RESEARCH COMMUNICATION

Galectin-9 Acts as a Prognostic Factor with Antimetastatic Potential in Hepatocellular Carcinoma

Zhao-Yang Zhang[1,2], Jia-Hong Dong[2]*, Yong-Wei Chen[2], Xian-Qiang Wang[2], Chong-Hui Li[2], Jian Wang[2], Guo-Qiang Wang[2], Hai-Lin Li[2], Xue-Dong Wang[2]

Abstract

Considerable research has been conducted concerning galectin-9 and carcinomas, but little information is available about any relation with the hepatocellular carcinoma. In this study, we employed a small interfering RNA (siRNA) targeting galectin-9 to down-regulate the expression in HepG2 cells. As a result, after galectin-9 expression was reduced, cell aggregation was suppressed, while other behaviour such as the proliferation, adhesion and invasion to ECM, cell-endothelial adhesion and transendothelial invasion of the cells were markedly enhanced. When tumors of 200 patients with hepatocellular carcinoma were tested for galectin-9 expression by immunohistochemistry, binding levels demonstrated intimate correlations with the histopathologic grade, lymph node metastasis, vascular invasion and intrahepatic metastasis (P<0.05). Moreover, survival analysis indicated that patients with galectin-9 expression had much longer survival time than those with negative lesions, and the Log-rank test indicated that this difference was statistical significant (P<0.0001). The Cox proportional hazards model suggested that negative galectin-9 expression in hepatocellular carcinoma represented a significant risk factor for patient survival. We propose that galectin-9 might be a new prognostic factor with antimetastatic potential in patients with hepatocellular carcinoma.

Keywords: Galectin-9 - hepatocellular carcinoma - metastasis - prognosis

Introduction

Hepatocellular carcinoma (HCC) is one of the critical global health issues, with about 600,000 new cases diagnosed each year (Hann et al., 2004), and it is the third most common cause of cancer-related deaths worldwide (Parkin et al., 2005), with the overall 5-year survival rate lower than 5% (Farazi et al., 2006). Although surgical resection provides an opportunity for cure, the outcome remains dismal due to frequent tumor recurrence and metastasis, and the five-year survival is only 15-40% after curative resection (Li N et al., 2009). Thus, suppression of invasion and metastasis has become a major goal in current HCC research, and accordingly, a new understanding of the molecular mechanisms controlling the development and progression of HCC is urgently needed for the efficient treatment of this deadly disease.

As a family of carbohydrate-binding proteins, galectins bind β-galactoside moieties with high affinity and specificity. To date, 15 members of the galectin family have been identified (Gray et al., 2004). Galectin-9 (Gal-9) is a member of them, and it was first identified as an eosinophil chemoattractant and activation factor (Matsumoto et al., 1998; Matsushita et al., 2000;

Matsumoto et al., 2002; Saita et al., 2002). Subsequent studies revealed that Gal-9, similar to other galectins, modulated a variety of biological functions, such as cell aggregation and adhesion, apoptosis of tumor cells, and others (Asakura et al., 2002; Hirashima et al., 2004).

Recently, much attention has been focused on the role of Gal-9 in malignant tumors. Kageshita et al confirmed that high Gal-9 expression in tumor cells was closely associated with reduced metastasis and low recurrence in patients with malignant melanoma (Kageshita et al., 2002), and similar findings were obtained by Irie et al in breast cancer (Irie et al., 2005). The study of Kadowaki T et al confirmed that Gal-9 signaling prolonged the survival of tumor-bearing mice (Kadowaki et al., 2012). In many solid cancers, the loss of Gal-9 expression is closely associated with metastatic progression (Wiersma et al., 2011). It was supposed that Gal-9 was involved in the suppression of tumor cell metastasis (Atsuya et al., 2008).

However, little work has been done to study the relationship between Gal-9 expression and hepatocellular carcinoma with both in vitro and in vivo approaches. The purpose of this study was to investigate the effect of Gal-9 in HCC progression and to determine whether it was able to act as a prognostic factor.

[1]Department of Hepatobiliary Surgery, Qilu Hospital of Shandong University, Jinan, Shandong, [2]Department of Hepatobiliary Surgery, Division of Surgery, Chinese PLA General Hospital, Beijing, China *For correspondence: dongjh301@yahoo.cn

Materials and Methods

Antibodies and reagents

The goat-anti-human polyclonal antibody against human Gal-9, which had been purified and verified no crossreactivity with other galectins, was obtained from B&D SYSTEMS (USA). The biotinylated mouse anti-goat IgG was obtained from Dako (Copenhagen, Denmark). Reagents for SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and molecular weight markers were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture and subcloning

The human hepatocellular carcinoma cell line HepG2 was obtained from American Type Culture Collection (ATCC), and maintained as monolayers in standard medium comprising Dulbecco's modified Eagle's medium (DMEM: 4.5 g/l of glucose) (Sigma, USA) containing 10% heat-inactivated fetal calf serum (FCS) (Sigma, USA) and supplemented with 20 mM HEPES, 100 IU/ml penicillin and 100 µg/ml streptomycin (Merck, Germany). The cells were incubated in 37 °C, 5% CO_2, and saturated humidity. Human umbilical vein endothelial cells (HUVEC) were also obtained from ATCC and were cultured in F12K medium supplemented with 0.1 mg/ml heparin (Sigma, USA), 0.03 mg/ml endothelial cell growth supplement (Sigma, USA), and 10% fetal calf serum at 37 °C.

Subclones of HepG2 cells were established by the limiting dilution method (Irie et al., 2005). In brief, a cell suspension was distributed into the wells of 96-well round-bottomed culture plates at a cell concentration of 0.5 cell per well, and only the wells containing a single cell were selected. Then we applied western blotting to detect the expression of Gal-9 in those cells, and selected the cells with the highest level of galectin-9 expression for transfection with small interfering RNA (siRNA) (GenePharma, Shanghai, China) targeting Gal-9 (Sense: 5' GUGCAGAGCUCAGAUUUCATT 3' Antisense: 5' UGAAAUCUGAGCUCUGCACTT 3'). The siRNA was designed to target the co-fragment of Gal-9L, Gal-9M and Gal-9S, and had no effects to other galectins (data not shown). The control cells were transfected with the scramble siRNA or cultured only with lipofectamine-2000 (Invitrogen).

Western blotting analysis

The cells were lysed in 500µl of extraction buffer (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $CaCl_2$, 1% Triton X-100, 0.1% SDS, 0.1% NP-40, 1 mM vanadate, 1µg/ml pepstatin, 1 mM PMSF, 5 µg/ml aprotinin, and 1µg/ml of leupeptin) for 30 min on ice. The extracts were cleared by centrifugation for 20 min at 10,000g. Proteins of the cells were normalized to 30µg/lane and were electrophoresed using SDS–PAGE, after which the proteins were transferred to nitrocellulose membranes (Amersham-Pharmacia Biotech, UK). The membranes were probed with the first antibody (1:300) overnight at 4°C followed by peroxidase-labelled secondary antibodies. Immunoreactive bands were visualized using DAB method, and the optical density was analysis with the Scion image software.

Cell proliferation assay

Cell viability was determined by MTT assay. In brief, cells were seeded into 96-well plates at 1×10^4 per well. After incubation for 48 h, 20 µl of MTT reagent (5 mg/ml; Sigma) was added to each well. Plates were incubated at 37 °C for 4h and then the supernatant was removed and 150 µl of dimethyl sulfoxide (Sigma) was used to dissolve the crystals. Absorbance was measured at 570 nm in a Microplate Reader (Bio-Tech Instruments, USA).

Cell adhesion Assay to ECM

Cells were harvested, washed thrice with PBS, resuspended in serum-free DMEM, and transferred to the wells (1×10^6 cells per well) of 96-well plates (Nunc, Roskilde, Denmark) that had been coated with 100 µL of 20 µg/mL matrigel (BD Biosciences, Franklin Lakes, NJ, USA). After incubation for 2h at 37 °C, the wells were washed 3 times with PBS and the remaining cells were fixed with 3% paraformaldehyde for 20 min at room temperature. Then the cells were stained with 0.5% crystal violet (in 20% methanol and 1% formaldehyde) and washed 3 times with PBS to remove free dye. The relative number of cells in each well was evaluated by measuring absorbance at 570 nm in a microplate reader (Bio Rad). Data were expressed as means of absorbance of triplicate wells.

Cell migration Assay

Cell migration was assessed in triplicates using a 48-well transwell setup (Neuro Probe, Cabin John, MD). In brief, $8\mu m$ pore-size polycarbonate filters (Poretics Corporation, Livermore, CA) were coated with 1 μg of matrigel (BD Biosciences). The lower wells were filled with DMEM containing 10% FBS. HepG2 cells (1×10^5 cells/well in DMEM with 0.5% FBS) were added to each of the upper wells. After incubation for 24h at 37 °C in 5% CO_2, nonmigratory cells in the upper chambers were removed with cotton swabs, and the cells migrated to the bottom side were stained with 0.1% crystal violet (Sigma) in 20% ethanol. Five fields were counted on each of the filters, and data were expressed as means of average cells per field.

Cell-endothelial adhesion assay

Subconfluent HepG2 cells were washed with PBS and labeled with 5 µg/ml DIO fluorescent cell labeling solution in serum-free DMEM for 30 min at 37 °C. The cells were washed with PBS and treated with a nonenzymatic cell dissociation solution (Sigma) that releases the cells from the culture plates while keeping the cell membrane proteins intact. After washing, 5×10^4 cells were added to HUVEC monolayer cultured on chamber slides for 1 h at 37 °C. The chamber slides were then gently washed with PBS and inverted for 10 min at room temperature. The slides were blinded and the fluorescently labeled cells remaining on the endothelial monolayer were counted in 10 randomly selected fields of view using fluorescent

microscopy, and data were expressed as means of average cells per field.

Cell trans-endothelial invasion assay

HUVECs were cultured in Transwell inserts with 8-μm-pore filters (Poretics Corporation, Livermore, CA) for 3d to allow tight formation of cell monolayers. Monolayer integrity was monitored by measuring trans-endothelial electrical resistance using a volt-ohm meter (EVOM, World Precision Instruments), and monolayers with trans-endothelial electrical resistance >800 Ω/cm^2 were used for trans-endothelial assessment (Qicheng Zhao et al. 2009). HepG2 cells, labeled with DIO, were applied to the HUVECs for 24 h at 37 °C. The cells at the upper side of the Transwell membrane were removed with a cotton swab and fluorescent cells migrated to the bottom side of the Transwell membrane were counted in 10 randomly selected fields of view using an fluorescence microscope, and data were expressed as means of average cells per field.

Patients and Specimens

A retrospectively compiled database was established of 200 consecutive patients with the first HCC lesions who underwent curative operations at the Chinese PLA General Hospital between 1995 and 2005. The median age of the patients was 51 (range 25-77) years. After surgery, the patients were followed in the surgical outpatient clinic. Patients with other malignancies or who died from causes related to the operations were excluded. Specimens were obtained during curative operations above, following the principles outlined in the Declaration of Helsinki. Before the research was conducted, all the patients had provided consent to use their samples for this research as consecutive samples, and the data to be published. This study was approved by the Ethics Committee of Chinese PLA General Hospital.

Immunohistochemistry

Briefly, for immunohistochemistry, formalin-fixed and paraffin-embedded tissues were cut into sections 2 μm thick, and the sections were deparaffinised, endogenous peroxidase activity was blocked, antigen was retrieved, and then the sections were incubated overnight at 4°C with primary anti-galectin-9 antibody (1:500). Negative controls were prepared using the identical concentration of goat immunoglobulin IgG (Dako). The following day, biotinylated anti-goat IgG (1:200) was applied to the sections, which were subsequently treated using horseradish peroxidase (HRP)-labelled strepto-antibiotin (Dako) for 15 min. The sections were then incubated for 15 min with a peroxidase solution (Dako), stained with haematoxylin, dehydrated, cleared, and mounted.

Assessment of Galectin-9 Staining in the Tissue Sections

Galectin-9 staining was mainly in the cytoplasm of the tumor cell. We assessed the Gal-9 staining using a method as previously described (Zhang et al., 2008). In brief, intensity of the staining: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining; ratio of staining cells: 0, no staining cells; 1, 2% staining cells; 2, 2~10%

staining cells; 3, 11~29% staining cells; 4, ≥ 30% staining cells. Adding the two scores, the last score of every section was obtained. We defined the staining class according to the last score as follows: negative, ≤ ; positive, > 2. Scoring was carried out in a double-blind manner by two independent investigators. Any disagreement was resolved by discussion to obtain a final score.

Statistical analysis

Data for the changes of Gal-9 level and other relative data were reported as the means ± SD, and were the representative of an average of at least three independent experiments. Statistical comparisons were made by t test and One-Way ANOVA, and P<0.05 was considered statistically significant. The chi-squared test was used to compare Gal-9 expression and the various clinical and pathological characteristics of the patients studied. Survival time was measured as the time from the date of surgery to disease-related death, and those who died from other reasons or were still alive when they were last seen were censored. Univariate survival analyses were carried out using the Kaplan-Meier method. Factors were compared using the Cox proportional hazards model and Log-rank tests. Multivariate survival analysis was carried out using the Cox proportional hazards model to identify independent predictors of survival. SPSS 14.0 was used.

Results

Cell aggregation was suppressed by Gal-9 silence
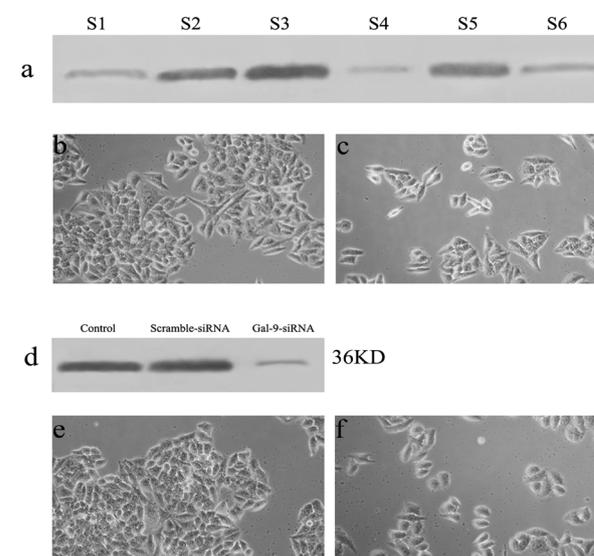
Figure 1. Relation Between Gal-9 Expression and Cell Aggregation in HepG2 Cells. (a) Western blotting analysis of Gal-9 expression in HepG2 subclones. We can see the highest expression of Gal-9 in subclone S3, and the lowest in subclone S4. (b) Image of HepG2 subclone S3 which exhibited tight cell aggregation with Gal-9 expression at high level (×400). (c) Image of HepG2 subclone S4 which exhibited no cell aggregation with Gal-9 expression at low level (×400). (d) Western blotting analysis of Gal-9 expression after transfection with siRNA. We can see that in the Gal-9-siRNA transfected cells, Gal-9 expression was suppressed significantly. (e) Image of HepG2 subclone S3 (control) which exhibited tight cell aggregation (×400). (f) Image of HepG2 subclone S3 (Gal-9-siRNA) which exhibited no cell aggregation (×400)
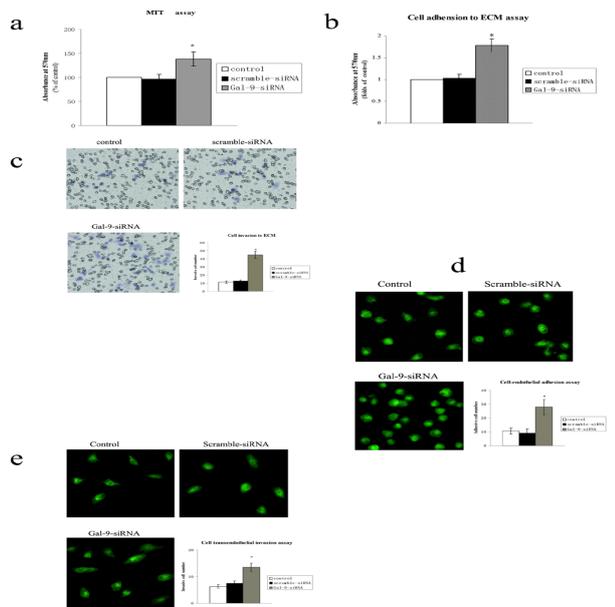
Figure 2. After Gal-9 Expression was Suppressed by siRNA, the Adhesive and Invasive Ability of HepG2 Cells Increased Not Only to ECM but also to Endothelial Cells. *P<0.01 vs control and scramble-siRNA cells. (a) Cell survival was determined by MTT assay. Values are expressed as a percentage of control. (b) Cell adhesion assay to ECM. Values are expressed as a fold of control. (c) Cell invasive assay to ECM. Values are expressed as means of average cells per field under light microscope. (d) Cell-endothelial adhesion assay. Values are expressed as means of average cells per field under fluorescent microscopy. (e) Cell trans-endothelial invasion assay. Values are expressed as means of average cells per field under fluorescent microscopy
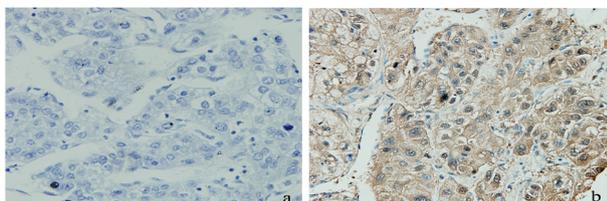
Figure 3. Immunohistochemical Staining of Hepatocellular Carcinoma Tissue for Gal-9 Expression. Representative Gal-9-negative (a) and Gal-9-positive (b) tumors are shown. Gal-9 was detected in the cytoplasm but not in the nucleus of positive cells. Magnification, ×400

We established 6 subclones (S1-S6) of HepG2 cells, and the results of Western Blotting indicated that the levels of Gal-9 protein were higher in those subclones that the cell proliferation exhibited evident aggregation than in those that did not. The subclone S3 exhibited the tightest aggregation and presented the highest level of galectin-9 (Figure 1A-C), and thus be selected for the following test. siRNA were transfected into S3 cells, and the cells were divided into 3 groups: control group (cultured only with lipofectamine-2000), scramble-siRNA group (transfected with the scramble siRNA) and Gal-9-siRNA group (transfected with the siRNA targeting Gal-9). The result of Western blotting showed that in Gal-9-siRNA group, Gal-9 expression was repressed obviously, and compared to the control and scramble-siRNA groups, the difference was significantly (Figure 1D).

Following the suppression of Gal-9, tight cell clusters

Table 1. Patient Characteristics and Galectin-9 Expression

Factor	n	Positive	Negative	P value
Age				
≥50	108	60	48	
<50	92	53	39	0.77
Gender				
F	56	32	24	
M	144	81	63	0.91
HBV				
yes	174	101	73	
no	26	12	14	0.26
AFP Level (ng/ml)				
<400	73	45	28	
≥400	127	68	59	0.27
Tumor size				
<5 cm	84	44	40	
≥5 cm	116	69	47	0.32
Histopathologic grade				
I	47	30	17	
II-III	124	73	51	
IV	29	10	19	0.03
Lymphonode metastasis				
yes	54	21	33	
no	146	92	54	0.002
Vascular invasion				
yes	61	27	34	
no	139	86	53	0.02
Intrahepatic metastasis				
yes	41	16	25	
no	159	97	62	0.01

disappeared in the proliferation of S3 cells (Figure 1E-F). These results suggested that galectin-9 might contribute to the aggregation of hepatocelluar carcinoma cells.

Suppression of Gal-9 expression increased the proliferation, adhesion and invasion to ECM, cell-endothelial adhesion and trans-endothelial invasion of the cells

The MTT assay showed (Figure 2A) that the survival rate of Gal-9-siRNA cells increased markedly, compared to the control and scramble-siRNA cells, the difference were significant (P<0.01). In cell adhesion and invasion assays, it indicated that Gal-9 suppression increased the adhesion and invasive abilities of HepG2 cells toward ECM, and the differences were significant (P<0.01) (Figure 2B-C).

Through cell-endothelial adhesion assay we can see that Gal-9 suppression increased the adhesive ability of HepG2 cells to HUVEC (P<0.01) (Figure 2D), and in the trans-endothelial invasion assay it indicated that Gal-9-siRNA cells showed stronger trans-HUVEC invasion than the control and scramble-siRNA groups (P<0.01) (Figure 2E).

Expression of galectin-9 in the Human Tissue Specimens

In the 200 hepatocellular carcinoma specimens, 113 (56.5%) were positive for Gal-9 expression. Gal-9 was detected in the cytoplasm but not in the nucleus of the cancer cells (Figure 3). Table 1 summarises the relationships between the clinical-pathological features and Gal-9 expression. A subsequent analysis showed that when correlating with the clinical-pathological features,

Galectin-9 Acts as a Prognostic Factor with Antimetastatic Potential in Hepatocellular Carcinoma

Table 2. Survival Estimates (and 95% confidence intervals) for Disease-related Deaths

Group	Median survival (months)	3-year survival rate (%)	5-year survival rate (%)
Gal-9 positive	63.0 (58.6-67.4)	67.1 (58.5-75.7)	55.3 (40.1-64.51)
Gal-9 negative	31.0 (21.9-40.1)	42.0 (31.6-52.4)	30.3 (20.5-40.1)

Log-rank test: negative vs positive (P<0.01)
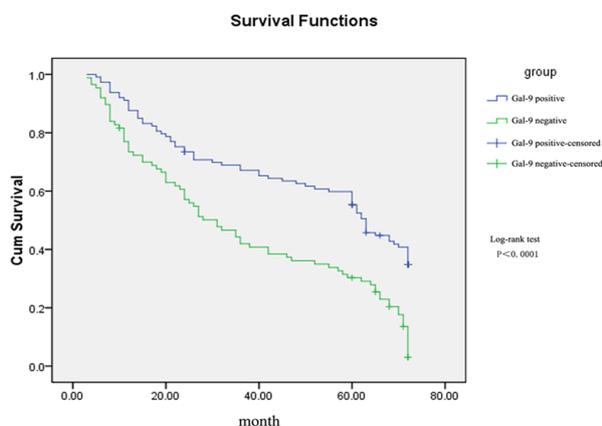
Figure 4. Kaplan-Meier Survival Analysis Using a Log-rank Test. The samples were grouped according to Gal-9 expression (positive or negative). The Log-rank P value is given (P<0.0001)

there was no evidence of a statistical difference between Gal-9-positive and -negative tumours regarding gender, age, tumor size, HBV infection and AFP level, but statistical differences existed regarding histopathologic grade, lymphonode metastasis, vascular invasion and intrahepatic metastasis.

Galectin-9 Expression and Patient Survival

We analyzed the data by considering only disease-related death as an event, censoring deaths unrelated to disease and the patients who were alive when they were last seen. The Kaplan-Meier plot (Figure 4) showed that patients who were Gal-9 positive had much longer survival times than those who were Gal-9 negative, and the Log-rank test indicated that this difference was statistical significant (P<0.0001). The survival estimates showed a striking difference in median survival between the positive and negative Gal-9 expression patients: the former averaged 63 months (95% confidence interval 58.6-67.4), whereas the latter averaged 31 months (95% confidence interval 21.9-40.1), and showed a 25% lower 5-year survival rate (Table 2). From a biological perspective, we concluded that negative expression of Gal-9 is a prognostic indicator of poor survival for patients with hepatocellular carcinoma.

In order to obtain a more precise estimate, the Cox proportional hazards model was used. The results indicated that in comparison with tumors expressing Gal-9, negative staining was associated with a hazard ratio of 1.73 (95% confidence interval 1.22-2.44), and the P value of 0.002 revealed a significant difference in survival. Other significant factors in univariate analyses were histopathologic grade, lymphonode metastasis, vascular invasion and intrahepatic metastasis, whereas in multivariate analysis, only Gal-9 expression, vascular invasion and intrahepatic metastasis retained prognostic significance for cancer survival (Table 3). This result

Table 3. Multivariate Analysis for Disease-related Deaths (Cox Regression Model)

Variable	P value	Hazard ratio	95% confidence interval
Age			
<50		1	
≥50	0.35	0.85	0.61-1.19
Gender			
F		1	
M	0.87	1.03	0.71-1.51
HBV			
no		1	
yes	0.1	1.48	0.92-2.36
AFP Level (ng/ml)			
<400		1	
≥400	0.59	0.91	0.64-1.29
Tumor size			
<5 cm		1	
≥5 cm	0.42	0.87	0.62-1.22
Histopathologic grade			
I		1	
II-IV	0.09	1.26	0.96-1.65
Lymphonode metastasis			
no		1	
yes	0.11	1.34	0.93-1.91
Vascular invasion			
no		1	
yes	0.001	1.86	1.30-2.68
Intrahepatic metastasis			
no		1	
yes	0.005	1.74	1.18-2.56
Gal-9 expression			
yes		1	
no	0.002	1.73	1.22-2.44

confirmed the findings of the Log-rank test in identifying Gal-9 expression as an independent variable, and importantly suggested that negative Gal-9 expression in hepatocellular carcinoma represented a significant risk factor for patient survival.

Discussion

In our study, we showed that suppression of Gal-9 expression by siRNA in HepG2 cells significantly weakened the cell aggregation, which indicated that Gal-9 contributed to the aggregation of tumor cells. In other words, the expression of Gal-9 perhaps made it difficult for the tumor cells to detach from primary site. It was consistent with the study of Irie et al. (2005) that exogenously added recombinant galectin-9 induced aggregation of breast cancer cells and the study of Kageshita et al. (2002) that Gal-9 expression promoted malignant melanoma cells aggregate. It was likely that in the process of migration, cancer cells should firstly detach from tumor tissue individually, and then migrate into lymphatic or blood vessels and metastasis to distant organs (Irie et al., 2005). Now that galectin-9 mediates

cell aggregation, we hypothesized that it might prevent metastasis of hepatocellular carcinoma.

Furthermore, we found that Gal-9 suppression increased the adhesion of HepG2 cells to ECM, which was consistent with the studies of Irie et al. (2005) and Nobumoto et al. (2008) that galectin-9 reduced adhesion of cancer cells to ECM. It is well known that cell–cell and cell–ECM interactions are involved in the establishment of metastasis (Pasco et al., 2004). Adhesion of cancer cells to ECM is an essential step in tumor cell invasion (Irie et al., 2005), because the adhesive interaction of metastatic tumor cells and components of ECM appears to be obligatory for successful target organ colonization (Engbring et al., 2003). Galectins are lectins that exhibit selective affinity for β-galactosides (Kilpatrick et al., 2002) and bind to a variety of β-galactoside-containing glycoproteins and glycolipids both on the cell surface and in ECM (Hsu et al., 2004). By binding to these glycoconjugates, galectins deliver signals intracellularly as well as mediate cell–cell and cell–ECM adhesion (Hughes, 2001). Meanwhile, our present study confirmed that Gal-9 suppression enhanced migration of HepG2 cells in ECM, which was consistent with the study of A. Nobumoto et al that galection-9 inhibited migration of B16F10 and Colon26 cells in ECM (Nobumoto et al., 2008), and it suggested that Gal-9 prevented a key step of tumor metastasis. However, it still requires our further study to get the intimate mechanisms.

One critical step in cancer metastasis is the adhesion of disseminating tumor cells to the blood vessel endothelium in distant organs (Miles et al., 2008), and invasion through vascular endothelium of these adhesive cells is also a vital step in cancer metastasis (Hanahan et al., 2000). Our present study confirmed that Gal-9 suppression increased the cell-endothelial adhesion and trans-endothlial invasion of HepG2 cells markedly, which indicated in an opposite view that Gal-9 performed an antimetastatic role in HepG2 cells. As for the molecular mechanisms, Miles FL et al thought that the process was regulated by the mechanical properties of the cancer cells and also by the specific expression of various adhesion molecules and/or ligands to adhesion molecules on the surface of cancer cells and endothelial cells (Miles et al., 2008). A. Nobumoto et al (2008) confirmed that secreted Gal-9 inhibited adhesion of B16F10 and Colon26 cells to vascular endothelium by blocking the binding between CD44 and hyaluronic acid through its lectin nature. Zhao et al. (2009) proved that galectin-3 reduced cell-endothelial adhesion and trans-endothelial invasion of malignant melanoma cells by modifying MUC1 localization on cancer cell surface. In our subsequent research, we will do more work to reveal the molecular mechanisms of Gal-9 regulating cell-endothelial adhesion and trans-endothelial invasion in cancer cells.

The process of metastasis involves five critical steps: detachment of cancer cells from primary sites, movement (invasion) of cancer cells in ECM, attachment of cancer cells to vascular endothelial cells at distal sites, invasion of cancer cells through vascular endothelium, and tumor growth with neovascularization, which are essential for accomplishing malignant tumor metastasis (Wada et al., 1997). According to the present study we can find that Gal-9 suppressed multiple steps of metastasis by promoting cell aggregation, inhibiting adhesion and invasion of cancer cells to ECM, blocking cell-endothelial adhesion and obstructing trans-endothelium invasion of the tumor cells, which supported the hypothesis that Gal-9 prevent metastasis of hepatocarcinoma cells. The following research about the relation between Gal-9 expression and patient survival further sustained it.

Analysis of the immunohistochemistry result combined with the clinical data in this study indicated that the total expression ratio of Gal-9 in hepatocellular carcinoma was 56.5%, and the concrete expression was associated with the histopathological grade, lymph node metastasis, vascular invasion and intrahepatic metastasis of the tumors. More important was that negative expression of Gal-9 was significantly associated with reduced survival time. These were consistent with our previous results in this study that Gal-9 suppressed multiple steps of metastasis of hepatocarcinoma cells in vitro. Irie A et al. (2005) confirmed that Gal-9 expression was correlated with histopathologic grade and distant metastasis, and Gal-9 acted as a prognostic role in breast cancer. Kageshita T et al. (2002) proved that high Gal-9 expression was inversely correlated with the progression of melanocytic tumors, and high Gal-9 expression in primary melanoma lesions links to a better prognosis.

In addition to the anti-metastatic roles of Gal-9 mentioned above, it had been reported that Gal-9 induced apoptosis in T cells (Wada et al., 1997; Tsuchiyama et al., 2000), malignant melanoma cells (Kageshita et al., 2002) and breast cancer cells (Irie et al., 2005), and the pro-apoptotic role was required for galectin-9–induced suppression of melanoma and breast cancers (Kageshita et al., 2002; Irie et al., 2005). Kobayashi et al. (2010) confirmed that Gal-9 exhibits anti-myeloma activity through JNK and p38 MAP kinase pathways. In our present study, we found that Gal-9 suppression reduced apoptosis in HepG2 cells at about 30% (data not shown). We have not known the molecular mechanisms for Gal-9 to induce apoptosis, but we think that the pro-apoptotic effect of Gal-9 might profit the suppression of hepatocellular carcinoma.

Tumor cells can escape the attack of the host immune system through various mechanisms, including immune evasion, immunosuppression, or others (Zou et al., 2005). Progressive tumor growth seems to be at least partly ascribed to tumor cell-induced down-regulation of T cell-mediated immune responses (Kim et al., 2006). It had been revealed that Gal-9 could increase Tim-3+ dendritic cells and CD8+ T cells and enhance antitumor immunity via Gal-9-Tim-3 interactions in mouse (Keiko et al., 2008). Whether Gal-9 also can regulate the antitumor immunity in hepatocellular carcinoma patients, more work is required.

In conclusion, galectin-9 suppressed metastasis of hepatocelllular carcinoma cells in multiple steps, including promoting aggregation, inhibiting adhesion and invasion to ECM, blocking cell-endothelial adhesion and obstructing trans-endothelium invasion of the cells. Together with the clinical data, we proposes that galectin-9 might be a new prognostic factor with antimetastatic potential in patients with hepatocellular carcinoma.

Acknowledgements

References

Asakura H, Kashio Y, Nakamura K, et al (2002). Selective eosinophil adhesion to fibroblast via IFN-γ-induced galectin-9. *J Immunol*, **169**, 5912-18.

Atsuya N, Keiko N, Souichi O, et al (2008). Galectin-9 suppresses tumor metastasis by blocking adhesion to endothelium and extracellular matrices. *Glycobiology*, **18**, 735-44.

Engbring JA, Kleinman HK (2003). The basement membrane matrix in malignancy. *J Pathol*, **200**, 465-70.

Farazi PA, DePinho RA (2006). Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer*, **6**, 674-87.

Gray CA, Adelson DL, Bazer FW, et al (2004). Discovery and characterization of an epithelial-specific galectin in the endometrium that forms crystals in the trophectoderm. *Proc Natl Acad Sci USA*, **101**, 7982-7.

Hanahan D, Weinberg RA (2000). The hallmarks of cancer. *Cell*, **100**, 57-70.

Hann HW, Lee J, Bussard A, et al (2004). Preneoplastic markers of hepatitis B virus-associated hepatocellular carcinoma. *Cancer Res*, **64**, 7329-35.

Hirashima M, Kashio Y, Nishi N, et al (2004). Galectin-9 in physiological and pathological conditions. *Glycoconj J*, **19**, 593-600.

Hsu DK, Liu FT (2004). Regulation of cellular homeostasis by galectins. *Glycoconj J*, **19**, 507-15.

Hughes RC (2001). Galectins as modulators of cell adhesion. *Biochimie*, **83**, 667-76.

Irie A, Yamauchi A, Kontani K, et al (2005). Galectin-9 as a prognostic factor with antimetastatic potential in breast cancer. *Clin Cancer Res*, **11**, 2962-8.

Kageshita T, Kashio Y, Yamauchi A, et al (2002). Possible role of galectin-9 in cell aggregation and apoptosis of human melanoma cell lines and its clinical significance. *Int J Cancer*, **99**, 809-16.

Keiko N, Tomohiro A, Souichi O, et al (2008). Galectin-9 Increases Tim-3+ Dendritic Cells and CD8+ T Cells and Enhances Antitumor Immunity via Galectin-9-Tim-3 Interactions. *J Immunol*, **181**, 7660-9.

Kilpatrick DC (2002). Animal lectins: A historical introduction and overview. *Biochim Biophys Acta*, **1572**, 187-97.

Kim R, Emi M, Tanabe K, et al (2006). Tumor-driven evolution of immunosuppressive networks during malignant progression. *Cancer Res*, **66**, 5527-36.

Kobayashi T, Kuroda J, Ashibara E, et al (2010). Galectin-9 exhibits anti-myeloma activity through JNK and p38 MAP kinase pathways. *Leukemia*, **24**, 843-50.

Li N, Lucy W, Herbert C, et al (2009). Down-regulation of Notch1 signaling inhibits tumor growth in human hepatocellular carcinoma. *Am J Transl Res*, **1**, 358-66.

Matsumoto R, Hirashima M, Kita H, et al (2002). Biological activities of ecalectin: a novel eosinophil-activating factor. *J Immunol*, **168**, 1961–67.

Matsumoto R, Matsumoto H, Seki M, et al (1998). Human ecalectin, a variant of human galectin-9, is a novel eosinophil chemoattractant produced by T lymphocytes. *J Biol Chem*, **273**, 16976-84.

Matsushita N, Nishi N, Seki M, et al (2000). Requirement of divalent galactoside-binding activity of ecalectin/galectin-9 for eosinophil chemoattraction. *J Biol Chem*, **275**, 8355-60.

Miles FL, Pruitt FL, van-Golen KL, et al (2008). Stepping out of the flow: capillary extravasation in cancer metastasis. *Clin Exp Metastasis*, **25**, 305-24.

Parkin DM, Bray F, Ferlay J, et al (2005). Global cancer statistics, 2002. *CA Cancer J Clin*, **55**, 74-108.

Pasco S, Ramont L, Maquart FX, et al (2004). Control of melanoma progression by various matrikines from basement membrane macromolecules. *Crit Rev Oncol Hematol*, **49**, 221-33.

Qicheng Zhao, Xiuli Guo, Gerard B Nash, et al (2009). Circulating Galectin-3 Promotes Metastasis by Modifying MUC1 Localization on Cancer Cell Surface. *Cancer Res*, **69**, 6799-806.

Saita N, Goto E, Yamamoto T, et al (2002). Association of galectin-9 with eosinophil apoptosis. *Int Arch Allergy Immunol*, **128**, 42-50.

Tsuchiyama Y, Wada J, Zhang H (2000). Efficacy of galectins in the amelioration of nephrotoxic serum nephritis in Wistar Kyoto rats. *Kidney Int*, **58**, 1941-52.

Wada J, Ota K, Kumar A, et al (1997). Developmental regulation, expression, and apoptotic potential of galectin-9, a β-galactoside binding lectin. *J Clin Invest*, **99**, 245-61.

Wiersma VR, de Bruyn M, Helfrich W, et al (2011). Therapeutic potential of Galectin-9 in human disease. *Med Res Rev*, doi: 10.1002/med. 20249.

Zhang ZY, Xu KS, Wang JS, et al (2008). Integrin alphanvbeta6 acts as a prognostic indicator in gastric carcinoma. *Clin Oncol (R Coll Radiol)*, **20**, 61-6.

Zou W (2005). Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer*, **5**, 263-74.