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RESEARCH

The Expression of S100A4 Protein in Human Intrahepatic Cholangiocarcinoma: Clinicopathologic Significance and Prognostic Value

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Abstract Intrahepatic cholangiocarcinoma(ICC) is a highly malignant adenocarcinoma arising from bile duct epithelial cells of the intrahepatic biliary system with early hematogenous and lymphatic extrahepatic spread. The current treatment methods for ICC are far from ideal. Identifying novel effective prognostic biomarkers which might be related to the development and progression of ICC may help provide new therapeutic strategies. Both calcium-binding protein S100A4 and Matrix metalloproteinase-9(MMP-9) are correlated with development and progression of many carcinomas. In the present study, we investigated expression of S100A4 as well as MMP-9 in ICC tissues from 65 patients using immunohistochemistry. The correlation of S100A4 and MMP-9 expression with clinicopathological features and prognosis of patients were analyzed. S100A4 and MMP-9 were positively expressed in 32(49.2 %) and 35(53.8 %) patients, respectively. The positive correlation between S100A4 and MMP-9 expression was statistically significant (P=0.018). S100A4 positive expression was significantly correlated with vascular invasion (P=0.008), lymph node metastasis (P=0.029) and the TNM stage (P=0.008). MMP-9 expression was not found to be correlated with any clinicopathological parameter. Patients with S100A4 positive expression had a significantly poorer overall survival rate than those with S100A4 negative expression (P=0.000). MMP-9 positive expression was also correlated with poor survival (P=0.044). However, only S100A4 expression (P=0.004) and the surgical margin (P=0.024) were significantly independent prognostic predictors by multivariate analysis. In conclusion, expression of S100A4 is correlated with MMP-9 expression and it could be a useful marker for predicting the progression, metastasis and prognosis of ICC.

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Introduction

Intrahepatic cholangiocarcinoma (ICC) is a highly malignant carcinoma arising from bile duct epithelial cells of the intrahepatic biliary system with early hematogenous and lymphatic extrahepatic spread. The incidence of ICC has increased worldwide in the past 3 decades, and it remains the second most common primary liver cancer [1–3]. Surgical resection of ICC represents the only potential curative therapeutic option. However, many patients are not candidates for surgical resection at clinical presentation, because it is difficult to diagnose ICC at an early stage, and prognosis is poor even after curative resection [4–6]. Moreover, advanced intrahepatic cholangiocarcinoma is for the most part unresponsive to systemic chemotherapy and radiotherapy regimens [7]. Therefore, novel molecular markers are required for early diagnosis of ICC and to predict tumor progression. Furthermore, the prognostic biomarkers might also serve as therapeutic targets.

S100A4 is a member of a family of S100 genes that encodes calcium-binding proteins of the EF-hand type [8]. S100A4 is involved in the regulation of a wide range of intracellular and extracellular biological effects, including cell proliferation, extracellular matrix remodeling, cell motility, cell detachment, and angiogenesis [9–13]. As a metastasis-related gene, it was reported that S100A4 is significantly correlated with aggressiveness and worse prognosis for

patients with different types of carcinoma, including colorectal, esophageal, pancreatic, bladder, gastric, and breast cancer [14–19]. A pilot study of hepatocellular carcinoma suggested that S100A4 is correlated with tumor differentiation, invasion, recurrence and overall survival and could be a useful marker of tumor aggressiveness and prognosis [20]. Recently, expression of S100A4 was detected in cholangiocarcinoma including the ICC, and nuclear expression of S100A4 is a strong predictor of metastasization and reduced survival after resection. Furthermore, nuclear S100A4 increases cholangiocarcinoma cell line invasiveness and metastasization [21].

Matrix metalloproteinase (MMP), a multigene family of zinc-dependent proteinases, have been shown to dissolve various components of the extracellular matrix (ECM) and are the principal mediators of alterations observed in the microenvironment during cancer progression [22, 23]. In the family, MMP-9 plays a key role in the catalytic activity of tumor cell invasion and metastasis and has been considered as a potential diagnostic and prognostic biomarker in the ICC [24].

Several experimental strategies have established an intimate connection between S100A4 and certain members of the MMP family [25, 26]. S100A4 stimulates expression and activity of several MMPs, and MMP activation is most likely critical for S100A4-induced metastasis. In human prostate cancer and osteosarcoma cells, the invasive capability is stimulated by S100A4, at least partly through transcriptional activation of MMP-9 [27–29]. In vitro experiments, down-regulation of S100A4 expression by small interfering RNA suppressed the proliferation, metastasis and angiogenesis of thyroid cancer cells through the inhibition of MMP-9 and VEGF expression [12].

In this study, we investigated immunohistochemical expression of S100A4 and MMP-9 and their correlation with the clinicopathological feature of ICC. The association between S100A4 and MMP-9 expression and postoperative survival of ICC patients was also evaluated to investigate the prognostic significance of S100A4 and MMP-9 expression levels.

Methods

Formalin-fixed and paraffin-embedded tissues obtained from 65 primary intrahepatic cholangiocarcinoma patients who underwent surgical resection from January 2007 to December 2012, were retrieved from the Department of Pathology, Provincial Hospital Affiliated to Shandong University. All the patients were staged according to the international TNM system defined by the World Health Organization Histological Classification of Tumors [30]. No patient received any preoperative therapy, such as chemotherapy or radiotherapy. Clinical and pathological variables of ICC patients, including age, sex, tumor size, macroscopic types, histological differentiation, surgical margin, vascular invasion, perineural invasion, intrahepatic metastasis, and lymph node metastasis, were retrospectively collected and are shown in Table 1. All the patients were followed-up. The mean follow-up period was 28.4±15.1 months (range, 5 to 66 months). This study was previously approved by the local ethics committee and informed written consent was obtained from each patient.

Immunohistochemical Staining

Immunohistochemistry using the streptavidin-biotin-peroxidase method was performed to observe expression of S100A4 and MMP-9 in tissue sections. Four micrometer-thick sections obtained from formalin-fixed, paraffin-embedded tissue blocks were baked at 60 °C for at least 2 h, and then deparaffinized with xylene and rehydrated through a graded alcohol series. The slides were placed in a glass box filled with 10 mmol/l citrate buffer (pH 6.0) and boiled for 15 min at 100 °C for antigen retrieval. Then the sections were allowed to cool in the box to room temperature. Endogenous peroxidase was blocked by using 3 % hydrogen peroxide for 10 min. 1 % goat serum was applied to sections to block nonspecific binding. Sections were then incubated overnight with the primary antibody (rabbit anti-S100A4 polyclonal antibody, 1:400 dilution, Abcam, U.K. and mouse anti-MMP-9 polyclonal antibody, 1:100 dilution, Santa Cruz, USA). Sections were thoroughly washed and then incubated at 37 °C with biotinylated goat anti-rabbit IgG or goat anti-mouse IgG for 30 min and streptavidin conjugated to horseradish peroxidase for 30 min. After being washed, the sections were exposed for 5–10 min to 3,3'-diaminobenzidine. Then, the slides were counterstained with hematoxylin, dehydrated, cleared, and mounted. For the negative control, the primary antibody was replaced by normal mouse serum.

The degree of immunostaining of the sections was evaluated by two independent observers in a blinded manner. For each section, five high-power fields (400 ×) were randomly selected. The percentage of immunostained tumor cells and staining intensity were assessed semi-quantitatively. For the evaluation of S100A4 expression, staining in the cytoplasm and/or the nuclei was considered positively immunostained. The tissue sections were scored based on the percentage of immunostained cells as: <5 %=0, 5-25 %=1, 26-50 %=2, or >51 %=3. Sections were also scored on basis of staining intensity as negative=0, weak =1, moderate =2, or strong =3. A final score was obtained by multiplying the intensity and percentage scores. Expression of S100A4 was categorized as either positive (≥2) or negative (<2) using a cut-off score of 2. Expression of MMP-9 was considered positively stained when moderate or strong cytoplasmic stained cells were greater than 10 % tumor cells according to previous reports [24].

Table 1 Relationship between S100A4, MMP-9 and clinicopathological features in human intrahepatic cholangiocarcinoma

	Cases	S100A4		*P*	MMP-9		*P*
	n	Positive	Negative		Positive	Negative	
Gender							
Male	29	14	15		16	13	
Female	36	18	18	0.690	19	17	0.847
Age (years)							
≤60	30	12	18		14	16	
>60	35	20	15	0.168	21	14	0.282
Tumor size (cm)							
≤4	27	14	13		12	15	
>4	38	18	20	0.722	23	15	0.200
Macroscopic types							
Mass-forming type	44	19	25		22	22	
Non-mass-forming type	21	13	8	0.158	13	8	0.368
Histological differentiation							
Well/Moderate	42	18	24		20	22	
Poorly/Undifferentiated	23	14	9	0.165	15	8	0.174
Surgical margin							
Positive	20	9	11		13	7	
Negative	45	23	22	0.649	22	23	0.229
Vascular invasion							
Positive	24	17	7		14	10	
Negative	41	15	26	0.008	21	20	0.579
Lymphatic invasion							
Positive	21	12	9		11	10	
Negative	44	20	24	0.378	24	20	0.870
Intrahepatic metastasis							
Positive	23	13	10		14	9	
Negative	42	19	23	0.384	21	21	0.401
Lymph node metastasis							
Positive	22	15	7		15	7	
Negative	43	17	26	0.029	20	23	0.097
T stage							
T1+T2	38	15	23		18	20	
T3+T4	27	17	10	0.062	17	10	0.214
TNM stage							
I+II	39	14	25		18	21	
III+IV	26	18	8	0.008	17	9	0.128

Statistical Analysis

The Chi-square test and Fisher exact test were used to examine the relationship between expression of S100A4, MMP-9, and clinicopathological features. The Log-rank test according to Kaplan–Meier survival analysis approach was used to compare the overall survival rate of patients. Univariate and multivariate analyses were done to assess the independent prognostic factors by using Cox proportional hazards regression model. Statistical analysis was carried out using the SPSS 17.0 Software. $P<0.05$ was considered statistically significant.

Results

Expression of S100A4 and MMP-9 in Primary Intrahepatic Cholangiocarcinoma

Representative positive staining for each protein is shown in Fig. 1. S100A4 expression was observed both in the cytoplasm and nuclei. A total of 32 (49.2 %) ICC patients had S100A4 positive expression. Furthermore, S100A4 expression was also found in tumor stromal cells including smooth muscle cells, endothelial cells of both arteries and veins,

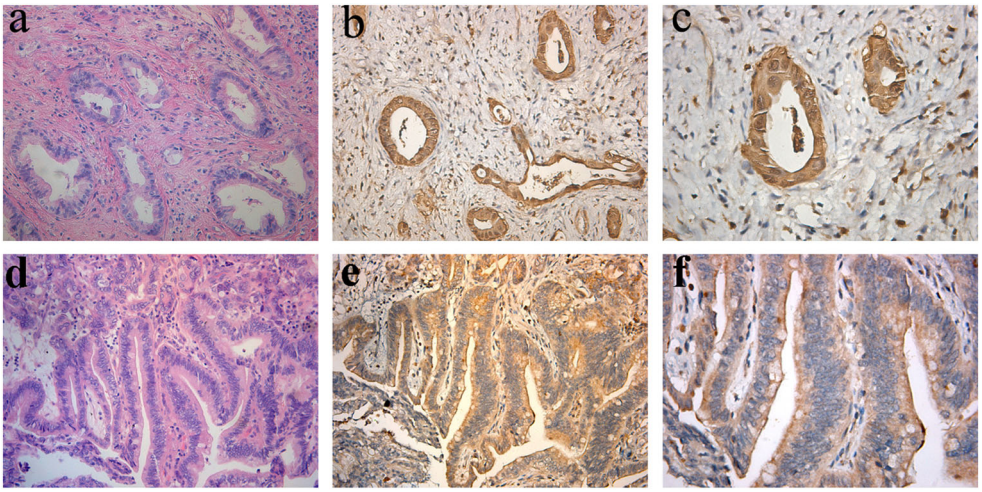

Fig. 1 Expression of S100A4 and MMP-9 in intrahepatic cholangiocarcinoma. H&E staining section shows histopathology of ICC (a, d). S100A4 expression was observed both in the cytoplasm and nuclei (b, c). MMP-9 expression was observed in the cytoplasm (e, f) (magnification: a, b, d, e×200; c, *f*×400)

fibroblast-like cells and lymphocytes. Positive staining of MMP-9 was seen in the cytoplasm of tumor cells in 35 (53.8 %) of the ICC patients.

In the 32 patients with S100A4 positive expression, 22 had MMP-9 positive expression. In the 33 patients with S100A4 negative expression, 20 had MMP-9 negative expression. The positive correlation between expression of S100A4 and MMP-9 was statistically significant (P=0.018, Table 2).

S100A4 and MMP-9 expression and clinicopathological characteristics

The correlation between S100A4 and MMP-9 expression and clinicopathological parameters was statistically analyzed (Table 1). Chi-square analysis showed that positive expression of S100A4 correlated with vascular invasion (P=0.008), lymph node metastasis (P=0.029) and TNM stage (P=0.008). Other factors including gender, age, tumor size, histological differentiation, surgical margin, macroscopic types, intrahepatic metastasis and T stage showed no significant correlation with expression of S100A4. MMP-9 expression was not significantly related to the clinicopathological parameters.

Table 2 Correlation between S100A4 expression and MMP-9 expression in human intrahepatic cholangiocarcinoma

	S100A4 expression		
	Positive	Negative	*P*
MMP-9 expression			
Positive	22	13	
Negative	10	20	0.018

Correlation Between S100A4 and MMP-9 Expression and Survival of Patients

During the follow-up period, a total of 39 (60.0 %) patients died. Patients with S100A4 positive expression had a significantly lower survival rate compared with patients with S100A4 negative expression. The survival rate at 3 years was 26.6 % in patients with S100A4 positive expression, and 65.0 % in patients with S100A4 negative expression (P=0.000, Fig. 2a). In terms of MMP-9 expression, the survival rate was lower for patients with MMP-9 positive expression than for those with MMP-9 negative expression. The 3-year survival rate of patients with MMP-9 positive expression was 37.7 %, compared to 53.5 % in patients with MMP-9 negative expression (P=0.044, Fig. 2b).

Univariate and Multivariate Analysis for Prognosis of Patients with ICC

Univariate and multivariate analyses were performed to assess the independent prognostic factors by using the Cox proportional hazards regression model. Univariate analysis showed that the significant prognostic factors included S100A4 expression (P=0.000), MMP-9 expression (P=0.050), vascular invasion (P=0.006), lymph node metastasis (P=0.007), surgical margin (P=0.007) and TNM stage (P=0.049) (Table 3). In the multivariate analysis, the six significant factors (S100A4 expression, MMP-9 expression, vascular invasion, lymph node metastasis, surgical margin and TNM stage) identified in the univariate analysis were analyzed. The multivariate analysis showed that S100A4 expression (P=0.004) and surgical margin (P=0.024) were independent prognostic factors of overall survival (Table 3).

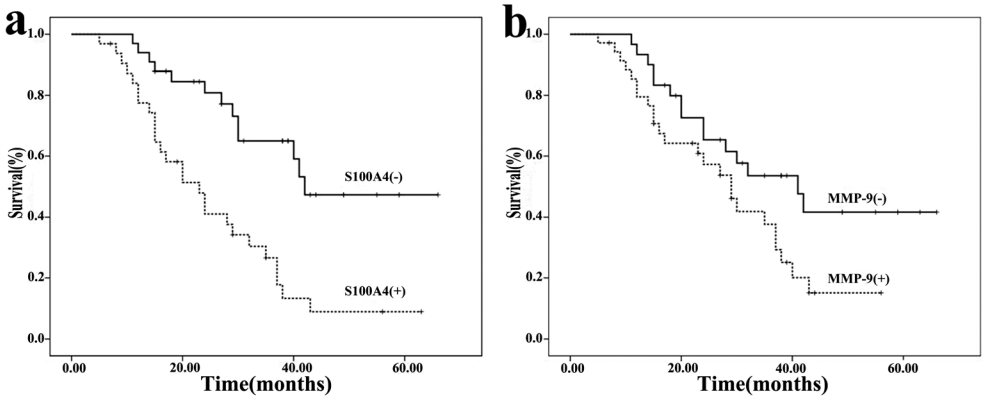

Fig. 2 Survival curves for patients with intrahepatic cholangiocarcinoma. The cumulative survival rate is significantly lower in patients with S100A4 positive expression than that in patients with S100A4 negative expression (a). The cumulative survival rate is significantly lower in patients with MMP-9 positive expression than that in patients with MMP-9 negative expression (b)

Discussion

Intrahepatic cholangiocarcinoma is considered to be a highly fatal carcinoma with poor prognosis because of early invasion, widespread metastasis, and the lack of effective therapy [1–7]. Identifying effective prognostic biomarkers which influence the invasion and metastasis of ICC, and investigating the molecular mechanisms underlying the progression of ICC are important for the diagnosis and therapy of ICC.

S100A4, as an identified metastasis-related gene, is localized in the nucleus, cytoplasm and extracellular space, and is involved in several steps of the metastatic cascade, including cell motility, invasion and angiogenesis [8, 9]. Previous studies of S100A4 expression have reported that over-expression of S100A4 may be correlated with tumor aggressiveness and poor prognosis in many types of carcinoma [14–19]. In a recent study, nuclear expression of S100A4 was reported to increase invasiveness and metastasization of cholangiocarcinoma [21]. However, ICC and extrahepatic cholangiocarcinoma(ECC) are anatomically different cancers and the incidence, mortality, risk factors and clinical features of ICC and ECC are different [1, 31]. To our knowledge, there are no reports individually evaluating expression of S100A4 and its clinicopathological and prognostic significance in ICC. In the present study, expression and prognostic value of S100A4 and its downstream target MMP-9 in ICC tissues were evaluated. The relationship between S100A4 and MMP-9 expression and clinicopathological factors in ICC were also examined.

In this study, S100A4 positive expression in primary ICC was confirmed. Thirty-two of 65 patients had S100A4 positive

Table 3 Univariate and multivariate analysis of prognostic factors for overall survival

	Univariate analysis			Multivariate analysis		
	RR	95%CI	*P*	RR	95%CI	*P*
Gender (male vs. female)	1.068	0.568–2.008	0.838	-	-	-
Age (≤60 vs. >60)	0.945	0.503–1.776	0.861	-	-	-
Tumor size (≤4 vs. >4)	1.001	0.530–1.889	0.998	-	-	-
Macroscopic types (mass-forming vs. non-mass-forming)	1.711	0.896–3.270	0.104	-	-	-
Histological differentiation (well/moderate vs. poorly/undifferentiated)	0.909	0.459–1.797	0.783	-	-	-
Surgical margin (positive vs. negative)	2.552	1.293–5.037	0.007	2.399	1.122–5.128	0.024
Vascular invasion (positive vs. negative)	2.432	1.285–4.601	0.006	1.661	0.848–3.256	0.139
-Lymphatic invasion (positive vs. negative)	1.490	0.773–2.871	0.233	-	-	-
Intrahepatic metastasis (positive vs. negative)	1.749-	0.930–3.289	0.083	-	-	-
Lymph node metastasis (positive vs. negative)	2.496	1.278–4.874	0.007	2.034	0.952–4.345	0.067
T stage (T1+T2 vs. T3+T4)	1.297	0.690–2.436	0.419	-	-	-
TNM stage (I+II vs. III+IV)	1.887	1.002–3.553	0.049	1.074	0.540–2.134	0.839
S100A4 expression (positive vs.negative)	3.324	1.693–6.527	0.000	2.989	1.417–6.305	0.004
MMP-9 expression (positive vs. negative)	1.913	1.000–3.660	0.050	1.140	0.554–2.346	0.722

expression, providing a positive rate of 49.2 %. Although previous results indicated that nuclear S100A4 was a strong predictor of metastasization and reduced survival after resection in cholangiocarcinoma [21], in our study, we did not distinguish between its nuclear or cytoplasmic expression, because S100A4 was located in both the cytoplasm and nuclei in most of the specimens. Furthermore, the positive staining in specimens was not restricted to only tumor cells, because highly expressed levels were also detected in tumor stromal tissues, in particular, smooth muscle cells, endothelial cells of both arteries and veins, fibroblast-like cells and lymphocytes. S100A4 protein was confirmed to play a pivotal role in the tumor-stroma interaction in vitro experiments [32]. The role of S100A4 positive cells in cholangiocarcinoma stromal tissues needs further research.

In addition, we evaluated the relationship of S100A4 expression and clinicopathological characteristics. S100A4 expression was associated with lymph node metastasis in ICC. The results are consistent with previously published studies in gastric cancer and colorectal cancer [14, 18]. However, positive expression of S100A4 was not correlated with tumor size which is inconsistent with the report of gastric cancer [18]. Vascular invasion and TNM stage were also found to be correlated with S100A4 expression. These factors are associated with tumor progression and invasion. It is plausible that ICC with high S100A4 expression has a more aggressive behavior. The analysis of S100A4 expression may be useful in predicting the aggressiveness of ICC. This result was different from Yasunori Sato's study [33], which indicated that there was no significant correlation between the expression of S100A4 and clinicopathological factors in the chalongiocarcinoma. The differences may because the relatively small number of cases in that study [33].

A large body of evidence has demonstrated that S100A4 stimulates the production of MMPs, thereby stimulating the remodeling of ECM [9]. MMP-9 plays an important role in this process, and is reported to correlate with the clinical prognosis of patients with intrahepatic cholangiocarcinoma and other types of tumors. S100A4 siRNA could downregulate expression of MMP-9 and inhibit tumor invasion in several tumor cell lines [12, 27–29]. S100A4 silenced cholangiocarcinoma cells showed significantly reduced MMP-9 secretion, accompanied with reduced cell motility and invasiveness [21]. In our study, the correlation between immunohistochemical expression of S100A4 and MMP-9 was evaluated in ICC tissues. It was found that increased S100A4 expression was significantly correlated with MMP-9 expression. This confirmed that S100A4 and MMP-9 have a synergistic role in ICC invasion and metastasis. However, the precise mechanism by which S100A4 participates in the regulation of MMP-9 needs further investigation.

Kaplan–Meier survival analysis showed that patients with S100A4 or MMP-9 positive expression had a significantly lower survival rate than patients with S100A4 or MMP-9 negative expression. However, only S100A4 expression was an independent prognostic factor according to univariate and multivariate survival analysis using the COX proportional hazard regression model. The surgical margin was another independent prognostic factor which is consistent with previous reports [34, 35]. Other previously reported clinicopathological factors, including tumor size, intrahepatic metastasis, lymph node metastasis, vascular invasion, and perineural invasion, were not confirmed in our study [4, 34, 35].

In conclusion, expression of S100A4 is a useful marker for predicting the progression, metastasis and prognosis of ICC. Furthermore, expression of S100A4 and MMP-9 were significantly correlated. S100A4 might have a synergistic effect with MMP-9 in the aggravation of ICC. However, the number of cases enrolled in our study was not large enough because intrahepatic cholangiocarcinoma is an uncommon tumor and the small number of enrolled cases may have increased the likelihood of systematic bias. A following study with a larger number of cases based on a multicenter survey is warranted for further investigation.

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