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Proteomic identification of glutamine synthetase as a differential marker for oligodendrogliomas and astrocytomas:

Laboratory investigation

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Abstract

Object—Astrocytomas and oligodendrogliomas are primary CNS tumors that remain a challenge to differentiate histologically because of their morphological variability and because there is a lack of reliable differential diagnostic markers. To identify proteins that are differentially expressed between astrocytomas and oligodendrogliomas, the authors analyzed the proteomic expression patterns and identified uniquely expressed proteins in these neoplasms.

Methods—Proteomes of astrocytomas and oligodendrogliomas were analyzed using 2D gel electrophoresis and subsequent computerized gel analysis to detect differentially expressed proteins. The proteins were identified using high-performance liquid chromatography accompanied by tandem mass spectrometry. To determine the role of the differentially expressed proteins in astrocytes, undifferentiated glial cell cultures were treated with dibutyryl–cyclic adenosine monophosphate (cAMP).

Results—Two-dimensional gel electrophoresis revealed that glutamine synthetase was differentially expressed in astrocytomas and oligodendrogliomas. Western blot and immunohistochemical analyses confirmed the increased expression of glutamine synthetase in

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Disclosure

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astrocytomas compared with oligodendrogliomas. Whereas glutamine synthetase expression was demonstrated across all grades of astrocytomas (Grade II–IV [15 tumors]) and oligoastrocytomas (4 tumors), it was expressed in only 1 oligodendroglioma (6% [16 tumors]). Treatment of undifferentiated glial cell cultures with dibutyryl-cAMP resulted in astrocyte differentiation that was associated with increased levels of glial fibrillary acidic protein and glutamine synthetase.

Conclusions—These data indicate that glutamine synthetase expression can be used to distinguish astrocytic from oligodendroglial tumors and may play a role in the pathogenesis of astrocytomas.

Keywords

oligodendroglioma; astrocytoma; proteomics; glutamine synthetase; 2D gel electrophoresis; mass spectrometry; oncology

Gliomas with astrocytic and/or oligodendrocytic differentiation account for approximately 30% of all adult brain tumors.[11] According to a population-based analysis by Ohgaki and Kleihues,[15] prognosis is not only associated with glioma grade but also with its differentiation characteristics. World Health Organization Grade II and III oligodendrogliomas are associated with higher sensitivity to chemotherapy[1,12] and longer patient survival (11.6 and 3.5 years, respectively) compared with WHO Grade II and III astrocytomas (5.6 years and 1.6 years, respectively). Subsequently, pathological diagnosis of astrocytoma compared with oligodendroglioma has direct impact on treatment and prognosis. However, the cytoarchitectural variability and inaccuracy of established markers (including GFAP) underlies the frequent difficulty in distinguishing between these neoplasms.[8,14,18]

Because astrocytomas and oligodendrogliomas are characterized by neoplastic transformation of progenitor cells of astrocytic or oligodendrocytic origin, respectively, [10,20] identification of unique protein markers should be possible. Specifically, identification of differentially expressed markers should make immunohistopathological distinction between these neoplasms more accurate and feasible. Furthermore, protein expression patterns can be identified through proteomic profiling, which combines the use of techniques including 2D-PAGE, mass spectrometry, and bioinformatics. To identify proteins that are differentially expressed between astrocytomas and oligodendrogliomas, we characterized the protein expression profiles in various grades of astrocytomas, oligodendrogliomas, and oligoastrocytomas.

Methods

Tissue Preparation

Pathological diagnosis of tumors was confirmed independently by 2 pathologists according to WHO histological classification. Tissue samples and clinical information were obtained as part of an institutional review board–approved study at the Surgical Neurology Branch in the National Institute of Neurological Disorders and Stroke and the Cleveland Clinic Foundation. Frozen samples were collected intraoperatively and immediately snap frozen in optimal cutting temperature compound (Sakura Finetek OCT 4583) and included 5 Grade II

astrocytomas, 5 Grade III astrocytomas, 5 Grade IV astrocytomas, 13 Grade II oligodendrogliomas, and 3 Grade III oligodendrogliomas. Additionally, paraffin slides of 2 Grade II and 2 Grade III oligoastrocytomas were obtained for immunohistochemical studies. Normal mouse cortex (1 sample) was embedded in optimal cutting temperature compound for frozen sectioning and immunostaining.

Two-Dimensional PAGE

We cut sections of frozen tissue from Grade II astrocytomas and Grade II oligodendrogliomas. Selective tissue microdissection was performed on the tissue sections, as previously described,[5] to avoid procurement of normal tissue or areas of necrosis, hemorrhage, and inflammation. For 2D-PAGE separation, a previously described technique was used.[16] Briefly, microdissected tissue was dissolved in Extraction Buffer-II containing 8 mol/L urea, 4% (wt/vol) Bio-Lyte 4/7, and 2 mmol/L tributyl phosphine (163–2103, Bio-Rad). Tissue samples were vigorously mixed and centrifuged at 12,000 rpm to remove insoluble debris. The resulting supernatant was combined with a rehydration buffer mixture containing 8 mol/L urea, 2% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 50 mmol/L dithiothreitol, and 0.2% (wt/vol) Bio-Lyte 4/7 ampholytes (163–2106, Bio-Rad); IPG (immobilized pH gradient) buffer, pH 4–7 (17–6000–86, GE Healthcare); and bromophenol blue. Rehydration was performed overnight in 11 cm pH 4–7 Immobiline Drystrips (18–1016–60, GE Healthcare) on a Reswelling Tray (GE Healthcare). Isoelectric focusing for the first dimension was performed with a Multiphore II Electrophoresis System (18–1018– 06, GE Healthcare). The strips were subjected to high voltages at 300–3500 V. Immobilized pH gradient strips were equilibrated with Equilibration Buffer I containing 6 mol/L urea, 2% SDS, 375 mmol/L Tris-HCL (pH 8.8), 20% glycerol and 2% (w/v) dithiothreitol; and Buffer II containing 6 mol/L urea, 2% SDS, 375 mmol/L Tris-HCL (pH 8.8), 20% glycerol, and 2.5% (w/v) iodoacetamide (Bio-Rad, Hercules) for 15 minutes each. Precast ExcelGel SDS gels (12%–14% Gradient gel; pH 4–7, 245 × 180 × 0.5 mm; GE Healthcare) were used for the second dimension of protein separation by a Multiphor II Flated System (GE Healthcare) under a constant voltage of 700 V for 3 hours. A silver staining kit (GE Healthcare) was used according to the manufacturer's instructions to detect protein spots. All samples were run in duplicate to confirm gel electrophoretic patterning.

Image Analysis and In-Gel Digestion

Intensities of protein spots on 2D gels were analyzed with Proteomweaver (Definiens) according to the manufacturer's protocol. Protein spots of interest with a statistically significant difference between the 2 tumor subtypes ($p < 0.05$) were excised from the gel and subjected to in-gel digestion with trypsin, based on a previously described procedure.[19]

Mass Spectrometry

Peptides from in-gel digests were analyzed using a ProteomeX LC/mass spectrometry system (ThermoElectron) operated in the high-throughput mode. Reversed-phase HPLC was carried out using a BioBasic-18 column (0.18 × 150 mm, ThermoElectron) eluted at 1–2 μl/minute with a gradient of 2%–50% B over 30 minutes. Mobile phase A was H_2O (0.1%

formic acid) and mobile phase B was CH_3CN (0.1% formic acid). Column effluent was analyzed on the LCQ Deca XP Plus (ThermoElectron) operating in the “Top Five” mode.

Protein Identification

Uninterpreted mass spectrometric spectra were searched against a human database using the BioWorks and SQUEST programs (ThermoElectron). Protein identification was accepted when mass spectrometric spectra of at least 2 peptides from the same protein exhibited at a minimum default Xcorr versus charge values set by the program (for Z = 1, 1.50; for Z = 2, 2.00; and for Z = 3, 2.50).

Western Blot Analysis

Sections of frozen tissue from normal brain, Grade II astrocytomas, Grade III astrocytomas, Grade IV astrocytomas, and Grade II oligodendrogliomas were homogenized in tissue protein extraction reagent (78510, Thermo Scientific) with Halt Protease/Phosphatase Inhibitor Cocktail (78420, Thermo Scientific) and centrifuged at 15,000 g at 4°C to remove insoluble debris. The supernatant was used as the lysate of the tissues. Thirty micrograms of each lysate was loaded onto 4%–20% SDS-polyacrylamide gel (Invitrogen), and the proteins were electrophoretically transferred to nitrocellulose membranes and blocked with 5% milk solution. Membranes were probed with antibodies against glutamine synthetase (ab49873, Abcam), GFAP (ab7260, Abcam), or β-actin (sc-47778, Santa Cruz Biotechnology). Proteins were detected using a horseradish peroxidase–conjugated secondary antibody and visualized with the SuperSignal West Pico Chemiluminescent Substrate (34077, Thermo Scientific).

Immunohistochemistry and Immunofluorescence

For frozen tissue, 10-μm sections were cut and fixed in Histochoice 108 (C999B53, Thomas Scientific). For paraffin-embedded tissue, 6-μm sections were cut, deparaffinized in xylene, rehydrated in graded alcohol baths, and processed for antigen retrieval using a citric acid buffer (C999B53, Dako). Primary antibodies were used against glutamine synthetase (ab49873, Abcam) and GFAP (ab7260, Abcam). Immunohistochemical staining used a streptavidin-biotin complex method with 3,3′-diaminobenzidine solution (Vector Labs, Inc.), which was catalyzed by biotinylated horseradish peroxidase.

Primary Culture of Glial Cells

Primary glial cell cultures were prepared as previously described.[7,9] Briefly, cells were prepared from the cerebral cortex of newborn C57BL/6 mice. Disassociated brain cells were seeded in 35-mm tissue culture dishes (Corning Inc.) and maintained in Dulbecco modified Eagle medium (Gibco Invitrogen Corp.) with 10% fetal bovine serum (Gibco) at 37°C in 5% CO_2. Cultures were fed twice a week and were used for experiments after they reached 2 weeks. To induce differentiation of astrocytes, cells were incubated in growth media with 25-mM dibutyryl-cAMP (Sigma) for 7 days. Cells were then harvested for Western blot analysis or fixed for immunostaining as described above.

Results

Two-Dimensional PAGE Proteomic Analysis

Using 2D-PAGE proteomic analysis, we compared protein expression patterns between astrocytomas (Grade II, 3 lesions) and oligodendrogliomas (Grade II, 3 lesions). Protein patterns were consistently expressed within each tumor type, and individual proteins within each tumor type were selected using 2D gel analysis software. This comparison revealed several differentially expressed proteins (Fig. 1) between astrocytomas and oligodendrogliomas. A subset of these proteins was successfully identified by HPLC mass spectrometry (Table 1) and included glutamine synthetase, an enzyme known to play an integral role in normal astrocyte processes including ammonia and glutamate metabolism (Fig. 2).

Glutamine Synthetase Expression

To assess the expression pattern of glutamine synthetase in gliomas of astrocytic and oligodendrocytic origin, we performed Western blot analysis on Grade II astrocytoma and Grade II oligodendroglioma samples. Levels of glutamine synthetase were markedly higher in astrocytomas than in oligodendrogliomas (Fig. 3A). To assess the quantitative impact of glutamine synthetase within grades of astrocytomas, we measured glutamine synthetase expression levels in astrocytomas of increasing grade (Grades II–IV, 5 samples each). Glutamine synthetase expression was similarly strong across all grades of astrocytoma (Fig. 3B).

Glutamine Synthetase Expression in Primary Gliomas

Immunohistochemical expression analysis of glutamine synthetase was performed in 15 astrocytomas (Grades II–IV, 5 samples each), 16 oligodendrogliomas (Grade II, 13 samples; and Grade III, 3 samples) and normal temporal lobe (1 sample). Four mixed oligoastrocytomas (Grades II and III, 2 samples each) were also analyzed for glutamine synthetase expression (Fig. 4). Immunohistochemical findings of these tumor and tissue samples are summarized in Table 2. When stained for glutamine synthetase, normal tissue demonstrated a diffuse expression pattern (Fig. 4A). Staining of Grade II and III astrocytomas revealed glutamine synthetase expression within individual cell bodies, but oligodendrogliomas exhibited little to no glutamine synthetase expression. Only 1 Grade II oligodendroglioma (6%) exhibited moderate staining for glutamine synthetase. Staining of oligoastrocytomas revealed that glutamine synthetase was confined to a focal population of cells that lacked the typical “fried egg” oligodendroglial morphology (Fig. 4B). Furthermore, glutamine synthetase staining within mixed oligoastrocytomas was stronger than GFAP staining, which identified a more diffuse population of cells yet excluded some cells that displayed the typical histological appearance of oligodendrogliomas.

Astrocyte Differentiation With Dibutyryl-cAMP

To investigate markers for the astrocyte lineage and for markers of reactive astrocytic change, we examined mouse astrocyte progenitor cells before and after treatment with dibutyryl-cAMP, a compound known to drive astrocytic differentiation.[2,6] Before dibutyryl-

cAMP treatment, glutamine synthetase expression was higher than that of GFAP in astrocyte progenitor cells. After treatment with dibutyryl-cAMP and glial cell differentiation, cellular levels of both GFAP and glutamine synthetase increased significantly (Fig. 5A and 5B). Staining of normal adult mouse brain revealed that the majority of glial cells expressed glutamine synthetase, but GFAP expression was found only in a small population of cells (Fig. 5C). Glial fibrillary acidic protein expression was scattered throughout mouse cortex, whereas glutamine synthetase expression was homogeneous in the same regions.

Discussion

Accurate pathological diagnosis of primary gliomas requires the distinction among astrocytomas, oligodendrogliomas, and mixed oligoastrocytomas. Often, identification is based on the interpretation of tissue morphology, cytoarchitecture, and immunohistochemical results. Individually, these tests cannot be used to make definitive decisions regarding pathological distinction between glioma subtypes (astrocytoma vs oligodendroglioma). Correct identification of gliomas with astrocytic and oligodendrocytic components is a challenging and subjective task that is associated with interobserver discordance of up to 50%.[3] Thus, there is a need for differential markers to distinguish between these glioma subtypes. To identify distinguishing markers between astrocytomas and oligodendrogliomas, we performed proteomic profiling of these glioma subtypes to uncover differentially expressed protein markers.

Using comparative proteomics, we visualized the protein expression pattern of low-grade astrocytomas and low-grade oligodendrogliomas. Several differentially expressed proteins were identified that comprised candidate markers that could be used to potentially distinguish the 2 glioma subtypes (Fig. 1). One of these proteins, glutamine synthetase, is an enzyme that plays a critical role in ammonia metabolism and glutamate recycling in astrocytes (Fig. 2).[4] Glutamine synthetase was found to be expressed in astrocytomas but not in oligodendrogliomas. Subsequently, this study focused its investigation on the specific expression pattern of this protein in astrocytomas and oligodendrogliomas of varying grades in an attempt to elucidate its potential use as a unique astrocytic biomarker across the spectrum of grades in these neoplasms.

To quantify glutamine synthetase expression in astrocytomas compared with oligodendrogliomas, we performed Western blot analysis in low-grade tumors of each subtype. Glutamine synthetase expression was markedly higher in astrocytomas than oligodendrogliomas, with little to no protein detectable in the latter (Fig. 3A). Further investigation demonstrated similar and strong glutamine synthetase expression across astrocytomas of varying grade (Fig. 3B). These results indicate that glutamine synthetase is a differentially expressed marker of astrocytomas of all grades. The expression of glutamine synthetase in astrocytomas in comparison with oligodendrogliomas highlights its potential use in the differential diagnosis of these tumors. Immunohistochemical staining or other techniques to detect glutamine synthetase expression could augment current pathological tests used to distinguish between primary glial tumors.

To further assess whether glutamine synthetase serves generally as a marker of the astrocytic lineage, we examined its expression in several tumor samples, including mixed oligoastrocytomas. This analysis revealed focal glutamine synthetase staining that likely represents the astrocytic components of such mixed tumors, as glutamine synthetase staining is not observed in oligodendrogliomas (Fig. 4). Classically, GFAP has been used in the identification of astrocytic components of mixed tumors to distinguish oligodendrogliomas from oligoastrocytomas,[13] but GFAP expression alone is insufficient to make the distinction between astrocytomas and oligodendrogliomas. We provide evidence that glutamine synthetase–positivity may be a distinguishing feature of oligoastrocytomas compared with oligodendrogliomas, which may represent an additional technique in identifying astrocytic components of such mixed glial tumors.

Previous in vivo studies have demonstrated that increases in glutamine synthetase activity during embryological development correlate with the differentiation status, rather than proliferative capacity, of astrocytes.[17] To determine whether glutamine synthetase is a marker of the astrocytic lineage in normal tissue, we measured glutamine synthetase expression levels before and after induction of differentiation of astrocyte precursor cells with dibutyryl-cAMP. Before treatment with dibutyryl-cAMP, glutamine synthetase expression was detectable in undifferentiated astrocyte precursor cells, whereas GFAP expression levels were significantly less. After differentiation, both GFAP and glutamine synthetase expression increased significantly (Fig. 5A) and terminally mature astrocytes expressed both GFAP and glutamine synthetase (Fig. 5B). These findings indicate that glutamine synthetase is a specific marker of the astrocytic lineage in both normal and pathological samples and could be a more reliable astrocytic marker than GFAP. While GFAP is widely recognized as a marker of reactive astrocytes,[2] glutamine synthetase may be a marker of both reactive and quiescent astrocytes, as glutamine synthetase–positive cells can be seen in normal adult mouse brain to a greater extent than GFAP-positive cells (Fig. 5C).

Conclusions

These findings indicate that glutamine synthetase is a useful marker that can aid in the differential diagnosis of astrocytomas and oligodendrogliomas. Moreover, it may have significant utility in the diagnosis of mixed glial tumors such as oligoastrocytomas. Further studies examining larger sample sizes will clarify the use of glutamine synthetase expression as an approach for glioma diagnosis among oligodendrogliomas, astrocytomas, and mixed oligoastrocytomas.

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Abbreviations used in this paper

cAMP cyclic adenosine monophosphate

GFAP	glial fibrillary acidic protein
HPLC	high-performance liquid chromatography
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate

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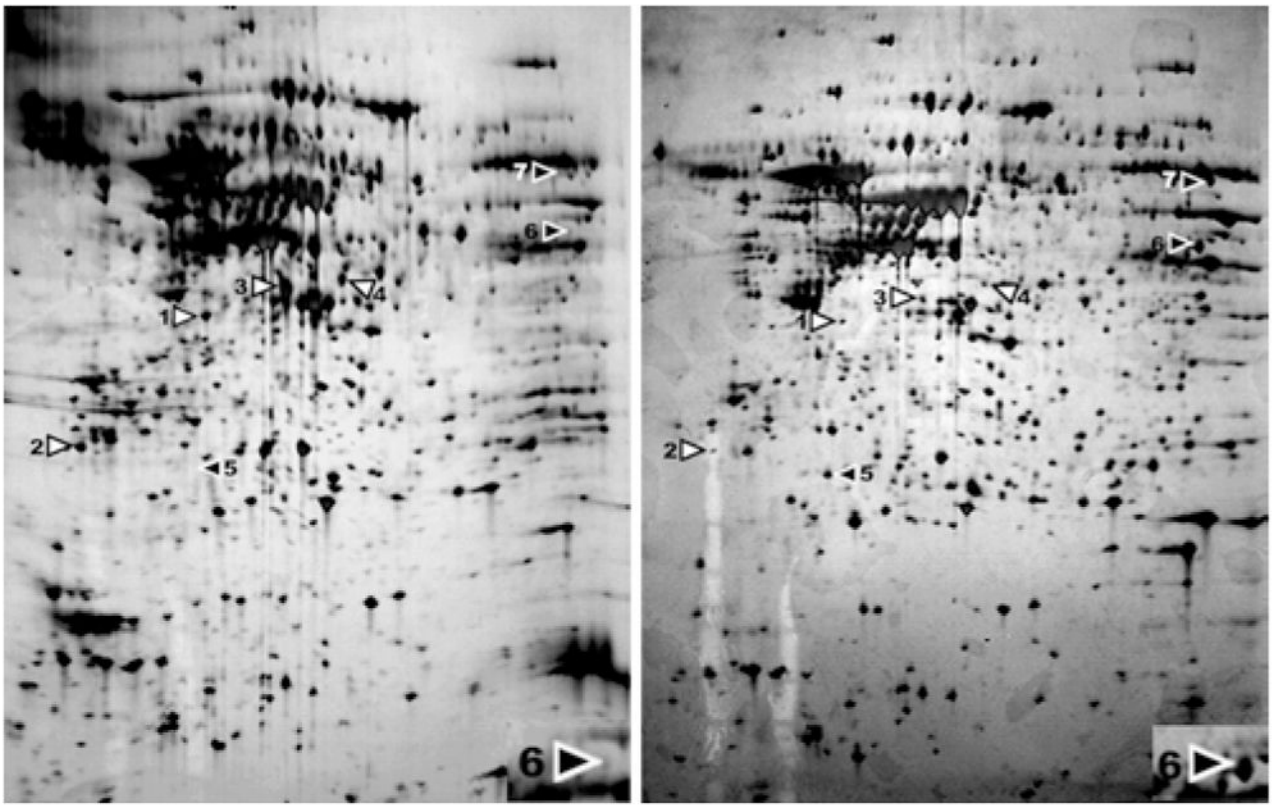

Fig. 1.

Proteomic comparison through 2D-PAGE. The proteomes of 3 WHO Grade II oligodendrogliomas and astrocytomas were resolved using 2D-PAGE and visualized through silver staining. Two representative gels are displayed. *Arrowheads* highlight differential proteins found across all samples analyzed.

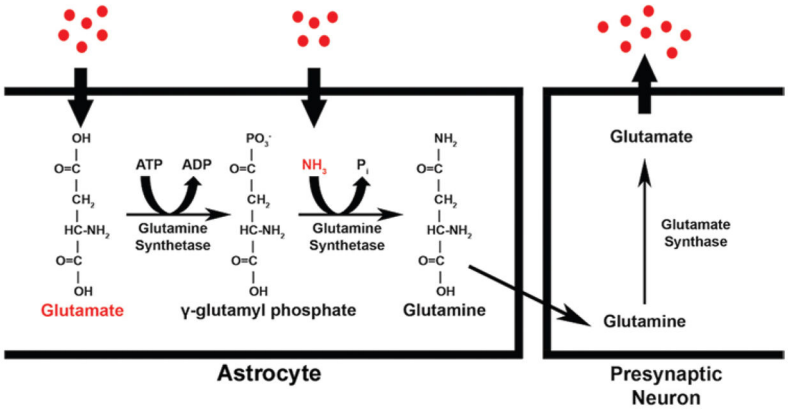

Fig. 2.

Schematic of glutamine synthetase reaction in the CNS. When expressed in astrocytes, glutamine synthetase is responsible for converting extracellular glutamate into glutamine through adenosine triphosphate hydrolysis and amination. This reaction is critical for ammonia metabolism in the CNS and also plays an important role in the recycling of glutamate as a neurotransmitter or production of glutamine for other metabolic or signaling activities.

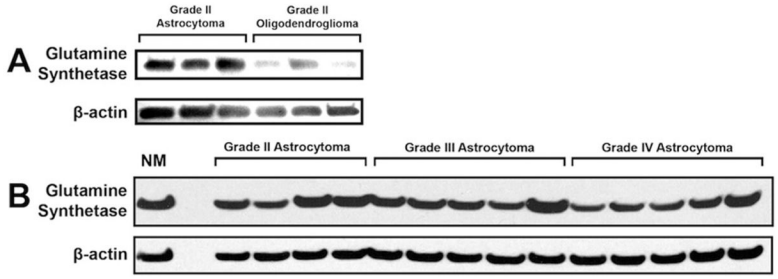

Fig. 3.

Western blot analysis comparing 3 WHO Grade II oligodendrogliomas and astrocytomas. **A:** Glutamine synthetase is expressed at higher levels in the astrocytomas. **B:** Glutamine synthetase expression is maintained across astrocytomas spanning WHO Grades II–IV.

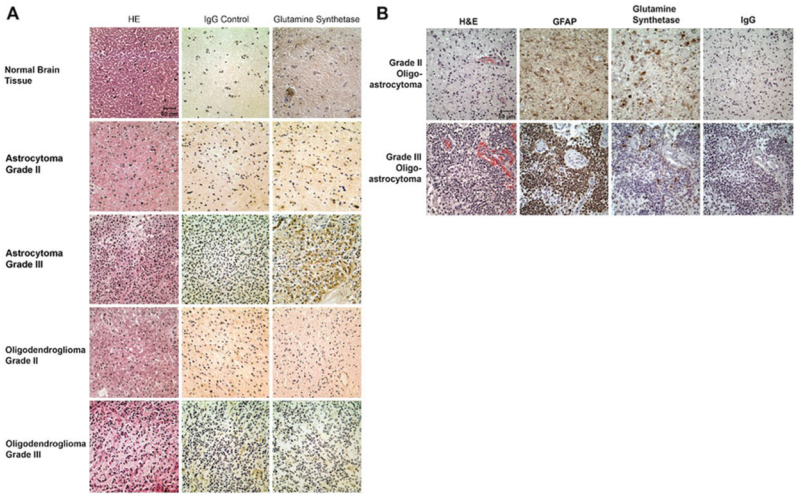

Fig. 4.

Immunohistochemical results. **A:** When staining normal brain, glutamine synthetase has a diffuse staining pattern. However, within astrocytomas, glutamine synthetase staining reveals individual cell bodies and stains a majority of the tumors cells within the sample. Little to no staining was observed in the majority of oligodendrogliomas. **B:** Within serially cut sections of formalin-fixed paraffin-embedded mixed oligoastrocytomas, glutamine synthetase staining was able to resolve individual tumor cells better than GFAP and also demonstrated higher selectivity in excluding tumor cells that had morphological traits traditionally associated with neoplastic oligodendroglia *(row 2, column 3).* Original magnification × 10.

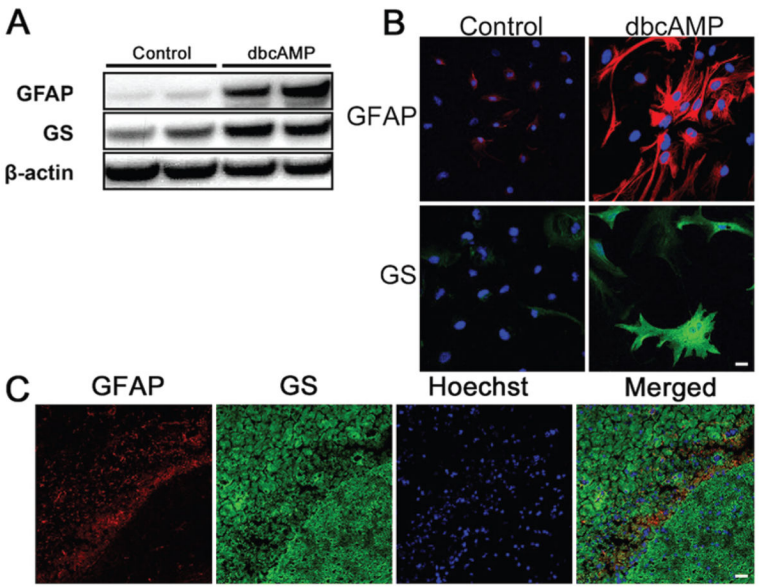

Fig. 5.

A: In murine primary astrocyte progenitor cells, glutamine synthetase (GS) is expressed in higher levels than GFAP and its level increases after cells are prompted to differentiate through treatment with dibutyryl-cAMP (dbcAMP). **B:** Immunofluorescent staining showing that glutamine synthetase and GFAP are highly expressed in terminal differentiated astrocytes. Bar = 10 mm. **C:** Immunofluorescent staining demonstrating glutamine synthetase widely expressed in glial cells in brain, whereas GFAP expression is only seen in a small population of glial cells. Bar = 50 μm.

TABLE 1

Proteins differentially expressed in Grade II oligodendrogliomas and Grade II astrocytomas*

Protein	Protein w/Increased Expression in Oligodendrogliomas	Potential Functions
1	NADP-regulated thyroid hormone–binding protein	promotes oligodendrocyte differentiation & proper myelination
2	neuron-specific β–III tubulin	structural protein
3	α–I tubulin	structural protein
4	neuron-specific β–III tubulin	structural protein
	Protein w/Increased Expression in Astrocytomas	Potential Functions
5	septin	cytoskeletal GTPase
6	glutamine synthetase	regulator of glutamatergic neurotransmission
7	cytosol aminopeptidase	unknown in astrocytes

*Proteins 1–4 had higher expression in oligodendrogliomas, whereas Proteins 5–7 had higher expression in astrocytomas.

TABLE 2

Summary of immunohistochemistry study findings on glutamine synthetase expression

Tumor Type	Total No. of Tumors	No. of Tumors w/Positive Glutamine Synthetase (%)
astrocytoma		
Grade II	5	5 (100)
Grade III	5	5 (100)
Grade IV	5	5 (100)
oligodendroglioma		
Grade II	13	1 (8)
Grade III	3	0 (0)
oligoastrocytoma		
Grade II	2	2 (100) focal
Grade III	2	2 (100) focal