Intranuclear accumulation of galectin-3 is an independent prognostic factor for patients with distal cholangiocarcinoma

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Abstract. Galectin-3 has been reported to be associated with the prognosis of patients with various malignancies; however, it has not yet been investigated in patients with extrahepatic cholangiocarcinoma (EHCC). Expression of galectin-3 was retrospectively examined in 58 patients with EHCC: 21 with perihilar cholangiocarcinoma and 37 with distal cholangiocarcinoma (DCC). The Cox proportional hazard model was used to identify independent prognostic factors. Intranuclear accumulation of galectin-3 (gal-3-INA) was associated with poorer overall survival (OS) in all patients (P=0.003), as well as in patients with DCC (P=0.004). Patients with gal-3-INA also exhibited a poorer disease-free survival (DFS) than those without gal-3-INA in all patients with EHCC (P<0.001), and in patients with DCC (P<0.001). Gal-3-INA was an independent prognostic factor of OS and DFS in all patients [OS: Hazard ratio (HR), 4.470; 95% confidence interval (CI), 1.759-11.357; P=0.002; and DFS: HR, 5.116; 95% CI, 2.025-12.925; P=0.001]. Gal-3-INA was also an independent prognostic factor in patients with DCC (OS: HR, 2.979; 95% CI, 1.035-8.570; P=0.043; and DFS: HR, 6.773; 95% CI, 1.558-29.439; P=0.011). In the analysis of patients with DCC, the number of patients with high galectin-3 expression (P=0.038), recurrence (P<0.001), distant metastases (P<0.001), R0 status (P=0.029) or microscopic vascular invasion (P=0.019) was significantly higher in the gal-3-INA-positive group than in the gal-3-INA-negative group. In conclusion, gal-3-INA was identified as a strong prognostic factor for OS and DFS in patients with DCC.

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Abbreviations: EHCC, extrahepatic cholangiocarcinoma; PCC, perihilar cholangiocarcinoma; DCC, distal cholangiocarcinoma; UICC, Union for International Cancer Control; gal-3-INA, intranuclear accumulation of galectin-3; OS, overall survival; DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; EMT, epithelial-to-mesenchymal transition; GSK-3β, glycogen synthase kinase-3β

Key words: galectin-3, distal cholangiocarcinoma, prognostic factor

Introduction

Extrahepatic cholangiocarcinoma (EHCC) is a relatively rare disease in Western countries, although its incidence is increasing: ~5,000 new cases are diagnosed every year in the USA (1). In Japan, however, EHCC has been reported to be associated with >18,000 mortalities annually (2). The disease is classified into two categories: Perihilar cholangiocarcinoma (PCC) and distal cholangiocarcinoma (DCC) (3). Surgical resection is the first-line treatment for the disease. However, the 5-year survival rate remains at 30-42% for PCC and 18-54% for DCC (4-12). Reported prognostic factors of EHCC include lymph node metastasis (11,13-21), the number of involved nodes (13,15,16,18,19), surgical margin status (12,22), Union for International Cancer Control (UICC) tumor-node-metastasis (TNM) factors and/or stage (23), lymphovascular invasion (23,24), perioperative blood transfusion (25) and comorbidity (26). Among these, lymph node metastasis and the number of involved nodes are considered to be the strongest prognostic factors. Therefore, the present study investigated the possibility of identifying a more effective prognostic factor.

Galectin-3, a β-galactoside binding lectin, exhibits pleiotropic biological functions, and has been implicated in cell growth, differentiation, apoptosis, adhesion, malignant transformation and RNA processing (27-30). Overexpression of galectin-3 was reported as a predictor of poorer prognosis in ovarian carcinoma (31), nasopharyngeal carcinoma (32), malignant melanoma (33), gallbladder carcinoma (34), osteosarcoma (35) and hepatocellular carcinoma (36). However, in pancreatic carcinoma (37), laryngeal squamous-cell carcinoma (38), gastric carcinoma (39), clear cell renal carcinoma (40) and breast carcinoma (41), its overexpression has been reported to be associated with improved prognosis. However, when considering the association between galectin-3 and cholangiocarcinoma, there are only a few studies on patients with intrahepatic cholangiocarcinoma (42-44). The present study focused on the ability of galectin-3 to prevent anoikis, which is a form of apoptosis that is induced when cells are exposed to a condition of no contact with each other or the

extracellular matrix, as is the case when cancer cells are not attached in lymphovascular vessels prior to the development of metastatic foci (45). Therefore, the present study focused on the association between EHCC prognosis and galectin-3 expression.

Patients and methods

Patients. A total of 63 patients with EHCC underwent surgical resection between January 1999 and January 2014. Among these, 3 patients were excluded due to surgery-associated mortality, and 2 patients were excluded, as follow-up was not possible. The remaining 58 patients with EHCC (21 PCC cases and 37 DCC cases) were enrolled in accordance with the guideline for informed consent and approval from the Ethics Committee of Fukushima Medical University (Fukushima, Japan). All patients gave written informed consent.

Clinicopathological features. Patient demographics are summarized in Table I. The final stage of patients was determined pathologically according to the UICC TNM classification system of malignant tumors (46). The mean observation period was 9.00 years (range, 1.09-19.00 years). The following factors were analyzed: Age (<75 vs. ≥75 years), sex, surgical procedure (bile duct resection only vs. pancreaticoduodenectomy or hepatectomy with extrahepatic bile duct resection), comorbidity (with vs. without systemic illnesses affecting surgical outcomes, including diabetes mellitus, hypertension, asthma, angina or ischemic heart diseases), postoperative complications (none vs. with pancreatic fistula or bile leakage), adjuvant chemotherapy (none vs. adjuvant chemotherapy, including tegaful/uracil or gemcitabine), pathological tumor aspects, T category (PCC, T0-T2a vs. T2b-T4b; DCC, T0-T2 vs. T3a-T4), pathological node status, N category (none vs. positive), number of involved nodes (≤2 vs. >2), M category (none vs. positive), stage (PCC, stage 0-II vs. stage IIIA-IVB; DCC, stage 0-IIB vs. stage III-IV), tumor differentiation (well-differentiated tubular or papillary vs. others), surgical margin status, R status (R0, no residual tumors vs. R1, existence of residual tumors), status of infiltration (well defined vs. infiltrative), microscopic lymphatic vessel invasion (none vs. positive), microscopic vascular invasion (none vs. positive), perineural invasion (none vs. positive), and serum levels of carcinoembryonic antigen and carbohydrate antigen 19-9 prior to surgery (within normal range vs. abnormal).

Immunohistochemistry. Galectin-3 expression was assessed by immunohistochemistry using an avidin-biotin-peroxidase complex method. Formalin-fixed, paraffin-embedded tissue samples were cut into 4 μm-thick sections. The sections were deparaffinized in xylene and rehydrated through a series of decreasing ethanol concentrations (100, 90, 80 and 60% ethanol). Subsequent to being rinsed three times in PBS, the sections were immersed in an absolute methanol solution containing 0.3% H_2O_2 for 30 min at room temperature to block endogenous peroxidase. Antigens were retrieved by autoclaving sections on slides in 0.01 M (pH 6.0) citrate buffer for 10 min. Subsequent to rinsing in PBS, the sections were incubated with polyclonal goat anti-galectin-3 antibody (dilution, 1:2,000; catalog no., AF1154; R&D Systems, Inc., Minneapolis, MN, USA) overnight at 4˚C. An additional wash in PBS was followed by treatment with peroxidase-labeled anti-goat antibody (Histofine Simple Stain Max-PO (G); catalog no., 414162; Nichirei Corporation, Tokyo, Japan) as the secondary antibody for 30 min at room temperature. The staining was visualized with 3,3'-diaminobenzidine (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Immunohistochemical evaluations were performed under a microscope (BX46; Olympus Corporation, Tokyo, Japan) (magnification, x100). A total of 1,000 tumor cells were counted to assess positive staining, and the percentages of positively stained cells were determined. The average percentage of the 58 specimens was 45.3%. Based on this result, the patients were divided into two groups: A low-galectin-3-expression group, in which <50% of the tumor cells were positive; and a high-galectin-3-expression group, in which ≥50% of the tumor cells were positive. When cancer cells with an intranuclear accumulation of galectin-3 (gal-3-INA) accounted for >5% of observed cells in an invasive front, the specimen was classified as intranuclear-accumulation positive.

Statistical analysis. Categorical variables were evaluated by the χ^2 test or the Fisher's exact test was applied when values were under 5. Survival time was calculated between the date of surgery and the date of the last follow-up. The final assessment of disease status was performed on April 30, 2015. OS and DFS were calculated using the Kaplan-Meier method, and differences between the groups were assessed by the log-rank test. Factors identified as significant by univariate analysis were then subjected to a multivariate analysis as previously reported (18,47) using the Cox proportional hazard model to identify independent predictors of recurrence and prognosis. All statistical calculations were performed using SPSS version 22 (IBM SPSS, Armonk, NY, USA).

Results

Galectin-3 expression. Fig. 1 shows the galectin-3 expression of patients with EHCC. In the specimens with gal-3-INA, galectin-3 existed in the nucleus as well as in the cytoplasm; however, in those without gal-3-INA, galectin-3 was only present in the cytosol.

OS. The median survival times of all patients, patients with PCC and patients with DCC were 2.320, 1.520 and 2.737 years, respectively. Fig. 2 shows the OS of all patients and patients with DCC, comparing patients with and without gal-3-INA. Patients with gal-3-INA had a significantly poorer prognosis than those without gal-3-INA in the total patients (median OS, 5.940 vs. 1.920 years; P=0.003) and patients with DCC (median OS, 13.160 vs. 2.100; P=0.004) groups. The results of univariate and multivariate analyses are shown in Table II. Analysis of all patients revealed that gal-3-INA [hazard ratio (HR), 4.470; 95% confidence interval (CI), 1.759-11.357; P=0.002] and tumor differentiation (HR, 2.344; 95% CI, 1.069-5.138; P=0.033) were independent prognostic factors. For the patients with PCC, T category (HR, 2.865; 95% CI, 0.944-8.694; P=0.063) and status of infiltration (HR, 7.861; 95% CI, 1.653-37.383, P=0.01) were independent prognostic

Table I. Profiles of patients.

Total	Total, n 58	PCC, n 21	DCC, n 37
Mean age ± SD (range), years	65.8±7.9 (36-86)	65.9±7.9 (52-78)	65.7±10.7 (36-86)
Sex			
Male	39	15	24
Female	19	6	13
Age, years			
<75	49	18	31
≥75	9	3	6
Galectin-3 expression			
Weak	22	10	12
High	36	11	25
Recurrence			
None	21	5	16
Positive	37	16	21
Distant metastasis			
None	28	7	21
Positive	30	14	16
Operation			
Bile duct resection	10	2	8
PD, PPPD or SSPPD	33	4	29
Hepatectomy with bile duct resection	11	11	0
HPD	4	4	0
Stage (PCC/DCC)			
0/0	1	1	0
I/IA	4	0	4
II/IB	27	15	12
IIIA/IIA	9	0	9
IIIB/IIB	11	0	11
IVA/III	4	3	1
IVB/IV	2	2	0
Adjuvant chemotherapy			
None	36	14	22
S-1 or gemcitabine	22	7	15

PCC, perihilar cholangiocarcinoma; DCC, distal cholangiocarcinoma; PD, pancreaticoduodenectomy; PPPD, pylorus-preserving pancreaticoduodenectomy; SSPPD, subtotal stomach-preserving pancreaticoduodenectomy; HPD, hepatopancreaticoduodenectomy; SD, standard deviation; S-1, an oral anticancer agent that contains tegafur, a prodrug of 5-fluorouracil, combined with two modulators (gimeracil and oteracil).

factors. In the analysis of patients with DCC, gal-3-INA was the only independent prognostic factor (HR, 2.979; 95% CI, 1.035-8.570; P=0.043).

DFS. The median DFS times of all patients, patients with PCC and patients with DCC were 1.840, 0.980 and 1.980 years, respectively. As shown in Fig. 2, the patients with gal-3-INA exhibited a poorer DFS than those without gal-3-INA in the analysis of all patients (median DFS, 11.960 vs. 0.970; P<0.001) and patients with DCC (median DFS, 11.960 vs. 1.200; P<0.001). The results of the univariate and multivariate analyses on DFS are shown in Table III. Analysis of all patients revealed that gal-3-INA (HR, 5.116; 95% CI, 2.025-12.925; P=0.001) and the number of involved nodes (HR, 2.493; 95% CI, 0.476-2.729; P=0.041) were independent prognostic factors. As for the patients with PCC, only the number of involved nodes was statistically significant (HR, 24.547; 95% CI, 2.458-245.18; P=0.006) in the univariate analysis. In the analysis of patients with DCC, gal-3-INA was the only independent prognostic factor (HR, 6.773; 95% CI, 0.558-29.439; P=0.011).

Subgroup analysis. Table IV shows the subgroup analysis on patients' demographics, according to the presence of gal-3-INA. In the analysis of patients with PCC, no statistically significant differences were observed, although the number of patients with a positive margin was lower in the gal-3-INA group than in the gal-3-INA-negative group. In the analysis of patients with DCC, the number of patients with higher galectin-3 expression, recurrence, distant metastases, R0 status or microscopic vascular invasion was significantly higher (P=0.029 and P=0.019, respectively) in the gal-3-INA-positive group than in the gal-3-INA-negative group, whereas the number of patients with postoperative complications was significantly lower (P=0.045) in the gal-3-INA-positive group than in the gal-3-INA-negative group. In the analysis of the total study population of 58 patients, the number of patients with higher galectin-3 expression (P=0.013), recurrence (P<0.001), distant metastases (P<0.001), exfoliation-margin negative (P=0.013), R0 status (P=0.009) or microscopic vascular invasion (P=0.033) was significantly higher in the gal-3-INA-positive group than in the gal-3-INA-negative group. No significant difference was observed among the subgroups in the other investigated categories: Age, sex, surgical procedure, comorbidity, adjuvant chemotherapy, TNM classification, number of involved nodes, tumor differentiation, microscopic lymphatic invasion or tumor markers.

Discussion

To the best of our knowledge, the present study reported for the first time the association between the expression of galectin-3 and the prognosis of EHCC. The results of the present study show that the gal-3-INA-positive group in patients with EHCC is associated with poorer prognosis than the gal-3-INA-negative group. In patients with DCC, gal-3-INA was the only independent prognostic factor. Overexpression of galectin-3 was reported as a predictor of poor prognosis in various malignancies, including ovarian carcinoma (31), nasopharyngeal carcinoma (32), malignant melanoma (33), gallbladder carcinoma (34), osteosarcoma (35) and hepatocellular carcinoma (36). However, galectin-3 overexpression was reported to be associated with improved prognosis in pancreatic carcinoma (37), laryngeal squamous-cell carcinoma (38), gastric carcinoma (39), clear cell renal carcinoma (40) and breast carcinoma (41). The reason for this contrast has been attributed to the idiosyncrasy of each malignancy (48). In the

Table II. Univariate and multivariate analyses of overall survival.

	Total (n=58)						PCC (n=21)						DCC (n=37)					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Sex (male vs. female)	0.840	0.419-1.688	0.625				0.518	0.141-1.902	0.322				0.778	0.301-2.006	0.603			
Age (<75 vs. ≥75 years)	0.339	0.103-1.113	0.075				0.319	0.041-2.466	0.273				0.363	0.084-1.573	0.175			
Comorbidity (none vs. positive)	0.831	0.430-1.606	0.583				0.385	0.117-1.273	0.118				0.895	0.346-2.314	0.819			
Operation method (BDR vs. others)	0.895	0.407-1.965	0.782				0.637	0.139-2.920	0.561				0.836	0.351-1.992	0.686			
Galectin-3 expression (low vs. high)	1.444	0.695-3.003	0.325				1.288	0.443-3.741	0.642				1.886	0.634-5.616	0.254			
gal-3-INA (none vs. positive)	2.866	1.375-5.975	**0.005**	4.470	1.759-11.357	**0.002**	1.697	0.462-6.194	0.423				3.750	1.433-9.811	**0.007**	2.970	1.035-8.570	**0.043**
Positive margin (none vs. positive)	1.531	0.631-3.717	0.346				1.25	0.383-4.077	0.711				1.327	0.304-5.790	0.707			
Adjuvant chemotherapy (none vs. positive)	1.309	0.664-2.582	0.437				1.53	0.506-4.623	0.451				1.214	0.491-3.002	0.675			
Location of main tumor (PCC vs. DCC)	0.566	0.291-1.102	0.094				NA						NA					
T category (PCC, T0-T2a vs. T2b-T4b; DCC, T0-2 vs. T3a-T4)	1.622	0.840-3.132	0.149				3.098	1.062-9.037	**0.038**	2.865	0.944-8.694	0.063	1.222	0.513-2.912	0.651			
N category (none vs. positive)	1.982	0.995-3.949	0.052				1.229	0.339-4.455	0.754				2.566	1.057-6.227	**0.037**	1.003	0.125-8.066	0.997
M category (none vs. positive)	2.579	1.227-5.423	**0.012**	1.265	0.472-3.392	0.640	2.159	0.581-8.021	0.250				NA					
Number of involved nodes ((≤2 vs. >2)	1.953	0.464-8.214	0.361				1.383	0.298-6.417	0.679				2.636	0.997-6.972	0.051			
Stage (PCC, 0-IIIA; vs. IIIB-IVB; DCC, 0-IIA vs. IIB-IV)	2.255	1.131-4.496	**0.021**	2.504	0.901-6.960	0.079	1.775	0.550-5.734	0.337				2.544	1.034-6.262	**0.042**	2.833	0.335-23.977	0.339
Tumor differentiation (well or moderate vs. poorly)	3.068	1.480-6.359	**0.003**	2.344	1.069-5.138	**0.033**	2.497	0.828-7.530	0.104				1.996	0.654-6.094	0.225			
Status of infiltration (well defined vs. infiltrative)	2.343	1.184-4.637	**0.014**	1.840	0.900-3.765	0.095	8.081	1.745-37.424	**0.008**	7.861	1.653-37.383	**0.010**	1.165	0.481-2.820	0.734			

Table II. Continued.

	Total (n=58)						PCC (n=21)						DCC (n=37)					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Microscopic lymphatic vascular invasion (none vs. positive)	2.773	1.069-7.196	**0.036**	0.952	0.328-2.765	0.928	0.629	0.171-2.320	0.486				6.104	1.401-26.597	**0.016**	3.186	0.655-15.507	0.151
Perineural invasion (none vs. positive)	4.348	1.332-14.198	**0.015**	2.946	0.841-10.312	0.091	2.376	0.527-10.715	0.260				3.980	0.925-17.114	0.063			
CEA (normal vs. abnormal)	0.641	0.153-2.684	0.543				3.229	0.681-15.307	0.140				0.041	0.000-23.159	0.323			
CA19-9 (normal vs. abnormal)	1.168	0.607-2.246	0.642				1.735	0.542-5.553	0.353				1.004	0.420-2.396	0.993			

HR, hazard ratio; CI, confidence interval; BDR, bile duct resection; PCC, perihilar cholangiocarcinoma; DCC, distal cholangiocarcinoma; NA, not applicable; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; gal-3-INA, intranuclear accumulation of galectin-3. Bold fonts indicate statistically significant P-values.

Table III. Univariate and multivariate analyses of disease-free survival.

	Total (n=58)						PCC (n=21)						DCC (n=37)					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
Category	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Sex (male vs. female)	0.877	0.439-1.752	0.710				0.628	0.194-2.030	0.437				0.991	0.399-2.461	0.985			
Age (<75 vs. ≥75 years)	0.632	0.245-1.629	0.342				0.502	0.112-2.246	0.367				0.704	0.206-2.404	0.576			
Comorbidity (none vs. positive)	0.913	0.476-1.751	0.785				0.385	0.117-1.273	0.118				0.616	0.253-1.498	0.285			
Operation method (BDR vs. others)	1.062	0.465-2.424	0.886				0.723	0.155-3.373	0.679				1.042	0.380-2.854	0.937			
Galectin-3 expression (low vs. high)	1.844	0.868-3.915	0.111				1.808	0.652-5.010	0.255				2.664	0.782-9.071	0.117			
gal-3-INA (none vs. positive)	0.966	2.470-14.411	**<0.001**	5.116	2.025-12.925	**0.001**	1.793	0.490-6.565	0.378				11.152	3.159-39.367	**<0.001**	6.773	1.558-29.439	**0.011**
Adjuvant chemotherapy (none vs. positive)	1.369	0.708-2.646	0.351				1.106	0.380-3.222	0.853				1.782	0.750-4.235	0.191			
Location of main tumor (PCC vs. DCC)	0.52	0.270-1.001	0.050				NA						NA					
T category (PCC, T0-T2a vs. T2b-T4b; DCC, T0-2 vs. T3a-T4)	1.503	0.785-2.876	0.219				3.108	0.964-10.021	0.058				1.483	0.627-3.506	0.370			
N category (none vs. positive)	1.697	0.836-3.445	0.143				2.551	0.675-9.643	0.167				1.967	0.812-4.766	0.134			
Number of involved nodes (≤2 vs. >2)	2.957	1.377-6.349	**0.005**	2.493	1.038-5.986	**0.041**	24.547	2.458-245.184	**0.006**				2.762	1.056-7.221	**0.038**	2.097	0.741-5.937	0.163
Stage (PCC, 0-IIIA vs. IIIB-IVB; DCC, 0-IIA vs. IIB-IV)	1.585	0.765-3.284	0.215				2.472	0.650-9.398	0.184				1.738	0.699-4.321	0.234			
Tumor differentiation (well or moderate vs. poorly)	2.276	1.050-4.936	**0.037**	1.140	0.476-2.729	0.768	2.046	0.641-6.530	0.227				2.018	0.660-6.164	0.218			

Table III. Continued.

	Total (n=58)						PCC (n=21)						DCC (n=37)					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
Category	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value
Status of infiltration (well defined vs. infiltrative)	1.869	0.970-3.600	0.062				2.960	0.976-8.980	0.055				1.424	0.601-3.373	0.422			
Microscopic lymphatic invasion (none vs. positive)	3.406	1.301-8.917	**0.013**	1.285	0.423-3.907	0.658	1.573	0.341-7.261	0.561				4.024	1.168-13.862	**0.027**	1.223	0.285-5.244	0.787
Microscopic vascular invasion (none vs. positive)	2.285	0.991-5.266	0.052				1.411	0.480-4.146	0.532				4.986	1.144-21.725	**0.032**	1.785	0.302-10.570	0.523
Perineural invasion (none vs. positive)	2.808	1.092-7.223	**0.032**	2.024	0.705-5.817	0.190	3.226	0.722-14.408	0.125				2.568	0.754-8.748	0.132			
CEA (normal vs. abnormal)	0.546	0.131-2.281	0.407				4.023	0.806-20.077	0.090				0.040	0.000-13.590	0.279			
CA19-9 (normal vs. abnormal)	1.411	0.739-2.694	0.297				1.020	0.376-2.771	0.968				1.513	0.642-3.568	0.344			

HR, hazard ratio; CI, confidence interval; BDR, bile duct resection; PCC, perihilar cholangiocarcinoma; DCC, distal cholangiocarcinoma; NA, not applicable; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; gal-3-INA, intranuclear accumulation of galectin-3. Bold fonts indicate statistically significant P-values.

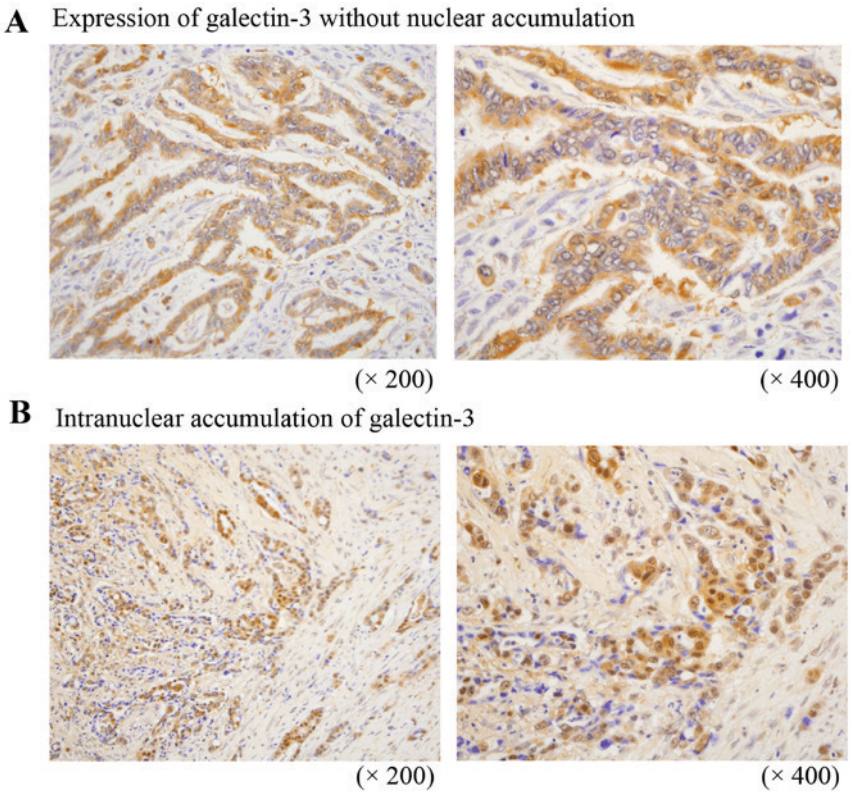

Figure 1. Galectin expression with and without gal-3-INA. (A) High expression level of galectin-3 (nuclear accumulation is not shown). (B) gal-3-INA. Magnification, x200 and x400. gal-3-INA, intranuclear accumulation of galectin-3.

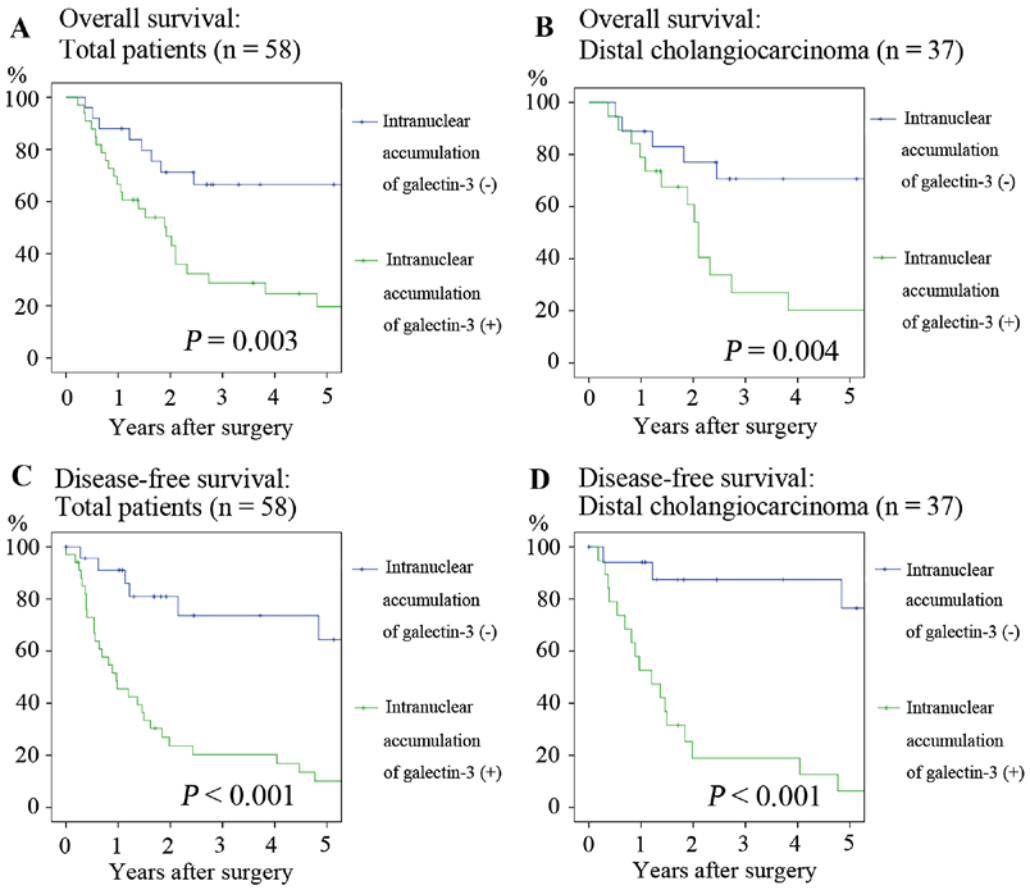

Figure 2. (A) Overall survival of all 58 patients and (B) patients with DCC, compared between patients with and without gal-3-INA. (C) Disease-free survival of all 58 patients and (D) patients with DCC, compared between patients with and without gal-3-INA. gal-3-INA, intranuclear accumulation of galectin-3; DCC, distal cholangiocarcinoma.

Table IV. Subgroup analysis according to gal-3-INA.

	PCC (n=21)			DCC (n=37)			Total (n=58)		
	gal-3-INA			gal-3-INA			gal-3-INA		
Variable	Negative (n=6)	Positive (n=15)	P-value	Negative (n=17)	Positive (n=18)	P-value	Negative (n=25)	Positive (n=33)	P-value
Galectin-3 expression			0.063			**0.038**			**0.013**
Low	5	5		9	3		14	8	
High	1	10		9	16		10	26	
Postoperative complication			0.331			**0.045**			0.506
-	4	5		8	15		12	20	
+	2	10		10	4		12	14	
Recurrence			0.115			**<0.001**			**<0.001**
-	3	2		14	2		17	3	
+	3	13		0	17		7	31	
Distant metastasis			0.120			**<0.001**			**<0.001**
-	4	3		18	2		22	5	
+	2	12		0	17		2	29	
Positive margin			**0.031**			1.000			0.291
-	2	13		16	17		18	30	
+	4	2		2	2		6	4	
Exfoliation margin			0.184			0.079			**0.013**
-	4	14		10	16		14	30	
+	2	1		8	3		10	4	
R status			0.146			**0.029**			**0.009**
0	2	11		10	17		12	28	
≥1	4	4		8	2		12	6	
Microscopic vascular invasion			0.354			**0.019**			**0.033**
-	3	4		7	1		10	5	
+	3	11		11	18		14	29	

PCC, perihilar cholangiocarcinoma; DCC, distal cholangiocarcinoma; gal-3-INA, intranuclear galectin-3 accumulation. Bold fonts indicate statistically significant P-values.

present study, the expression level of galectin-3 had no association with prognosis, while it was augmented in tumor cells, compared with that in adjacent normal bile duct epithelia (data not shown). Little attention has been paid to the subcellular distribution of galectin-3 in association with patient prognosis, whereas overexpression of galectin-3 has been reported to promote various functions in tumor cells, including anti-apoptosis, resistance to therapeutic agents, proliferation and migration (27-29,49).

To establish metastatic foci, tumor cells must survive certain conditions, including isolation from cell-to-cell contact or cell-to-matrix adhesion. This potential cancer cell development may be attained through the epithelial-to-mesenchymal transition (EMT) (45,50). Previously, inhibition of the kinase activity of glycogen synthase kinase-3β (GSK-3β) was shown to result in the induction of EMT through the carboxyl terminus of heat shock protein 70-interacting protein-mediated degradation of Slug (51). Galectin-3 contains a consensus sequence of GSK-3β phosphorylation (52). Nuclear import-export of galectin-3 was reported to be dependent on this phosphorylation by GSK-3β (53). Galectin-3 was also reported to be an important partner for the inactive form of GSK-3β to drive oncogenic transformation (54). By contrast, transforming growth factor-β (TGF-β) is a major inducer of EMT (55). Previously, TGF-β-induced EMT was reported to be reduced in mice deficient in galectin-3 (56). Therefore, galectin-3 may serve a role in the induction of EMT by inhibiting GSK-3β activity, resulting in tumor cell survival in lymphatic or blood vessels, where tumor cells have no contact with each other or the matrix. In the subgroup analysis conducted in the present study, recurrence (P<0.001), distant metastasis (P<0.001), R1 status (P=0.009), and microscopic vascular invasion (P=0.033) had significantly higher prevalence in patients with gal-3-INA than in those without gal-3-INA. These results support the hypothesis that gal-3-INA serves a role in EMT induction. When the presence of gal-3-INA was examined in 3 patients

with lymph node metastasis who had undergone surgery after 2006, gal-3-INA was observed in 5/6 involved nodes (88.9%) (data not shown). However, the association between the nuclear accumulation of galectin-3 and EMT induction has not yet been elucidated. Additional investigation, in the form of large-scale study and *in vitro* studies, is required to confirm this hypothesis.

The reported prognostic factors of EHCC include lymph node metastasis, number of involved nodes, surgical margin status, UICC TNM factors, UICC TNM stage, perineural invasion, adjuvant chemotherapy and comorbidity (11-26). In the present study, these factors had a statistical significance in certain univariate analyses. However, they were not able to overcome the effect of the presence of gal-3-INA. In the present study, only 23 patients undertook adjuvant chemotherapy, which did not improve the prognosis of the patients. If recent advances with gemcitabine- and/or S1-based chemotherapy were applied to patients with gal-3-INA, the OS and DFS may have been improved. Therefore, gal-3-INA may become one of the biomarkers that indicates the necessity of adjuvant chemotherapy.

The present study had certain limitations. Firstly, the authors recognize that this is a retrospective and small study. Furthermore, the present study includes a number of patients (n=14) whose observation period following surgery had not yet surpassed 5 years. Of these 14 patients, 7 patients were living without any recurrence (observation period: 1.1, 1.9, 2.3, 2.4, 2.8, 2.8 and 2.8 years, respectively). Since EHCC is known to recur even after >5 years, these patients must be carefully followed up. However, in the analysis of the DFS of 44 patients observed for >5 years, gal-3-INA was observed to be an independent prognostic factor by multivariate analysis (HR, 3.088; 95% CI, 1.246-7.651, P=0.03).

In conclusion, the presence of gal-3-INA is a prognostic factor for patients with DCC. This performs a role in developing metastatic foci, resulting in poor prognosis. Elucidating the mechanisms of the translocation of galectin-3 into the nucleus may improve the prognosis of patients with DCC.

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