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RESEARCH

Clinicopathologic Significance of Sox2, CD44 and CD44v6 Expression in Intrahepatic Cholangiocarcinoma

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Abstract Embryonic stem cells (ESC) and cancer stem cells (CSC) have a capacity for self-renewal and differentiation into multiple cell lineages. Sox2 plays a critical role in ESC and has been shown to participate in carcinogenesis and tumor progression in many human cancers. CD44 and CD44v6 are putative CSC markers and their association with tumor progression, metastasis, and tumor relapse after treatment has been demonstrated. We evaluated the immunoexpression of Sox2, CD44, and CD44v6 in 85 cases of Intrahepatic cholangiocarcinomas (IHCC) and assessed their prognostic significance. Sox2 expression showed a significant association with lymph node metastasis (p=0.025), T4 stage (p=0.046), and worse overall survival (p=0.047). Greater expression of Sox2 was observed in IHCC with poor differentiation, vascular invasion, and stage IV, without statistical significance (p>0.05). CD44 expression showed an association with periductal infiltrative type (p=0.034), poor differentiation (p=0.012), and vascular invasion (p=0.009). CD44v6 expression was evident in patients with stage IV (p=0.019). These results demonstrated that Sox2 expression is associated with aggressive behavior and poor overall survival in IHCC.

Keywords Intrahepatic cholangiocarcinoma · Sox2 · CD44 · CD44v6 · Survival

Abbreviations

ESC	Embryonic stem cell
CSC	Cancer stem cell
IHCC	Intrahepatic cholangiocarcinoma
DFS	Disease-free survival
OS	Overall survival

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Introduction

Intrahepatic cholangiocarcinoma (IHCC) arises from the intraductal biliary epithelium and peribiliary glands and accounts for approximately 10–15 % of primary liver cancers, and its incidence has increased in recent years [1]. However, despite advances in surgical and therapeutic strategies, the majority of IHCC patients showed a poor outcome. Recently, research for correlation between embryogenesis and oncogenesis has being widely conducted. Embryonic stem cells (ESC) and cancer stem cells (CSC) are defined according to their ability for self-renewal and differentiation into multiple cell lineages [2]. Thus, ESC and CSC have been known to have similarities, such as proliferative potential, differentiation potential, and common signaling pathways [3]. Transcription factors are critical molecular switches regulating ESC fate, which may also function in renewal of cancer cells [4, 5]. Sox2, a transcription factor, plays an important role in regulation of pluripotency and self-renewal in ESC [3]. Recent studies have reported participation of Sox2 in carcinogenesis and tumor progression in several human cancers [3, 6–16]. However, the role of Sox2 in IHCC has not yet been studied. CD44, a cell-surface glycoprotein, is involved in many cellular processes, including cell–cell adhesion, growth, survival, differentiation, and mortality [17, 18]. Up-regulation of CD44 has been reported in many gastrointestinal tumors [19]. A CD44 isoform containing variant exon 6 (CD44v6) is a cell surface protein which has been reported to show correlation with tumor invasion, progression, and metastasis in many human carcinomas [20, 21].

In this study, our aim was to investigate expression of Sox2, CD44, and CD44v6 and evaluate the correlation of

their expression with prognostic factors and clinicopathological parameters.

Materials and Methods

Patients and Clinicopathologic Factors

Data were obtained on 85 consecutive patients who underwent resection at Yeungnam University Hospital from July 1988 to June 2012. Clinicopathologic parameters, including age, gender, tumor size, differentiation, presence of vascular invasion, presence of lymph node (LN) metastasis, pathologic tumor stage, stage, disease-free survival (DFS), and overall survival (OS) were evaluated by review of medical records and pathologic reports. Overall patients' survival was defined as the time from surgical resection to death of patients or patients' last follow-up. Follow-up lasted through June 2012 (range 0–180 months, mean 24.98 months). The study was approved by the Human Ethics Review Board of our hospital (YUH13-0424-O54).

Tissue Microarray Construction

Five tissue microarray (TMA) blocks were made with 83 cases; three 2 mm cores were obtained from the most representative tumor area of each case and arrayed in a new recipient block. For the other two cases, full-face sections from ordinary tissue blocks were used. Normal liver, gastric cancer and breast cancer tissues were used as controls.

Immunohistochemistry

After deparaffinization and rehydration, 4 μm sections were immunostained for Sox2 (1:60, clone SP76, rabbit monoclonal antibody, Cell Marque, USA), CD44 (1:100, clone MRQ-13, mouse monoclonal antibody, Cell marque, USA), and CD44v6 (1:500, clone VFF-18, mouse monoclonal, Abcam®, UK). Staining was performed on the Ventana BenchMark® platform automated slide stainer (Ventana Medical Systems, Tucson, AZ, USA) using the onboard heat-induced epitope retrieval method in high pH CC1 buffer (99 °C, 1 h). The antibodies were incubated at 37 °C for 60 min (Sox2), 32 min (CD44), and 20 min (CD44v6). The

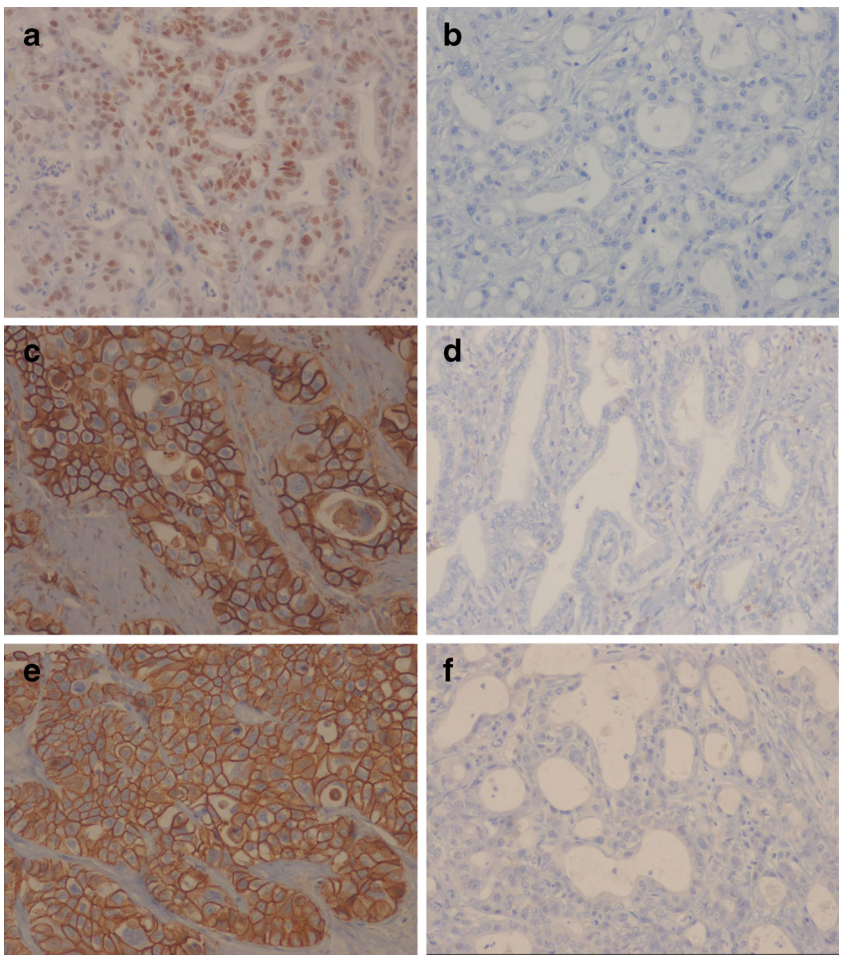

Fig. 1 Immunohistochemical staining results. Sox2-**a**, **b**; **a** diffuse nuclear expression, **b** no expression. CD44-**c**, **d**; **c** diffuse membranous expression, **d** no expression. CD44v6-**e**, **f**; **e** diffuse membranous expression, **f** no expression

staining was visualized using the UltraView™ DAB detection Kit (Automated BenchMark®, Ventana), which included a hydrogen peroxide substrate and 3,3′-diaminobenzidine chromogen solution. According to the definition of positive staining in previous reports, when more than 10 % of tumor cells showed staining in the nucleus (Sox2) and in the membrane (CD44 and CD44v6), we considered expression [10, 18]. If staining was heterogeneous, scoring was based on the predominant staining intensity.

Statistical Analysis

SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used in performance of statistical comparisons. Pearson's Chi-square test and Fisher's exact test were performed in order to examine associations between clinicopathological parameters and expression. DFS and OS were calculated using the Kaplan–Meier method. The Cox proportional Hazard Model was used for evaluation of the association between clinicopathological parameters and survival. We obtained the hazard ratio (HR) and associated 95 % confidence interval (CI) for each factor. A p-value <0.05 was considered statistically significant.

Results

Clinicopathologic Characteristics

Fifty three male and 32 female patients with a median age of 60.5 years (range 39–76 years) were included in this study. Regarding histologic subtypes, there were 66 well/moderately differentiated adenocarcinomas (77.6 %) and 19 poorly

Table 1 Comparison between Sox2, CD44, CD44v6 expression and clinicopathological parameters

Factors	Sox2 expression		p	CD44 expression		p	CD44v6 expression		P
	Negative	Positive		Negative	Positive		Negative	Positive	
Size			0.614			0.103			0.724
<5 cm	33 (84.6 %)	6 (15.4 %)		33 (84.6 %)	6 (15.4 %)		31 (86.1 %)	5 (13.9 %)	
≥5 cm	37 (80.4 %)	9 (19.6 %)		32 (69.6 %)	14 (30.4 %)		41 (91.1 %)	4 (8.9 %)	
Gross type			1.000			0.034			0.118
Mass forming	58 (81.7 %)	13 (18.3 %)		54 (76.1 %)	17 (23.9 %)		62 (89.9 %)	7 (10.1 %)	
Periductal infiltrate	4 (80.0 %)	1 (20.0 %)		2 (40.0 %)	3 (60.0 %)		3 (60.0 %)	2 (40.0 %)	
Intraductal	8 (88.9 %)	1 (11.1 %)		9 (100 %)	0 (0 %)		7 (100 %)	0 (0 %)	
Differentiation			0.076			0.012			1.000
Well/moderate	57 (86.4 %)	9 (13.6 %)		55 (83.3 %)	11 (16.7 %)		55 (88.7 %)	7 (11.3 %)	
Poor	13 (68.4 %)	6 (31.6 %)		10 (52.6 %)	9 (47.4 %)		17 (89.5 %)	2 (10.5 %)	
Vascular invasion			0.053			0.009			1.000
Present	43 (76.8 %)	13 (23.2 %)		38 (67.9 %)	18 (32.1 %)		24 (88.9 %)	3 (11.1 %)	
Absent	27 (93.1 %)	2 (6.9 %)		27 (93.1 %)	2 (6.9 %)		48 (88.9 %)	6 (11.1 %)	
Perineural invasion			0.482			0.489			0.485
Present	35 (79.5 %)	9 (20.5 %)		35 (79.5 %)	9 (20.5 %)		36 (85.7 %)	6 (14.3 %)	
Absent	35 (85.4 %)	6 (14.6 %)		30 (73.2 %)	11 (26.8 %)		36 (92.3 %)	3 (7.7 %)	
Tumor stage[a]			0.046			0.078			0.557
pT1	18 (90.0 %)	2 (10.0 %)		19 (95.0 %)	1 (5.0 %)		18 (94.7 %)	1 (5.3 %)	
pT2	42 (80.8 %)	10 (19.2 %)		37 (71.2 %)	15 (28.8 %)		44 (88.0 %)	6 (12.0 %)	
pT3	10 (90.9 %)	1 (9.1 %)		7 (63.6 %)	4 (36.4 %)		8 (80.0 %)	2 (20.0 %)	
pT4	0 (0 %)	2 (100 %)		2 (100 %)	0 (0 %)		2 (100 %)	0 (0 %)	
Lymph node metastasis			0.025			0.802			0.728
Present	29 (72.5 %)	11 (27.5 %)		30 (75.0 %)	10 (25.0 %)		33 (86.8 %)	5 (13.2 %)	
Absent	41 (91.1 %)	4 (8.9 %)		35 (77.8 %)	10 (22.2 %)		39 (90.7 %)	4 (9.3 %)	
Stage[b]			0.058			0.070			0.019
I	18 (94.7 %)	1 (5.3 %)		18 (94.7 %)	1 (5.3 %)		18 (100 %)	0 (0 %)	
II	19 (86.4 %)	3 (13.6 %)		14 (63.6 %)	8 (36.4 %)		18 (85.7 %)	3 (14.3 %)	
III	27 (81.8 %)	6 (18.2 %)		26 (78.8 %)	7 (21.2 %)		29 (93.5 %)	2 (6.5 %)	
IV	6 (54.5 %)	5 (45.5 %)		7 (63.6 %)	4 (36.4 %)		7 (63.6 %)	4 (36.4 %)	

[a,b] Pathologic tumor stages and stage according to the AJCC Staging System, 7th edition

differentiated adenocarcinomas (22.4 %). Fifty six (65.9 %) patients had tumors with vascular invasion. Neural invasion was observed in 44 patients (51.8 %). Resection margin involvement by cancer was observed in 33 patients (38.8 %). Forty (47.1 %) patients showed metastasis in regional lymph nodes. Regarding pathologic tumor (T) stage, T1 was 20 (23.5 %) patients, T2 was 52 (61.2 %) patients, T3 was 11 (12.9 %) patients, and T4 was 2 (2.4 %) patients. Regarding stage, 19 (22.4 %) patients were stage I, 22 patients (25.9 %) were stage II, 33 patients (38.8 %) were stage III, and 11 patients (12.9 %) were stage IV.

Correlation Between Sox2, CD44, and CD44v6 Expression and Clinicopathologic Factors

A summary of the relationships between clinicopathological parameters and Sox2, CD44, and CD44v6 expression is shown in Fig. 1 and Table 1. Sox2 expression was observed in 15 cases (17.6 %) and was evident in patients with poor OS (p=0.047), not DFS (p=0.113) (Fig. 2). There was significant expression of Sox2 with LN metastasis (p=0.025) and advancing T stage (p=0.046). Greater expression of Sox2 was observed in poorly differentiated IHCC, compared with well and moderately differentiated, but without statistical significance (p=0.076). Sox2 expression was observed more often in IHCC with vascular invasion (p=0.053) and advancing stage (p=0.058), without statistical significance.

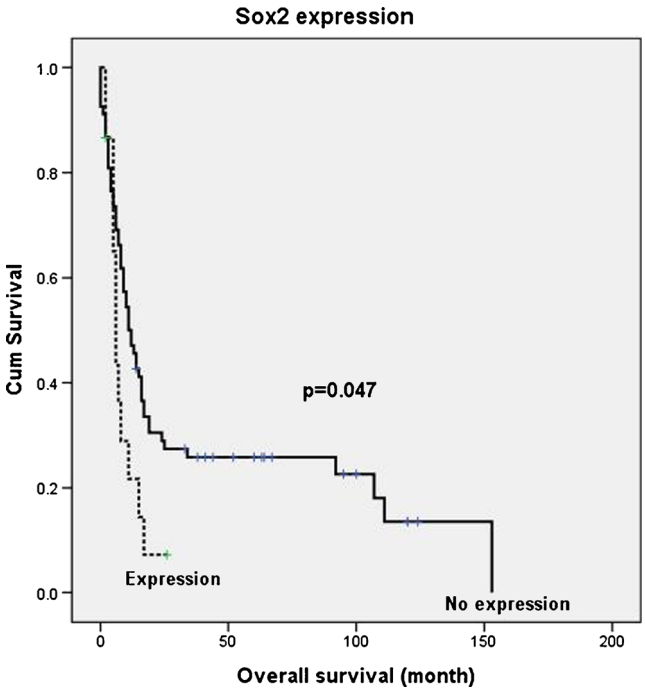

Fig. 2 Cumulative overall survival curves regarding Sox2 expression. Intrahepatic cholangiocarcinoma with Sox2 expression showed a worse overall survival compare to no expression

CD44 expression was absent in the normal bile duct epithelium and hepatocytes and was observed in 20 cases (23.5 %) of IHCC. CD44 expression was evident in IHCC with periductal infiltrative type (p=0.034), poor differentiation (p=0.012), and vascular invasion (p=0.009).

Among the 85 cases, in four cases there was no sufficient tissue for interpretation of CD44v6 expression, then these were excluded. CD44v6 expression was observed in nine cases (11.1 %) and was evident in stage IV IHCC (p= 0.019). CD44 and CD44v6 expression did not show association with OS and DFS. No correlation was observed between Sox2, CD44, and CD44v6. In multivariate analysis, lymph node metastasis was an independent prognostic factor for OS (p=0.003, 95 % CI 2.472, 1.370–4.459), but not DFS (p= 0.092, 95 % CI 1.748, 0.912–3.350).

Discussion

Embryonic stem cells and CSCs have the properties of self-renewal and differentiation and are resistant to chemotherapy and radiotherapy [6]. Sox2 is an important transcription factor for maintenance of embryonic stem cell pluripotency and self-renewal and plays a key role during organogenesis and in embryonic development [2]. Sox2 is expressed in various phases of embryonic development and its expression has been studied in many human cancers, including breast, lung, colon, and nasopharyngeal carcinoma, hepatocellular carcinoma, and glioma, and was generally reported to show an association with aggressive behavior or poor prognosis [3, 6–8, 11, 12]. Therefore, we investigated the clinicopathological significance of Sox2 expression in IHCC. To the best of our knowledge, this study is the first report on Sox2 expression with its clinicopathological significance in IHCC.

Greater expression of Sox2 was observed in basal-phenotype breast cancer and showed an association with metastatic potential [9, 10]. In colon cancer, Sox2 was highly expressed in tumors with lymph node metastasis and distant spread [8, 11]. In hepatocellular carcinoma, Sox2 expression showed correlation with metastasis and poor survival [12, 22]. In bladder cancer, greater expression of Sox2 was observed in tumors with large size, high nuclear grade, and high Ki-67 labeling index [2]. However, the prognostic value of Sox2 expression was controversial, depending on the type of cancers and researchers. In non-small cell lung cancers, some researchers demonstrated an association of Sox2 expression with better outcome [7, 14]. In lung squamous cell carcinoma, Sox2 expression showed an association with carcinogenesis and a lack of differentiation, however, its expression showed an association with better overall survival [7]. In gastric cancer, Sox2 overexpression inhibited tumor cell growth through cell-cycle arrest and apoptosis [15]. In contrast, Matsuoka et al. [16] reported significant correlation of Sox2 positive

tumors with worse survival than Sox2 negative tumors. In this study, Sox2 expression showed correlation with worse overall survival and was evident in aggressive clinicopathological parameters, such as LN metastasis and pT4 stage. Although there was no statistical significance, greater expression of Sox2 was observed in IHCC with poor differentiation, vascular invasion, and advanced stage. Results of our study suggest that Sox2 expression is associated with aggressive behavior and poor survival in IHCC.

CD44 expression rate was reported as 18 % to 49 % in IHCC [17, 23]. Nanashima et al. [17] reported that CD44 expression was observed in 18 % (7/38) and showed significant association with periductal infiltrative growth; this finding is consistent with our result. Pongcharoen et al. [23] reported that greater CD44 expression was observed in an invasive front and showed correlation with poor differentiation and mass-forming type. This difference was the result of classification of tumor growth type. Pongcharoen et al. classified just two types, mass-forming and intraductal type without periductal infiltrative type. Although no statistical significance was observed between CD44 expression and stage, CD44 expression was increased in advancing stage.

Correlation between CD44v6 and clinicopathological parameters has been shown to be diverse according to expression or loss of expression in various human cancers. In colorectal and gastric cancers, lack of CD44v6 expression showed an association with poor prognostic factors, such as early recurrence, lymph node metastasis, or worse survival [20, 24–26]. However, in adenocarcinoma of lung, CD44v6 expression showed correlation with lymph node metastasis and tumor size [27]. In cholangiocarcinoma, including intrahepatic and extrahepatic CC, CD44v6 has been shown not to be related to tumor progression [28]. In this study, CD44v6 expression only showed correlation with advancing stage. There was no correlation with other clinicopathologic parameters.

In conclusion, Sox2 expression showed correlation with aggressive clinicopathologic behavior, such as lymph node metastasis, pT4, and poor overall survival; however, it was not an independent prognostic factor. CD44 expression showed an association with periductal infiltrative type, poor differentiation, and vascular invasion, but not with survival. CD44v6 expression only showed correlation with advancing stage. Lymph node metastasis was an independent prognostic factor in multivariate analysis. These results demonstrated that Sox2 expression is associated with aggressive behavior and poor overall survival in IHCC.

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Conflict of Interest The authors declare that they have no conflict of interest.

Author Contribution Study design, analysis, and interpretation: Mi Jin Gu.
Acquisition of data: Byung Ik Jang.
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