Clinical Significances of Cancer Stem Cells Markers in Patients with Intrahepatic Cholangiocarcinoma who Underwent Hepatectomy

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Abstract. *The present study aimed to elucidate the relationship between cancer stem cells markers (CSCs), according to cell adhesion molecule (CD44) and glioma-associated oncogene homolog-1 (GLI1) expression, and clinicopathological factors and prognosis in 38 patients with intrahepatic cholangiocarcinoma (ICC) who underwent hepatectomy. CD44 and GLI1 expression was examined by immunohistochemical staining methods. The relationship with tumor angiogenesis or proliferation activity was also analyzed. Positivity of CD44 was 18% and that of GLI1 was 39%, but there was no significant correlation between the expressions of both. On macroscopic findings, CD44 expression in the periductal infiltration-type of ICC was significantly higher than in other types ($p<0.01$), and this type showed significantly worse survival after hepatectomy. Positive expression of GLI1 was significantly associated with older age. Although expression of neither CD44 nor GLI1 was significantly associated with disease-free survival, positive expression of both CD44 and GLI1 led to a significantly lower 3-year disease-free survival rate (0%; $p<0.05$). With respect to 5-year overall survival after hepatectomy, expression of both CD44 and GLI1 was not significantly associated with survival rate. CSCs might be useful markers for tumor-free survival in patients with ICC after hepatectomy and further investigation in larger series is warranted.*

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Peripheral intrahepatic cholangiocarcinoma (ICC) is a relatively rare type of primary liver cancer that shows highly malignant behavior (1-3). Hepatic resection is currently the only curative option for radical treatment of ICC. However, the recurrence rate after resection remains high and patient survival is unsatisfactory (2). Although some conventional clinicopathological and surgical factors in ICC have been shown to be related to tumor relapse and shorter patient survival, accurate prediction of prognosis for ICC is currently difficult (4-6).

Cancer stem cells (CSCs) are cancer cells possessing characteristics associated with normal stem cells and can develop into various cell phenotypes found in a particular cancer sample (7). CSCs generate tumors by processes of self-renewal and differentiation into multiple cell types, which may cause tumor progression, metastasis and tumor relapse after treatments (7). Various CSC markers including cell adhesion molecule CD44, CD24, CD133, CD166, and Epithelial cell adhesion molecule (EpCAM) have been examined in solid carcinomas (8). The CD44 antigen is a cell-surface glycoprotein involved in cell–cell interactions and cell adhesion, which is used extensively in combination with other putative markers to isolate CSCs from solid tumors (8). Expression of CD44 reflects malignant behavior such as tumor progression, metastasis or poor prognosis in solid carcinomas (9).

Glioma-associated oncogene homolog-1 (GLI1) is one of the transcription factors that mediates the Hedgehog (Hh) signaling pathway, which is an essential pathway involved in the development of cancer (10). Inappropriate activation of the sonic Hh–GLI signalling pathway occurs in several types of glioma and skin cancer and GLI1 was also found to have a relationship with malignant behavior in some carcinoma types (11). We hypothesized that an increased expression of CSCs might stimulate tumor growth and invasion, and may play a significant role in poor prognosis of patients with ICC, as the roles of CD44 and GLI1 expression have not been yet fully clarified.

In the present study, to clarify our hypothesis, we examined the relationship between prognosis and expression of CD44 and GLI1 in ICC using immunohistochemical staining methods and conventional clinicopathological factors of 38 patients with ICC, with a minimum follow-up period of 12 months.

Materials and Methods

Patients. A total of 38 consecutive patients (21 males, 17 females) with ICC who were admitted to the Nagasaki University Graduate School of Biomedical Sciences (NUGSBS) between 1997 and 2011 (minimum follow-up 12 months) were analyzed retrospectively in this study. Data were retrieved from both anesthetic and patient charts to cover the period of hospitalization following hepatectomy. The study design was approved by the Ethics Review Board of our Institute and a signed consent for clinical research, tissue or blood samples was obtained from each patient. Follow-up included measurement of serum tumor markers and abdominal computed tomography (CT) every 3-6 months. Pathological and morphological parameters, macroscopic findings and for use of Japanese tumor–node–metastasis (TNM) stage were used, as defined by the Liver Cancer Study Group of Japan (12).

Immunohistochemical staining. Resected specimens were fixed in 10% formalin and embedded in paraffin. Thin sections (4 μm) were de-paraffinized twice using xylene and rehydrated in an ethanol series (100%, 90%, and 80%). Sections were placed in 0.01 mol/l trisodium citrate dehydrate buffer (pH 6.0), then treated in a microwave oven for 10 min at 500 W. Sections were then treated with 3% of H_2O_2 for 30 min to inhibit intrinsic peroxidase.

For CD44, in order to inhibit non-specific reaction, specimens were treated by normal goat serum for 10 min. A 100-fold diluted antibody to CD44 (#3570S, mouse monoclonal antibody; 156-3C11; Cell Signaling Technology®, Inc., Danvers, MA, USA) was used as a primary antibody and reacted overnight at 4˚C. The Simple Stain max PO (mouse, monoclonal; Cosmo Bio, Carlsbad, CA, USA) was used as a secondary antibody and reacted for 30 min. For GLI1, in order to inhibit non-specific reaction, specimens were treated with normal rabbit serum for 10 min. A 100-fold diluted antibody to GLI1 (ab92611; Abcam®, Cambridgeshire, UK) was used as a primary antibody and reacted overnight at 4˚C. The EnVision+ System horseradish peroxidase (HRP)-labeled polymer anti-rabbit (Code K4002, Dako, Carpinteria, CA) was used as a secondary antibody and reacted for 30 min.

For CD34 and proliferating cell nuclear antigen (PCNA) staining, tissue sections were digested with 0.2% trypsin in 0.01 mol/l phosphate-buffered saline (PBS) for 20 min at 37˚C. Sections were incubated overnight at 4˚C with a mouse monoclonal antibody against CD34 (1:25, QB-END/10; Novocastra Laboratories, Newcastle, UK), or for 1 h at room temperature with 1:100 diluted monoclonal mouse antibody against PCNA (PC-10; Dako), as the primary antibody. This was followed by reaction with biotinylated anti-immunoglobulin and a reagent using labeled streptavidin–biotin (LSAB) kit peroxidase (Dako). The peroxidase reaction was visualized with 0.01% H_2O_2 and 3,3'-diaminobenzidine under light microscopy (×200) (13).

For CD44 and GLI1 (Figure 1 a and b), staining of fewer than 10% of cells was defined as negative expression, that between 10-50% was defined as positive expression (1+), and that over 50% was defined as strongly-positive expression (2+), respectively. For microvessel count (MVC) using CD34 staining, an average count was determined for the five most vascular areas in the section examined under ×200 magnification. The average percentage of cells exhibiting positive PCNA nuclear expression among 1,000 nuclei in five areas of the tumor was estimated.

The arterial attenuation index of the tumor was indicated by examining Hounsfield unit (HU) by the high resolution computed tomography and its software.

Statistical analysis. Continuous data are expressed as mean±standard deviation. Data from different groups were compared using one-way analysis of variance and examined with the Student's *t*-test or Dunnett's multiple comparison test. Categorical data were analyzed using the Fishers' exact test. Survival rates were calculated according to the Kaplan–Meier method, and differences between groups were tested for significance using the log-rank test. Two-tailed values of $p<0.05$ were considered statistically significant. The Statistical Package for the Social Science (SPSS) version 18.0 software (SPSS, Chicago, IL, USA) was used for all statistical analyses.

Results

Patient prognosis. Among the 38 patients, disease-free 3-, and 5-year survival rates were 34%, and 28%, respectively, and the median disease-free survival was 63 months. Overall 5-year survival rates were 29% and the median overall survival was 71 months. A total of 27 patients (71%) displayed tumor recurrence after hepatectomy, which were liver metastasis in 12, local recurrence in four, peritoneal dissemination in six, lymph node metastasis in three, lung in one and bone in one.

Expression of cancer stem cells and relationship with clinicopathological features. Positive expression of CD44 was found in seven (18%) and that of GLI1 was found in 11 (39%). Both parameters were negative in 22 cases (58%), only CD44 positive in five (13%), only GLI1-positive in nine (24%) and both positive in two (5%), respectively. The median MVC by CD34 was 126±52/mm^2 and median PCNA labeling index of 149±78/field (×200) was applied as a cutoff value. The arterial attenuation index was 15.9±33.5 HU. The relationship between CD44 and GLI1 expression, and no significant correlation between these expressions was observed (Table I). GLI1 1+ and 2+ were grouped as positive expression in the subsequent analysis.

The relationship between expression of both factors and clinicopathological features was examined and the results are shown in Table II. For CD44 expression, prevalence of CD44 expression in the periductal infiltration type of ICC was significantly higher than that in other types. Expression of CD44 was not significantly associated with other parameters of ICC, nor with prevalence of tumor relapse. In GLI1 expression, positive expression of GLI1 was significantly

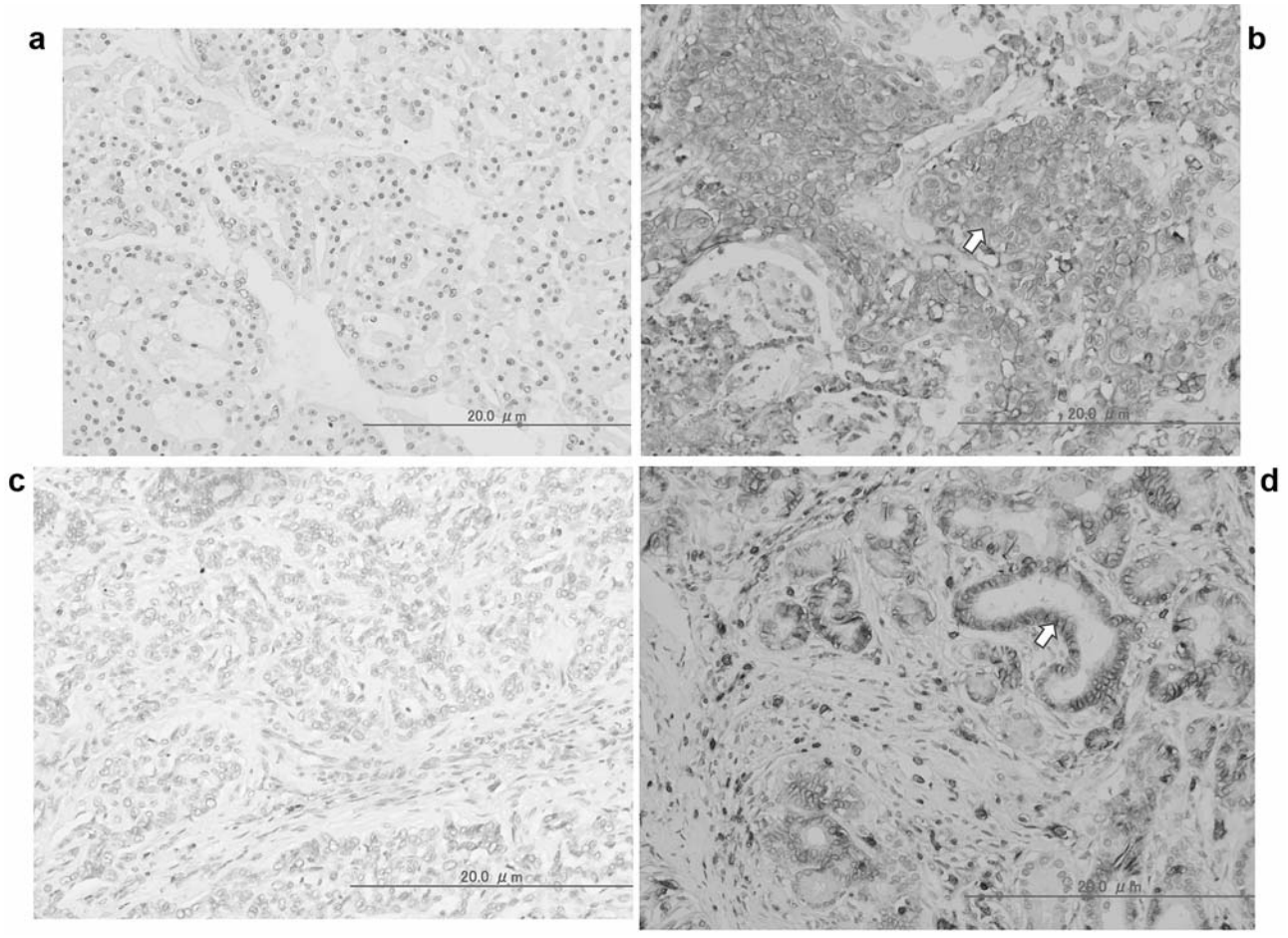

Figure 1. *Representative figures of cell adhesion molecule CD44 and Glioma-associated oncogene homolog-1 (GLI1) expression. Cases of negative (a) and positive (b) expressions of CD44, and negative (c) and positive (d) expressions of GLI1. Findings at ×200 magnification.*

associated with age in comparison with negative expression. Expression of GLI1 was not significantly associated with other parameters of ICC or prevalence of tumor relapse.

Expression of cancer stem cells and relationship with patient prognosis. Table III shows the relationship between disease-free and overall survival rate and clinicopathological factors including expression of CD44 and GLI1. For periductal infiltrating-type ICC, intrahepatic metastasis was significantly associated with lower 3-year disease-free survival rate after hepatectomy ($p<0.05$). In addition, higher carcinoembryonic antigen (CEA) levels, node metastasis and lower MVC tended to be associated with lower 3-year disease-free survival ($p<0.10$). Although the expression of either CD44 or GLI1 led to a significantly associated with disease-free survival, positive expression of both CD44 and GLI1 showed a significantly lower 3-year disease-free survival rate ($p<0.05$). With respect to 5-year overall survival after hepatectomy, higher CEA levels, periductal infiltrating-type, intrahepatic metastasis, and tumor size were significantly associated with lower 5-year survival rate. Expression of either CD44 or GLI1 or both CD44 and GLI1 was not significantly associated with 5-year overall survival rate.

Table I. *Correlation between cell adhesion molecule CD44 and Glioma-associated oncogene homolog-1 (GLI1) expression by immunohistochemical staining.*

	GLI1			
	Negative	Positive (1+)	Positive (2+)	*p*-Value
CD44				
Negative	22	7	2	
Positive	5	2	0	0.764

Table II. *Correlation between cell adhesion molecule CD44 and Glioma-associated oncogene homolog-1 (GLI1) expression and clinicopathological parameters or postoperative recurrence rate in intrahepatic cholangiocarcinomas.*

	CD44		*p*-Value	GLI1		*p*-Value
	Negative (n=31)	Positive (n=7)		Negative (n=27)	Positive (n=11)	
Gender						
Male (n=17)	15 (88)	2 (12)		10 (58)	7 (42)	
Female (N=21)	16 (76)	5 (24)	0.423	17 (81)	4 (19)	0.298
Age (years)	67± 9	74± 9	0.115	66± 8	77± 4	0.0004
Cholecysto- or hepatolithiasis, yes (n=7)	6 (19)	1 (14)	1.0	4 (57)	3 (43)	0.665
Chronic viral hepatitis, yes (n=18)	16 (89)	2 (11)	0.410	12 (44)	6 (55)	0.836
Serum tumor marker						
CEA (ng/ml)	68±237	6.6±5.1	0.923	34±138	109±338	1.000
CA19-9 (U/ml)	642±1295	203±253	0.826	677±1335	208±343	0.887
AFP (ng/ml)	25±98	15±24	0.441	7±10	73±182	0.685
Tumor size (cm)	6.9±6.5	3.7±2.5	0.101	4.8±2.7	9.9±9.9	0.197
Child classification						
A	30 (97)	7 (100)	0.865	26 (96)	11 (100)	0.772
B	1 (3)	0		1 (4)	0	
Macroscopic finding[a]						
Mass-forming type (n=31)	29 (94)	2 (6)		23 (74)	8 (26)	
Periductal infiltrating type (n=5)	0	5 (100)	*p*=0.0021	2 (40)	3 (60)	0.191
Intraductal growth type (n=2)	2 (100)	0		2 (100)	0	
Histological differentiation						
Well (n=8)	8 (100)	0		4 (100)	4	
Moderately (n=22)	16 (73)	6 (27)	0.208	17 (73)	5 (27)	0.333
Poorly (n=8)	7 (88)	1 (13)		6 (88)	2 (13)	
Intrahepatic metastasis, yes (n=10)	7 (70)	3 (30)	0.552	8 (80)	2 (20)	0.748
Lymph node metastasis, yes (n=15)	14 (93)	1 (7)	0.280	10 (67)	5 (33)	0.908
Serosal invasion, yes (n=15)	12 (80)	3 (20)	1.0	9 (60)	6 (40)	0.397
Tumor–node–metastasis stage[b]						
I (n=3)	3 (100)	0		2 (67)	1 (34)	
II (n=2)	2 (100)	0		2 (100)	0	
III (n=14)	12 (86)	2 (14)		11 (79)	3 (21)	
IV (n=19)	14 (74)	5 (26)	0.556	12 (63)	7 (37)	0.614
Knodell's histological fibrosis grade						
0, 1 (n=26)	20 (77)	6 (23)		19 (73)	7 (24)	
2 (n=7)	6 (86)	1 (14)		5 (71)	2 (29)	
3, 4 (n=5)	5 (100)	0	0.453	3 (60)	2 (40)	0.840
CD34 (/mm^2)	116±47	132±46	0.514	109±35	144±69	0.325
PCNA (cells/1000 cells)	142±84	125±69	0.900	143±83	105±42	0.600
Postoperative tumor recurrence, yes (n=27)	21 (78)	6 (22)	0.627	18 (67)	9 (33)	0.589

Values in parentheses are percentages. CEA: Carcinoembryonic antigen, *CA19-9*: carbohydrate antigen 19-9, *AFP*: alpha-feto protein, *PCNA:* proliferating cell nuclear antigen. [a]Macroscopic classification of intrahepatic cholangiocarcinoma (12). [b]Japanese TNM stage for classification of intrahepatic cholangiocarcinoma (12).

Discussion

Various molecular factors in ICC have been reported as candidates for prognostic markers (14-17). In both preliminary studies and the present study, we focused on microvessel counts or tumor enhancement patterns by computed tomography as a marker of tumor angiogenesis for ICC (14, 18). This parameter can be conventionally and easily examined using immunohistochemistry at any institute, and we propose the inclusion of this examination in conventional pathological diagnosis to predict for tumor malignancy. Tumor angiogenesis might be related to patient prognosis in ICC for those who undergo radical hepatectomy, although results have not been similar between investigators (17-19). However, few new markers associated with prognosis or malignant behavior of ICC have been determined (20-22). Therefore, in this study, we focused on CSCs markers, which have recently been shown as a promising parameter for revealing malignancy in digestive tract carcinomas (23).

Table III. *Relationship between clinicopathological factors and survival rates in intrahepatic cholangiocarcinoma.*

	Disease-free survival rates (3 years, %)	*p*-Value	Overall survival rates (5 years, %)	*p*-Value
Gender				
Male	45	0.273	25	0.711
Female	67		37	
Age, years				
≤60	50	0.379	38	0.709
>60	43		26	
Chronic viral hepatitis				
No	40	0.556	17	0.567
Yes	58		40	
CEA (ng/ml)				
≤10	56	0.071	37	0.0057
>10	0		0	
Macroscopic finding				
MF	65		40	
PDI	0	0.033	0	0.030
IG	100		100	
Lymph node metastasis				
No	67	0.070	46	0.148
Yes	0		0	
Intrahepatic metastasis				
No	62	0.0033	36	0.039
Yes	0		0	
Size of tumor (cm)				
≤5	53	0.840	44	0.033
>5	38		7	
Histological differentiation				
Well	75		50	
Moderate	51	0.171	30	0.245
Poor	0		0	
CD34 (/mm^2)				
≤140	25	0.080	14	0.085
>140	75		50	
Blood loss (ml)				
≤1000	68	0.455	45	0.318
>1000	25		13	
CD44				
Negative	38	0.415	33	0.335
Positive	0		25	
GLI1				
Negative	33	0.537	29	0.767
Positive	15		15	
CD44-GLI1				
N-N (n=22)	57		35	
P-N (n=5)	38	0.0435	0	0.227
N-P (n=8)	33		25	
P-P (n=3)	0		0	

CEA: Carcinoembryonic antigen, *CD34*: microvessel counts, *CD44*: cell adhesion molecule, *GLI1*: Glioma-associated oncogene homolog-1.

It remains unclear, however, whether various candidate markers indicate if CSCs or progenitor cells will develop into treatment-resistant cancer cells. CD44 has been used to examine CSCs and is expressed in various parts of digestive organs (9, 24). GLI1 is also a candidate CSC marker and is associated with the Hh signaling pathway (10, 11). The mechanism of this marker has not been well-clarified. We proposed a correlation between both markers before this study; however, we did not observe a significant correlation in the present study. Mechanism of effects to cancer growth by CD44 and Gli1 might be different between each other and, therefore, we separately examined each parameter in the next analysis.

Regarding the cause of carcinogenesis in ICC, existence of biliary stones, chronic viral hepatitis and patient occupation have been proposed to be related factors (1, 4, 17, 25). In the present study, expression of CSC markers was not associated with these factors and we were unable to speculate on the mechanism of specific carcinogenesis in ICC. The risk of ICC may increase in relation to aging (26) and GLI1 was significantly correlated with older age in our findings. Levi *et al*. and other investigators reported a relationship between increases in CD44 or CD166 expression with patient aging, which might be a cause of increase of carcinogenesis (27, 28). Similar to these reports, in this study, CD44 expression tended to be correlated with older age, but this association was not statistically significant. Carlson *et al*. and Mimeaulta *et al*. pointed out that aging associated with carcinogenesis might be influenced by DNA damage or imbalance of gene signaling transduction in cells such as the one shown by the Hh signaling pathway (29). *GLI1*, a glioma-associated oncogene, plays an important role in this signaling pathway (10, 30). Malignant transformation of glioma is frequent in elderly patients and accumulation of GLI1 is also observed (31).

Increase of positivity of CSCs was associated with malignant behavior and poor prognosis in solid tumors with the exception of glioma (32, 33). We hypothesized that CSC markers might be associated with poor prognostis in ICC, however, CD44 expression was only significantly associated with macroscopic findings of ICC (12). On the other hand, GLI1 expression was not associated with any tumor-related factors. Our previous study regarding CD44v6 showed a strong correlation with malignancy in metastatic colonic liver adenocarcinomas (34). In the present study, CD44 positivity was not observed in the early stage according to the Japanese TNM classification (12), but this result was not statistically significant. Significant CD44 positivity was observed in the periductal infiltrating-type by macroscopic findings (12), and this type exhibited a highly malignant potential in ICC (35-37). This type may infiltrate along intrahepatic ducts and invade adjacent lymphatic or venous ducts and perineural tissues, which can lead to node metastasis or distant metastasis. On the other hand, the intraductal growth-type has the best prognosis and CD44 positivity was not observed; some CD44 positivity was observed in the mass-forming-type. Intermediate malignant activity is observed in the mass-forming-type, and some patients have relatively better survival (38). Therefore, the mechanism of malignant potential in these three types might be different. Among these, CD44 positivity was most frequent in the ICC types with the most malignant potential. CD44 expression reflects cell-cell adhesion through the tissue matrix (39) and, therefore, this might indicate aggressive infiltration to biliary tracts. Microvessel density, as indicated by CD34 and PCNA, is a remarkable prognostic factor in malignant tumors, including ICC (17, 18, 40). However, CD44 positivity was not correlated with these parameters. GLI1 positivity was not associated with macroscopic findings, tumor stage, microvessel density, or proliferative activity and, therefore, the association between CSCs and these parameters is not supported in this study.

The goal of this study was to clarify the relationship between promising biological parameters and survival in patients with ICC. Predictive factors for patient prognosis have been proposed by many investigators, but no consensus has been so far reached (15-17, 20). The present study showed that periductal infiltrating-type and intrahepatic metastasis were associated with lower disease-free survival rates, as previous reports (35-37). Expression of either CD44 or GLI1 was not significantly associated with a disease-free survival rate. However, the combined expression of both markers was associated with significantly poor disease-free survival. Shimada *et al*. also reported that expression of CCC markers was associated with tumor recurrence in patients with ICC (21). In the analysis of overall survival, positivity for CSC marker expression was not significantly associated with overall survival rates. However, as there were no survivors with positive expression of both CD44 and GLI1 at five years, other stronger prognostic factors might influence survival. In the present preliminary study, the number of participants was limited and therefore multivariate analysis was not performed. To resolve this problem, a further study in a larger population of patients at multiple institutes is necessary. As ICC is not a common disease, a nation-wide comprehensive study to investigate various candidate factors is necessary.

ICC is a malignant tumor originating from the intrahepatic bile duct, and comprises cells that mostly resemble those of the peripheral bile ducts (41). However, ICC is not always uniform, and is classified into subgroups according to macroscopic findings, with the clinical and pathological characteristics and prognosis differing significantly among subgroups. Diagnosis of ICC subtypes would be useful in predicting prognosis or selecting adjuvant treatments with hepatic resection. When the relationship between malignant behaviors of ICC and markers of CSCs is identified, CSC-targeting drugs may be clinically applicable in the future, as well as in other malignancies.(42)

In conclusion, the present study examined the relationship between CD44 and GLI1 expression as markers of CSCs with clinicopathological features and survival in 38 patients with ICC. CD44 and GLI1 were not closely-related. As a tumor biological factor, CD44 was associated with the periductal infiltrating-type of ICC, the most malignant phenotype. GLI1 was associated with increased patient age. Positive expression of both CD44 and GLI1 might represent tumor malignant potential and therefore may be useful as prognostic factors of tumor-free survival in ICC, but not as factors of overall

survival. Further study in a larger population of patients with ICC undergoing surgical resection is warranted to clarify the role of CSCs in this disease.

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