

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

TATSUO SHIMURA¹, YASUhide KOFUNATO¹, RYO OKADA¹, REI YASHIMA¹,
YOSHIHISA KOYAMA¹, KENICHIRO ARAKI², HIROYUKI KUWANO² and SEIICHI TAKENOSHITA¹

¹Department of Organ Regulatory Surgery, Fukushima Medical University, Fukushima 960-1295;

²Department of General Surgical Science, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

Received February 5, 2016; Accepted March 9, 2017

DOI: 10.3892/ol.2017.6252

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.

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

Correspondence to: Professor Tatsuo Shimura, Department of Organ Regulatory Surgery, Fukushima Medical University, 1 Hikarigaoka, Fukushima 960-1295, Japan
E-mail: tshimura@fmu.ac.jp

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-3 β , glycogen synthase kinase-3 β

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

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 tegafur/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% H₂O₂ 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 \pm SD (range), years	65.8 \pm 7.9 (36-86)	65.9 \pm 7.9 (52-78)	65.7 \pm 10.7 (36-86)
Sex			
Male	39	15	24
Female	19	6	13
Age, years			
<75	49	18	31
\geq 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.

Category	Total (n=58)						PCC (n=21)						DCC (n=37)					
	Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
	HR	CI	P-value	HR	CI	P-value	HR	CI	P-value	HR	CI	P-value	HR	CI	P-value	HR	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	0.470-14.411	<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.

Category	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
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.

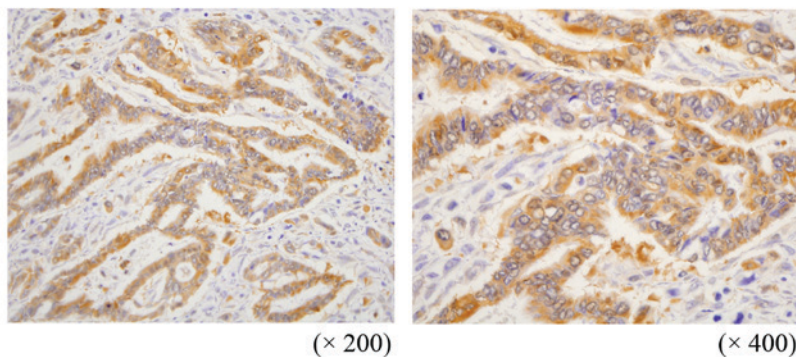
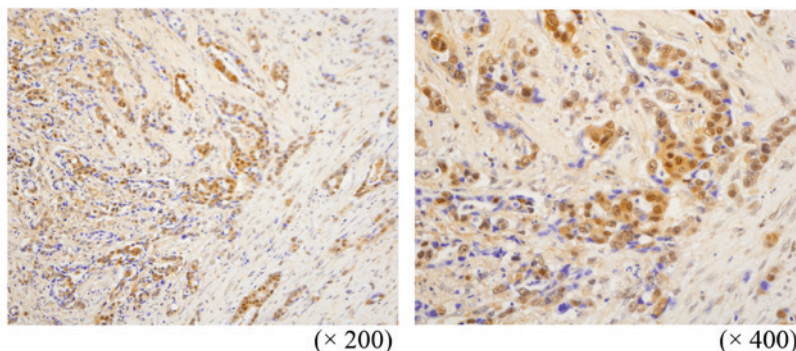
A Expression of galectin-3 without nuclear accumulation**B** Intracellular accumulation of galectin-3

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, intracellular accumulation of galectin-3.

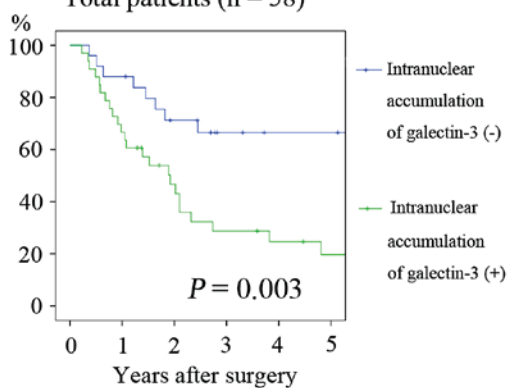
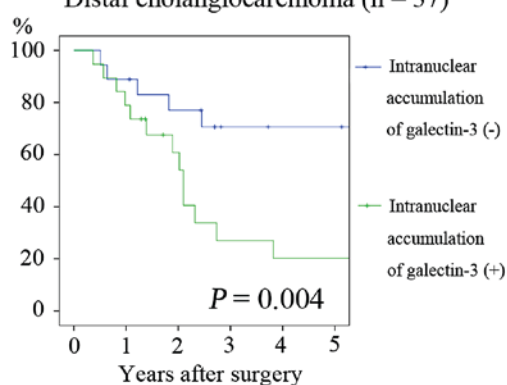
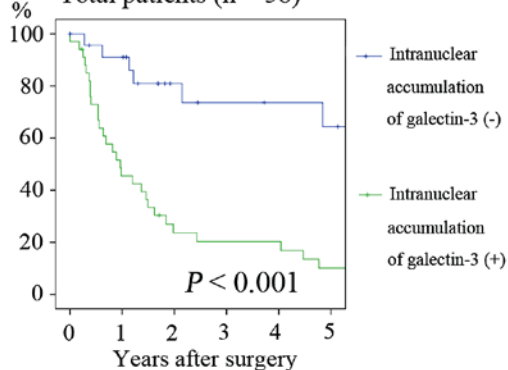
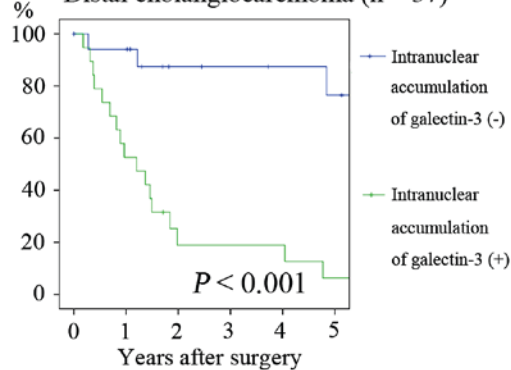
A Overall survival:
Total patients (n = 58)**B** Overall survival:
Distal cholangiocarcinoma (n = 37)**C** Disease-free survival:
Total patients (n = 58)**D** Disease-free survival:
Distal cholangiocarcinoma (n = 37)

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, intracellular accumulation of galectin-3; DCC, distal cholangiocarcinoma.

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

Variable	PCC (n=21)			DCC (n=37)			Total (n=58)		
	gal-3-INA			gal-3-INA			gal-3-INA		
	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-3 β (GSK-3 β) 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-3 β phosphorylation (52). Nuclear import-export of galectin-3 was reported to be dependent on this phosphorylation by GSK-3 β (53). Galectin-3 was also reported to be an important partner for the inactive form of GSK-3 β 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-3 β 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.

Acknowledgements

The authors thank Mr Katsuharu Saito (laboratory technician of the Department of Organ Regulatory Surgery, Fukushima Medical University, Fukushima Japan) for performing immunohistochemical staining and for his assistance in capturing images.

References

- Khan SA, Thomas HC, Davidson BR and Tayler-Robinson SD: Cholangiocarcinoma. *Lancet* 366: 1303-1314, 2005.
- Katanoda K, Hori M, Matsuda T, Shibata A, Nishino Y, Hattori M, Soda M, Ioka A, Sobue T and Nishimoto H: An updated report on the trends in cancer incidence and mortality in Japan, 1958-2013. *Jpn J Clin Oncol* 45: 390-401, 2015.
- Nakeeb A, Pitt HA, Sohn TA, Coleman J, Abrams RA, Piantadosi S, Hruban RH, Lillemoe KD, Yeo CJ and Cameron JL: Cholangiocarcinoma. A spectrum of intrahepatic, perihilar, and distal tumors. *Ann Surg* 224: 463-475, 1996.
- Dinant S, Gerhards MF, Rauws EA, Busch OR, Gouma DJ and van Gulik TM: Improved outcome of resection of hilar cholangiocarcinoma (Klatskin tumor). *Ann Surg Oncol* 13: 872-880, 2006.
- DeOliveira ML, Cunningham SC, Cameron JL, Kamangar F, Winter JM, Lillemoe KD, Choti MA, Yeo CJ and Schulick RD: Cholangiocarcinoma: Thirty-one-year experience with 564 patients at a single institution. *Ann Surg* 245: 755-762, 2007.
- Seyama Y, Kubota K, Sano K, Noie T, Takayama T, Kosuge T and Makuuchi M: Long-term outcome of extended hemihepatectomy for hilar bile duct cancer with no mortality and high survival rate. *Ann Surg* 238: 73-83, 2003.
- Neuhaus P, Jonas S, Settmacher U, Thelen A, Benckert C, Lopez-Hänninen E and Hintze RE: Surgical management of proximal bile duct cancer: Extended right lobe resection increases respectability and radicality. *Langenbecks Arch Surg* 388: 194-200, 2003.
- Kawasaki S, Imamura H, Kobayashi A, Noike T, Miwa S and Miyagawa S: Results of surgical resection for patients with hilar bile duct cancer: Application of extended hepatectomy after biliary drainage and hemihepatic portal vein embolization. *Ann Surg* 238: 84-92, 2003.
- Murakami Y, Uemura K, Sudo T, Hayashidani Y, Nakamura H, Nakashima A and Sueda T: Gemcitabine-based adjuvant chemotherapy improves survival after aggressive surgery for hilar cholangiocarcinoma. *J Gastrointest Surg* 13: 1470-1479, 2009.
- Hirano S, Kondo S, Tanaka E, Shichinohe T, Tsuchikawa T, Kato K, Matsumoto J and Kawasaki R: Outcome of surgical treatment of hilar cholangiocarcinoma: A special reference to postoperative morbidity and mortality. *J Hepatobiliary Pancreat Sci* 17: 455-462, 2010.
- Sakamoto Y, Kosuge T, Shimada K, Sano T, Ojima H, Yamamoto J, Yamasaki S, Takayama T and Makuuchi M: Prognostic factors of surgical resection in middle and distal bile duct cancer: An analysis of 55 patients concerning the significance of ductal and radial margins. *Surgery* 137: 396-402, 2005.
- Murakami Y, Uemura K, Hayashidani Y, Sudo T, Hashimoto T, Ohge H and Sueda T: Prognostic significance of lymph node metastasis and surgical margin status for distal cholangiocarcinoma. *J Surg Oncol* 95: 207-212, 2007.
- Kiriya M, Ebata T, Aoba T, Kaneoka Y, Arai T, Shimizu Y and Nagino M: Nagoya Surgical Oncology Group: Prognostic impact of lymph node metastasis in distal cholangiocarcinoma. *Br J Surg* 102: 399-406, 2015.
- Kim HJ, Kim CY, Hur YH, Koh YS, Kim JC, Kim HJ and Cho CK: The prognostic factors for survival after curative resection of distal cholangiocarcinoma: Perineural invasion and lymphovascular invasion. *Surg Today* 44: 1879-1886, 2014.
- Sasaki R, Takahashi M, Funato O, Nitta H, Murakami M, Kawamura H, Suto T, Kanno S and Saito K: Prognostic significance of lymph node involvement in middle and distal bile duct cancer. *Surgery* 129: 677-683, 2001.
- Yoshida T, Matsumoto T, Sasaki A, Morii Y, Aramaki M and Kitano S: Prognostic factors after pancreatoduodenectomy with extended lymphadenectomy for distal bile duct cancer. *Arch Surg* 137: 69-73, 2002.
- Hong SM, Cho H, Lee OJ and Ro JY: The number of metastatic lymph nodes in extrahepatic bile duct carcinoma as a prognostic factor. *Am J Surg Pathol* 29: 1177-1183, 2005.
- Murakami Y, Uemura K, Hayashidani Y, Sudo T, Ohge H and Sueda T: Pancreatoduodenectomy for distal cholangiocarcinoma: Prognostic impact of lymph node metastasis. *World J Surg* 31: 337-344, 2007.
- Kawai M, Tani M, Kobayashi Y, Tsuji T, Tabuse K, Horiuchi T, Oka M, Yamaguchi K, Sakata Y, Shimomura T and Yamaue H: The ratio between metastatic and examined lymph nodes is an independent prognostic factor for patients with resectable middle and distal bile duct carcinoma. *Am J Surg* 199: 447-452, 2010.
- Tamandl D, Kanczicek K, Gruenberger B, Koelblinger C, Maresch J, Jakesz R and Gruenberger T: Lymph node ratio after curative surgery for intrahepatic cholangiocarcinoma. *Br J Surg* 96: 919-925, 2009.
- Aoba T, Ebata T, Yokoyama Y, Igami T, Sugawara G, Takahashi Y, Nimura Y and Nagino M: Assessment of nodal status for perihilar cholangiocarcinoma: Location, number, or ratio of involved nodes. *Ann Surg* 257: 718-725, 2013.
- Murakami Y, Uemura K, Sudo T, Hashimoto Y, Nakashima A, Kondo N, Sakabe R, Ohge H and Sueda T: Prognostic factors after surgical resection for intrahepatic, hilar, and distal cholangiocarcinoma. *Ann Surg Oncol* 18: 651-658, 2011.
- Kwon HJ, Kim SG, Chun JM, Lee WK and Hwang YJ: Prognostic factors in patients with middle and distal bile duct cancers. *World J Gastroenterol* 20: 6658-6665, 2014.
- Nagahashi M, Shirai Y, Wakai T, Sakata J, Ajioka Y, Nomura T, Tsuchiya Y and Hatakeyama K: Depth of invasion determines the postresectional prognosis for patients with T1 extrahepatic cholangiocarcinoma. *Cancer* 116: 400-405, 2010.

25. Kimura N, Toyoki Y, Ishido K, Kudo D, Yakoshi Y, Tsutsumi S, Miura T, Wakiya T and Hakamada K: Perioperative blood transfusion as a poor prognostic factor after aggressive surgical resection for hilar cholangiocarcinoma. *J Gastrointest Surg* 19: 866-879, 2015.
26. Fernández-Ruiz M, Guerra-Vales JM and Colina-Ruizdelgado F: Comorbidity negatively influences prognosis in patients with extrahepatic cholangiocarcinoma. *World J Gastroenterol* 15: 5279-5286, 2009.
27. Akahani S, Nangia-Makker P, Inohara H, Kim HR and Raz A: Galectin-3: A novel antiapoptotic molecule with a functional BHI (NWGR) domain of Bcl-2 family. *Cancer Res* 57: 5272-5276, 1997.
28. Danguy A, Camby I and Kiss R: Galectins and cancer. *Biochem Biophys Acta* 1572: 285-293, 2002.
29. Davidson PJ, Davis MJ, Patterson RJ, Ripoché MA, Poirier F and Wang JL: Shuttling of galectin-3 between the nucleus and cytoplasm. *Glycobiology* 12: 329-337, 2002.
30. Lin HM, Pestell RG, Raz A and Kim HR: Galectin-3 enhances cyclin D(1) promoter activity through SP1 and a cAMP-responsive element in human breast epithelial cells. *Oncogene* 21: 8001-8010, 2002.
31. Kim MK, Sung CO, Do IG, Jeon HK, Song TJ, Park HS, Lee YY, Kim BG, Lee JW and Bae DS: Overexpression of galectin-3 and its clinical significance in ovarian carcinoma. *Int J Clin Oncol* 16: 352-358, 2011.
32. Acikalin MF, Etiz D, Gurbuz MK, Ozudogru E, Canaz F and Colak E: Prognostic significance of galectin-3 and cyclin D1 expression in undifferentiated nasopharyngeal carcinoma. *Med Oncol* 29: 742-749, 2012.
33. Brown ER, Doig T, Anderson N, Brenn T, Doherty V, Xu Y, Bartlett JM, Smyth JF and Melton DW: Association of galectin-3 expression with melanoma progression and prognosis. *Eur J Cancer* 48: 865-874, 2012.
34. Yang LP, Jiang S, Liu JQ, Miao XY and Yang ZL: Up-regulation of galectin-3 and sambucus nigra agglutinin binding site is associated with invasion, metastasis and poor-progression of the gallbladder adenocarcinoma. *Hepatogastroenterology* 59: 2089-2094, 2012.
35. Zhou X, Jing J, Peng J, Mao W, Zheng Y, Wang D, Wang X, Liu Z and Zhang X: Expression and clinical significance of galectin-3 in osteosarcoma. *Gene* 546: 403-407, 2014.
36. Jiang SS, Weng DS, Wang QJ, Pan K, Zhang YJ, Li YQ, Li JJ, Zhao JJ, He J, Lv L, *et al*: Galectin-3 is associated with a poor prognosis in primary hepatocellular carcinoma. *J Transl Med* 12: 273, 2014.
37. Shimamura T, Sakamoto M, Ino Y, Shimada K, Kosuge T, Sato Y, Tanaka K, Sekihara H and Hirohashi S: Clinicopathological significance of galectin-3 expression in ductal adenocarcinoma of the pancreas. *Clin Cancer Res* 8: 2570-2575, 2002.
38. Piantelli M, Iacobelli S, Almadori G, Iezzi M, Tinari N, Natoli C, Cadoni G, Lauriola L and Ranelletti FO: Lack of expression of galectin-3 is associated with a poor outcome in node-negative patients with laryngeal squamous-cell carcinoma. *J Clin Oncol* 20: 3850-3856, 2002.
39. Okada K, Shimura T, Suehiro T, Mochiki E and Kuwano H: Reduced galectin-3 expression is an indicator of favorable prognosis in gastric cancer. *Anticancer Res* 26: 1369-1376, 2006.
40. Merseburger AS, Kramer MW, Hennenlotter J, Serth J, Kruck S, Gracia A, Stenzl A and Kuczyk MA: Loss of galectin-3 expression correlates with clear cell renal carcinoma progression and reduced survival. *World J Urol* 26: 637-642, 2008.
41. Yamaki S, Fujii T, Yajima R, Hirakata T, Yamaguchi S, Fujisawa T, Tsutsumi S, Asao T, Yanagita Y, Iijima M and Kuwano H: Clinicopathological significance of decreased galectin-3 expression and the long-term prognosis in patients with breast cancer. *Surg Today* 43: 901-905, 2013.
42. Shimonishi T, Miyazaki K, Kono N, Sabit H, Tuneyama K, Harada K, Hirabayashi J, Kasai K and Nakanuma Y: Expression of endogenous galectin-1 and galectin-3 in intrahepatic cholangiocarcinoma. *Hum Pathol* 32: 302-310, 2001.
43. Junking M, Wongkham C, Sripa B, Sawanyawisuth K, Araki N and Wongkham S: Decreased expression of galectin-3 is associated with metastatic potential of liver fluke-associated cholangiocarcinoma. *Eur J Cancer* 44: 619-626, 2008.
44. Wongkham S, Junking M, Wongkham C, Sripa B, Chur-In S and Araki N: Suppression of galectin-3 expression enhances apoptosis and chemosensitivity in liver fluke-associated cholangiocarcinoma. *Cancer Sci* 100: 2077-2084, 2009.
45. Simpson CD, Anyiwe K and Schimmer AD: Anoikis resistance and tumor metastasis. *Cancer Lett* 272: 177-185, 2008.
46. Sobin LH, Gospodarowicz MK and Wittekind C: Union for International Cancer Control (UICC): TNM classification of malignant tumors. 7th edition. Wiley-Blackwell, New York, 2010.
47. van Domberg R, Hoeks S, Kardys I, Lenzen M and Boersma E: Tools and techniques-statistics: How many variables are allowed in the logistic and Cox regression models? *EuroIntervention* 9: 1472-1473, 2014.
48. Thijssen VL, Heusschen R, Caers J and Griffioen AW: Galectin expression in cancer diagnosis and prognosis: A systematic review. *Biochim Biophys Acta* 1855: 235-247, 2015.
49. Kobayashi T, Shimura T, Yajima T, Kubo N, Araki K, Wada W, Tsutsumi S, Suzuki H, Kuwano H and Raz A: Transient silencing of galectin-3 expression promotes both in vitro and in vivo drug-induced apoptosis of human pancreatic carcinoma cells. *Clin Exp Metastasis* 28: 367-376, 2011.
50. Cao Z, Livas T and Kyprianou N: Anoikis and EMT: Lethal 'liaisons' during cancer progression. *Crit Rev Oncog* 21: 155-168, 2016.
51. Kao SH, Wang WL, Chen CY, Chang YL, Wu YY, Wang YT, Wang SP, Nesvizhskii AI, Chen YJ, Hong TM and Yang PC: GSK3 β controls epithelial-mesenchymal transition and tumor metastasis by CHIP-mediated degradation of Slug. *Oncogene* 33: 3172-3182, 2014.
52. Shimura T, Takenaka Y, Fukumori T, Tsutsumi S, Okada K, Hogan V, Kuwano H and Raz A: Implication of galectin-3 in Wnt signaling. *Cancer Res* 65: 3535-3537, 2005.
53. Song S, Mazurek N, Liu C, Sun Y, Ding QQ, Liu K, Hung MC and Bresalier RS: Galectin-3 mediates nuclear β -catenin accumulation and Wnt signaling in human colon cancer cells by regulation of glycogen synthase kinase-3 β activity. *Cancer Res* 69: 1343-1349, 2009.
54. Mendonça DF, Chammas R, Liu FT, Nonogaki S, Cardoso SV, Loyola AM and de Faria PR: The inactive form of glycogen synthase-3 β is associated with the development of carcinoma in galectin-3 wild-type mice, but not in galectin-3-deficient mice. *Int J Clin Exp Pathol* 5: 547-554, 2012.
55. Katsuno Y, Lamouille S and Derynck R: TGF- β signaling and epithelial-mesenchymal transition in cancer progression. *Curr Opin Oncol* 25: 76-84, 2013.
56. MacKinnon AC, Gibbons MA, Farnworth SL, Leffler H, Nilsson UJ, Delaine T, Simpson AJ, Forbes SJ, Hirani N, Gaudie J and Sethi T: Regulation of transforming growth factor- β 1-driven lung fibrosis by galectin-3. *Am J Respir Crit Care Med* 185: 537-546, 2012.