Abstract. Galectin-9 (Gal-9), a member of the β-galactoside-binding galectin family, plays a role in immune response, apoptosis, cell proliferation and cell death. Recent studies have shown that abnormal expression of Gal-9 is involved in certain primary cancers. The present study is the first investigation of the role of Gal-9 gene expression in clinically diagnosed primary gastric cancer tissues. Gal-9 mRNA expression was assessed in 44 clinically diagnosed frozen primary cancer tissue samples using quantitative PCR (qPCR). Analysis of the qPCR data revealed a significant reduction (>2-fold decreased) of Gal-9 gene expression in gastric cancer tissues in 77% (34/44) of patients. In patients with gastric cancer, although no statistically significant difference was found between adjacent (<2 cm away from the cancer tissue) and normal tissues (>5 cm away from the cancer tissue), a >2-fold reduction in Gal-9 expression was observed in the adjacent tissues of 34% of the patients. Compared to matched normal or adjacent tissues, the gene expression of Gal-9 was significantly decreased in tumor tissues (p<0.001). The correlation of Gal-9 expression and clinicopathological features in gastric cancer was analyzed according to the TNM classification system using AJCC stage grouping. In patients with gastric cancer, clinical staging, tumor pathological stage (pT stage), tumor cell differentiation, lymph node metastasis and survival rate were found to be associated with Gal-9 expression. However, no significant association was found between Gal-9 expression and distant metastasis (p>0.05). No significant difference was found between patients of different genders, levels of cell differentiation, distant metastasis status or different survival time of patients. Compared to normal tissues, >2-fold reduction of Tim-3 expression in gastric cancer tissues occurred in 59% of patients, but no correlation was found between Gal-9 and Tim-3 in gastric cancer. These results strongly suggest that Gal-9 is involved in tumorigenesis of gastric cancer.

Introduction

Galectins have been characterized based on their β-galactoside-binding affinity, and their proteins have an evolutionarily conserved carbohydrate recognition domain (CRD) (1,2). Galectin family proteins can be classified by structure into three subtypes: chimeric galectin, prototype galectin, and tandem-repeat galectin (3,4). Galectin-9 (Gal-9), as a tandem-repeat galectin, was first identified in a patient with Hodgkin’s disease (5). The structure of Gal-9 is similar to that of other members of the galectin family, such as galectin-4 and galectin-8, which contained two homologous CRD domains separated by a linker peptide (6,7). There are three isoforms of Gal-9. Based on the size of the linker peptide connecting two CRDs, they were named long-sized Gal-9 (Gal-9L), medium-sized Gal-9 (Gal-9M), and short-sized Gal-9 (Gal-9S) (8). All these isoforms are transcriptionally active and protein-coding (9), but little is known about their differences in cellular functions. Research has revealed that overexpression of Gal-9L decreased E-selectin levels, while Gal-9M or Gal-9S increased E-selectin levels in LoVo cells, suggesting three different isoforms of Gal-9 might exert distinct biological functions (10).

Increasing amounts of evidence suggest that Gal-9 has a variety of biological functions. Experimental results have shown that Gal-9 induces apoptosis when added to various types of cells, such as T-cells and different types of leukemia cells (11,12). Gal-9-induced apoptosis may be associated with the Ca²⁺-calpain-caspase-1 pathway and Jun NH₂-terminal kinase (JNK) and/or p38 MAPK signaling pathways (13,14). Previous results have shown the involvement of Gal-9 in tumor cell adhesion, aggregation, and proliferation. For example, high levels of Gal-9 expression in melanoma and MCF-7 cells causes formation of colonies and clusters, but cells that lack Gal-9 expression do not (11,15). Further studies
have confirmed that abnormal expression of Gal-9 is involved in the regulation of cell adhesion and aggregation. This view was supported by findings reported by Zhang et al, who observed that the loss of Gal-9 in hepatocellular carcinoma cells in vitro increased the activity of adhesion and invasion of these cells (16). Marked differences were found between Gal-9 expression in cancer and in normal tissues. Differential Gal-9 protein expression was observed in different tumor tissues. Gal-9 protein was strongly and homogeneously expressed in melanocytic nevi but downregulated in melanoma cells, especially in metastatic lesions. High levels of Gal-9 expression are inversely correlated with the progression of primary melanoma lesions (11). However, the lack of Gal-9 expression was correlated with distant metastasis in a majority of patients with breast cancer (15). Similarly, Gal-9 was evidently detected in normal epithelium and endocervical glands, but those in cervical intraepithelial neoplasia and cervical squamous cell carcinoma cells were significantly faint (17). To be clear, so far, the expression of Gal-9 in tumor tissues has only been investigated in limited types of tumors, and all experiments are based on histochemical staining to determine the expression level of Gal-9 as an indicator. Here, we first measured Gal-9 gene expression levels in clinically diagnosed primary gastric cancer tissues by quantitative PCR (qPCR) and the relationship between mRNA expression levels of Gal-9 and clinicopathological features in gastric cancer was analyzed. The correlation between Gal-9 and Tim-3 was also assessed in gastric cell lines.

Materials and methods

Tissue collection. Forty-four patients with primary gastric cancer were enrolled in this study. All patients underwent radical surgery between September 2008 and July 2013 at the First Hospital of Jilin University and did not receive any adjuvant therapy before the surgical operation. Gastric cancer and corresponding adjacent (<2 cm away from the tumor area) and normal tissues (>5 cm away from the tumor area) were collected from patients. The median age of the patients was 64 years (range 44-84 years). Written informed consent was obtained from all participants, and the study was approved by the Institutional Ethics Board of the School of Medicine, Jilin University. Medical records of the patients, including age and gender, tumor staging, pathological diagnosis, and surgical records were reviewed. Tumors were staged according to the 2010 TNM classification system using the American Joint Committee on Cancer (AJCC) stage grouping (18).

Antibodies. Anti-Galectin-9 (17938-1-AP) and anti-Tim-3 (11872-1-AP) polyclonal antibodies were purchased from Proteintech Group (Wuhan, China). Anti-GAPDH was raised against bacterially expressed proteins (Jilin University, Changchun, China).

Construction of galectin-9 (Gal-9) expression plasmids. Expression vectors for human Gal-9 were constructed by inserting cDNA into the Xhol/BamHI sites of pcDNA3.1(-). The integrity of the DNA sequence was confirmed by DNA sequencing (GenBank accession no. BAB83624). Reverse transcription PCR (RT-PCR). Total RNA from gastric cancer, adjacent, and normal tissues was isolated using TRIzol® LS reagent (Invitrogen, CA, USA). Then, 1 µg of total RNA from each sample was used as a template to produce cDNA with a PrimeScript 1st Strand cDNA Synthesis kit (Takara, Dalian, China). Human Gal-9, Tim3 and GAPDH mRNA levels were analyzed using quantitative PCR (qPCR) with an Eco Real-Time PCR system (Illumina, CA, USA). All PCR reactions were finished as follows: initial denaturation step at 95˚C for 30 sec, followed by 40 cycles of denaturation at 95˚C for 5 sec, annealing at 60˚C for 30 sec, and extension at 72˚C for 30 sec. Primer sets used for PCR were as follows: Gal-9, 5’-CTTTCATCACACCATTCTG-3’ (forward) and 5’-ATGGGAACCTCTGACACTG-3’ (reverse). This pair of primers can detect three different isoforms of Gal-9 and produce a 91-bp product, β-actin, 5’-ATGGGTCAGAAGGATTCCTATGT-3’ (forward) and 5’-AGCCACACGCGACTCATT-3’ (reverse) produce a 153-bp product. Tim-3, 5’-CAGATCTGGCTAAATGGG-3’ (forward) and 5’-CTTGGCTGTTTGTAGCAC-3’ (reverse) produce a 160-bp product.

Cell culture and transient transfection. Human gastric cancer cell lines SGC-7901 and MGC-803 were obtained from Department of Gastrointestinal Surgery, First Hospital of Jilin University. Human gastric mucosal cell line GES-1 was provided by the Cancer Hospital of Beijing University. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) with 5% glucose and 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in 10-cm dishes at 37˚C in a humidified atmosphere of 5% CO2. For transient transfection, cells were cultured in 6-well tissue culture plates (~2.5x104 cells/well) in DMEM medium containing 10% fetal bovine serum. Then cells were transfected with Gal-9 cDNAs. After 48 h of transfection, cells were harvested and lysed for western blotting and total RNA isolation.

Preparation of whole cell extracts and western blotting (WB). First, 100 µg of gastric cancer, adjacent or normal tissue samples were homogenized with liquid nitrogen and solubilized in 200 µl cold PBS containing 1.0% Nonidet P-40, 0.5% Na-deoxycholate, 0.1% SDS, 0.05 mM PMSF and protease inhibitor cocktail. The homogenate was swirled and kept on ice for 30 min. Whole-cell extracts were sonicated (Scientz-IID, Ningbo, China) for 10 sec with 50% initial cycle and centrifugation at 13,000 × g for 30 min. Equal total amounts of protein from tissue whole-cell lysates were mixed with 4X SDS-containing sample buffer and boiled for 5 min at 95°C. Proteins were then separated by 12% SDS-PAGE. Specific proteins were detected by WB using Galectin-9, Tim-3, and GAPDH polyclonal antibodies.

Statistical analysis. The western blot images were scanned and quantified with Quantity One Basic software (Bio-Rad, USA). Differences in gene and protein expression between tumor and normal tissues or tumor and adjacent tissues or adjacent and normal tissues were statistically analyzed using SPSS 17.0 (SPSS, Inc., Chicago IL, USA). Statistical comparisons were analyzed using the Student's t-test. Values of p<0.05 were considered statistically significant.
Results

**Downregulation of Gal-9 gene expression in gastric cancer.** To investigate the involvement of Gal-9 gene expression in the pathogenesis of primary gastric cancer, 44 clinical gastric cancer tissues and matched adjacent (<2 cm away from the tumor) and normal (>5 cm away from the tumor) tissues were used (Fig. 1A). Levels of expression of Gal-9 were measured by qPCR. Compared to matched normal or adjacent tissues, the gene expression of Gal-9 was significantly decreased in gastric cancer tissues (p<0.001 both) (Fig. 1C). Analysis of the mRNA expression of 44 samples showed significant (>2-fold decreased) downregulation of Gal-9 mRNA in 77% (34/44) of patients, whereas 2% (1/44) of patients showed significant (>2-fold increased) upregulation of Gal-9. Interestingly, Gal-9 expression in adjacent tissues had also a reduction (>2-fold decrease) in 34% (15/44) of samples (Fig. 1B). To determine whether the reduction of Gal-9 mRNA expression resulted in decreased Gal-9 protein levels, aliquots of whole cell extract from eight selected gastric cancer and corresponding normal or adjacent tissues were analyzed by western blotting with the indicated antibodies (Fig. 1D). As expected, there was significantly less Gal-9 protein in gastric cancer samples than in matched normal tissues (p<0.05). However, there was no significant difference between gastric cancer tissues and adjacent tissues (p>0.05).

Gal-9 gene expression and clinicopathological features of gastric cancer. To expand upon the observations given above and to determine the relationship between Gal-9 gene expression and clinicopathological parameters, qPCR results were examined according to the clinical characteristics of gastric cancer patients. The analysis showed that a lower expression of Gal-9 was associated with a higher incidence of lymph node metastasis, advanced tumor stage, and poorer overall survival (Fig. 1). These findings suggest that Gal-9 may play a role in the progression and prognosis of gastric cancer.
cancer. Gastric tumors were staged according to the 2010 TNM classification system using American Joint Committee on Cancer (AJCC) stage grouping (18). A summary of clinical characteristics of the patients, including age, gender, cell differentiation, and survival, is shown in Table I. Less Gal-9 expression was observed in cancer tissues than in both normal and adjacent tissues in both the >65 and ≤65 age groups, but the difference was significantly less pronounced in patients ≤65 years than in patients >65 years (p<0.05). However, there was no significant difference by gender, cell differentiation, or survival time.

Detailed statistical analyses were performed in order to further explore the correlation between Gal-9 expression and clinical features. The correlation between Gal-9 expression and clinical staging of cancer is shown in Fig. 2. There was significantly less (>2-fold) Gal-9 mRNA than in normal tissues in both normal and adjacent tissues in both the >65 and ≤65 age groups, but the difference was significantly less pronounced in patients ≤65 years than in patients >65 years (p<0.05). However, there was no significant difference by gender, cell differentiation, or survival time.

Analysis of the pathological stage showed significantly low levels of Gal-9 expression in pT2- and pT3-stage gastric cancer compared to normal (p<0.01) or adjacent (p<0.01 and p<0.05, respectively) tissues (Fig. 3B). A >2-fold reduction of Gal-9 mRNA was found in 75% (6/8) of pT2 and 73% (16/22) of pT3 (Fig. 3A). qPCR data were also analyzed based on adjacent lymph node metastasis and distant metastasis. A significant downregulation of Gal-9 mRNA in N2 (p<0.05) and N3 (p<0.001) of lymph node metastasis groups was observed (Fig. 4A). Despite the remarkably decrease in Gal-9 expression in patients with gastric cancer, no statistically significant difference was not found between with or without metastasis groups (Fig. 4B). In addition, a significant observation was discovered in the Gal-9 expression (Fig. 4C and D). Low levels of Gal-9 mRNA expression were observed in patients who survived for less (p<0.01) or more (p<0.01) than one year. Of all patients evaluated here, 66% survived less than one year.

Gal-9 and Tim-3 in gastric cancer. Gal-9 has been identified as a Tim-3 ligand and the binding of Gal-9 to Tim-3 induces cell death in Th1 cells (19). In order to assess the correlation between Gal-9 and Tim-3 in gastric cancer, Tim-3 gene expression was examined in 44 paired clinically diagnosed primary
Table I. Relationship between hGal-9 gene expression (qPCR) and clinicopathological characteristics of gastric cancer.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Case (n)</th>
<th>Normal mean ± SD</th>
<th>Adjacent mean ± SD</th>
<th>Cancer mean ± SD</th>
<th>p-value nor vs. adj</th>
<th>p-value nor vs. can</th>
<th>p-value adj vs. can</th>
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<tr>
<td>All</td>
<td>44</td>
<td>19.1±2.23</td>
<td>14.5±2.07</td>
<td>3.99±1.69</td>
<td>0.373</td>
<td>0.000477</td>
<td>0.000753</td>
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<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>≤65</td>
<td>21</td>
<td>23.3±2.23</td>
<td>14.4±2.11</td>
<td>5.35±1.67</td>
<td>0.303</td>
<td>0.0315</td>
<td>0.00389</td>
</tr>
<tr>
<td>&gt;65</td>
<td>23</td>
<td>15.3±2.22</td>
<td>14.6±2.05</td>
<td>2.75±1.70</td>
<td>0.909</td>
<td>0.00211</td>
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</tr>
<tr>
<td>Male</td>
<td>30</td>
<td>16.5±2.29</td>
<td>11.1±2.06</td>
<td>3.79±1.79</td>
<td>0.0739</td>
<td>1.35E-05</td>
<td>5.76E-05</td>
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<tr>
<td>Female</td>
<td>14</td>
<td>24.7±2.08</td>
<td>21.9±2.14</td>
<td>4.43±1.48</td>
<td>0.857</td>
<td>0.118</td>
<td>0.0415</td>
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<td>Differentiation</td>
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<tr>
<td>Well</td>
<td>2</td>
<td>10.3±1.95</td>
<td>10.6±1.66</td>
<td>25.1±13.0</td>
<td>0.982</td>
<td>0.518</td>
<td>0.503</td>
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<tr>
<td>Moderate</td>
<td>17</td>
<td>15.5±1.88</td>
<td>12.7±1.87</td>
<td>16.1±2.19</td>
<td>0.579</td>
<td>0.0083</td>
<td>0.519</td>
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<tr>
<td>Poorly</td>
<td>25</td>
<td>4.06±1.22</td>
<td>269±1.26</td>
<td>4.88±1.89</td>
<td>0.461</td>
<td>0.0199</td>
<td>0.0156</td>
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<tr>
<td>Survival of patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>&gt;12 months</td>
<td>15</td>
<td>15.0±2.17</td>
<td>13.9±1.99</td>
<td>4.03±1.43</td>
<td>0.836</td>
<td>0.00719</td>
<td>0.0195</td>
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<tr>
<td>≤12 months</td>
<td>29</td>
<td>21.3±2.27</td>
<td>14.9±2.13</td>
<td>3.98±1.82</td>
<td>0.385</td>
<td>0.00797</td>
<td>0.00726</td>
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</table>

Gastric tumor compared to normal tissues; *p<0.05; †p<0.01; ‡p<0.001. Gastric tumor compared to adjacent tissues; ^p<0.05; ¶p<0.01; ‡‡p<0.001. Analysis according to age group; ††p<0.05.

Figure 4. Gal-9 gene expression, metastasis of gastric cancer, and impact of Gal-9 gene expression levels on patient survival. (A) Statistical analysis of qPCR data according to the lymph node status. N0, no nearby lymph node metastasis; N1, 1-2 nearby lymph node metastasis; N2, 3-6 nearby lymph node metastasis; N3, >7 nearby lymph node metastasis. The significant difference between cancer and normal tissues is expressed as *p<0.05 and ‡‡‡p<0.001. (B) Relative mRNA levels of Gal-9 with or without distant metastasis. qPCR results were analyzed according to distant metastasis of gastric cancer. (C) Levels of Gal-9 mRNA expression and patient survival. The y-axis shows the ratio of expression of Gal-9 in gastric cancer versus matched normal tissues. The x-axis shows patient survival in months. (D) Differences in Gal-9 mRNA expression in patients who survived for different lengths of time. The 100% stacked column charts are here used to compare the numbers of cases of differentially expressed Gal-9 mRNAs. The total numbers of cases of differentially expressed mRNAs (increase, <2-fold reduction and >2-fold reduction) in gastric cancer tissues represents 100%.
gastric cancer and matched normal tissues using qPCR. As shown in Fig. 5A, cancerous tissues showed less expression of Tim-3 than in the matched normal tissues (>2-fold decreased) in 59% of patients. However, Tim-3 expression was significantly increased (>2-fold) in 25% of patients. Statistical analysis confirmed the absence of any significant difference between the tumor and matched normal tissues (p>0.05) (Fig. 5B). To further determine whether there is any regulatory relationship between Gal-9 and Tim-3 in cells, experiments were performed using gastric cancer cell lines as models. Gal-9 and Tim-3 protein expression levels were evaluated in gastric cancer cells SGC-7901 and MGC-803 (GES-1 gastric mucosal cell as control). Fig. 5C shows the protein expression levels of Gal-9 and Tim-3 by western blotting. To determine whether Tim-3 expression was regulated by Gal-9, MGC-803 cells were transiently transfected with 0.8 and 1.6 µg of Gal-9 cDNAs. Protein expression levels of Gal-9 increased dose-dependently (Fig. 5D top and middle). However, neither gene nor protein expression of Tim-3 was affected by the transient transfection of gastric cancer MGC-803 cells with Gal-9 cDNA (Fig. 5D, bottom; gene expression data not shown).

**Discussion**

The galectin genes are evolutionarily conserved from viruses to mammals (20). To date, 15 mammalian galectins have been identified, all of which contain one or two CRDs. Prototype galectins such as galectin-1, -2, -5, -7, -10, -11, -13 and -14 contain only one CRD. In contrast, tandem-repeat type galectins including galectin-4, -6, -8, -9, and -12 have two separate CRDs connected by linker peptides. Galectin-3 is the only chimeric type galectin. It contains a proline-glycine rich N-terminal tail fused to a CRD (3,4). The wide distribution of galectins and the variety of binding partners led them to function in multiple biological reactions, including mRNA splicing, cell apoptosis, cell cycle regulation, cell adhesion and migration, and cell differentiation (21-24).

Galectin-9 (Gal-9) is widely distributed in tissues involved in the immune system and in tissues of endodermal origin, such as spleen, thymus, liver, intestine, and stomach tissues. Low levels of Gal-9 expression were observed in breast, lung, renal, adrenal, prostate, skin, cervical, oral, brain, ovarian, and liver cancer cell lines, but not in leukemia or colon cancer.
cell lines (4). The expression of Gal-9 in tumor tissues has only been pathologically investigated in certain limited tumor types. Research published by several groups show levels of Gal-9 expression to be lower in cancer tissues than in normal tissues (11,15). These findings were consistent with the present results. In this study, the gene expression of Gal-9 was first investigated in clinically diagnosed primary gastric cancer tissues using qRT-PCR. Significant (>2-fold decreased) downregulation of Gal-9 mRNA was observed in 77% (34/44) of patients with gastric cancer. It is noteworthy that the abnormal expression of Gal-9 had already appeared in adjacent tissues (<2 cm away from the cancer tissue). Compared to matched normal tissues, although no statistically significant difference was found between adjacent and normal tissues, decreased expression of Gal-9 (>2-fold) in adjacent tissues had already emerged in 34% (15/44) of patients with gastric cancer. Loss of Gal-9 expression was found to be consistently correlated with distant metastasis (11,15). The results of the present study show low levels of expression of Gal-9 mRNA to be associated with clinical staging, tumor pT stage, cell differentiation, lymph node metastasis, and patient survival. However, no significant association was observed between Gal-9 expression and distant metastasis (p=0.0616, p>0.05). Recently, Jiang et al reported that although the Gal-9 shows pathologically higher levels of expression in gastric cancer than in normal tissues, low levels of Gal-9 expression are correlated with poor cancer prognosis (25). Combined with our findings, suggesting that low expression of Gal-9 is involved in tumorigenesis of gastric cancer.

Tim-3, a member of the T cell Ig and mucin domain (Tim) family, was found to be specifically expressed on terminally differentiated CD4+ Th1 cells, but not on Th2 cells (26). Cumulative findings indicate that the interaction of Tim-3 and its natural ligand Gal-9, exerts a crucial role in immune regulation. A recent study shows that the Tim-3/Gal-9 signaling pathway plays a critical role in the homeostasis of hepatic NKT cells through activation-induced apoptosis and secondary proliferation (27). In the present study, significantly less Tim-3 mRNA expression (>2-fold) was observed in gastric cancer tissues in 59% of patients, but higher than normal expression of Tim-3 (>2-fold) was also observed in 25% of the patients. Cell experiments showed no correlation between Gal-9 and Tim-3 in gastric cancer. Further investigation might be needed to determine the functional mechanism of Tim-3/Gal-9 in gastric cancer.

In conclusion, downregulation of Gal-9 mRNA was observed in gastric cancer tissues, and statistical analysis demonstrated the molecular mechanism underlying this process. These results showed that the loss of Gal-9 expression may be involved in the progression of gastric cancers. Although the Gal-9 gene was found to be involved in gastric cancer, further studies are required to address the many remaining questions, such as the effects of alternative splicing of Gal-9 in different tumor models.

Acknowledgements

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