

# CD133 expression and $\alpha$ -fetoprotein levels define novel prognostic subtypes of HBV-associated hepatocellular carcinoma: A long-term follow-up analysis

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**Abstract.** Hepatocellular carcinoma (HCC) is a highly heterogeneous type of tumor, which may be caused by the stem/progenitor cell features of particular HCC cells. Recent studies have subclassified HCC into different prognostic subtypes according to just one stemness-associated marker. However, one stemness-associated marker is not sufficient to clearly define cancer stem cells, or to decipher the heterogeneous nature of HCC. For a more precise subtype classification for prognostic application, a combination of multiple stemness-associated markers is required. Cluster of differentiation 133 (CD133) and  $\alpha$ -fetoprotein (AFP) are common stemness-associated markers for HCC that have not yet been employed for HCC subtype classification. In the present study, CD133 expression was assessed by immunohistochemistry in 127 hepatitis B virus-associated HCC tumor specimens. Based on CD133 immunostaining and serum AFP levels, the HCC cases were subclassified into four subtypes, which demonstrated different clinicopathological features and varying prognoses. Among the four subtypes, the number of tumor lesions, histological grade and vascular invasion were significantly different ( $P=0.002$ ,  $P=0.018$  and  $P=0.022$ , respectively). CD133<sup>+</sup>AFP<sup>+</sup> HCC was associated with a relatively poor prognosis, CD133<sup>+</sup>AFP<sup>-</sup> HCC was associated with a relatively good prognosis, while CD133<sup>+</sup>AFP<sup>-</sup> HCC and CD133<sup>-</sup>AFP<sup>+</sup> HCC were associated with an intermediate

prognosis. These prognostic values were confirmed by borderline or statistical significance (between all groups, overall survival,  $P=0.061$ ; recurrence-free survival,  $P=0.015$ ). These results define a novel and simple system, based on CD133 and AFP, for classifying HCC into four distinct prognostic subtypes. This classification system may aid the assessment of patients with HCC for personalized therapy.

## Introduction

Hepatocellular carcinoma (HCC) is a highly malignant type of cancer that is the 5th most commonly diagnosed cancer and the second leading cause of cancer-associated mortality worldwide (1). Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are major risk factors for the development of HCC (2). Due to the high incidence of HBV and HCV infection, the incidence of HCC is increasing, particularly in Southeast Asia and sub-Saharan Africa (3). At present, surgical resection and liver transplantation remain the most effective therapies for HCC. Although improvements have been made regarding HCC diagnosis and systematic therapies, the prognosis is not favorable (three-year survival rate, 30-40%) (4,5). Cancer metastasis and recurrence are the major causes for HCC-associated mortality. The resistance of HCC to anticancer therapy results from its highly heterogeneous character (6). Cancer metastasis and recurrence, and embryonic development have many analogous properties, including dynamic cell motility, cellular plasticity and integral interaction with the microenvironment. Past research has identified that the heterogeneity of HCC may result from the presence of hepatic cancer cells with stem/progenitor features (7).

Cancer stem cells (CSCs) are a subpopulation of cancer cells that can self-renew and differentiate to produce heterogeneous tumors. CSCs may cause cancer relapse and metastases, due to their resistance to drugs and radiation therapy. Past research has indicated the existence of CSCs in various cancers by isolation using cell surface stemness-associated markers, including markers for leukemia (CD34), breast cancer (CD44) and colon cancer (CD133) CSCs (8-10). Several stemness-associated markers of HCC have been proposed for subtype classification, including CD133, epithelial cell

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adhesion molecule (EpCAM), keratin 19, and  $\alpha$ -fetoprotein (AFP), which have been individually associated with a poorer prognosis (11-13). However, subtype classification based on only one stemness-associated marker may not be sufficient to clearly define CSCs or decipher the heterogeneous nature of HCC. The combination of multiple stemness-associated markers is required to achieve a more precise subtype classification, which may enable the prognostic stratification and effective assessment of patients with HCC for personalized therapy.

Yamashita *et al* (7) identified novel HCC subtypes using EpCAM expression and serum AFP levels to subclassify HCC into the four groups: EpCAM<sup>+</sup>AFP<sup>+</sup>, EpCAM<sup>+</sup>AFP<sup>-</sup>, EpCAM<sup>-</sup>AFP<sup>+</sup>, and EpCAM<sup>-</sup>AFP<sup>-</sup>. These four subtypes exhibited distinct gene expression patterns, with features resembling certain developmental stages of hepatic lineages. The EpCAM<sup>+</sup>AFP<sup>+</sup> subtype exhibited the poorest prognosis and displayed hepatic stem cell-like traits, whereas the EpCAM<sup>+</sup>AFP<sup>-</sup> subtype was associated with a more favorable outcome and exhibited mature hepatocyte-like features. The Wnt/ $\beta$ -catenin signaling pathway, critical for maintaining embryonic stem cells (14), was activated in the EpCAM<sup>+</sup>AFP<sup>+</sup> subtype, but not in the EpCAM<sup>+</sup>AFP<sup>-</sup> subtype, indicating that these four subtypes required different therapeutic interventions. CD133 is one of the most commonly used stemness-associated markers in HCC (15). To the best of our knowledge, HCC subtype classification based on a combination of CD133 expression and serum AFP levels has not been previously reported.

In the present study, a CD133/AFP classification system to subdivide HCC into four different subtypes is defined for the prediction of the prognosis and clinicopathological features.

## Subjects and methods

**Patients and clinicopathological information.** Tissue samples were obtained, with informed consent, from 127 patients with HBV-associated HCC who underwent curative hepatectomies at the Prince of Wales Hospital (Hong Kong, China) from November 1995 to January 2013. The present study strictly abided by the Reporting Recommendations for Tumor Marker Prognostic Studies guidelines (16) and the Transparent Reporting of a multivariable prediction model for Individual Prognosis or Diagnosis Statement (17). The study was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee (Hong Kong, China). The patient eligibility criteria were as follows: i) To have been diagnosed with HCC for the first time, without distant metastasis and to have not received any anticancer therapy before surgery; ii) to have undergone a liver function test revealing Child-Pugh grade A disease and tumor-negative resection margins following the curative hepatectomy; iii) to have no other malignant tumors, autoimmune diseases, or serious heart, lung, kidney or blood diseases; iv) to be seropositive for hepatitis B surface antigen and seronegative for HCV; and v) to have available follow-up information. All HCC specimens were histologically documented by two independent pathologists blinded to all patient identities and clinical outcomes. Serum AFP levels were evaluated by an electrochemiluminescence immunoassay (E170 Analytics;

Table I. Main demographic, biochemical and clinical characteristics of the 127 patients with HCC.

Variable	Unit	Value
Age, median (range)	years	57.3 (13-84)
Sex (n, %)		
Male		103 (81.1)
Female		14 (18.9)
Albumin, median (range)	g/l	38.2 (29-46)
ALT, median (range)	U/l	49.8 (11-227)
Total bilirubin, median (range)	g/l	10.5 (3-20)
HCC diameter, median (range)	cm	5.3 (1.1-15)
AFP, median (range)	ng/ml	92 (2-699,800)

HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; AFP,  $\alpha$ -fetoprotein.

Roche Diagnostics, Indianapolis, IN, USA) as previously described (18) prior to hepatectomy. The details of other biochemical markers, including albumin, alanine aminotransferase and bilirubin, were acquired from the patients' medical records. All patients' clinicopathological data are summarized in Table I.

**Follow-up information.** Patients visited the outpatient clinic every 3 months in the first year after surgery, every 4 months in the second year after surgery, and every 6 months thereafter; tumor recurrence was diagnosed by contrast-enhanced computed tomography or magnetic resonance images, which were obtained at least every three months during the post-operative follow-up. All patients were followed-up until 8th November 2014 or the event of mortality. Information regarding patient mortality was obtained from the Social Security Death Index, medical records or notification from the family of the deceased.

**Immunohistochemical analysis.** Immunohistochemical staining was performed as described by Kang *et al* (19). The primary antibody was anti-human CD133 mouse monoclonal antibody (cat. no. MAB4399; Millipore, Billerica, MA, USA). CD133 expression levels were semi-quantitatively scored from 0-3, according to the proportion of tumor cells with positive cytoplasmic/membranous staining, as follows: 0 (negative), staining in <1% of tumor cells; 1, weak staining in  $\geq 1\%$ ; 2, moderate staining in  $\geq 1\%$ ; and 3, strong staining in  $\geq 1\%$  of tumor cells. Staining scores of 2 and 3 were defined as positive staining, whereas 0 and 1 were regarded as negative staining (11).

**Statistical analysis.** Continuous data were expressed as the median (range). The association of CD133 expression or AFP level with clinicopathological characteristics was examined using  $\chi^2$  tests. Overall survival (OS) was defined as the time from curative hepatectomy until 8th November 2014 or the event of mortality. Recurrence-free survival (RFS) was defined as the time from curative hepatectomy to the first recurrence. The Kaplan-Meier method was used to calculate OS and RFS, and the log rank-test was applied to determine the differences

Table II. Associations of serum AFP levels and CD133 protein expression in surgical specimens of HCC with clinicopathological characteristics.

Clinicopathological parameter	AFP (ng/ml), n		P-value	CD133, n		P-value
	≤400	>400		Negative	Positive	
Total	80	47		75	52	
Age, years			0.275			0.614
≥50	60	31		55	36	
<50	20	16		20	16	
Sex			0.377			0.316
Male	63	40		63	40	
Female	17	7		12	12	
No. of tumor lesions			0.280			<0.001 <sup>a</sup>
Solitary	63	33		65	31	
Multiple	17	14		10	21	
Cirrhosis			0.738			0.396
Absent	35	22		36	21	
Present	45	25		39	31	
Tumor size			0.094			0.650
≥5 cm	32	26		33	25	
<5 cm	48	21		42	27	
AFP						0.927
>400 μg/l	-	-		28	19	
≤400 μg/l	-	-		47	33	
Differentiation			0.022 <sup>a</sup>			0.487
Well/moderate	73	36		63	46	
Poor	7	11		12	6	
Vascular invasion			0.016 <sup>a</sup>			0.054
Absent	70	33		65	38	
Present	10	14		10	14	

<sup>a</sup>P<0.05. AFP, α-fetoprotein; HCC, hepatocellular carcinoma; CD, cluster of differentiation.

between survival curves. Our previous study (13) demonstrated that an AFP cut-off value of 400 ng/ml was optimal to assess the link between AFP levels and OS/RFS, rather than 300 ng/ml as recommended by the existing literature (20). Therefore, the AFP cut-off value was defined as 400 ng/ml in the present study. All statistical analyses were performed using the SPSS software package (version 16.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**Patient characteristics and clinicopathological features.** A total of 127 eligible patients with HBV-associated HCC were recruited to the present study. Their characteristics are summarized in Tables I and II. The mean follow-up time was 106.3 (range, 24-215) months.

The distribution of AFP levels was skewed, with a range of 2-699,800 (median, 92) ng/ml. A serum AFP cut-off value of (400 ng/ml) was used to classify HCC as AFP<sup>+</sup> or AFP<sup>-</sup>. AFP<sup>+</sup>

HCC was associated with poorer histological differentiation (P=0.022) and vascular invasion (P=0.016). No associations were identified between AFP and patient age, sex, number of tumor lesions (solitary or multiple), cirrhosis (absence or presence) or tumor size (≤5 vs. >5 cm; Table II).

The CD133 cytoplasmic/membranous expression was observed as negative, weak, moderate or strong staining (Fig. 1). Positive CD133 expression was detected in 52 out of 127 of the patients with HCC. Positive CD133 staining was detected more often in patients with multiple tumor lesions (P<0.001), and was trending towards an association with vascular invasion status (P=0.054). No association between CD133 expression and other clinicopathological features was identified (Table II).

According to CD133 expression and serum AFP levels, all 127 HBV-associated HCC cases were subclassified into four groups: i) CD133<sup>+</sup>AFP<sup>+</sup>, 19 cases (15.0%); ii) CD133<sup>-</sup>AFP<sup>+</sup>, 28 cases (22.0%); iii) CD133<sup>+</sup>AFP<sup>-</sup>, 33 cases (26.0%) and iv) CD133<sup>-</sup>AFP<sup>-</sup>, 47 cases (37.0%; Table III). Among these four HCC subtypes, the number of tumor lesions, the histological grade and vascular invasion were significantly different



Table III. Clinicopathological characteristics of HCC subtypes defined by AFP and CD133 expression.

Clinicopathological parameter	HCC subtype				P-value
	AFP-CD133 <sup>-</sup>	AFP-CD133 <sup>+</sup>	AFP <sup>+</sup> CD133 <sup>-</sup>	AFP <sup>+</sup> CD133 <sup>+</sup>	
Age, years	47	33	28	19	0.526
≥50	35	25	20	11	
<50	12	8	8	8	
Sex					0.594
Male	38	25	25	15	
Female	9	8	3	4	
No. of tumor lesions					0.002 <sup>a</sup>
Solitary	41	22	24	9	
Multiple	6	11	4	10	
Cirrhosis					0.843
Absent	22	13	14	8	
Present	25	20	14	11	
Tumor size, cm					0.312
≥5	17	15	16	10	
<5	30	18	12	9	
Differentiation					0.018 <sup>a</sup>
Well/moderate	44	29	19	17	
Poor	3	4	9	2	
Vascular invasion					0.022 <sup>a</sup>
Absent	44	26	21	12	
Present	3	7	7	7	

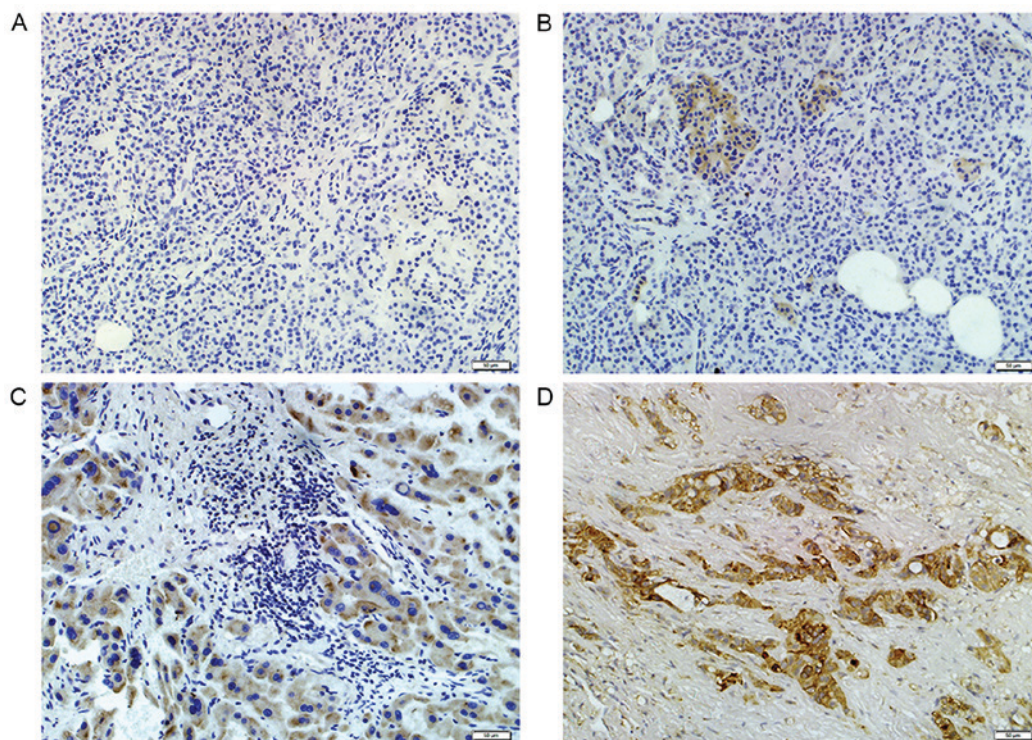
<sup>a</sup>P<0.05. HCC, hepatocellular carcinoma; AFP, α-fetoprotein.

Figure 1. Immunohistochemical analyses of the expression of CD133 in hepatocellular carcinoma. Representative images of (A) negative cytoplasmic/membranous staining; (B) weak cytoplasmic/membranous staining; (C) moderate cytoplasmic/membranous staining; (D) strong cytoplasmic/membranous staining. Magnification, x200 (scale bar, 50 μm). CD, cluster of differentiation.

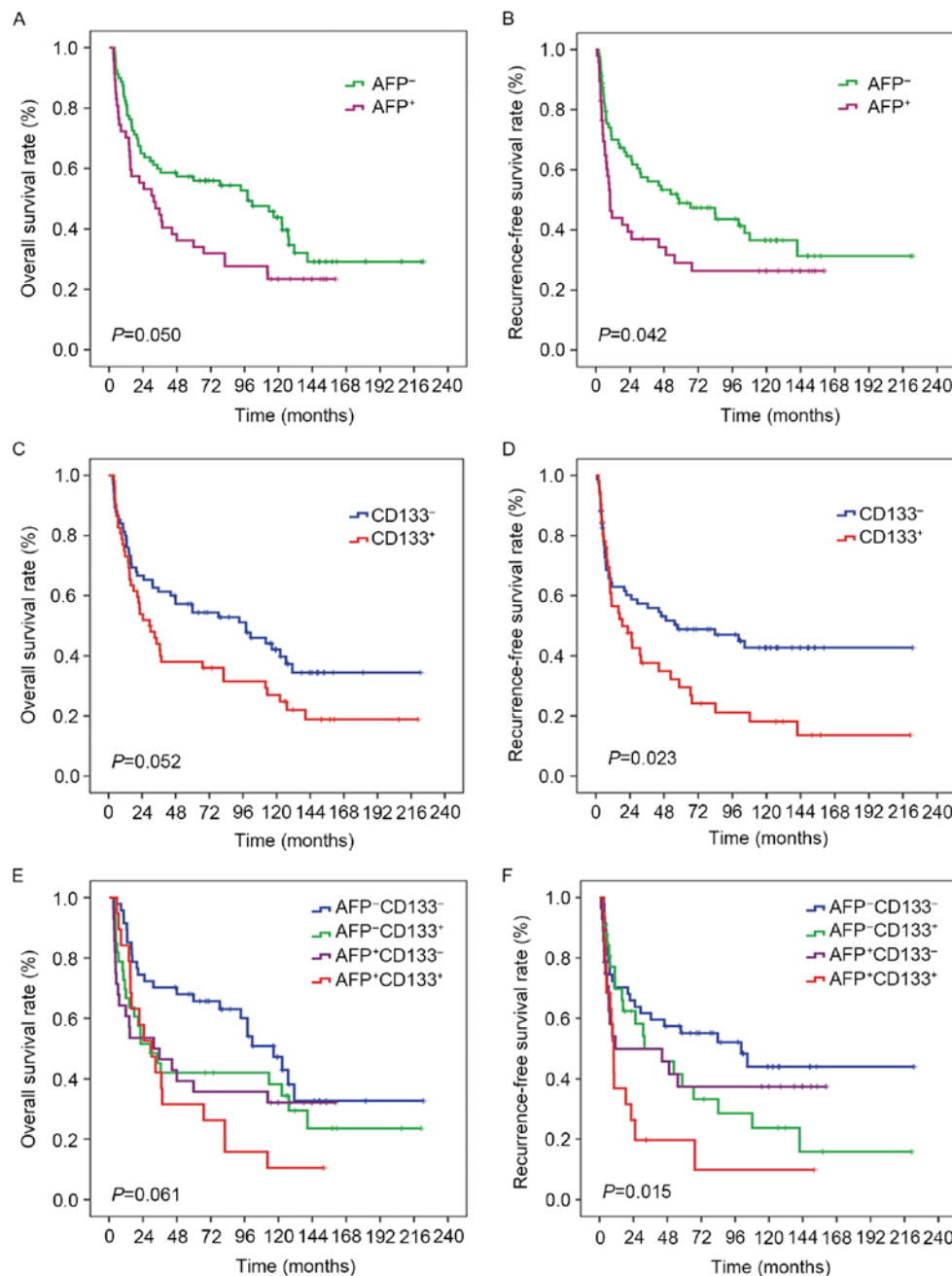


Figure 2. Prognostic outcomes of different HCC subtypes. Kaplan-Meier plots for the association of AFP with (A) OS and (B) RFS; CD133 with (C) OS and (D) RFS; CD133 and AFP with (E) OS and (F) RFS, in 127 patients with HCC. HCC, hepatocellular carcinoma; AFP,  $\alpha$ -fetoprotein; OS, overall survival; RFS, recurrence-free survival; CD, cluster of differentiation.

( $P=0.002$ ,  $P=0.018$  and  $P=0.022$ , respectively). Patients classified with  $CD133^+AFP^+$  HCC were more liable to developing multiple tumor lesions and vascular invasion compared with patients classified with  $CD133^-AFP^-$  HCC, which typically presented with single tumor lesions without vascular invasion.  $CD133^+AFP^-$  HCC was also associated with multiple tumor lesions, and  $CD133^-AFP^+$  HCC was associated with the patients demonstrating 'poor' histological differentiation, with the highest frequency of all types (32.1%; 9/28).

**Prognosis of HCC subtypes defined by AFP or CD133.** OS and RFS curves according to AFP levels (cut-off value, 400 ng/ml) are illustrated in Fig. 2A and B. A significantly

increased risk of mortality and recurrence was identified in AFP-positive patients compared with AFP-negative patients ( $P=0.050$  and  $P=0.042$ , respectively). Fig. 2C and D includes OS and RFS curves based on the expression of CD133; patients with CD133-positive tumors had a higher risk of mortality that was trending towards significance ( $P=0.052$ ) and a significantly higher risk of disease recurrence ( $P=0.023$ ) than CD133-negative patients.

**Prognosis of HCC subtypes defined by AFP and CD133.** Kaplan-Meier survival and recurrence analysis were performed to assess the prognosis of the four HCC subtypes. The result of Kaplan-Meier OS and RFS analyses (Fig. 2E and F) revealed

that CD133<sup>+</sup>AFP<sup>+</sup> HCC was associated with a relatively poor prognosis, CD133<sup>-</sup>AFP<sup>-</sup> HCC was associated with a relatively good prognosis, and CD133<sup>+</sup> AFP<sup>-</sup> HCC and CD133<sup>-</sup>AFP<sup>+</sup> HCC were associated with intermediate prognoses. Statistical analysis demonstrated a borderline result for the association with OS ( $P=0.061$ ) and statistical significance for RFS ( $P=0.015$ ).

## Discussion

HCC is a highly heterogeneous type of tumor. Several studies have demonstrated that the presence of HCC cells with stem/progenitor features may lead to tumor heterogeneity (12,21). Experimental evidence has indicated that liver stem cells can develop into hepatoblasts, differentiate into hepatocytic or biliary lineage progenitor cells, and mature into hepatocytes (22,23). CD133 or AFP have been used to identify hepatic CSCs (24,25). CD133 is a five-pass transmembrane cell surface glycoprotein that is expressed in normal and malignant stem cells of hematopoietic, neuronal/glia, renal/prostatic and hepatic/endothelial lineages (26-30). Other studies have indicated that CD133-positive cells separated from HCC cell lines are able to self-renew and differentiate (25,31), suggesting that CD133 is a common and robust HCC CSC marker. AFP is a biomarker for HCC, expressed in fetal livers and HCC tumors, but not in the adult liver, analogous to the expression of CD133 (32). A previous study has indicated that AFP-producing cells in primary liver cancers may exhibit CSC-like properties (24). Therefore, the combination approach of using CD133 and AFP to define CSCs in HCC is reasonable.

The present study revealed that patients with AFP<sup>+</sup> HCC exhibited poorer histological differentiation, positive vascular invasion and an increased risk of mortality and recurrence. CD133-immunopositivity was more frequently observed in patients with multiple tumor lesions, vascular invasion and higher risks of mortality and recurrence. Therefore, AFP and CD133 were identified as independent poor prognosis factors for patients with HCC, which corroborates the results of previous studies (13,33). A poor prognosis may be associated with the existence of CSCs (AFP<sup>+</sup> or CD133<sup>+</sup>), which facilitate cancer therapy resistance and accelerate cancer progression. The combination of CD133 and AFP may be necessary for a more precise classification of prognosis-associated subtypes.

HCC was classified into four subtypes in the present study based on CD133 expression and serum AFP levels. The four HCC subtypes corresponded to different clinicopathological features and prognosis. Among the four subtypes, CD133<sup>+</sup>AFP<sup>+</sup> HCC was associated with the poorest prognosis and CD133<sup>-</sup>AFP<sup>-</sup> HCC with the most favorable prognosis.

This classification system is similar to the model of Yamashita *et al* (7), which demonstrated that EpCAM<sup>+</sup>AFP<sup>+</sup> HCC, associated with the poorest prognosis, displayed hepatic stem cell-like traits, whereas EpCAM<sup>-</sup>AFP<sup>-</sup> HCC, associated with a more favorable prognosis, exhibited the features of mature hepatocytes. We hypothesize that the four subtypes classified based on AFP and CD133 may also resemble different hepatocyte lineages, and that this classification system may reflect the cellular origin of the heterogeneous nature of HCC. The present study indicates that the expression of various stem cell markers in CSCs may be different in

each HCC subtype, possibly resulting from the heterogeneity of the activated CSC signaling pathways. Yamashita *et al* (21) indicated that EpCAM<sup>+</sup>AFP<sup>+</sup> HCC cells displayed hepatic CSC-like traits, including the abilities to self-renew and differentiate by the activation of Wnt/ $\beta$ -catenin signaling. Therefore, experimental design to test this hypothesis should consider the molecular pathways potentially involved in the maintenance of CD133<sup>+</sup>AFP<sup>+</sup> HCC cells.

The present study is constrained by a number of limitations. The sample size of the study is relatively small due to the rigorous eligibility criteria for the selection of patients with HBV-associated HCC; however, including such a homogeneous group of patients provides internal validity to the results. Furthermore, study regarding why CD133<sup>+</sup>AFP<sup>+</sup> HCC is associated with a poor prognosis is required. Future studies by this group will aim to explore whether CD133<sup>+</sup>AFP<sup>+</sup> HCC cells exhibit hepatic stem cell-like traits and determine the associated molecular pathways that are activated to sustain the CSC features of CD133<sup>+</sup>AFP<sup>+</sup> HCC cells.

In conclusion, the present study defined a novel and simple classification system for subclassifying HCC into four different prognostic subtypes based on CD133 expression and AFP serum levels. This classification system may allow the provision of personalized therapy to patients with HCC.

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