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CD44 Expression Is a Prognostic Factor in Patients with Intrahepatic Cholangiocarcinoma After Surgical Resection

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Abstract. *Background/Aim: Cancer stem cells (CSC) plays an important role in various kinds of cancers. The aim of this study was to clarify the role of CD44 expression in intrahepatic cholangiocarcinoma (IHCC) as a marker of CSCs. Materials and Methods: Thirty-five patients with IHCC patients who underwent hepatectomy were evaluated. CD44 expression was determined immunohistochemically. The patients were divided into a CD44-positive group (n=22) or CD44-negative group (n=13). Clinicopathological variables including prognosis were compared between the two groups. Results: The CD44-positive group had a worse prognosis than the CD44-negative group (5-year survival: 19.3% vs. 55.5%, respectively, p=0.016), although no difference in the background variables was observed. In multivariate analysis, CD44-positivity was identified as an independent prognostic factor (hazard ratio=3.676, p=0.034). Conclusion: These data suggest that CD44-positivity might be a candidate CSC marker in IHCC and a prognostic indicator.*

Intrahepatic cholangiocarcinoma (IHCC) is a primary adenocarcinoma of the liver arising from the intrahepatic bile ducts, and the second most common primary hepatic tumor after hepatocellular carcinoma (HCC). The incidence of IHCC has been reported at only about 4.1% of primary liver carcinoma cases (1), and curative surgical treatment is considered to be the only truly effective treatment (2-5). However, patients with IHCC have an extremely poorer prognosis compared to other malignancies, even if curative resection has been performed (6-10).

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Cancer stem cells (CSCs) have been reported to play an important role in various kinds of cancers, and abundant evidence has reported that stem cell properties, such as self-renewal, unlimited proliferation and differentiation, are highly relevant to the biology of several human cancer types (11-14). For solid tumors, the repertoire of cell surface markers currently used to identify human CSCs includes CD44, CD133, epithelial surface antigen (ESA), and CD24, either on their own or in combination. Considering these findings, it is extremely important to elucidate the prognostic factors and establish an effective treatment strategy to overcome the poor prognosis of IHCC, paying attention to the existence of CSCs. We previously reported that CD133 positivity was independently related to worse prognosis, and tended to correlate with higher incidence of intrahepatic metastasis and expression of hypoxia-inducible factor-1a in patients with IHCC (15).

The CD44 antigen is a cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration (16-18). Al-Hajj *et al*. first reported the potential role of CD44 antigen of breast cancer cells, where CD44-positive cells were breast CSC that possessed higher tumorigenicity and metastatic potential (19). CD44 as a marker was also used to isolate prostate (20-22), pancreatic (23, 24), colorectal (25, 26), and hepatocellular (27) CSCs.

The aim of this study was, therefore, to investigate the correlation between CD44 expression and clinicopathological characteristics, including prognosis, as an important CSC marker in IHCC.

Patients and Methods

Patients. Among thirty-five patients with IHCC who had undergone surgical resection at our Institute from 1992 to 2009, with available surgical specimens for immunohistochemistry, who survived surgery without complications such as postoperative liver failure, were included in this study. This study was authorized in advance by the Institutional Review Board of the University of Tokushima Graduate School (approved number: 266), and all patients provided written informed consent. The participants in this study were 22 men and 13 women, with a mean age of 68.0 years, ranging from 43 to 84 years.

Table I. *Clinicopathological characteristics according to CD44 expression.*

Factor		CD44 expression		*p*-Value
		Positive (n=22)	Negative (n=13)	
Age	Median±SD, years	66.1±10.1	69.1±10.1	0.3478
Gender, n	Male/female	15/7	7/6	0.4803
Hepatic viral infection	Negative/HBV/HCV/combined	17/2/2/1	8/3/2/0	0.5171
CEA, ng/ml	Median (range)	2.5 (0.6-10.0)	1.9 (1.0-80.2)	0.3785
CA19-9, ng/ml	Median (range)	708.0 (3.9-25100.0)	288.0 (5.0-4568.0)	0.8780
Stage, n	I, II/III, IV	4/18	4/9	0.3915
Curability, n	A, B/C	15/7	9/4	0.9485
Location, n	Hilar/peripheral	10/12	4/9	0.3915
Tumor diameter, n	<5/≥5 cm	13/9	6/5 (unknown 2)	0.8036
Macroscopic type, n	MF/MF+PI	9/13	5/8	0.8863
Differentiation, n	Diff./undiff	9/13	5/8	0.8864
LN metastasis, n	Negative/positive	14/8	8/5	0.9012
Vessel infiltration, n	Negative/positive	8/14	7/6	0.3126
Intrahepatic metastasis, n	Negative/positive	16/6	10/3	0.7838

CEA: Carcinoembryonic antigen, CA19-9: carbohydrate antigen 19-9, HBV/HCV: hepatitis B virus/ hepatitis C virus, MF: mass-forming type, MF+PI: mass-forming + periductal infiltrative type, Diff: differentiated, Undiff: undifferentiated, LN: lymph node.

Staging and curability were defined according to the Classification of Primary Liver Cancer by the Liver Cancer Study Group of Japan (28). Regarding the stage, T-factor was determined by tumor number (single or not), size (no more than 2 cm) and vascular infiltration (present or absent). The stage was finally determined by T-, N- and M-factors. Curability was defined as follows: Curability A, no residual tumor for stage I and II patients; curability B, no residual tumor for Stage III and IV disease; and curability C, definite residual tumors.

Consequently, 24 patients (68.6%) underwent resections with curability A or B. None of the patients had received chemotherapy or irradiation before or after surgical resection. The 3- and 5-year survival rates of the whole patient cohort were 34.6% and 34.6%, respectively. The mean follow-up period was 27.0 months (range: 2.4-110.6 months).

CD44 immunohistochemical staining and assessment. Four-micrometer-thick sections were cut from archival formalin-fixed paraffin-embedded tissue blocks. The samples were deparaffinized and dehydrated using a graded series of ethanol solutions. Endogenous peroxidase activity was halted through the administration of 0.3% hydrogen peroxidase and methanol for 20 minutes. After rinsing in phosphate-buffered saline (PBS), the tissue sections were processed in a 0.01 M citrate buffer (pH 6.0) inside a heat-resistance plastic container. The sections were then irradiated in a domestic microwave oven for 20 min. After microwave irradiation, the slides were allowed to cool at room temperature. The sections required a primary mouse monoclonal antibody to CD44 (Abcam51037, diluted 1:100 in PBS; Abcam Inc, Cambridge, UK) overnight at 4˚C. After overnight rinsing, the sections were incubated using Dako REAL™ Envision™/HRP, Rabbit/Mouse (Dako, Glostrup, Denmark) for 45 min followed by three washes in PBS. After washing in PBS, peroxidase labeling was developed by incubating the section in 3.3'-diaminobenzidine tetrahydrochloride (DAB) for 5 min. Finally, nuclear counterstaining was completed using Mayer's hematoxylin solution. All cell counts were performed using a Nikon Digital Camera DXM 1200F photomicroscope (NIKON instruments Inc. NY, USA) at a magnification of ×200 (×20 objectives and ×10 eyepiece).

Regarding the assessment of staining, normal bile duct epithelium was entirely negative in the non-cancer part. The tumor was defined as positively stained when any cells staining in cytoplasm were seen in the tumor (Figure 1), according to other previous studies (11, 18, 25). Positive CD44 expression in cancer cells was present in 22 (62.9%) out of 35 cases.

Statistical analysis. All statistical analysis was performed using statistical software (JMP 8.0.1., Cary, NC, USA). Relationships between CD44 expression and the clinicopathological variables were analyzed with the chi-square test and Mann–Whitney *U*-test. Survival curves were calculated using the Kaplan–Meier method and compared using the log-rank test. All factors found to be significant by univariate analysis were included in the Cox's proportional hazards model of multivariate analysis to identify independent factors influencing survival. Statistical significance was defined as $p<0.05$.

Results

Correlation between CD44 expression and clinico-pathological valuables. Table I presents the comparison of clinicopathological characteristics according to CD44 expression. There were no significant relationships for clinicopathological variables according to CD44 expression. However, CD44 positivity was significantly associated with poorer prognosis after surgery compared to the CD44-negative group ($p=0.016$), and 5-year survival rate was

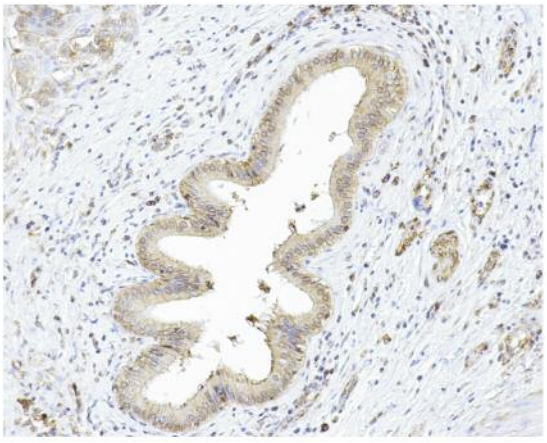

Figure 1. *CD44 expression in tumor tissue of intrahepatic cholangiocarcinoma. The expression of CD44 was recognized in the cytoplasm of cancer cells (×200).*

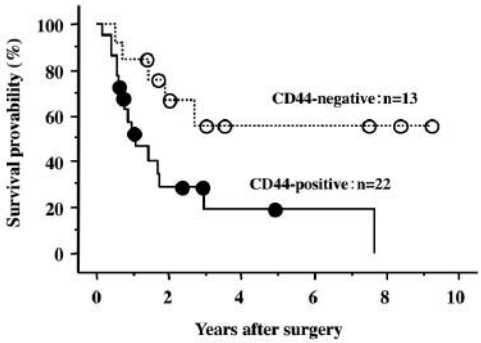

Figure 2. *Overall survival curves according to CD44 expression. The survival of patients in the CD44-positive group was significantly poorer than that of those in the CD44-negative group ($p<0.05$).*

55.5% and 19.3%, respectively (Figure 2). Regarding relapse-free survival, CD44 positivity did not affect the surgical outcome.

Univariate and multivariate analysis of prognostic factors. Univariate analysis revealed that staging (stage III, IV; $p=0.004$), curability (C; $p=0.012$), tumor size (≥5 cm; $p=0.009$) and lymph node metastasis (positive; $p=0.002$) were found to be significant prognostic factors for overall survival, as well as CD44 expression (Table II).

CD44 positivity was entered in a proportional hazards model along with staging, curability, tumor size and lymph node metastasis. CD44 positivity was found to be an independent prognostic factor ($p=0.034$) (Table II).

Table II. *Univariate and multivariate analyses of prognostic factors for overall survival.*

	Univariate		Multivariate	
Factor	*p*-Value	HR	95% CI	*p*-Value
Stage: III, IV	0.004	6.803	0.827-55.556	0.076
Curability: C	0.012	1.805	0.568-5.747	0.317
Tumor diameter: ≥5 cm	0.009	3.243	1.097-9.584	0.033
LNM: Positive	0.002	1.972	0.628-6.189	0.245
CD44 expression: Positive	0.016	3.676	1.105-12.195	0.034

CI: Confidence interval; HR: hazard ratio; LNM: lymph node metastasis.

Discussion

In this study, the impact of the CD44 positivity in IHCC was demonstrated, and it was revealed that CD44 positivity significantly correlated with malignant behavior of IHCC, being associated with worse prognosis. We previously demonstrated the role of CD133 expression as a CSC marker in patients with IHCC (15). Likewise, we revealed that the CD44 expression was identified as an independent prognostic factor in this study, and certified the utility of this molecule as another CSC marker for IHCC.

A number of recent studies have demonstrated in solid tumors the presence of CSCs, which share many characteristics with tissue stem cells, such as self-renewal and differentiation, and are primarily responsible for sustaining the growth of tumors (11-14). They are essential for tumor growth and metastasis formation even after prolonged periods of tumor dormancy. Therefore, it is a technical challenge to identify and to characterize CSCs due to the rarity of CSCs in the tissue of origin and the lack of specific markers. CSCs have been identified particularly in solid tumors, such as malignant melanoma, colorectal, pancreatic, prostate, breast and hepatocellular carcinoma (22-27, 29), and the following markers, either on their own or in combination, were adopted as CSC markers: CD44, CD133, ESA, CD24, and CD166. Among these markers, CD133 and CD44 showed overlapping expression in various tumors and CSC. We previously reported the utility of CD133 expression as a promising CSC marker in IHCC (10).

CD44, originally described as a leukocyte-homing receptor, as a transmembrane glycoprotein participates in many cellular processes, including growth, survival, differentiation, and motility (16-18). It is a unique adhesion molecule and plays a role in cancer cell migration and matrix adhesion in response to the cellular microenvironment, thus enhancing cellular aggregation and tumor cell growth (30). These adhesive activities of CD44 could well be important for CSC

properties, however, no single CSC-specific marker has yet been identified. Therefore, for identification of more putative CSCs, the following combinations of several CSC markers have been explored in several malignant tumors. Regarding combination in conjunction with CD44, such as prostate cancer initiating cells (CICs) were $CD44^{+}\alpha2\beta1^{high}CD133^{+}$ (31, 32), breast CICs were $CD44^{+}CD24^{-}$ epithelial cell adhesion molecule (EpCAM)+ $CD44^{+}CD24^{-}$ phenotypes (33), colorectal CICs were $EpCAM^{+}CD44^{+}CD166^{+}$ (34), and pancreatic CICs were $CD44^{+}EpCAM^{+}CD24^{+}$ (17).

In this study, we did not assess the combination of CD44 and other CSC markers. However, we did demonstrate that single CD44 positivity was an independent prognostic factor. Although further investigation is necessary to confirm the true role CD44 in IHCC, this molecular expression takes part in tumor malignant behavior including the relevance of CSCs. In conclusion, our data suggest that CD44-positivity is a promising CSC marker, and a new prognostic indicator of IHCC.

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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