Galectin-3 as an Immunohistochemical Tool to Distinguish Pilocytic Astrocytomas from Diffuse Astrocytomas, and Glioblastomas from Anaplastic Oligodendrogliomas

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The distinction of astrocytomas and oligodendrogliomas, mainly pilocytic astrocytomas (PILOs) from infiltrating astrocytomas and oligodendrogliomas (ODs), and high-grade oligodendrogliomas from glioblastomas (GBMs), poses a serious clinical problem. There is no useful immunohistochemical (IHC) marker to differentiate these gliomas, and sometimes the differential diagnosis between them is arbitrary. We identified galectin-3 (Gal-3) as a possible tool to differentiate them based on gene expression profiles of GBMs. We confirmed the differential expression in 45 surgical samples (thirteen GBMs; seven PILOs; five grade II ODs; five anaplastic oligodendrogliomas [AODs], including two Oligo-astrocytomas; 8 diffuse astrocytomas [ASTs], and 7 non-neoplastic samples) by quantification of Gal-3 gene expression by real-time quantitative PCR (rt-PCR). Higher expression of Gal-3 was observed in GBMs and PILOs than in OD, AODs, and ASTs. The IHC expression of Gal-3 was evaluated in 90 specimens (fifteen PILOs, fourteen ASTs, 10 anaplastic astrocytomas, fifteen GBMs, eleven ODs, fifteen AODs, and 10 dysembryoplastic neuroepithelial tumors). The mean labeling score for Gal-3 determined according to the percentage of labeled cells in the tumor bulk was significantly different in GBMs versus AODs and in PILOs versus ASTs. Hence, Gal-3 is differentially expressed in central nervous system tumors, making IHC detection of Gal-3 a useful tool in distinguishing between these gliomas.

INTRODUCTION

The distinction of pilocytic astrocytomas (PILOs) from infiltrating astrocytomas, oligodendrogliomas (ODs), and other low-grade lesions, including dysembryoplastic neuroepithelial tumors (DNTs), can sometimes be difficult. Similarly, the discrimination of high-grade oligodendroglioma from glioblastoma (GBM) poses a serious clinical problem, since anaplastic oligodendroglioma (AOD) may respond to chemotherapy with a relatively long survival time, in contrast to GBM with its poor prognosis (39). Although several studies have focused on the search for biological markers for oligodendrogial neoplasm (13, 14, 19, 28, 29, 31, 33, 36, 43, 51), there is no immunohistochemical marker that distinguishes between these entities.

The comparison of the gene expression profile of GBM with non-neoplastic brain tissue by Serial Analysis of Gene Expression (SAGE) (NCBI web site: http://www.ncbi.nlm.nih.gov/SAGE) reveals the overexpression of galectin-3 (Gal-3), a 31 kDa β-galactoside-binding lectin (18). Gal-3 has been observed in macrophage, endothelial cell, lymphocyte and different tumors, including central nervous system tumors (CNS) tumors (8, 32, 42), and plays a role in cell growth, adhesion, migration, and cell death (2, 12, 21, 22, 39, 50). The CNS contains large amounts of glycoconjugates, and gliaoma cells produce and secrete extracellular matrix (ECM) components involved in cell adhesion and migration (44). These specific characteristics, associated with the fact that Gal-3 mediates cell-cell and cell-matrix interactions, have lead to several studies of Gal-3 expression in glial tumors of the CNS. However, the role of this lectin in these tumors has not been fully addressed, while some of the previous data are conflicting (4, 5, 10, 16, 24, 47). Additionally, there is no systematic study using molecular and immunohistochemical (IHC) approaches to compare Gal-3 expression in astrocytic and oligodendrogial neoplasms of different grades of malignancy.

Thus, in the present study we report the distinct gene expression of Gal-3 in surgical specimens of astrocytomas and oligodendrogliomas, on the basis of quantitative real-time polymerase chain reaction (rt-PCR), and propose that IHC expression of Gal-3 is a useful tool to differentiate GBM from AOD, and grade I and II astrocytomas, even in small samples obtained by stereotactic biopsies.

MATERIAL AND METHODS

Tissue samples. Tumor samples were obtained from 90 patients with astrocytomas, oligodendrogliomas of different grades of malignancy, and DNTs. All tumor specimens were examined microscopically and graded according to the latest WHO classification of CNS tumors (23) by 2 independent neuropathologists with full diagnostic agreement. The tumors were graded as: PILOs (n = 15), diffuse astrocytoma, grade II (ASTs) (n = 14), anaplastic astrocytoma, grade III (AAs) (n = 10), GBMs, grade IV (n = 15), ODs, grade II (n = 11, including one oligoastrocytoma), AODs, grade III (n = 15, including one anaplastic oligoas-
tromyoma), and ten DNTs. The age ranges of the patients with these 7 tumor groups were, respectively: 3 to 56 (median: 14 years), 7 to 46 (median: 34 years), 9 to 35 (median: 30 years), 36 to 81 (median: 61 years), 7 to 46 (median: 48 years), 20 to 73 (median: 46 years), and 18 to 50 (median: 31 years) (Table 1). RNA was extracted from 38 tissue samples for molecular analysis: ten ODs and AODs (including 2 cases of oligoastrocytomas, one of grade II and another of grade III), seven PILOs, eight ASTs, and thirteen GBMs. Additionally, non-neoplastic brain tissues were obtained from 7 individuals submitted to temporal lobe resection of epilepsy surgery.

**Immunohistochemical detection of Gal-3**. Sections were deparaffinized and subjected to heat-induced epitope retrieval by steaming for 40 minutes at 98°C in a citrate buffer (0.001M, pH 6.0). After blocking of nonspecific binding sites using 2% normal horse serum in PBS (pH 7.4) for 60 minutes, primary antibody (anti-Gal-3, monoclonal, 1:200, Novocastra®) was applied overnight at room temperature. Labeling was detected by adding biotinylated secondary antibody for 30 minutes (anti-human universal antibody, 1:200, Novocasra®) with avidin-biotin complex, and 3, 3’-diaminobenzidine. Sections were then counterstained with Harris hematoxylin. Negative controls were performed by omitting the primary antibody from the immunohistochemical reagents.

**Microscopic evaluation.** The descriptive analysis was supplemented by a quantitative evaluation of Gal-3 label. The percentage of labeled cells (considering nuclei and cytoplasm, and excluding endothelia) in the tumor bulk was assessed using labeling scores determined simultaneously by 2 observers as follows: 0 = negative, 1 = up to 10% of labeled cells; 2 = 11% to 25% of labeled cells; 3 = 26% to 75% of labeled cells; and 4 = more than 75% of labeled cells. The mean labeling score (MLS) for Gal-3 was determined for each tumor group (Table 1).

Surgical material preparation for gene expression studies. During surgery, tissue samples from tumors were collected, frozen in liquid nitrogen and stored at -70°C in appropriate vials. Guided by the microscopic examination of cryostatic sections, the samples were microdissected by apposition, in order to remove areas of necrosis, cellular debris and any non-neoplastic tissue. A diagnostic confirmation was performed during this procedure. The tumor areas of interest were collected in microvials, kept under dry ice throughout all the manipulation and stored at -70°C until final usage.

**RNA isolation and cDNA synthesis.** RNA was isolated using the classic Trizol method (Invitrogen®, Paisley, United Kingdom) and preliminary evaluation of RNA integrity was done by RNA Gel Electrophoresis. cDNA was synthesized from 2 µg of total RNA by extension with random hexamer primers (Invitrogen®, Paisley, United Kingdom), and 200U of Superscript II reverse transcriptase (Invitrogen®, Paisley, United Kingdom).

**Real-Time TaqMan assay.** rt-PCR for Gal-3 was performed using Assay-by-Design (Applied Biosystems®, United Kingdom) TaqMan primers and probe, following the technique described previously in detail (17). Primers used were gal3F 5′-GCCAC TGGCC CCTAT GG-3′, gal3R 5′-AGGCA CAATC AGTGG C-3′, MGBNFQ. Primers were positioned 60 nucleotides apart (Invitrogen®), United Kingdom). The primer used was gal3, with 3, 3′-diaminobenzidine. Sections were then counterstained with Harris hematoxylin. Negative controls were performed by omitting the primary antibody from the immunohistochemical reagents.

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**Table 1.** Gender, age at the time of surgery, tumor location, and immunohistochemical data of Gal-3 labeling in the tumor bulk and the endothelium of astrocytomas and oligodendrogliomas of different malignancy grades and dysgenetic astrocytomas.
Molecular expression of Galectin-3. The mRNA levels for Gal-3 measured by rt-PCR in PILOs and GBMs were similar, being 5.02- and 6.06-fold lower in OD than normal samples. Gal-3 transcript levels are different in PILOs (p = 0.0036) and GBMs (p = 0.0145), but not in AODs (p = 0.074) and ASTs (p = 0.258) when compared with normal CNS tissues. A significant difference was found when Gal-3 transcript levels were compared between PILOs and ASTs (p = 0.0016) and between AODs and GBMs (p = 0.029).

Statistical analysis. Student's t-test or a normal reference distribution was used to construct 95% confidence intervals for the differences in the different grade astrocytomas and normal CNS samples. Differences were considered to be statistically significant at p < 0.05.

RESULTS

Molecular expression of Galectin-3. The mRNA levels for Gal-3 measured by rt-PCR in PILOs and GBMs were similar, being 5.02- and 6.06-fold higher than in normal CNS tissues. Two micrograms of total RNA from oligodendrogliomas (OD), anaplastic oligodendrogliomas (AOD), glioblastomas (GBM), pilocytic astrocytomas (PILO), astrocytomas (AST) and normal central nervous system samples were reverse transcribed and 100 ng of cDNA were analyzed by rt-PCR. Expression levels (log_{10} 2\Delta CT) were calculated based on GAPDH expression levels of each sample. The horizontal bar shows the median of each group. The level of Gal-3 was 1.52-fold lower in AODs, 3.3-fold lower in AODs, and -5.6-fold lower in OD than normal samples. Gal-3 transcript levels are different in PILOs (p = 0.0036) and GBMs (p = 0.0145), but not in AODs (p = 0.074) and ASTs (p = 0.258) when compared with normal CNS tissues. A significant difference was found when Gal-3 transcript levels were compared between PILOs and ASTs (p = 0.0016) and between AODs and GBMs (p = 0.029).

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Immunohistochemical expression of Galectin-3. The results of IHC staining for Gal-3 in astrocytomas, oligodendrogliomas of different malignancy grades and DNTs are presented in Table 1. A distinct Gal-3 expression was observed in PILOs (Figure 2C, D) compared to ASTs (Figure 2E), AAs, and DNTs (Figure 2F). PILOs presented a diffuse Gal-3 expression (Figure 2C) in tumor cells, both in the nuclei and cytoplasm.
the cytoplasm (Figure 2D), whereas in ASTs (Figures 2E) and AAs, no tumor cells expressed Gal-3, and few DNTs presented score 1 Gal-3 reactivity, both in the nuclei and the cytoplasm of tumor cells (Table 1). The endothelium in PILOs did not stain for Gal-3 (Figure 2D), in contrast to non-tumoral adjacent brain parenchyma, which exhibited strong immunoreactivity for Gal-3 in vessels (Figure A), and reactive astrocytes (Figure 2B). The majority of the endothelium of tumor vessels in ASTs was positive for Gal-3 (Figure 2E, asterisk), but the expression was variable in AAs and DNTs (Figure 2F, asterisk). A weak Gal-3 expression in the tumor cells of the astrocytic component in a case of grade II Oligoastrocytoma was detected. However, there was no reactivity in the oligodendroglial counterpart of the tumor (Figures 2G, H).

Diffuse extracellular immunopositivity was observed in all cases of PILOs and GBMs, being less intense than the immunostaining detected in the nuclei and the cytoplasm. GBMs presented less extracellular positivity than PILOs.

In contrast to the positive immunostaining of PILOs and GBMs at tumor cores, the non-neoplastic areas presented Gal-3 expression only in vessels and any arising reactive astrocytes (Figure 2A, B), where no expression was observed extracellularly.

The mean labeling scores (MLS) obtained in the tumor bulk were significantly different in PILOs (3.2 ± 0.2 SD) and GBMs (3.5 ± 0.2 SD) in comparison with all other gliomas analyzed, which were uniformly negative, except for a case of oligodendroglia that was labeled with a 2 score in the astrocytoma component, and few cases of AOD with a 1 score either in the nuclei or the cytoplasm of tumor cells.

**DISCUSSION**

The present study demonstrates the distinct gene expression of Gal-3 in CNS tumors, with significantly higher mRNA levels in GBMs and PILOs, in contrast with the low or absent expression in diffuse astrocytomas, oligodendrogliomas, and DNTs. The mean levels for the PILO and GBM groups were respectively 5.02- and 6.2-fold higher than the levels observed for normal samples. In addition, the Gal-3 transcript levels in anaplastic oligodendrogliomas and grade II astrocytomas did not
differ from the non-neoplastic CNS tissues. Accordingly, the immunohistochemical staining with a monoclonal antibody reflected these results at the protein level and forms the basis for a simple laboratory approach to help toward distinguishing these tumors.

The pattern of IHC expression was heterogeneous in GBMs, present mainly in the tumor cells forming pseudopalisades around areas of necrosis and in bizarre tumor cells. In contrast, PILOs showed diffuse staining of Gal-3, present in the majority of cells in the tumor bulk. In oligodendrogliomas, regardless of the malignancy grade, the IHC expression of Gal-3 was not observed in tumor cells, even in pseudopalisades, and only infiltrating macrophages, occasional reactive astrocytes entrapped in the tumor bulk, and endothelial cells stained positively. As two-thirds of oligodendrogliomas respond to chemotherapy, patients with these tumors have longer survival time in comparison with ordinary GBMs (11, 38, 40). Differential diagnosis between tumors is therefore important, mostly in cases where the setting of classical morphological characteristics is not presented.

By the same token, PILOs exhibit diffuse staining of Gal-3 whereas ASTs, and DNTs do not. The IHC detection of Gal-3 is of diagnostic interest, as it can sometimes be difficult to discriminate from infiltrating astrocytomas, oligodendrogliomas and other low grade lesions such as DNTs, which present diverse biological behavior, prognosis and treatment.

Perhaps the high expression of Gal-3 in 2 tumors with such distinct PILO and GBM could be a coincidence, or may depend on common causes and mechanisms. The exact meaning of the high Gal-3 expression remains an open question. Galectins are a large family of structurally related beta-galactoside-binding proteins, recognized by their affinity for poly-N-acetyllactosamine-enriched glycoconjugates and sequence similarities in the carbohydrate recognition domain, that participate in the control of cell differentiation, proliferation, activation and cell death in different tissues (2, 6, 26, 41). Gal-3, one of the fourteen-member family, is found in both the extracellular (cell surface and medium) and in the intracellular (nucleus and cytoplasm) compartments (20), where it exists in a nonphosphorylated (concentrated in the nucleus) and phosphorylated form (nucleus and cytoplasm) (7). The intracellular localization of Gal-3 is consistent with several interacting ligands: Bcl-2, CBP70, Chrp, Gemin4 and cytokeratin (15, 30, 35, 46, 49).

Although GBMs and PILOs express Gal-3 with similar labeling pattern, ie, both in the nuclei and the cytoplasm of tumor cells, expression in GBMs were found mainly in the pseudopalisading of tumor cells around serpiginous areas of necrosis and in multinucleated giant tumor cells, whereas the staining was diffuse in PILOs, with no “hot spots” as seen in GBMs. Pseudopalisading glioma cells are the boundaries of the hypoxic tissue microenvironment. Expression of Gal-3 in these cells may have a protective role, since Gal-3 has been reported as an anti-apoptotic molecule (50). On the other hand, it has recently been reported that glial tumor cells in these pseudopalisading areas are migrating away from hypoxic regions. The strong staining here may be therefore also related to cell motility (3). In fact Gal-3 mediates cell-cell and cell-matrix interaction, particularly adhesion to laminin, fibronectin (45), integrins (34), and binds specifically to the neuronal adhesive glycoproteins L1, N-CAM and myelin-associated glycoprotein MAG (39). Further studies detailing the interaction of Gal-3 with the above pathways would clarify whether this lectin is also involved in cell motility.

Our present data conflict with some earlier reports describing decreasing Gal-3 immunopositivity from PILO to GBM (5, 16). However, our immunohistochemical results were substantiated by mRNA expression profiles. Interestingly OD and AOD, in contrast to WHO grade I and

Figure 3. H&E stained section showing a glioblastoma (GBM) (A, ×200) and respective Gal-3 expression along the pseudopalisading (B, ×100). Microvascular proliferation showed lack of Gal-3 reactivity (C, ×200), whereas the nuclei and the cytoplasm of tumor cells showed conspicuous Gal-3 expression (D, ×400), including the multinucleated giant cell (D, insert ×400). Anaplastic oligodendroglioma (AOD) presented absence of Gal-3 expression in tumor cells, even in the pseudopalisading (E, ×200; F, ×400). Only histiocytes (E, #), reactive astrocytes (F, arrow heads) and vessels (F, *) exhibited immunoreactivity for Gal-3.
IV astrocytomas, showed no Gal-3 immunostaining, whereas Deininger et al (10) described fewer Gal-3 positive cells in WHO grade II than in grade III primary oligodendrogliomas. At least part of these contradictory results may be due to the use of different techniques, such as distinct antigen retrieval procedures, and the variable amounts of Gal-3 expressed by distinct tumor clones (16). The difficulties in establishing the diagnosis based on morphological characteristics may also explain these distinct results, mainly in those tumors primarily classified as OD/AOD and subsequently as GBM on relapses.

Another interesting finding was the lack of Gal-3 immunoreactivity in endothelial cells in PILOs and GBMs, in contrast to other astrocytomas and oligodendrogliomas. For GBMs, a constant finding was the complete absence of Gal-3 staining in vessels exhibiting microvascular proliferation, whereas in PILOs the single endothelial layer did not express this lectin. Our results of the expression of Gal-3 in the endothelium in astrocytomas are similar to those reported in the literature (4, 14). In ODs, the Gal-3 expression in the endothelial vessels was variable, however we found lesser staining in high-grade tumors, akin to that reported by Deininger et al (10). Moreover, it is intriguing that contrast-enhancing tumors on neuroimaging, namely PILOs and GBMs, did not express Gal-3 in endothelial cells. We speculate that this finding could be related to some alteration of the blood brain barrier by the pathological vasculature.

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