available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Decreased expression of galectin-3 is associated with metastatic potential of liver fluke-associated cholangiocarcinoma

Mutita Junking[a,c], *Chaisiri Wongkham*[a,c], *Banchob Sripa*[b,c], *Kanlayanee Sawanyawisuth*[a,c], *Norie Araki*[d], *Sopit Wongkham*[a,c,*]

[a]*Department of Biochemistry, Faculty of Medicine, Khon Kaen University, 123 Mitraparb Road, Muang, Khon Kaen 40002, Thailand*
[b]*Department of Pathology, Faculty of Medicine, Khon Kaen University, Thailand*
[c]*Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand*
[d]*Department of Tumor Genetics and Biology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan*

ARTICLE INFO

Article history:
Received 11 November 2007
Received in revised form
5 January 2008
Accepted 8 January 2008
Available online 12 February 2008

Keywords:
Bile duct
Cholangiocarcinoma
Galectin
Galectin-3
LGALS3
Liver fluke
Metastasis
Migration

ABSTRACT

Galectin-3, a β-galactoside-binding lectin, is a multifunctional protein implicated in a variety of biological functions, including tumour cell adhesion, proliferation, differentiation, cancer progression and metastasis. The present study was performed to clarify the impact of galectin-3 expression on patients with liver fluke-associated cholangiocarcinoma. Galectin-3 expression was examined immunohistochemically in 53 patients with intrahepatic cholangiocarcinoma, who had undergone surgery without pre-operative therapy. All bile duct epithelium expressed galectin-3 with different intensities, according to the different histological subtypes. The poorly-differentiated type expressed galectin-3 less intensely than the papillary, well- to moderately-differentiated types ($P = 0.012$). We observed the association of low galectin-3 expression with lymphatic invasion ($P = 0.002$). Suppression of galectin-3 expression in two human cholangiocarcinoma cell lines using siRNA targeted to galectin-3 significantly increased cell migration and invasion without alterations in cell proliferation. Regulation of galectin-3 expression may therefore be an alternative therapeutic approach to control metastasis of cholangiocarcinoma.

1. Introduction

Galectins are a family of carbohydrate-binding proteins that bind β-galactoside moieties with high affinity and specificity. To date, 16 members of the galectin family have been identified,[1] of which galectin-3 (Gal-3) is one of the most frequently reported on, due to its involvement in cancer progression and metastasis.[2] Gal-3 is a multifunctional protein implicated in a variety of biological functions, including tumour cell adhesion, proliferation, differentiation, angiogenesis, cancer progression and metastasis.[3]

Gal-3 is a chimeric gene product consisting of three distinct structural domains, including a short amino-terminal domain which controls its cellular targeting, a repetitive, collagen-like sequence which serves as a substrate for the metalloproteinase matrix and a carboxy-terminal domain containing a carbohydrate-binding site.[4] Thus, the pleiotropic biological functions of Gal-3 depend on its subcellular

* *Corresponding author:* Address: Department of Biochemistry, Faculty of Medicine, Khon Kaen University, 123 Mitraparb Road, Muang, Khon Kaen 40002, Thailand. Tel.: +66 43 348 386; fax: +66 43 348 375.
E-mail address: sopit@kku.ac.th (S. Wongkham).
0959-8049/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2008.01.014

location. For example, extracellular Gal-3 has been implicated in cell migration, adhesion and cell–cell interactions,[5] whereas intracellular Gal-3 exhibits anti-apoptotic activity.[6,7] Gal-3 is also found in the nucleus as a nuclear matrix protein involved in pre-mRNA splicing[8] and it may regulate gene expression at the transcription level.[7,9]

Cholangiocarcinoma (CCA) is a malignancy of bile duct epithelia and the most common liver cancer amongst both males and females in Northeast Thailand.[10] The incidence of CCA has also been increasing in industrialised countries.[11] The established risk factors for CCA in the West include primary sclerosing cholangitis, Caroli's disease, congenital choledochal cysts and chronic intrahepatic lithiasis,[11] whereas liver fluke (*Opisthorchis viverrini*) infection is the major risk factor in Southeast Asia.[10] The strong association between the occurrence of CCA and of liver fluke infection has been demonstrated both in experimental animal[12] and in epidemiological studies.[13–15] The mechanism by which liver fluke infection contributes to the carcinogenesis of CCA is not well understood. However, the bile duct injury resulting from liver fluke inhabitation, and the immunopathologic processes due to host immune responses to that inhabitation are suspected to be the main causes of carcinogenesis in liver fluke-associated CCA.[10]

CCA is a highly invasive/metastasising malignancy that is difficult to be diagnosed until the advanced or disseminated stage, resulting in poor prognosis. A number of studies have examined the prognostic factors of CCA; however, the exact mechanism underlying the aggressiveness of this cancer has not been clarified. We recently established a serial analysis of gene expression (SAGE) of the primary and corresponding metastatic tumours from a Thai male with CCA (http://cgap.nci.nih.gov). Using a SAGE Digital Gene Expression Displayer, we found a differentially low expression of Gal-3 (LGALS3; SAGE tag: TTCACTGTGA) in the metastatic tumour versus the primary tumour. This is interesting since evidence indicates a correlation of Gal-3 expression to neoplastic progression in various cancers.[16,17]

Even though there is substantial evidence regarding Gal-3 expression in cancer, it is yet to be determined as to how Gal-3 functions in cancer. Nevertheless, some common characteristics of Gal-3-expressing cancer cells, such as uncontrolled proliferation, disturbed adhesiveness and resistance to apoptosis, have been reported. The relevance of these observations to the mechanistic role of Gal-3 in the carcinogenesis and progression of CCA has not been examined. Herein, however, we present evidence that down-regulation of Gal-3 in CCA tissue is associated with poor differentiation of cells and lymphatic invasion. In addition, we further explore the clinicopathological significance of low Gal-3 expression in CCA.

2. Materials and methods

2.1. Human CCA specimens

The 53 paraffin-embedded liver tissues from the primary tumours of intrahepatic CCA patients were obtained from the specimen bank of the Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Thailand. Our research protocols were approved by the Human Research Ethics Committee, Khon Kaen University (#HE471214) and informed consent was obtained from each subject before surgery.

All CCA subjects were hepatectomised and the operative procedures for all patients were intended to cure. Pre-operative diagnosis and staging of CCA patients were evaluated from clinical features, chest X-ray, ultrasonography, computed tomography (CT) and magnetic resonance cholangiopancreatography (MRCP). The age, sex, tumour location, tumour size, histological grading and pTNM stage[18] were evaluated from the medical charts and pathologic records. Normal, hyper- and dys-plastic biliary epithelia were examined from non-cancerous portions of CCA liver tissues.

2.2. Immunohistochemistry

Gal-3 was detected on the formalin-fixed, paraffin-embedded sections using standard immunohistochemistry protocols. Specifically, the paraffin sections were deparaffinised, then hydrated, and the endogenous peroxidase was blocked with H_2O_2. After blocking with normal horse serum, the sections were incubated with 1:1500 mouse anti-Gal-3 (Chemicon, Temecula, CA, United States) at 4 °C overnight, followed by 1:200 goat antimouse IgG (H + L) (Zymed Laboratories, San Francisco, CA, USA). Peroxidase activity was observed using diaminobenzidine tetrahydroxychloride solution (DAB, Dako, Glostrup, Denmark) as the substrate. The sections were counterstained with haematoxylin. The positive staining was eliminated when phosphate-buffered saline was applied instead of the primary antibody. Colon tissues were used as the positive control.

The slides were evaluated independently by two investigators who had no prior knowledge of the clinicopathological and survival data. The frequency of Gal-3 positive cells was semi-quantitatively scored on the basis of the percentage of positive cells as 0% = negative; 1–25% = +1; 26–50% = +2; and >50% = +3. The intensity of Gal-3 expression was scored as weak = 1, moderate = 2 and strong = 3. The average Gal-3 expression of each section was calculated as intensity multiplied by frequency and categorised as low ($\leqslant$2) or high (>2).

2.3. Cell lines and culture condition

Two respective CCA cell lines, KKU-100 and KKU-M214, were established as described by Sripa and colleagues.[19] KKU-100 was established from primary tumour of a 65-year-old Thai woman with CCA of porta-hepatis-poorly-differentiated tubular adenocarcinoma, whereas KKU-M214 was from the primary tumour of a 52-year-old Thai male with well-differentiated type-mass forming CCA. CCA cells were cultured in HAM-F12 (Life Technologies, Rockville, MD, USA) supplemented with 10% w/v foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C and 5% CO_2.

2.4. Transient knockdown of Gal-3 using siRNA

CCA cells (8×10^4 cells in 2 ml complete culture medium) were seeded into a 6-well plate for 24 h before transfection. The siRNA specific for human *Gal-3* derived from the mRNA

sequence (5′-GGTGCCTCGCATGCTGATAAC-3′) of human *Gal*-3 (siGal-3) was obtained from JbioS Inc., Saitama, Japan. The siGal-3 was dissolved in annealing buffer, reheated to 95 °C for 1 min and then incubated for 1 h at 37 °C, following the protocol described previously.[20] Transfection of CCA cells with 100 pM siGal-3 was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. As a mock control, cells were treated with (2 μg) Lipofectamine 2000 under identical conditions. After siRNA transfection, the plates were incubated at 37 °C for 24–72 h for further analysis.

2.5. Protein extraction and Western blot analysis

Total cell lysate extractions and Western blots were performed as previously described.[21] The blots were then probed with 1:10,000 anti-Gal-3 antibody (Chemicon, Temecula, CA, USA) or with 1:5000 anti-β actin antibody (Sigma–Aldrich, St. Louis, MO, USA) as an internal control. The immuno-reactive proteins were visualised by Western Lightning Chemiluminescence Reagents (PerkinElmer Ltd., Boston, MA, USA). Quantitative analysis of Gal-3/β-actin expression was performed using Gel-Pro analyser (Media Cybernetics, Silver Spring, MD, USA).

2.6. Cell proliferation assay

CCA cells (10^4 cells/100 μl) in a culture medium were seeded into a 96-well plate and incubated at 37 °C, 5% CO_2. The number of cells was determined using sulforhodamine B (SRB) assay at 24-h intervals for 3 d, as described previously.[21] Briefly, cells were fixed in 10% cold trichloroacetic acid for 1 h at 4 °C and stained with 0.4% w/v SRB in 1% v/v acetic acid for 30 min. After washing the excess dye with 1% acetic acid, the SRB stained cells were solubilised with 200 μl of 10 mM unbuffered Tris–base solution and the absorbance was measured at 540 nm.

2.7. Migration and invasion assay

Migration and invasion were determined using Transwell® (8 μm pore size, Corning Inc., NY, USA). Briefly, 4×10^4 of CCA cells or 48 h siGal-3 transfected cells in serum-free HAM-F12 medium were seeded into each of the upper chamber and aliquots of HAM-F12 supplemented with 10% w/v foetal calf serum were placed in the lower compartment of the chamber. After incubation at 37 °C for 12 h for the KKU-100 or 10 h for the KKU-M214, cells in the upper surface of the filter were scraped off using a cotton swab. Cells, which migrated to the underside of the filter, were fixed with 25% v/v methanol for 15 min and stained with 0.5% w/v crystal violet in 25% v/v methanol. The number of migrated cells was counted under a microscope by an independent investigator. Mean values of nine low-power fields (100× magnification) were determined. Assays were done in triplicate and two independent experiments were repeated.

For invasion assay, 4×10^4 CCA cells or siGal-3 transfected cells in complete medium were seeded into each of the 0.3-mg, pre-coated Matrigel-culture inserts (Becton-Dickinson, San Jose, CA, USA). The chamber was incubated at 37 °C, 5% CO_2, 20 h for the KKU-100 or 18 h for the KKU-M214. The number of invaded cells was determined in the same way as stated for the migration assay.

2.8. Statistical analysis

Statistical analyses were performed using Sigma Stat version 3.1 software (Systat Software UK Limited., London, United Kingdom). Expression of Gal-3 was assessed for association with various clinical and pathological parameters using the χ^2-test. Patient survival was calculated from the time of surgical resection to death and the survival curves were calculated according to Kaplan–Meier, with a log-rank test. Results from migration and invasion experiments are presented as means ± SD and the significance of the differences was addressed by Student's *t*-test. A *P*-value <0.05 was considered statistically significant.

3. Results

Of the 53 CCA patients studied, 74% were male, resulting in a male to female ratio of 2.8: 1. In this series, advanced (IVA and IVB) (88%) and intrahepatic mass-forming CCA (68%) predominated. The common metastatic sites of CCA patients in this study were diaphragm, omentum and gallbladder.

3.1. Immunohistochemical expression of Gal-3 in CCA

Immunohistochemical analysis, with anti-Gal-3 antibody of normal, hyper/dys-plastic and tumour tissues of bile duct epithelium of CCA patients, showed diffuse and strong cytoplasmic immuno-reactivity. Intraluminal macrophages but not hepatocytes were also positive for Gal-3 (Fig. 1A). The majority of bile duct epithelia exhibited cytoplasmic Gal-3, whereas only 14 of 53 cases (26.4%) had cytoplasmic with weak nuclear immuno-reactivity. Strong Gal-3 expression was observed in normal bile duct epithelium (Fig. 1A) and moderately- and well-differentiated malignant bile ducts (Fig. 1B and C). Significantly, lower Gal-3 expression was found in the poorly-differentiated type of CCA ($P = 0.012$) and was associated with lymphatic invasion ($P = 0.002$) (Fig. 1D, thin arrow) (Table 1). We did not find any correlation between the expression level or localisation of Gal-3 and other clinical and pathological findings, *e.g.* tumour staging, tumour size, vascular and neural invasions, level of serum liver enzymes or jaundice (total bilirubin >2 mg/dl).

Cumulative survival was compared amongst CCA patients with low and high expression of Gal-3. Patients with survival under 30 d were labelled 'peri-operative death' ($n = 2$) and excluded from the analysis. The median follow-up duration of patients in this study was 224 d. All patients died by the end of the follow-up period. The median survival time was 324 d (95% confidence interval (CI), 172–476 d) for CCA patients with low Gal-3 expression and 557 d (95% CI, 274–839 d) for those with high Gal-3 expression. However, we did not find a significant difference in the survival times of CCA patients with low and high Gal-3 expression (log-rank, $P = 0.281$) (Fig. 2). Multivariate Cox regression analysis revealed that tumour staging was an independent prognostic indicator for the overall survival of intrahepatic CCA patients after hepatectomy

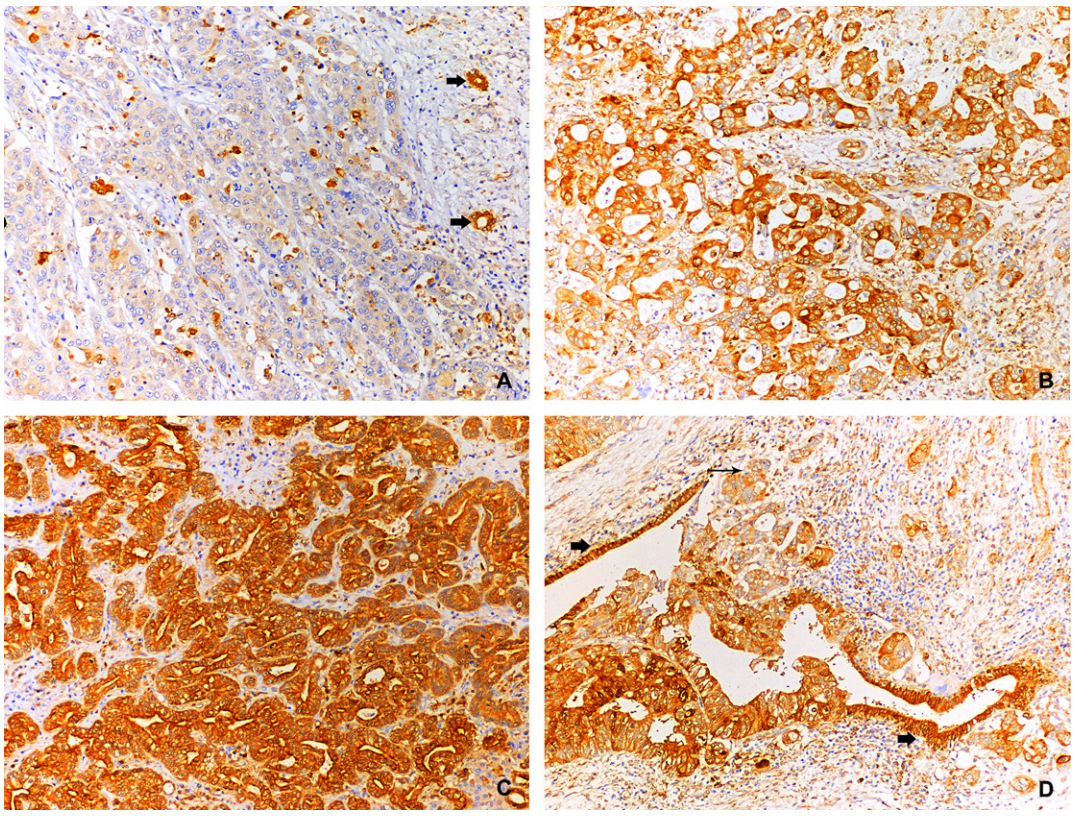

Fig. 1 – Immunohistochemistry of Gal-3 in CCA tissues. Normal bile ducts (thick arrows) and intraluminal macrophages (A), moderately- (B), and well-differentiated CCA (C) show strong expression of Gal-3. Lymphatic metastatic CCA (thin arrow) shows less Gal-3 staining compared to the normal bile ducts (thick arrow) (D). Original magnification, 100×.

(Table 2). Patients with stage IVA had better prognosis than those with stage IVB.

3.2. *Specific knockdown of Gal-3 enhances cell motility and invasion*

To address the functional importance of Gal-3, we employed RNAi to deplete the expression of Gal-3 in two CCA cell lines (*viz.*, KKU-100 and KKU-M214). β-Actin was detected to assess the specificity of siGal-3 and the equality of protein loading in the immunoblotting analysis. The optimal level of siGal-3 (100 pM) was transfected into the cells to minimise potential off-target effects of siRNA, as shown by the expression of β-actin. As determined by Western blotting with Gal-3 antibody, cells treated with siRNA targeting 5′- GTTATCAGCATGCGAGG-CACC-3′ for 72 h significantly suppressed Gal-3 expression to 20% of the control (Fig. 3) without any observed change in the level of β-actin and cell phenotype. Mock transfection did not affect the expression level of Gal-3 or β-actin in CCA cell lines. This *in vitro* suppression of Gal-3 expression may mimic the low level of Gal-3 expression found in poorly-differentiated CCA.

siRNA knockdown of Gal-3 in CCA cells for 72 h did not affect cell proliferation as monitored by SRB assay. Mock or si-Gal-3 transfected cells exhibited similar proliferation rates (Fig. 4). No apoptotic cell-death characteristics were observed under a phase contrast microscope after either treatment. Thus, the subsequent investigations of cell migration and invasion were done within 72 h after transient knockdown with siGal-3.

The reduction of Gal-3 expression resulted in a significant increase over the parental controls in the number of cells which had migrated and invaded. The numbers of migrated cells in the siGal-3 treated groups (40 ± 11 for KKU-100 and 31 ± 9 for KKU-M214) were considerably higher than those of the controls (22 ± 5 for KKU-100, $P = 0.004$ and 19 ± 5 for KKU-M214, $P = 0.023$) (Fig. 5). A similar observation was made from the invasion assay. Significantly, more numbers of invaded cells were found in CCA cells treated with siGal-3 (142 ± 34 for KKU-100 and 135 ± 23 for KKU-M214) than the controls (21 ± 8 for KKU-100, $P < 0.001$ and 111 ± 18 for KKU-M214, $P = 0.004$) (Fig. 6).

4. Discussion

Although most galectins are ubiquitously expressed in various normal human tissues, in most cancers, galectins are either silenced or up-regulated.[22] Our data, obtained by evaluating Gal-3 expression in neoplastic bile duct epithelial tissues, show that this protein is generally expressed in normal, precancerous and malignant tumours. The intensity of Gal-3 immuno-reactivity, however, is different according to the histological grading subtype of CCA. Gal-3 was highly expressed in papillary, well- to moderately-differentiated carcinomas but down-regulated in poorly-differentiated tumours. Down-regulation of galectin-3 expression in

Table 1 – Pathological features of CCA patients and expressions of Gal-3 in primary tumour tissues

		Gal-3 expression		
	n	Low	High	*P*
Age				
⩽56	26	9 (34.6%)	17 (65.4%)	0.471
>56	27	13 (48.1%)	14 (51.9%)	
Sex				
Male	39	15 (38.5%)	24 (61.5%)	0.663
Female	14	7 (50.0%)	7 (50.0%)	
Staging				
II–III	6	2 (33.3%)	4 (66.7%)	0.494
IVA	8	2 (25.0%)	6 (75.0%)	
IVB	39	18 (48.7%)	21 (51.3%)	
Gross type				
Intraductal growth type	13	4 (30.8%)	9 (69.2%)	0.104
Mass-forming type	36	18 (50.0%)	18 (50.0%)	
Mixed type	4	0 (0%)	4 (100%)	
Histological grading				
Invasive papillary	13	4 (30.8%)	9 (69.2%)	0.012
Well- to moderately-differentiated	25	7 (28.0%)	18 (72.0%)	
Poorly-differentiated	9	8 (88.9%)	1 (1.1%)	
Mixed	6	3(50.0%)	3 (50.0%)	
Vascular invasion				
No	20	10 (50.0%)	10 (50.0%)	0.491
Yes	33	12 (36.4%)	21 (63.6%)	
Neural invasion				
No	32	13 (40.6%)	19 (59.4%)	0.902
Yes	21	9 (42.8%)	12 (57.2%)	
Lymphatic invasion				
No	13	0 (0%)	13 (100%)	0.002
Yes	40	22 (55.0%)	18 (45.0%)	

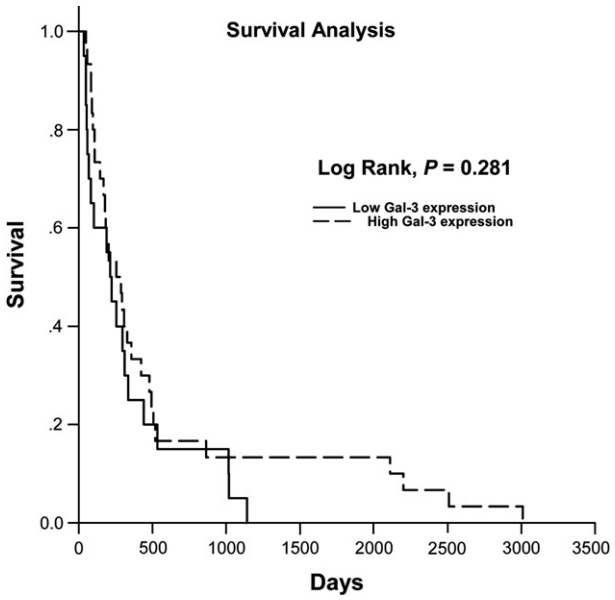

Fig. 2 – Survival curves using Kaplan–Meier method. Survival curves showed that patients with high Gal-3 expression have no significant different survival compared to those who had low Gal-3 expression (*P* = 0.281).

poorly-differentiated CCA seems to be a common phenotype of this CCA subtype since similar observation was also reported for intrahepatic CCA of Japanese patients who had no history of liver fluke infection.[23] In the present study, associations of low Gal-3 expression with poorly-differentiated CCA and lymphatic invasion were observed. These observations implied that low Gal-3 expression may correlate with unfavourable outcome since these two parameters are known to result in a poor prognosis for CCA patients.[24,25] However, the correlation of Gal-3 expression and patients' survival could not be demonstrated in the present study. This is probably due to the small number of CCA patients with low Gal-3 expression and/or with poorly-differentiated type CCA. In addition, the majority of the patients (88%) in the present study were in advanced stage with metastasis. Nevertheless, down-regulation of Gal-3 in more aggressive phenotypes has been described for CCA[23] and several human cancers, *e.g.* breast,[4] colon,[26] ovarian[27] and thyroid[28] carcinomas.

Many reports indicate multiple roles for Gal-3 in cancer pathogenesis, proliferation and metastasis; however, altering Gal-3 expression can result in increased or decreased proliferation, differentiation and metastasis in a specific way, depending on the properties of the particular cell type. The finding that down-regulation of Gal-3 expression is associated with a more aggressive type of CCA and positive lymphatic invasion prompted us to investigate the effects of Gal-3 suppression on cell growth and metastasis of CCA cell lines. Since RNA interference allows in-depth study of the molecular mechanisms of a specific gene target, we used siRNA duplexes to specifically examine the roles of Gal-3 in growth, migration and invasion of CCA cell lines *in vitro*. Our results did not support the association of Gal-3 expression and cell growth as depletion of Gal-3 by siRNA had no effect on the proliferation of the CCA cell lines studied. siRNA silencing of Gal-3 expression, however, significantly enhanced cell migration and invasion of CCA cells; thus, suppression of Gal-3 may be critical for migration and invasion of CCA.

Gal-3 functions differ depending on the site of its localisation and cell types. Expression of Gal-3 in breast carcinoma cell lines leads to rapid spread of the cells.[29] In prostate and several other cancers, however, cytosolic accumulation of Gal-3 promoted metastasis, angiogenesis and abolition of anchorage dependence, while its nuclear localisation inhibited metastasis and anchorage independence and promoted apoptosis.[26,30,31] In our study, Gal-3 was mostly found in cytoplasmic compartments of CCA tissues and can possibly be implicated in increasing cell motility and invasion. This finding is in agreement with studies on prostate cancer in which cytoplasmic Gal-3 induced significantly increased Matrigel invasion, anchorage-independent growth and *in vivo* tumour growth and angiogenesis.[30]

The role of Gal-3 in cell migration and invasion may be attributed to the cytoplasmic and extracellular Gal-3. Gal-3 can bind some cytokeratins bearing a terminal α1,3 linked *N*-acetylgalactosamine residue in cytoplasm and may modulate cell motility.[32] On the other hand, extracellular Gal-3 may exhibit numerous autocrines and paracrines which affect and mediate CCA phenotypes and functions by means of cell–cell

Table 2 – Multivariate analysis by a Cox Proportional Hazards Regression model

Variable	HR (hazard ratio)	95% confidence interval (CI)	*P*
Sex			
Female	1		
Male	1.155	0.57–2.31	0.685
Age			
>56	1		
<56	0.973	0.42–2.25	0.949
Gal-3 expression			
Low	1		
High	1.054	0.45–2.46	0.901
Gross type			
Intraductal growth type	1		
Mass forming	1.16	0.85–4.61	0.450
Mixed	1.26	0.33–4.85	0.726
Histological grading			
Invasive papillary	1		
Well- to moderately-differentiated	0.649	0.11–3.59	0.620
Poorly-differentiated	0.554	0.19–1.56	0.265
Mixed	0.792	0.24–2.59	0.701
Staging			
II–III	1		
IVA	0.214	0.05–0.78	0.020
IVB	1.09	0.38–3.07	0.867
Vascular invasion			
No	1		
Yes	0.635	0.30–1.13	0.216
Neural invasion			
No	1		
Yes	0.862	0.31–2.36	0.716
Lymphatic invasion			
No	1		
Yes	0.52	0.23–1.18	0.118

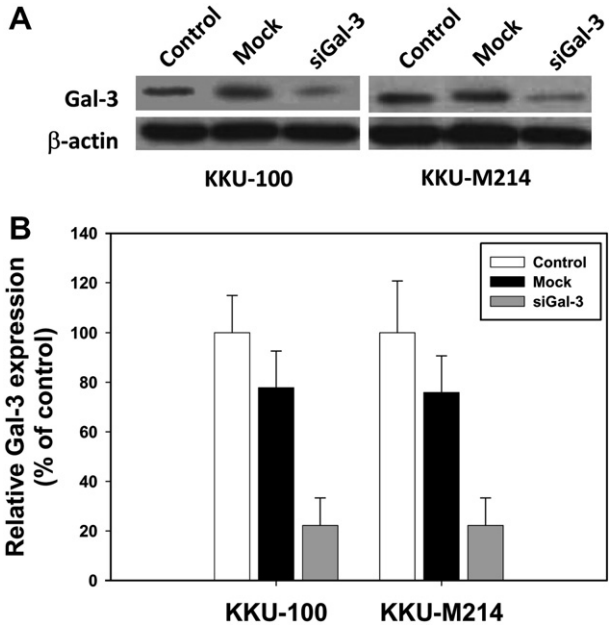

Fig. 3 – siGal-3 suppresses Gal-3 expression in CCA cell lines (*viz.*, KKU-100 and KKU-M214). (A) Expression of Gal-3 protein by immunoblotting with anti-Gal-3. (B) Relative Gal-3 expression levels (% of parental control) after siGal-3 transfection for 72 h.

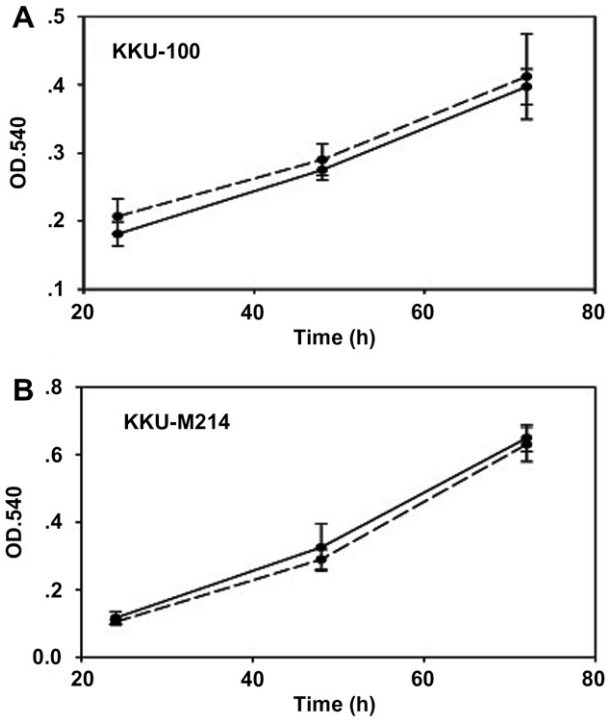

Fig. 4 – Effect of siGal-3 on cell proliferation. The number of cells was determined by SRB assay. CCA cell lines KKU-100 (A) or KKU-M214 (B) were treated with either lipofectamine (solid line) or siGal-3 (dashed line) for 24, 48 and 72 h. Each point represents averaged values from triplicates.

and cell–matrix contacts. The effect of Gal-3 as a modulator of cell adhesion is based on its multivalent properties and on its ability to bind cell surface glycoproteins and glycosylated components of the extracellular matrix. Gal-3 was shown to bind laminin, fibronectin, hensin, elastin, collagen IV and tenascin. Gal-3 also binds certain integrins ($\alpha1\beta1$ integrin and α subunit of $\alpha M\beta1$ integrin), the main membrane molecules involved in cell adhesion.[33] Gal-3 can inhibit or potentiate cell adhesion of different cell types depending on its concentration. Suppression of cellular Gal-3 expression may reduce extracellular Gal-3, minimise the interaction of cell to extracellular matrix and subsequently reduce cell adhesion, which in turn would promote cell migration, as evidenced in our study. The precise mechanism(s) by which Gal-3 regulates these processes in CCA, however, needs confirmation.

Gal-3 expression depends on a complex, fine-tuned mechanism involving numerous transcription factors and signalling pathways, which varies with cell type, external stimuli and environmental conditions. The present study demonstrates the down-regulation of Gal-3 expression in poorly-differentiated and lymphatically invasive CCA. Our data, which show that the suppression of Gal-3 expression results in sig-

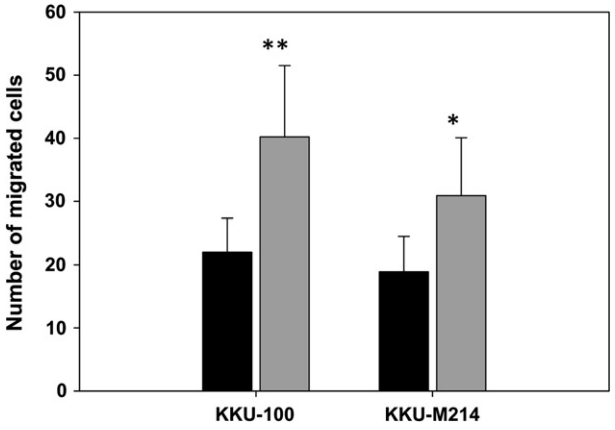

Fig. 5 – Effect of siGal-3 on cell motility. Numbers of migrated cells were compared between mock- (black bar) and siGal-3-treated CCA cells (grey bar). The data are the averaged values from triplicates and represent one of the two independent experiments. $^{*}P = 0.023$, $^{**}P = 0.004$.

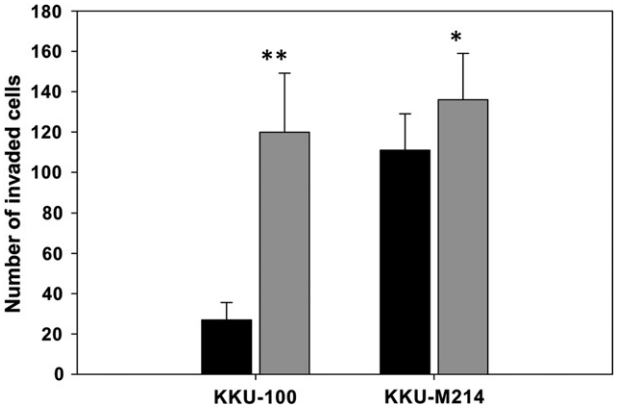

Fig. 6 – Effect of siGal-3 on cell invasion. The numbers of cells invading the Matrigel were compared between the mock- (black bar) and siGal-3-transfected CCA cells (grey bar). The data are the averaged values from triplicates and represent one of the two independent experiments. $^{*}P = 0.004$; $^{**}P < 0.001$.

nificantly enhanced cell migration and invasion *in vitro*, indicate that a decrease in Gal-3 expression may be an important factor influencing tumour progression in CCA. These results suggest that Gal-3 could be functionally involved in CCA progression and metastasis; however, further studies are necessary to detail the exact mechanism by which Gal-3 down-regulation influences the CCA phenotype.

Conflict of interest statement

None declared.

Acknowledgements

This project was co-supported by the Research Strengthening Grant 2006 from National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA); the Royal Golden Jubilee PhD Program (PHD/0140/2546 to Junking M. and Wongkham S.); and the Ministry of Education, Science and Culture of Japan (Araki N.) and JSPS-Asia Africa Science Platform Program (Khon Kaen University and Kumamoto University). We thank Mr. Bryan Roderick Hamman and Mr. Anthony Wayne Wilson for assistance with the English language presentation of this manuscript.

REFERENCES

1. Gray CA, Adelson DL, Bazer FW, Burghardt RC, Meeusen TE, Spencer TE. Discovery and characterization of an epithelial-specific galectin in the endometrium that forms crystals in the trophectoderm. *Proc Natl Acad Sci USA* 2004;**101**(21):7982–7.
2. Raz A, Zhu DG, Hogan V, et al. Evidence for the role of 34-kDa galactoside-binding lectin in transformation and metastasis. *Int J Cancer* 1990;**46**(5):871–7.
3. Castronovo V, Van Den Brule FA, Jackers P, et al. Decreased expression of galectin-3 is associated with progression of human breast cancer. *J Pathol* 1996;**179**(1):43–8.
4. Gong HC, Honjo Y, Nangia-Makker P, et al. The NH_2 terminus of galectin-3 governs cellular compartmentalization and functions in cancer cells. *Cancer Res* 1999;**59**(24):6239–45.
5. Ochieng J, Furtak V, Lukyanov P. Extracellular functions of galectin-3. *Glycoconj J* 2004;**19**(7–9):527–35.
6. Yu F, Finley Jr RL, Raz A, Kim HR. Galectin-3 translocates to the perinuclear membranes and inhibits cytochrome c release from the mitochondria. A role for synexin in galectin-3 translocation. *J Biol Chem* 2002;**277**(18):15819–27.
7. Takenaka Y, Fukumori T, Yoshii T, et al. Nuclear export of phosphorylated galectin-3 regulates its antiapoptotic activity in response to chemotherapeutic drugs. *Mol Cell Biol* 2004;**24**(10):4395–406.
8. Patterson RJ, Wang W, Wang JL. Understanding the biochemical activities of galectin-1 and galectin-3 in the nucleus. *Glycoconj J* 2004;**19**(7–9):499–506.
9. Shimura T, Takenaka Y, Tsutsumi S, Hogan V, Kikuchi A, Raz A. Galectin-3, a novel binding partner of beta-catenin. *Cancer Res* 2004;**64**(18):6363–7.
10. Sripa B, Kaewkes S, Sithithaworn P, et al. Liver fluke induces cholangiocarcinoma. *PLoS Med* 2007;**4**(7):e201.
11. Khan SA, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. *J Hepatol* 2002;**37**(6):806–13.
12. Thamavit W, Bhamarapravati N, Sahaphong S, Vajrasthira S, Angsubhakorn S. Effects of dimethylnitrosamine on induction of cholangiocarcinoma in *Opisthorchis viverrini*-infected Syrian golden hamsters. *Cancer Res* 1978;**38**(12):4634–9.
13. Vatanasapt V, Tangvoraphonkchai V, Titapant V, Pipitgool V, Viriyapap D, Sriamporn S. A high incidence of liver cancer in Khon Kaen Province, Thailand. *Southeast Asian J Trop Med Public Health* 1990;**21**(3):489–94.
14. Vatanasapt V, Uttaravichien T, Mairiang EO, Pairojkul C, Chartbanchachai W, Haswell-Elkins M. Cholangiocarcinoma in north-east Thailand. *Lancet* 1990;**335**(8681):116–7.
15. Sithithaworn P, Haswell-Elkins MR, Mairiang P, et al. Parasite-associated morbidity: liver fluke infection and bile duct cancer in northeast Thailand. *Int J Parasitol* 1994;**24**(6):833–43.

16. Brustmann H. Galectin-3 and CD1a-positive dendritic cells are involved in the development of an invasive phenotype in vulvar squamous lesions. *Int J Gynecol Pathol* 2006;**25**(1):30–7.
17. Song YK, Billiar TR, Lee YJ. Role of galectin-3 in breast cancer metastasis: involvement of nitric oxide. *Am J Pathol* 2002;**160**(3):1069–75.
18. Greene FL, Page DL, Fleming ID, et al. *AJCC cancer staging manual*. 6th ed. New York: Springer-Verlag; 2002.
19. Sripa B, Leungwattanawanit S, Nitta T, et al. Establishment and characterization of an opisthorchiasis-associated cholangiocarcinoma cell line (KKU-100). *World J Gastroenterol* 2005;**11**(22):3392–7.
20. Elbashir SM, Harborth J, Weber K, Tuschl T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* 2002;**26**(2):199–213.
21. Sawanyawisuth K, Wongkham C, Pairojkul C, et al. Methionine aminopeptidase 2 over-expressed in cholangiocarcinoma: potential for drug target. *Acta Oncol* 2007;**46**(3):378–85.
22. van den Brule F, Califice S, Castronovo V. Expression of galectins in cancer: a critical review. *Glycoconj J* 2004;**19**(7–9): 537–42.
23. Shimonishi T, Miyazaki K, Kono N, et al. Expression of endogenous galectin-1 and galectin-3 in intrahepatic cholangiocarcinoma. *Hum Pathol* 2001;**32**(3):302–10.
24. Su CH, Tsay SH, Wu CC, et al. Factors influencing postoperative morbidity, mortality, and survival after resection for hilar cholangiocarcinoma. *Ann Surg* 1996;**223**(4):384–94.
25. Kawarada Y, Yamagiwa K, Das BC. Analysis of the relationships between clinicopathologic factors and survival time in intrahepatic cholangiocarcinoma. *Am J Surg* 2002;**183**(6):679–85.
26. Lotz MM, Andrews Jr CW, Korzelius CA, et al. Decreased expression of Mac-2 (carbohydrate binding protein 35) and loss of its nuclear localization are associated with the neoplastic progression of colon carcinoma. *Proc Natl Acad Sci USA* 1993;**90**(8):3466–70.
27. van den Brule FA, Berchuck A, Bast RC, et al. Differential expression of the 67-kD laminin receptor and 31-kD human laminin-binding protein in human ovarian carcinomas. *Eur J Cancer* 1994;**30A**(8):1096–9.
28. Takano T, Miyauchi A, Yoshida H, Kuma K, Amino N. Decreased relative expression level of trefoil factor 3 mRNA to galectin-3 mRNA distinguishes thyroid follicular carcinoma from adenoma. *Cancer Lett* 2005;**219**(1):91–6.
29. Matarrese P, Tinari N, Semeraro ML, Natoli C, Iacobelli S, Malorni W. Galectin-3 overexpression protects from cell damage and death by influencing mitochondrial homeostasis. *FEBS Lett* 2000;**473**(3):311–5.
30. Califice S, Castronovo V, Bracke M, van den Brule F. Dual activities of galectin-3 in human prostate cancer: tumor suppression of nuclear galectin-3 vs tumor promotion of cytoplasmic galectin-3. *Oncogene* 2004;**23**(45):7527–36.
31. Honjo Y, Inohara H, Akahani S, et al. Expression of cytoplasmic galectin-3 as a prognostic marker in tongue carcinoma. *Clin Cancer Res* 2000;**6**(12):4635–40.
32. Goletz S, Hanisch FG, Karsten U. Novel alpha-GalNAc containing glycans on cytokeratins are recognized invitro by galectins with type II carbohydrate recognition domains. *J Cell Sci* 1997;**110**(Pt 14):1585–96.
33. Dumic J, Dabelic S, Flogel M. Galectin-3: an open-ended story. *Biochim Biophys Acta* 2006;**1760**(4):616–35.