Brief Report

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The expression and clinicopathological role of CDX2 in intrahepatic cholangiocarcinoma

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Summary **The aim of this study was to examine the expression and clinicopathological role of caudal homeobox 2 (CDX2) in intrahepatic cholangiocarcinoma (ICC). CDX2 expression was determined immunohistochemically in 93 patients with ICC. The association between CDX2 expression and clinicopathological features of ICC was also examined in patients with ICC. Immunohistochemical staining for CDX2 was noted in 27 patients (29.03%); patients with CDX2-positive tumors had significant survival advantages over those with CDX2-negative tumors (median survival was 40 months for patients with CDX2-positive tumors and 13 months for patients with CDX2-negative tumors; the hazard ratio was 0.36, the 95% confidence interval was 0.22-0.59, and $p < 0.001$). The rate of CDX2 expression was 13.46% in patients with lymphatic invasion and 48.78% in patients without lymphatic invasion ($\chi^2 = 13.88$, $p < 0.01$); positivity for CDX2 expression was significantly higher in patients with well-differentiated or moderately differentiated tumors than that in patients with poorly differentiated tumors (41.7% in patients with well-differentiated tumors, 47.6% in patients with moderately differentiated tumors, and 20.0% in patients with poorly differentiated tumors; Mann-Whitney *U* test, $p = 0.01$). In addition, CDX2 expression differed significantly in patients with ICC due to hepatolithiasis and patients with ICC not due to hepatolithiasis (36.51% and 13.33%, respectively, $\chi^2 = 5.30$, $p = 0.02$). Positivity for CDX2 expression resulted in significant survival advantages for patients with ICC. CDX2 might be used as a prognostic marker in patients with ICC.**

Keywords: Caudal homeobox 2 (CDX2), intrahepatic cholangiocarcinoma, immunohistochemistry, hepatolithiasis, clinicopathological features, prognosis

1. Introduction

Intrahepatic cholangiocarcinoma (ICC) accounts for approximately 10% to 20% of primary liver cancers, second only to hepatocellular carcinoma (*1-5*). Depending on its anatomical location, ICC is classified into one of three categories of cholangiocarcinoma (intrahepatic, hilar, or extrahepatic). Histologically, ICC is considered to be the least common of the three categories (*4*). The incidence and mortality of ICC have been increasing worldwide over the past 30 years, while the incidence of all other forms of cholangiocarcinoma has been declining slightly (*5-11*). Thus far, several risk factors for ICC have been identified, including liver fluke infection, primary sclerosing cholangitis, and hepatolithiasis; hepatolithiasis has been found to be etiologically related to the development of ICC (*12-17*). Surgery is the main form of treatment and it offers the hope of prolonged survival for patients with ICC. However, postoperative long-term outcomes are poor, with a 5-year survival rate of around 30% to 35% (*18*). Similarly, locoregional neoadjuvant or palliative therapies have not been found to offer any significant survival advantages (*19-22*).

Advances in tumor biology have greatly facilitated the use of bio-molecular markers from biopsy, serum,

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or postoperative specimens to predict tumor behavior. Therefore, novel prognostic predictors for early diagnosis, prevention, and treatment of ICC need to be identified.

A member of the caudal-related homeobox gene family, caudal homeobox 2 (CDX2) is commonly expressed in small and large epithelial cells (*23*). CDX2 has been found to play important roles in the proliferation and differentiation of intestinal epithelial cells (*23,24*). Ectopic CDX2 expression is regarded as a sensitive marker indicating intestinal metaplasia in Barrett's esophagus and the stomach, and this finding has been corroborated both in vivo and in vitro (*25-29*). The prognostic value of CDX2 has also been examined in malignancies; CDX2 was found to be expressed in gastric cancer, colon cancer, esophageal carcinoma, and other tumors such as intrahepatic intraductal papillary neoplasia (*25,30-35*). However, CDX2 expression and its prognostic significance in ICC has not been widely reported.

Accordingly, the current study immunohistochemically evaluated the expression of CDX2 and its prognostic value in ICC. In addition, the association between CDX2 expression and clinicopathological features of ICC was also examined.

2. Materials and Methods

2.1. *Patients and tumor samples*

The study was reviewed and approved by the Ethics Committee of the PLA General Hospital (Beijing, China). Subjects were enrolled over a 6-year period (from 2011 to 2016). Subjects were patients with pathologically confirmed ICC who underwent radical resection at the PLA General Hospital. To ensure the verifiability of this study, the following exclusion criteria were used when selecting patients: *i*) patients with additional malignancies in other organs or systems; *ii*) patients with a pathological diagnosis of combined hepatocellular carcinoma and cholangiocarcinoma; *iii*) evidence of cancer cells in the surgical margins; and *iv*) patients lost to follow-up. Patients meeting any of the four exclusion criteria were excluded. Demographic data were collected and clinicopathological features were examined in all patients enrolled in this study using a computerized medical database, perioperative records and pathology reports were thoroughly reviewed, and a supplementary follow-up was conducted by telephone.

2.2. *Immunohistochemistry*

Tissues were immunohistochemically stained using the Envision two-step method (*36*). Briefly, specimens were fixed in formalin and embedded in paraffin to prepare 4-μm-thick sections as previously described (*36*). Slides were serially incubated (40 min each, at room temperature) with CDX2 primary antibody (1:150, ZETA, USA). Each incubation with primary antibody was followed by 40-min of incubation at room temperature with horseradish peroxidase-labeled polymer. The stain was developed by incubation with diaminobenzidine substrate-chromogen for 5-10 min, and a hematoxylin counterstain was applied. Both negative and positive controls were used.

2.3. *Scoring of staining*

Staining was graded for intensity (0, negative; 1, weak; 2, moderate; and 3, strong), and the percentage of cells stained was determined (0, < 5%; 1, 6-25%; 2, 26-50%; 3, > 50%). The final score was calculated as the combined staining score (percentage of cells + intensity). A score less than 1 was defined as negative expression, and a score equal to or greater than 1 was defined as positive expression (*35*).

2.4. *Definition*

In the current study, hepatolithiasis was defined as concretions existing in the intrahepatic bile ducts; ICC due to hepatolithiasis refers to ICC as a result of hepatolithiasis, and ICC not due to hepatolithiasis refers to ICC not resulting from hepatolithiasis (*37-39*). Based on the macroscopic appearance described by the Liver Cancer Study Group of Japan, ICC consists of 3 gross subtypes: the mass-forming subtype, the periductal infiltrating subtype, and the intraductal growth subtype. Lymphatic invasion mostly refers to lymph node involvement at the site of the hepatoduodenal ligament (involvement of regional lymph nodes (N1 disease) according to the 8th edition of the American Joint Committee on Cancer Staging System).

2.5. *Statistical analysis*

Statistical analyses were performed using SPSS v14.0 (IBM, Armonk, NY, USA). Categorical variables are expressed as a percentage. Continuous variables are expressed as the mean and standard deviation. Categorical variables were compared using the χ^2 test or Fisher's exact test, as appropriate; continuous variables were compared using the Mann-Whitney *U* test. Long-term survival was estimated using the Kaplan-Meier method and compared using the log rank test. A two-tailed *p* value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. *Demographic data and clinicopathological characteristics of patients*

Strict application of the exclusion criteria resulted in a

Table 1. Correlation between CDX2 expression according to immunohistochemistry and clinicopathological features of ICC

Items	Total	CDX2 immunohistochemistry expression		*p* value
		Positive	Negative	
Age (year, mean ± SD)		53 ± 7.8	51 ± 7.0	0.23[#]
Gender				0.78[&]
Male	60	18 (30.0%)	42 (70.0%)	
Female	33	9 (27.3%)	24 (72.7%)	
Tumor nodules				0.30[&]
Single	69	22 (31.9%)	47 (68.1%)	
Multiple	24	5 (20.8%)	19 (79.2%)	
Hepatolithiasis				0.02[&]
With hepatolithiasisPresent	63	23 (36.5%)	40 (63.5%)	
Without hepatolithiasisAbsent	30	4 (13.3%)	26 (86.7%)	
Gross subtype				0.63[*]
Mass-forming	55	18 (32.7%)	37 (67.3%)	
Peri-ductal infiltrating	13	2 (15.4%)	11 (84.6%)	
Intraductal papillary	25	7 (28.0%)	18 (72.0%)	
Lymphatic invasion				< 0.01[&]
PositivePresent	52	7 (13.5%)	45 (86.5%)	
NegativeAbsent	41	20 (48.8%)	21 (51.2%)	
Differentiation				0.01[*]
Well differentiated	12	5 (41.7%)	7 (58.3%)	
Moderately differentiated	21	10 (47.6%)	11 (52.4%)	
Poorly differentiated	60	12 (20.0%)	48 (80.0%)	
TNM Staging				0.72[*]
1	49	17 (34.7%)	32 (65.3%)	
2	27	6 (22.2%)	21 (77.8%)	
3	17	4 (23.5%)	13 (76.5%)	

CDX2: caudal homeobox 2; SD, standard deviation; [#], Student's *t* test; [*], Mann–Whitney *U* test; &, χ^2 test.

total of 93 patients with pathologically confirmed ICC in this study. Corresponding tissue samples (paraffin-embedded sections) were obtained from surgically resected specimens from the 93 patients. Demographic data and clinicopathological characteristics of patients are shown in Table 1. The 93 patients with ICC consisted of 60 males and 33 females with a mean age of 51.6 years (standard deviation: 7.3 years). Multiple tumor nodules were present in 24 of the 93 patients, and 63 patients had underlying hepatolithiasis. Fifty-five of the 93 patients had the mass-forming gross subtype, 13 had the peri-ductal infiltrating subtype, and 25 had the intraductal papillary subtype. Lymphatic invasion was present in 52 patients and absent in 41. Twelve patients had well-differentiated tumors, 21 had moderately differentiated tumors, and 60 had poorly differentiated tumors. Forty-nine tumors were T1, 27 were T2, and 17 were T3. The follow-up ranged from 6 months to 86 months in length.

3.2. *CDX2 expression according to immunohistochemistry*

CDX2 staining was immunohistochemically assessed in all 93 specimens of ICC. As shown in Figure 1, CDX2 protein was mainly concentrated in the nuclei of carcinoma cells. CDX2 staining was noted in 27 specimens (29.03%). Subjects were divided into patients with ICC due to hepatolithiasis and those with ICC not due to hepatolithiasis. Positive CDX2 expression was noted in 23 of 63 patients with ICC due to hepatolithiasis (36.51%) and in 4 of 30 patients with ICC not due to hepatolithiasis (13.33%). The rate of expression differed significantly in the two groups (χ^2 = 5.30, p = 0.02).

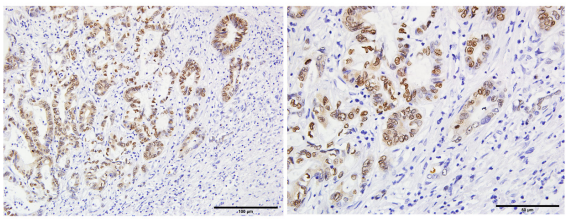

Figure 1. CDX2 expression in ICC. A Strong immunostaining of the nuclei of carcinoma cells in yellow or brown. (left, ×100; right, ×200)

3.3. *Survival analysis*

Patients were categorized into two groups, those with CDX2-positive tumors and those with CDX2-negative tumors. Patients with CDX2-positive tumors had a median survival of 40 months, whereas those with CDX2-negative tumors had a median survival of a mere 13 months; as shown in Figure 2, patients with CDX2-positive tumors had a significantly lower likelihood of dying compared to those with CDX2-negative tumors (hazard ratio (HR) 0.36, 95% confidence interval (95% CI) 0.22-0.59; p < 0.001).

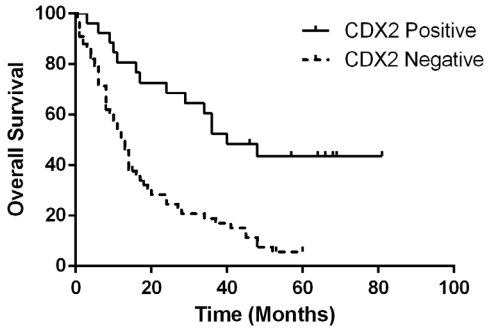

Figure 2. Kaplan-Meier survival analysis by CDX2 status. The y-axis represents the percentage of patients, and the x-axis represents their survival in months. The solid line represents patients with CDX2-positive tumors, who tended to survive longer than patients with CDX2-negative tumors represented by the dotted line ($p < 0.001$).

3.4. *Correlation between CDX2 expression and clinicopathological features*

The current study noted a significant correlation between CDX2 expression and lymphatic invasion; the rate of CDX2 expression was 13.46% in patients with lymphatic invasion and 48.78% in patients without lymphatic invasion ($\chi^2 = 13.88$, $p < 0.01$). In addition, positivity for CDX2 expression was significantly higher in patients with well-differentiated or moderately differentiated tumors than that in patients with poorly differentiated tumors (41.7% in patients with well-differentiated tumors, 47.6% in patients with moderately differentiated tumors, and 20.0% in patients with poorly differentiated tumors; Mann-Whitney U test, $p = 0.01$). There were no statistical differences between positive CDX2 staining and gender, the number of tumor nodules (single or multiple), tumor gross subtype, and TNM stage.

The prognostic value of CDX2 has been noted in relation to certain malignancies. However, its predictive significance in ICC has yet to be determined. The current study is as a relatively large-scale study that immunohistochemically evaluated the expression of CDX2 and its prognostic value in predicting ICC. Results indicated that patients with CDX2-positive tumors had significant survival advantages over those with CDX2-negative tumors (HR 0.36, 95% CI 0.22-0.59). Moreover, CDX2 staining was positive in 29.03% of the patients with ICC, and this finding agrees with figures reported in the literature (*40-42*). A correlation between CDX2 expression and the presence of hepatolithiasis, the presence of lymphatic invasion, and the extent of tumor differentiation was also noted. Interestingly, expression differed significantly in patients with ICC due to hepatolithiasis and patients with ICC not due to hepatolithiasis (36.51% *vs*. 13.33%, $\chi^2 = 5.30$, $p = 0.02$). Taken together, these findings suggest that CDX2 might serve as a marker of prognostic significance for patients with ICC in the future.

The detailed mechanisms that account for the correlation between CDX2 expression and survival disadvantages in patients with ICC have not been specifically studied. The following could be confounding variables.

Studies have indicated that positive expression of CDX2 is related to inhibition of invasion and metastasis by ICC, and CDX2 is considered to be an independent indicator of improved long-term survival (*41,43*). As reported previously, 16.46% of patients with biliary tract carcinoma had positive expression of CDX2 (*41*). In addition, a study has indicated that CDX2 was expressed in 37.3% of extrahepatic biliary tract carcinoma and more frequently in tumors with papillary growth and no vascular invasion (*42*). Similar results were reported by Jinawath *et al*. (*33*). A study by Li *et al*, reported that expression of CDX2 was negatively correlated with tumor size and lymph node metastasis in biliary tract tumors; increased CDX2 expression was correlated with a better prognostic outcome (*40*). Similarly, the rate of positive CDX2 expression was 29.03% in the current study; CDX2 expression was also negatively associated with lymphatic invasion, but there was no significant relationship between CDX2 expression and morphological tumor type or TNM staging. Such discrepancies could be due to multiple factors, such as patient selection bias. CDX2 expression is correlated with a greater degree of differentiation in gastric and gallbladder adenocarcinoma (*29,40,44*), and the current study yielded a similar finding. Taken together, the aforementioned mechanisms or hypotheses might explain the current finding that patients with CDX2-positive tumors had superior survival.

That said, the rate of CDX2 expression in patients with ICC due to hepatolithiasis was significantly higher than that in patients with ICC not due to hepatolithiasis. This finding implies the possible involvement of CDX2 in the pathogenesis of hepatolithiasis leading to ICC. Hepatolithiasis was deemed to be a confirmed risk factor for ICC. Mucin hypersecretion or aberrant expression of mucin 2 and mucin 5AC in the intrahepatic biliary system is closely associated with the lithogenesis of hepatolithiasis (*45*). Two studies in Japan confirmed that aberrant expression of CDX2 is closely correlated with the overexpression of mucin 2 or mucin 5AC in mucinous ICC due to hepatolithiasis, suggesting its role in intestinal differentiation and its association with carcinogenesis in these tumors (*31,33*). One might conclude that patients with ICC due to hepatolithiasis had a higher rate of CDX2 expression. However, the detailed mechanism for this expression needs to be studied further.

The current study has several limitations. The detailed or definitive molecular mechanisms accounting for the correlation with CDX2 expression were not explored, thus reducing the reliability and consistency of the current results. Moreover, CDX2 expression was

not examined in patients with hepatolithiasis alone, and those patients could serve as a control or parallel group. Nevertheless, the current study has helped to lay out a persuasive argument for the prognostic significance of CDX2 in ICC.

In conclusion, positive CDX2 expression resulted in significant survival advantages in ICC. CDX2 might be used as a prognostic marker in patients with ICC.

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