Effects of DNA Methylation on Galectin-3 Expression in Pituitary Tumors

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Abstract
Galectin-3 (Gal-3), a β-galactoside-binding protein is expressed in a specific cell-type manner in pituitary tumors. Here we questioned the mechanism of Gal-3 expression in pituitary tumors, by using methylation-specific PCR and DNA sequence analyses to analyze the methylation status of the promoter region of the LGALS3 gene. DNA analysis of a human pituitary tumor, breast carcinoma cell lines, and thyroid carcinoma cell lines showed that in cells expressing Gal-3 protein, the LGALS3 gene was unmethylated, whereas in Gal-3 null cells, the promoter of the LGALS3 gene was methylated. Treatment of cells with 30 μmol/L 5-aza-2′-deoxycytidine induced Gal-3 mRNA and protein expression. Among pituitary tumors, 30% (7/23), mainly in follicle-stimulating hormone/luteinizing hormone–producing (38%) and null cell (57%) adenomas, the promoter of the LGALS3 was found to be methylated and silenced, although prolactin- and adrenocorticotropic hormone–producing tumors, which were unmethylated, expressed the Gal-3 protein. These results show for the first time that Gal-3 expression is regulated in part by promoter methylation in pituitary as well as in other tumors. Because it is functionally involved in cancer progression and metastasis, Gal-3 may serve as a possible therapeutic target in the treatment of pituitary tumors. (Cancer Res 2005; 65(4): 1136–40)

Introduction
Galectins are a family of carbohydrate-binding proteins with a high affinity for β-galactoside (1). Members of the family have specific sequence homologies for specific carbohydrate-binding motifs (1). Although there are at least 14 members of the family that are involved with β-1,3 galactosylation (2), Galectin-3 (Gal-3) is one of the most frequently investigated members of the group due to its involvement in cancer progression and metastasis (3–8). The human Gal-3 is a 31-kDa chimeric protein with three distinct structural domains including a 12-amino acid NH₂-terminal domain that controls cellular targeting, a collagen-like sequence rich in glycine, tyrosine, and proline which functions as a substrate for matrix metalloproteinases, and a carboxyl-terminal domain similar to Galectin-1 with a globular structure containing a carbohydrate-binding site (3). Gal-3 is highly expressed in a variety of human cancers and tumor cell lines including thyroid, breast, colon, pancreas, liver, and kidney (3–6). Gal-3 is involved in diverse biological processes including cell growth, differentiation, cell adhesion, angiogenesis, tumor progression, apoptosis, and metastasis (7, 8). We have recently reported that Gal-3 is expressed in anterior pituitary cells and tumors and its expression was restricted to adrenocorticotropic hormone (ACTH)- and prolactin-producing tumors (9). To examine the mechanisms regulating Gal-3 expression in normal and neoplastic pituitaries, we analyzed the LGALS3 gene (10) for possible genetic and epigenetic alterations including the methylation status of the promoter region. Our results show, for the first time, CpG island methylation in the LGALS3 promoter region of a significant percentage of tumors which did not express Gal-3 protein indicating that epigenetic regulation is important in Gal-3 expression in pituitary tumors.

Materials and Methods
Cell Culture. The following cell lines and culture conditions were used, HP75 pituitary cell line developed in our laboratory (9), MDA-MB468, MDA-MB231 breast carcinoma and the HeLa cell lines obtained from American Type Culture Collection (Manassas, VA). All of these lines were cultured in DMEM with 15% horse serum, 2.5% FCS, and 1% antibiotics (Invitrogen, Carlsbad, CA). SKBR3 breast carcinoma cells (from ATCC) were cultured in McCoy’s 5A medium modified with 10% fetal bovine serum (Invitrogen) and maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air.

The NPA and TPC1 thyroid papillary carcinoma cells were obtained from Dr. Yuri Nikiforov at the University of Cincinnati, Cincinnati OH. The TT1 follicular thyroid carcinoma cell line was developed in our laboratory. All three thyroid cell lines were cultured in DMEM with 15% horse and 2.5% FCS (Invitrogen).

The SKBR3 cells were treated with 30 μmol/L 5-aza-2′-deoxycytidine (5-Aza-CdR; Sigma Chemical Company, St. Louis, MO) for 4 and 8 days in three independent experiments. Control dishes received DMEM. Cells were harvested and used for DNA, RNA, protein analysis, and for immunostaining (9, 11) at the end of the experiments.

DNA Preparations. Genomic DNA was extracted from frozen pituitary tissues and cell lines. Tissue was incubated in a solution containing 10 mmol Tris-HCl (pH 7.4), 25 mmol EDTA, and 10 mmol NaCl at 37 °C for 30 minutes. Proteinase K (Roche Diagnostics, Almeda, CA) and SDS (Sigma) were added to the solution to a final concentration of 0.2 mg/mL and 0.8%, respectively, and the tissues incubated overnight at 55 °C. After incubation, 1 volume phenol/chloroform/isoamyl alcohol (1:1:1) was added, and the aqueous layer containing the DNA was transferred to a separate tube. One volume of chloroform/isooamyl alcohol (1:1) was then added, and the aqueous layer again removed. Genomic DNA was precipitated by the addition of 2.5 volumes of 100% ethanol (Sigma) and 1 vol. 3 M sodium acetate (pH 5.2) was added, and the aqueous layer was removed. Genomic DNA was dissolved in 100 μl water and quantitated with a spectrophotometer.

Bisulfite Modification. Two micrograms of genomic DNA from each tissue specimen or cell line was bisulfite-modified using the EZ DNA methylation kit (Zymo Research, Orange, CA) according to the manufacturer’s instructions. One microgram CPGenome Universally Methylated DNA (Chemicon International, Temecula, CA) was used as a

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Galectin-3 DNA Methylation

Results

Galectin-3 Methylation and Protein Expression in Cell Lines. Gal-3 expression was examined in a series of cell lines including a human pituitary (HP75), HeLa, three breast carcinoma cell lines (MDA-MB-468, MDA-MB-231, and SKBR3) and three thyroid carcinoma cell lines (PTC-1, NPA, and TT1). All but one of these cell lines expressed Gal-3 protein and the LGALS3 gene promoter region was unmethylated (Fig. 1). The TT1 cell line showed both methylated and unmethylated CpG islands (Fig 1A) and expressed lower levels of Gal-3 protein compared with the other cell lines (Fig 1B). The SKBR3 breast carcinoma cell line did not express Gal-3, and methylation-specific PCR and sequencing showed that the promoter region of the LGALS3 gene in this cell line was methylated. Treatment of SKBR3 cells with 30 mol/L of 5-Aza-CdR for 4 and 8 days resulted in Gal-3 mRNA and Gal-3 protein expression (Fig. 2).

Methylation-Specific PCR and Gal-3 Protein Expression in Pituitary Tumors. Methylation-specific PCR using specific primers showed that normal pituitary and, prolactin-, growth hormone (GH)- and ACTH-producing tumors were unmethylated.
as determined after methylation-specific PCR followed by cloning and sequencing. Overall, 30% (7/23) of the pituitary tumors were methylated, whereas 38% of the follicle-stimulating hormone/luteinizing hormone (FSH/LH)-producing and 57% of the null cell adenomas were methylated in the LGALS3 promoter region (Table 1; Fig. 3A).

Immunohistochemical analysis revealed that Gal-3 was expressed in a subset of cells in the normal anterior pituitary (data not shown). Adenomas producing prolactin and ACTH were positive for Gal-3, whereas adenomas producing FSH/LH, GH, and most null cell adenomas were negative for Gal-3 (Table 1). Western blotting supported the immunohistochemical findings (Fig. 3B). There was some variation in the amount of Gal-3 protein expression in the two prolactin adenomas shown, although both adenomas were unmethylated in the LGALS3 promoter region (Fig. 3A and B). Sequencing showed that the percentage of the total of 36 cytosine sites that were methylated ranged from 67% to 93% (mean of 84%) for the FSH/LH-producing adenomas, and from 10% to 93% (mean of 46%) for null cell adenomas (Fig. 3C).

Amplification of a 488 bp fragment in the promoter region of the LGALS3 gene did not show any mutations in this region in a normal pituitary, in a null cell adenoma, and in an ACTH-producing carcinoma (data not shown). Reverse transcription-PCR analysis and sequencing of the amplified product of total RNA from a normal pituitary and in an ACTH-producing carcinoma did not show any mutations in the coding regions of the LGALS3 gene (data not shown).

**Table 1. Analysis of Normal and Neoplastic Pituitaries for Gal-3 Methylation and Protein Expression**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Diagnosis</th>
<th>Gal-3 Methylation-specific PCR* expression</th>
<th>Gal-3 Protein expression</th>
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<tbody>
<tr>
<td>1</td>
<td>Normal pituitary</td>
<td>U+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Normal pituitary</td>
<td>U+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Normal pituitary</td>
<td>U+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Prolactin adenoma</td>
<td>U+</td>
<td>+</td>
</tr>
<tr>
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<td>Prolactin adenoma</td>
<td>U+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Prolactin adenoma</td>
<td>U+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>ACTH adenoma</td>
<td>U+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>ACTH adenoma</td>
<td>U+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>ACTH carcinoma</td>
<td>U+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>GH adenoma</td>
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<td>11</td>
<td>GH adenoma</td>
<td>U+</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
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<td>M–</td>
<td>–</td>
</tr>
<tr>
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<td>FSH/LH adenoma</td>
<td>U ND</td>
<td>–</td>
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<td>26</td>
<td>Null cell adenoma</td>
<td>U+</td>
<td>–</td>
</tr>
</tbody>
</table>

**Note:** ND, not done because sufficient protein was not available. Gal-3 protein was analyzed by Western blotting and/or immunohistochemistry.

*Gal-3 methylation was analyzed by methylation-specific-PCR and sequencing of all cases using at least three clones for analysis.

†Positive (+) or negative (−) for Gal-3 protein expression.

**Discussion**

Galectins are involved in many biological processes including cell growth, adhesion, apoptosis, tumor progression, and metastasis (3, 7, 8). Gal-3 is expressed in many human tumors and cell lines (4–8). We recently observed a differential expression of Gal-3 in pituitary tumors (9); however, the mechanisms regulating Gal-3 gene expression in these tumors is still unknown.

In the present study, we show for the first time that Gal-3 expression in cell lines and in pituitary tumors is related to the promoter methylation status of the LGALS3 gene. These findings help to explain the observed differential expression of Gal-3 protein in pituitary tumors (9). However, promoter methylation (13) is probably not the only factor regulating Gal-3 protein expression in pituitary tumors, because some FSH/LH-producing and null cell tumors were unmethylated and did not express Gal-3 protein. Similarly, the two GH-producing adenomas which did not express Gal-3 proteins were unmethylated. These observations

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**Figure 2.** Analysis of SKBR3 breast carcinoma cell line by reverse transcription-PCR and Western blotting. A, reverse transcription-PCR analysis shows that 30 μmol/L 5-Aza-CdR treatment for 4 and 8 days led to expression of Gal-3 mRNA in the SKBR3 cells. B, Western blot shows that the untreated SKBR3 cells did not express Gal-3 (left), although treatment with 30 μmol/L 5-Aza-CdR for 4 and 8 days results in Gal-3 expression. The HeLa cell line was used as a positive control. β-Actin was used to check for equal loading of the gel.
suggest that additional mechanisms such as histone acetylation may also contribute to the expression of Gal-3 in pituitary as well as other tumors. Our preliminary experiments using sodium butyrate to treat the SKBR3 and other methylated cell lines did not increase the level of Gal-3 expression compared with 5-Aza-CdR (data not shown). However, the role of promoter methylation in regulating Gal-3 mRNA and protein expression was shown by treatment of the Gal-3 null SKBR3 breast carcinoma cell line, which did not express Gal-3 mRNA or protein, with 5’ Aza-CdR which induced Gal-3 expression in these cells.

Recent studies of other members of the galectin family including galectin-1 and galectin-7 suggest that methylation is important for the regulation of galectin expression (14–16). Benvenuto et al. (14) used restriction endonucleases and sodium bisulfite analysis of genomic DNA from expressing and unexpressing cell lines to show a correlation between gene activity and demethylation of the 5’ region of the LGALS-1 gene. Moisan et al. (15) recently showed that treatment of nonaggressive lymphomas with 5-Aza CdR–induced galectin-7 gene expression. Arar et al. (16) observed that both expressing (BB-1) and nonexpressing (RCMV-5) cell lines were able to activate a reporter cell gene under the control of the regulatory gene of the LGALS3 gene in transfection experiments. It was suggested that this phenomenon was the result of a repressor acting on a genomic region not present in the reporter plasmid or by DNA methylation (16). Our own data shows, for the first time, that DNA methylation is involved in the regulation of Gal-3 expression in at least some pituitary tumors, in breast carcinoma, and in thyroid carcinoma cell lines.

Our current findings provide further insight into earlier observations that Gal-3 was differentially expressed in pituitary tumors (9). The nonfunctional tumors including FSH/LH and null cell adenomas which did not express Gal-3 proteins were frequently methylated, whereas the functional prolactin- and ACTH-producing tumors which expressed Gal-3 were unmethylated. In addition to its role as an antiapoptotic molecule (8), Gal-3 has a critical role in tumor progression and metastasis. Interestingly, the two main subtypes of pituitary tumors expressing Gal-3 are the ones associated with pituitary carcinomas, which are aggressive neuroendocrine tumors (17). These findings suggest that the expression of Gal-3 in prolactin- and ACTH-producing pituitary tumors may be associated with progression to carcinomas in association with other genetic alterations in these tumors (18), because prolactin- and ACTH-producing carcinomas constitute the majority of reported pituitary carcinomas (17).

An increasing number of studies have shown that epigenetic changes with DNA methylation probably have an important role in pituitary tumorigenesis (18, 19). These genes have included INK4A (p16), GADD45-g, RBL, DAPK, and others such as KIP1 (p27; refs. 18, 19). One unique aspect of LGALS3 promoter methylation in the pituitary which differs from the model of
Herman and Baylin (13) is that the normal pituitary prolactin- and ACTH-producing cells also expressed Gal-3, indicating maintenance of cell and tumor type specificity in the expression of this gene product in pituitary adenomas and carcinomas. In addition to abnormal epigenetic methylation, which is observed as a pathologic process (13), methylation in endocrine tissues has also been observed as a physiologic regulatory process. For example, during the regulation of the hormonal control of prolactin and GH gene expression in rat pituitary gland during gestation and lactation, there are changes in the expression of prolactin and GH gene by altered methylation patterns (20). Because Gal-3 has a role in tumor progression and metastasis, the expression of Gal-3 in these cell types probably contribute, along with other genetic alterations, to the progression to pituitary carcinomas.

In summary, this study shows that cell specific expression of Gal-3 in various human tumor cell lines and in pituitary tumors is regulated by DNA methylation of the promoter region. It is possible that because of Gal-3 expression and function in tumor progression and metastases, the unmethylated state of some pituitary tumors may contribute to the higher incidence of pituitary carcinomas observed in functional (prolactin- and ACTH-producing) tumors. These results suggest that because it is functionally involved in cancer progression and metastasis, Gal-3 may serve as a possible therapeutic target in the treatment of pituitary tumors.

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