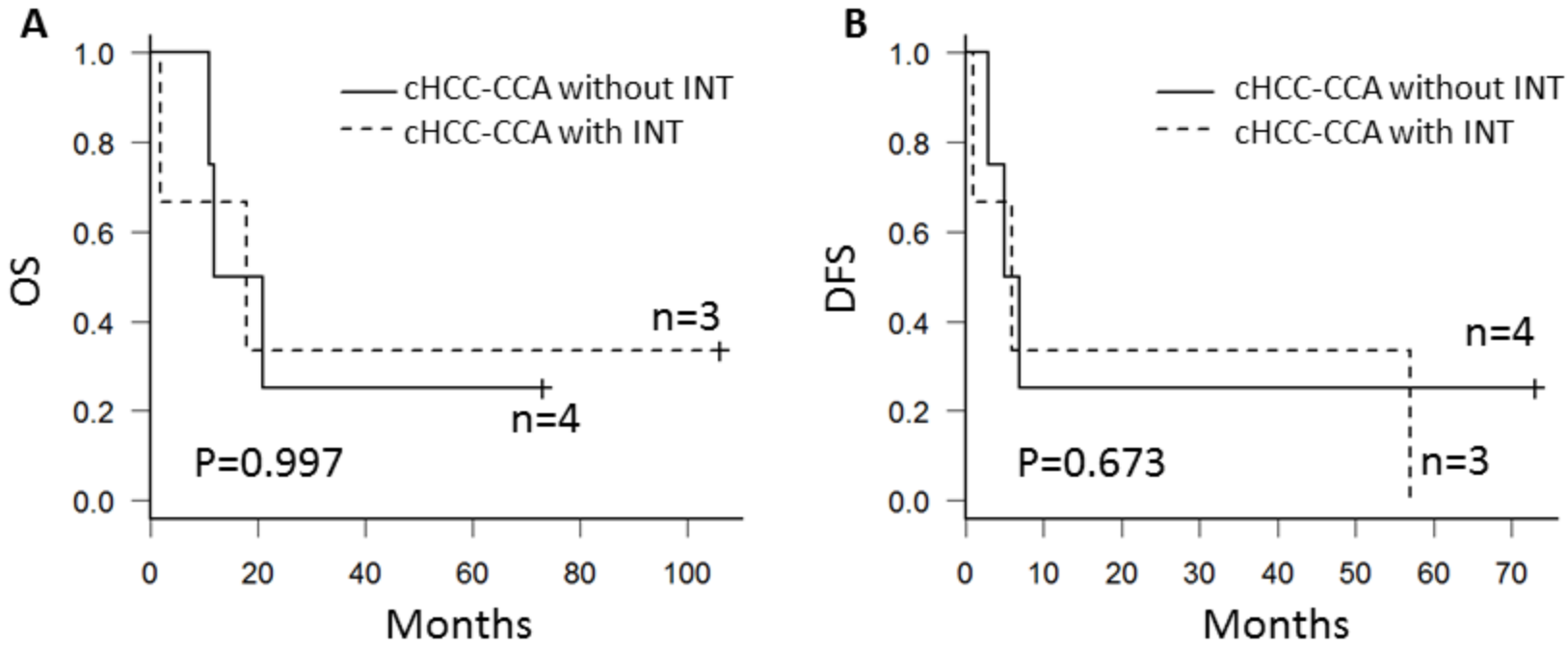

A
OS
1.0
0.8
0.6
0.4
0.2
0.0
cHCC-CCA without INT
cHCC-CCA with INT
n=3
n=4
P=0.997
0
20
40
60
80
100
Months
B
DFS
1.0
0.8
0.6
0.4
0.2
0.0
cHCC-CCA without INT
cHCC-CCA with INT
n=4
n=3
P=0.673
0
10
20
30
40
50
60
70
Months

Figure 3

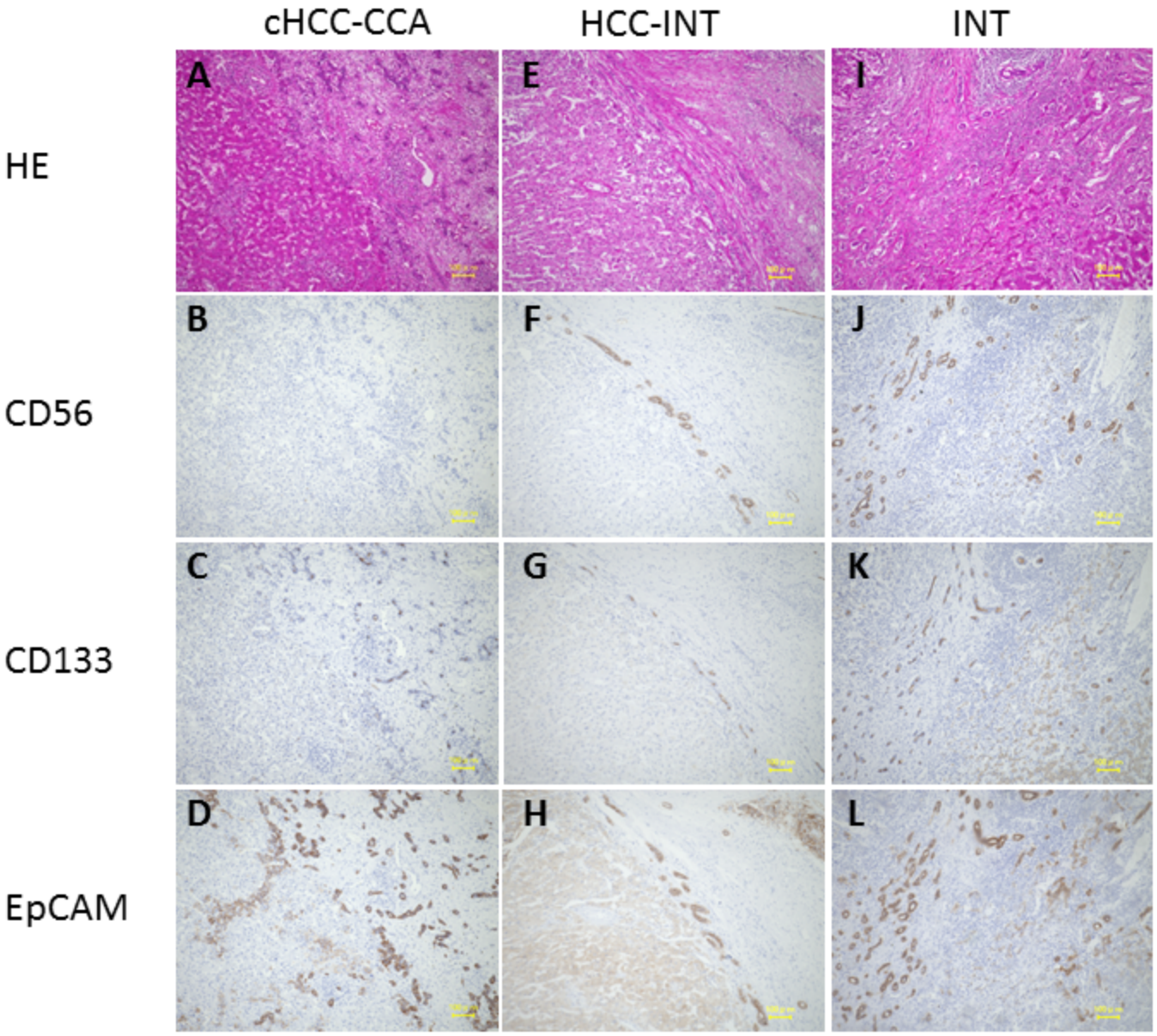


Figure 4

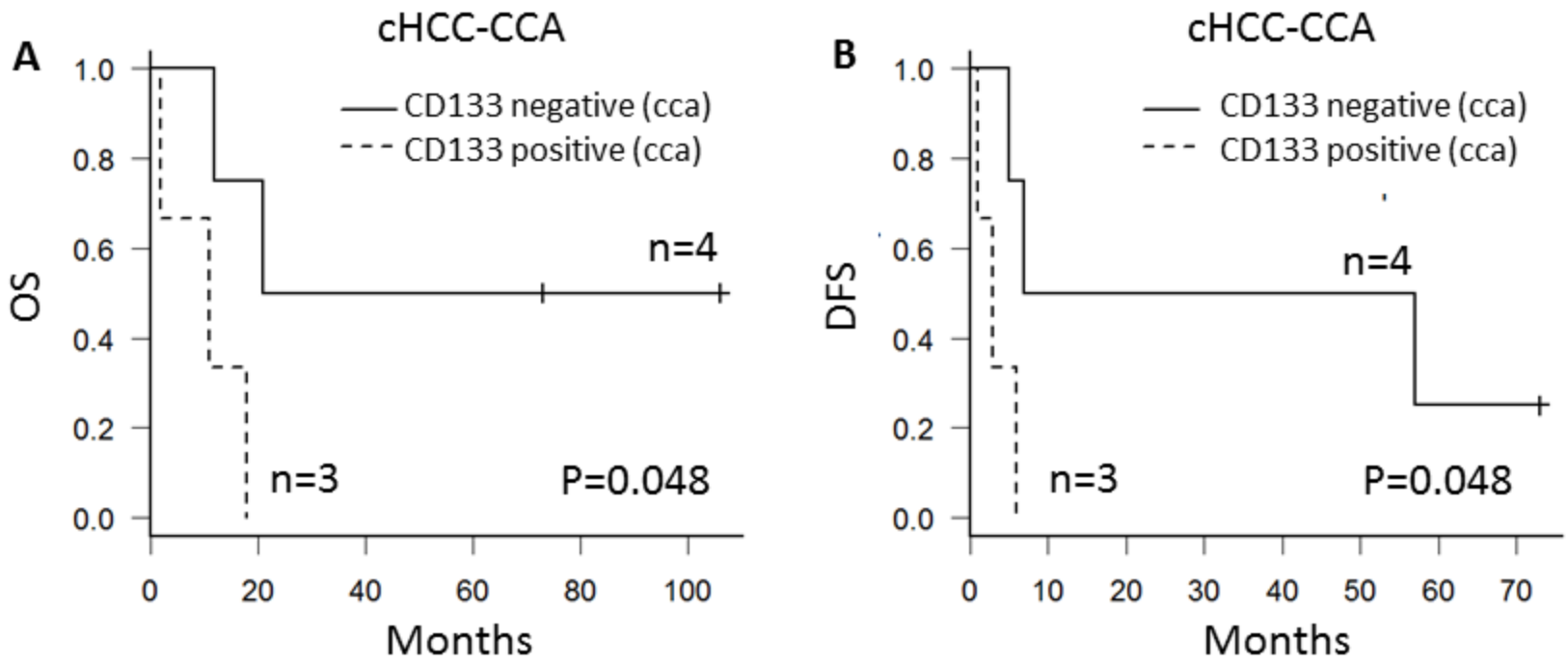
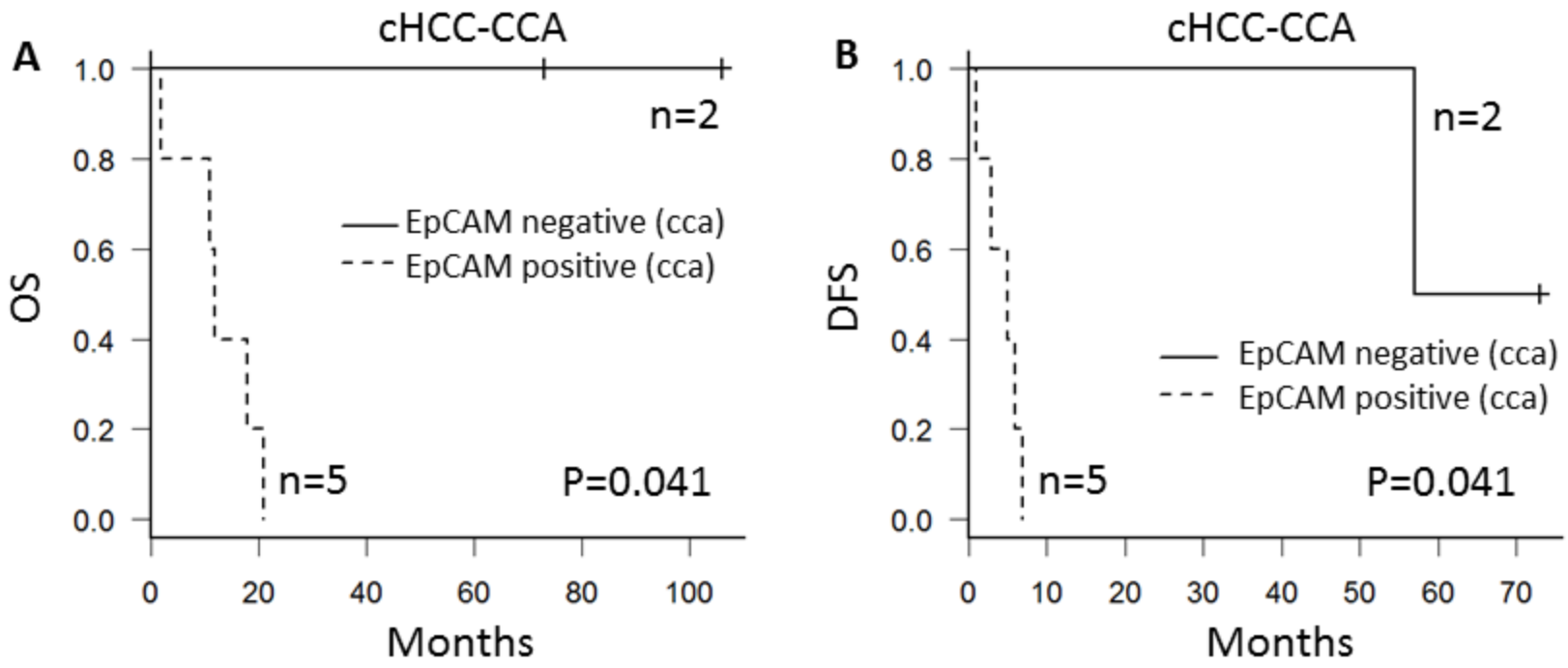

A
cHCC-CCA
CD133 negative (cca)
CD133 positive (cca)
OS
1.0
0.8
0.6
0.4
0.2
0.0
n=4
n=3
P=0.048
0
20
40
60
80
100
Months
B
cHCC-CCA
CD133 negative (cca)
CD133 positive (cca)
DFS
1.0
0.8
0.6
0.4
0.2
0.0
n=4
n=3
P=0.048
0
10
20
30
40
50
60
70
Months

Figure 5


A
cHCC-CCA
OS
1.0
0.8
0.6
0.4
0.2
0.0
n=2
EpCAM negative (cca)
EpCAM positive (cca)
n=5
P=0.041
0
20
40
60
80
100
Months
B
cHCC-CCA
DFS
1.0
0.8
0.6
0.4
0.2
0.0
n=2
EpCAM negative (cca)
EpCAM positive (cca)
n=5
P=0.041
0
10
20
30
40
50
60
70
Months