GAL3 Protein Expression is Related to Clinical Features of Prolactin-Secreting Pituitary Microadenoma andPredicts its Recurrence after Surgical Treatment

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Key Words
Galectin-3 • Pituitary adenomas • Prognosis

Abstract

\textbf{Background:} Previous in vitro study showed that Galectin-3 (Gal-3) protein plays an important role in pituitary tumorigenesis, however, the association of Gal-3 expression with the clinical feature and prognosis of pituitary tumor in a clinical setting remains unknown. \textbf{Methods:} We enrolled 220 patients with prolactin-secreting pituitary adenomas (PA) who previously had transsphenoidal pituitary surgery. The Gal-3 expression was detected in the patients’ PA samples using immunohistochemistry and those patients were followed up. A prolactin-secreting PA cell line, the MMQ cell line, was used to study the in vitro effect of Gal-3 on proliferation, migration and invasion of PA cells using small interfering RNA (siRNA) transfection technique. The in vivo tumorgenesis in nude mice was also studied. \textbf{Results:} We found that Gal-3 expression was not related to age and sex, but positively associated with tumor invasion (P<0.001), tumor sizes (P<0.001) and pre-operative prolactin levels (P<0.001). The multivariate Cox analysis showed that the Gal-3 expression was closely associated with the recurrence of PA after the surgical treatment (HR =3.15, P=0.002). The in vitro studies showed that Gal-3 knock-down by the siRNA technique significantly inhibited the proliferation, migration and invasion ability of the MMQ cells, whereas Gal-3 siRNA transfection induced apoptosis of the MMQ cells. The in vivo tumorgenesis assay showed that Gal-3 siRNA transfection significantly inhibited the tumor volume in vivo compared to transfection of the control siRNA (P<0.001). \textbf{Conclusion:} Gal-3 regulates proliferation, apoptosis, migration and invasion of the MMQ cells. Gal-3 may be used as a tissue marker to evaluate the clinical feature and prognosis of PA patients.

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Introduction

Pituitary tumors occur in nearly 20% of the population and represent approximately 10% of surgically resectable intracranial tumors. Despite the histologically benign nature, pituitary adenomas (PA) is frequently invasive to the surrounding structures, making it hard to remove with the surgical treatment. The preoperative diagnosis of cavernous sinus invasion remains difficult and controversial, and there are currently no reliable histological or molecular markers that predict patients' responses to treatment and prognosis.

Galectin-3 (Gal-3) is a 31 kDa β-galactoside-binding lectin that is immunohistochemically expressed in macrophages, lymphocytes, and endothelial cells, and in numerous types of cancer cells [1, 2]. The expression of Gal-3 has been considered as a potential diagnostic and/or prognostic marker in stomach, colon-rectum, parathyroid, thyroid, breast, salivary glands, bladder, head and neck, prostate, and liver cancers [3-8]. Increasing evidence has revealed that Gal-3 protein plays an important role in pituitary tumorigenesis [9]. Immunohistochemical and Western blot analysis showed that human normal and tumor pituitary lactotroph (PRL) and corticotroph (ACTH) hormone-producing cells expressed Gal-3 [10]. Gal-3 has an important role in pituitary cell proliferation and tumor progression [11].

However, most of the above mentioned studies were conducted in vitro; the association of Gal-3 expression with the clinical characteristics of PA and its prognosis remains unknown. In this study, we investigated the correlation between the expression of Gal-3 in the tumor tissues and the clinical characteristics and outcomes of PA after the surgical treatment.

Materials and Methods

Enrollment and follow-up

A total of 220 patients diagnosed with prolactin-secreting PA were enrolled in this study. All patients underwent magnetic resonance imaging (MRI) at the time of diagnosis and again before the surgical treatment. The tumor size was determined by MRI and was classified as following: microadenomas (diameter, <1 cm), macroadenomas (>1 cm and <4 cm), and giant adenomas (>4 cm). PA with invasion of cavernous sinus, dura, sphenoid bone or nasopharynx was regarded as invasive adenomas. The age, sex and pre-operative prolactin levels were acquired from the medical records.

All selected patients underwent the transsphenoidal pituitary surgery to remove the tumor. Patients were followed up in out-patient clinics for an average of 4.5 year period (0.5-6.0 years). Patients with normal plasma prolactin levels, without clinical symptoms, and visible radiological tumor remnants, and not requiring dopamine agonist therapy were considered as remission. Recurrence was defined as an increase in plasma prolactin levels, with or without radiological evidence of any tumor mass, after a previous remission. The study was approved by the ethics committees of our hospital and written informed consent was obtained from each participant.

Immunohistochemistry

All PA tumor samples were obtained at the time of pituitary surgery after acquiring written informed consent from patients. All tissues were fixed in 10% buffered formalin, dehydrated, and embedded in paraffin. Histological examination and immunohistochemistry were performed. After antigen retrieval, tissue sections were incubated with anti-Gal-3 monoclonal antibody (Santa Cruz, 1:1000 dilutions). Positive and negative controls were run concomitantly in all analyses.

Cell culture and small interfering RNA (siRNA) transfection

A PA cell line, MMQ cell line, was obtained from the Cell Resource Center of the Chinese Academy of Medical Science (Beijing, China). Cells were cultured in medium and seeded at 24 h before small interfering RNA (siRNA) transfection. Synthetic Gal-3 siRNA (Japan Bio Services Co., LTD., Saitama, JAPAN) was transfected for 6 hours using the Lipofectamine™ 2000 transfection reagent (Life Technologies™, Carlsbad, CA, USA) according to the manufacturer's instructions.
Western Blot Analysis

The expression of Gal-3 was detected using the Western blot assay 48 hours after siRNA infection. The cells were lysed and samples (15 μg of proteins) were electrophoresed on a 10–12% SDS-PAGE gel and electroblotted onto the nitrocellulose membrane. The membrane was blocked in 5% non-fat milk and incubated with primary antibodies overnight at 4°C. Primary antibodies used were anti-Gal-3 (Santa Cruz, CA), anti-matrix metalloproteinases 2 (Anti-MMP2, Santa Cruz, CA) and anti-matrix metalloproteinases 9 (Anti-MMP9, Santa Cruz, CA). The intensity of bands was quantified using the densitometric analysis. Results were expressed as the ratio of intensity to that of its internal control.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining for apoptosis

Apoptosis of tumor cells after siRNA transfection was determined by the TUNEL assay using an in situ cell death detection kit conjugated with horse-radish peroxidase (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer’s instructions. Briefly, fixed cells were washed, permeabilized, and then incubated with terminal deoxynucleotidyl transferase end-labeling cocktail for 60 min at 37°C. The samples were then incubated with 50 μL of converter-POD, and in turn 3,3’-diaminobenzidine (DAB) substrate solution. The slides were analyzed under a light microscope using an inverted Nikon TE300 (Nikon, Melville, NY, USA).

Cell Proliferation Assay

Cellular proliferation was measured using a WST-8 Cell Counting Kit-8 (Beyotime, Nantong, China) after siRNA transfection. 3×10^3 cells were seeded in 96-well plates and incubated for 24 h. 10 μl CCK-8 solution was added to each well and the cultures were incubated at 37°C for 1 h. Absorbance at 450 nm was measured on an ELX-800 spectrometer reader (Bio-Tek Instruments, Winooski, USA).

Cell Migration Assay

After siRNA transfection, cell migration was determined using a modified two-chamber migration assay with a pore size of 8 μm. For migration assay, 1×10^5 cells were seeded on the upper compartment of 24-well Transwell culture chamber and 600 μl of complete medium was added to the lower compartment. After 24-hour incubation, cells were fixed with methanol. Non-traversed cells were removed from the upper surface of the filter carefully with a cotton swab. Traversed cells on the lower side of the filter were stained with crystal violet and counted.

Cell Invasion Assay

The invasion assay was performed using a modified two-chamber plates. 30 μl of 50 mg/ml Matrigel (BD Biosciences, Mississauga, Canada) in serum-free medium was added to the upper compartment of 24-well Transwell culture chamber. 1×10^5 cells suspended in 200 μl of serum-free medium were seeded on the upper compartment, and 600 μl of complete medium was added to the lower compartment. After 24-hour incubation at 37°C, cells were fixed with methanol. Non-invaded cells were removed from the upper surface of the filter carefully with a cotton swab. Invaded cells on the lower side of the filter were stained with crystal violet and counted.

In vivo tumorgenesis

Six-week-old nude mice were inoculated subcutaneously on the hind flank with MMQ cells (1×10^6) transfected with Gal-3 siRNA and control siRNA. The tumor size was assessed by caliper measurements twice a week. Mice were sacrificed four weeks after tumor cell inoculation and tumors were excised for further analysis. Tumor volumes were calculated using the following formula: (cubic millimeters) = (length × width^2) × 0.5. Mouse weights were recorded every 2 days [12].

Statistical analysis

All data were analyzed using the SPSS statistics software (version 16.0, Chicago, IL, USA). Association between the expression of Gal-3 expression status and the clinical parameters was studied using the chi-square, Fisher’s extract, or independent t tests. The log-rank test was used to analyze survival differences. Multivariate analysis was performed using the Cox proportional hazards model in forward stepwise. In vivo proliferation migration, invasion, and tumorgenesis were measured in nude mice and compared between
Fig. 1. The typical Figs of Gal-3 expression in PA tumor samples. Left: low Gal-3 expression. Right: high SIRT1 expression. Gal-3 was mainly expressed in cytoplasm of PA samples, but not in nucleus.

Table 1. The association between Gal-3 and clinical characteristics of PA patients stratified by Gal-3 expressions

<table>
<thead>
<tr>
<th></th>
<th>High expression n=94</th>
<th>Low expression n=126</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>40.4±3.4</td>
<td>41.2±4.1</td>
<td>0.386</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>50</td>
<td>70</td>
<td>0.416</td>
</tr>
<tr>
<td>Male</td>
<td>44</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Tumor invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence</td>
<td>46</td>
<td>31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Absence</td>
<td>48</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microadenomas</td>
<td>21</td>
<td>65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Macroadenoma</td>
<td>40</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Giant adenomas</td>
<td>33</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Pre-operative Prolactin</td>
<td>2459±347mU/l</td>
<td>1659±265mU/l</td>
<td>&lt;0.001</td>
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</table>

Table 2. Multivariate Cox proportional regression analysis

<table>
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<th></th>
<th>HR</th>
<th>95%CI</th>
<th>P</th>
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<tr>
<td>Gal-3 expression</td>
<td>3.15</td>
<td>2.15–3.97</td>
<td>0.002</td>
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<tr>
<td>Tumor invasion</td>
<td>2.68</td>
<td>1.98–3.76</td>
<td>0.007</td>
</tr>
<tr>
<td>Tumor size</td>
<td>2.11</td>
<td>1.67–4.28</td>
<td>0.012</td>
</tr>
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</table>

The MMQ cells treated with the Gal-3 and control siRNA using the t tests. A P value of <0.05 was considered statistically significant.

Results

The association between Gal-3 expression and tumor invasion

Immunohistochemical analysis shows positive immunoreactivity to anti-Gal-3 antibody in the cytoplasm of PA cells instead of nucleus (Fig. 1). The association between Gal-3 and clinical characteristics of PA patients stratified by Gal-3 expressions are shown in Table 1. High expression of Gal-3 was detected in 94 patients whereas low expression of Gal-3 in 126 patients. The mean age of patients at surgery was 40.8 ± 10.1 years (range; 19.6–57.6 yr).
MRI examination shows that tumor invasion was identified among 179 (44.75%) patients. A total of 21 patients had microadenomas, 40 had macroadenomas and 33 had giant adenomas. We found that Gal-3 expression was not related to age and sex, but positively associated with tumor invasion (P<0.001), tumor sizes (P<0.001) and pre-operative prolactin levels (P<0.001).

The Gal-3 expression predicts the prognosis of PA

Follow-up study shows that 35 out of 94 patients had recurrent tumor (recurrent rate: 37.2%) among patients with high Gal-3 expression, while 22 out of 126 patients had recurrent...
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The mean relapse-free survival period (RFS) in the high Gal-3 expression patients was 40.8±3.5 months, which was significantly shorter than those with the low Gal-3 expression (52.4±3.5 months, P<0.001 by the log rank analysis).

The multivariate analysis was performed by using the Cox proportional hazard model to evaluate the impact of Gal-3 expression on the prognosis of PA (Table 2). Our data showed that the Gal-3 expression (HR =3.15, P=0.002), tumor invasion (HR =2.68, P=0.007, Table 2) and tumor size (HR =2.11, P=0.012, Table 2) were closely associated with the recurrence of PA after the surgical treatment.

Gal-3 Regulates Proliferation, Migration and Invasion of PA Cells

Western blot results confirmed significant reduction of Gal-3 protein expression in the MMQ cells transfected with Gal-3 siRNA compared with cells transfected with the control siRNA (Fig. 2a). MMP2 and MMP9 are two major factors related to tumor cell invasion. Compared to cells transfected with the control siRNA, cells treated with the Gal-3 siRNA showed significantly reduced MMP2 and MMP9 expressions (Fig. 2b).

The results of MTT assays revealed that the proliferation rate was significantly inhibited by the Gal-3 siRNA transfection (absorbance at 562 nm: 0.87±0.16 vs. 0.45±0.11, P<0.001, Fig. 3a). The cell migration assay showed that Gal-3 knockdown by siRNA transfection significantly decreased the cells migration ability of the MMQ cells by (82±8 vs.48±13, P<0.001, Fig. 3b). Furthermore, silencing of the Gal-3 gene by siRNA dramatically inhibited the invasive ability invasion of the MMQ cells (95±12 vs.58±8, P<0.001, Fig. 3c). In contrast, the Gal-3 siRNA transfection dramatically increased the apoptosis rate in of cells compared to transfection of the control siRNA (7.5±1.1 vs.19.7±2.4, P<0.001, Fig. 3d).

The in vivo tumorigenesis assay showed that nude mice receiving the Gal-3 siRNA-treated cells (Fig. 4b) had the markedly reduced tumor volume compared to those receiving the control siRNA-treated cells (Fig. 4a). In addition, transfection of the Gal-3 siRNA significantly inhibited the tumor volume in vivo compared transfection of the control siRNA (P<0.001).
Discussion

Gal-3 is a member of a growing family of multifunctional proteins with at least one characteristic carbohydrate-recognition domain (CRD) [13-15]. Elevated Gal-3 levels have been associated with a wide range of neoplasms and events that may promote tumor progression and metastasis [15-17]. The potential diagnostic usage of Gal-3 has been supported by studies in thyroid, pulmonary, gastric, colon cancer and anaplastic large-cell lymphoma, although inconsistent results have been suggested from different studies [18, 19]. According to recent studies, Gal-3 play an important role in malignant potentials of the CNS tumors, pituitary cell proliferation and tumor progression; and ACTH pituitary carcinoma expresses a higher level of Gal-3 in comparison to pituitary adenoma [17, 20]. Here, we investigated the association between Gal-3 and the prognosis of patients with pituitary adenomas, and intended to explore its potential diagnostic and therapeutic implications.

In this study, we found that the higher Gal-3 expression group was significantly correlated with more frequent local pituitary adenoma invasion compared with the lower expression group. A statistically significant difference in the Gal-3 expression level was also observed among patients diagnosed with microadenoma, macroadenoma, and giant adenoma via MRI. This observation correlates well with unfavorable prognosis of pituitary adenoma, indicated by less PFS time and a higher risk of tumor recurrence in patients with a higher Gal-3 expression level.

Both our in vitro and the in vivo assays have also suggested that Gal-3 knock-down by the siRNA technique significantly inhibited proliferation, migration and invasion of the MMQ cells, and inhibited the tumor growth in nude mice, respectively. This is consistent with a previous study conducted in the pituitary cell line, HP75, indicating a similar interference of cell growth and apoptosis by the Gal-3-specific siRNA [17]. We also measured the protein expression level of MMP2 and MMP9 in addition to Gal-3 following the siRNA treatment. Cells treated with the Gal-3 siRNA presented with the reduced expression of MMP2 and MMP9 compared to cells transfected with the control siRNA. The MMPs are a group of matrix-degrading zinc-dependent proteolytic enzymes that cleave galectins including Gal-3 between G\textsuperscript{32}-A\textsuperscript{33} and A\textsuperscript{62}-Y\textsuperscript{63}, resulting in -27 and -22 kda peptides, respectively [21, 22]. Studies have shown that cleavage of Gal-3 can be used as a surrogate diagnostic marker for in vivo activities of MMP-2 and MMP-9 [21]. On the other hand, MMP-2 and MMP-9 have been well studied in association with cancer invasion and metastases [20]. In this study, the decreased expression of MMP2 and MMP9 was correlated to the reduced Gal-3 level and may be associated with significantly inhibited proliferation, migration and invasion of the MMQ cells using the si-RNA technique. Introduction of siRNA by transfection of the target cells is a potent approach to inhibit the synthesis of endogenous cellular proteins [23]. Promise of siRNA-based therapy has been shown in many diseases including cancer [23-26]. SiRNA therapy targeting Gal-3 hence may potentially contain progression of pituitary adenoma in conjunction with the surgery treatment and warrants further investigation.

Furthermore, our immunohistochemical analysis demonstrated that Gal-3 is predominantly present in cytoplasm of pituitary adenoma cells rather than nucleus. Similar findings have been suggested from the studies of tongue [27] and prostate cancer [28]. In those studies, a poorer disease-free survival was associated with the reduced expression of Gal-3 in the nucleus, and the increased disease progression was attributed to the cytoplasmic expression of Gal-3, respectively. Gal-3 is located in both intracellular and extracellular spaces, including the cell surface or the extracellular matrix [15, 29]. Its localization is dependent on the tissue, cell type, the proliferative state of the cells and the levels of differentiation [29-31]. Information on cellular localization of Gal-3 may also be valuable in understanding progression of pituitary adenoma cells because of its roles in tumor cell proliferation and differentiation.

Gal-3 is not an oncogene and the mechanisms involved in its tumorgenesis in pituitary adenomas remains to be studied. So far, different mechanisms have been proposed, including carbohydrate-mediated homotypic aggregation, angiogenesis, and inhibition of apoptosis.
[15, 29]. It may also be attributed to promotion of tumor transformation and metastasis through its regulation on cell cycle [29]. According to a multistep theory, molecular genetic alteration is the initial event that leads to cell transformation in pituitary tumor, and progression of tumor cells are further dependent on hormonal and/growth factor stimulation [32-36]. A series of cyclins and cyclin-dependent kinases (cdks) are known to play a central role in the regulation of cell cycle progression [34, 37], and their activity is modulated by cdk inhibitors. Mice lacking p27<sup>kip1</sup>, a cdk inhibitor, have an increased propensity to develop multiorgan neoplasia, such as pituitary tumor [38-40]. Interestingly, mutations of the p27<sup>kip1</sup> gene do not seem to play a role in human pituitary carcinogenesis [34, 40], however Gal-3 expression was markedly elevated in p27-null mice that develop pituitary hyperplasia and intermediod lobe of ACTH cell pituitary tumors [17, 41, 42].

The true incidence of PA may be underestimated since certain types of tumors are in slow growth and do not give rise to clinical symptoms [34]. To date, only a few molecular events leading to pituitary tumors have been proposed [17, 43]. Although the underlying actions involved in Gal-3 is vastly unclear, our study supports the potential application of Gal-3 in providing information on progression and invasion of pituitary adenomas when combined with clinical data. The current study is designed to provide direct and clinical evidence suggesting that Gal-3 may play an important role in prognosis of pituitary adenoma. According to this study and others, it is rationale to engage further studies on siRNA-based treatment with patients who histologically express a high level of Gal-3 in pituitary adenoma.

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