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The Oligodendroglial Lineage Marker OLIG2 Is Universally Expressed in Diffuse Gliomas

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Abstract. Astrocytomas, oligodendrogliomas, and oligoastrocytomas, collectively referred to as diffuse gliomas, are the most common primary brain tumors. These tumors are classified by histologic similarity to differentiated astrocytes and oligodendrocytes, but this approach has major limitations in guiding modern treatment and research. Lineage markers represent a potentially useful adjunct to morphologic classification. The murine bHLH transcription factors *Olig1* and *Olig2* are expressed in neural progenitors and oligodendroglia and are essential for oligodendrocyte development. High OLIG expression alone has been proposed to distinguish oligodendrogliomas from astrocytomas, so we critically evaluated OLIG2 as a marker by immunohistochemical and oligonucleotide microarray analysis. OLIG2 protein is faithfully restricted to normal oligodendroglia and their progenitors in human brain. Immunohistochemical analysis of 180 primary, metastatic, and non-neural human tumors shows OLIG2 is highly expressed in all diffuse gliomas. Immunohistochemistry and microarray analyses demonstrate higher OLIG2 in anaplastic oligodendrogliomas versus glioblastomas, which are heterogeneous with respect to OLIG2 levels. OLIG2 protein expression is present but inconsistent and generally lower in most other brain tumors and is absent in non-neuroectodermal tumors. Overall, OLIG2 is a useful marker of diffuse gliomas as a class. However, expression heterogeneity of OLIG2 in astrocytomas precludes immunohistochemical classification of individual gliomas by OLIG2 alone.

Key Words: Antibody; Astrocytoma; Glioma; Lineage; Olig2; Progenitor.

INTRODUCTION

Astrocytomas, oligodendrogliomas, and oligoastrocytomas, collectively referred to as diffuse gliomas, are rare neoplasms, but they are a leading cause of cancer-related death in the United States due to their invasive nature and resistance to conventional anti-tumor therapies. However, recent progress has shown that oligodendrogliomas and potentially oligoastrocytomas may exhibit dramatic responses to specific chemotherapeutic agents (1–3). Thus, potentially important prognostic and therapeutic decisions are based on the histopathologic classification of these tumors. Currently, gliomas are classified based on their histologic similarity to differentiated astrocytes or oligodendrocytes (4), but it is increasingly recognized that this classification method has major limitations in guiding patient treatment and basic research (5). Advances in classification are likely to arise from an improved understanding of the molecular and neurodevelopmental basis of these tumors (6).

Recent scientific advances in neural stem cell biology (7, 8) have led to renewed speculation that diffuse gliomas may arise from neuroectodermal stem or progenitor cells (9–11). A common origin for diffuse gliomas would imply the shared expression of progenitor cell markers across different classes of gliomas. Although expression of some neural progenitor markers has been identified in several classes of tumors, their expression is inconsistent and is not restricted to neuroectodermally derived malignancies (12, 13). Indeed, no single marker currently effectively marks or distinguishes the entire class of tumors known as diffuse gliomas.

The *Olig* family of bHLH transcription factors are essential regulators of ventral neuroectodermal progenitor cell fate and oligodendrocyte development in the murine CNS (14–16). We previously identified *OLIG2* gene expression in oligodendrogliomas and high-grade astrocytomas by RNA in situ hybridization (ISH) (17). A subsequent study has also shown *OLIG* RNA expression in oligodendrogliomas and glioblastomas by RT-PCR (18). These results were in contrast to those of Marie et al, who described *OLIG2* expression as restricted to oligodendrogliomas and a recent study by Ohnishi et al, which suggested that high *OLIG2* RNA and protein levels alone might reliably distinguish individual oligodendrogliomas from glioblastomas (19, 20). Unfortunately, the observations in each of these previous studies were limited in part by technique or the small number of tumors examined. As such, fundamental biological questions remain regarding 1) the status of OLIG2 in astrocytomas, 2) the presence of OLIG2 heterogeneity within tumor classes

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and individual tumors, and 3) the potential of OLIG2 as a diagnostic marker.

To address these questions and more comprehensively establish the nature of OLIG2 expression in human tumors, we have generated and tested an antibody that effectively and specifically recognizes OLIG2 protein in mouse and human tissue. This reagent was utilized to critically evaluate OLIG2 as a lineage marker and to compare OLIG2 expression in human tumors by immunohistochemical and oligonucleotide microarray analysis.

MATERIALS AND METHODS

Antibody Generation and Characterization

Rabbit polyclonal antisera were generated by standard techniques (21). A GST-OLIG2 fusion protein containing 107 amino-terminal amino acids of the murine OLIG2 protein was used as antigen. This region of OLIG2 has only 19 and 22 percent identity at the amino acid level to the related proteins OLIG1 and OLIG3, respectively, and does not include the bHLH portion of the protein. Western blot screening of antisera from 4 injected rabbits identified antisera DF308 as having strong and specific activity to OLIG2. Immunoglobulin was isolated using protein-A sepharose column chromatography as described previously (21).

Tissue Collection

Mouse CNS tissues were collected in accordance with National Research Council recommendations for the care and use of animals. Tissues were fixed in paraformaldehyde and embedded for paraffin or frozen sectioning as previously described (22). Olig2$^{-/-}$ mice were generated as previously described (16). Human tissue samples were obtained in accordance with the rules and regulations for human tissue collection and their use in these studies was approved by the Institutional Review Boards of the Brigham and Women's Hospital, Massachusetts General Hospital and the Boston Children's Hospital Boston. Each sample for immunohistochemical analysis had received a clinical neuropathologic diagnosis and was independently reconfirmed by a neuropathologist (K.L.L.). The histopathologic classification of ''classic'' tumors for microarray analysis is previously described (23).

Western Blot Analysis

Western blot analysis was performed as previously described (24). A eukaryotic expression construct containing mouse OLIG2 was overexpressed in COS-1 cells and total protein from these transiently transfected cells was harvested at 2 days by standard protocols (21). Total protein extracts from adult human brain (neocortex), newborn (P0) Swiss wild type mouse whole brain, and human oligodendroglioma and anaplastic oligodendroglioma surgical tissue samples were made, quantitated, and exactly 40 μg of total protein were loaded per lane. OLIG2 was detected using Protein A purified DF308 at a dilution of 1:5,000 followed by detection using an anti-rabbit secondary antibody conjugated to alkaline phosphatase and the nitro-blue tetrazolium/bromochloroindolyl phosphate (NBT/BCIP) detection system. GST-OLIG1 includes the 66 amino terminal amino acids of murine OLIG1 and does not include the bHLH domain.

Immunohistochemistry and In Situ Hybridization

Immunohistochemistry with DAB detection on human and mouse paraffin sections was performed using the DAKO Envision+ system (DakoCytomation, Carpinteria, CA, #K4009) according to the manufacturer's specifications with the exception that primary antibody incubation was performed overnight at 4°C and secondary antibody incubation time was performed for 1 to 2 h at room temperature. Immunohistochemistry with fluorescent detection was performed by blocking sections for 30 min in 5% normal goat serum/PBS, incubation with primary antibody for a minimum of 3 h and incubation with anti-mouse Cy2 and anti-rabbit Cy3 based secondary antibodies for 1 h at room temperature prior to staining with DAPI and mounting in 90% glycerol. Heat based antigen retrieval (10 mM Citrate, 99°C, 30 min) was performed on all sections to enhance immunodetection. Antibodies were used at the following dilutions: OLIG2 1:40,000 (DF308, rabbit polyclonal); Glial fibrillary acidic protein (GFAP) 1:1,000 (#G3893, mouse monoclonal, Sigma, St. Louis, MO); anti-neuronal nuclei (NeuN) 1:100 (#MAB377, mouse monoclonal, Chemicon, Temecula, CA); Ki-67/MIB-1 1:100 (#MM1, mouse monoclonal, Novocastra, Newcastle upon Tyne, UK), Pax6 1:1,000 (mouse monoclonal, gift of Dr. P. Gruss, Max Planck Institute, Germany), platelet derived growth factor receptor alpha (PDGFRα) 1:200 (SC338, rabbit polyclonal, Santa Cruz, Santa Cruz, CA). Double label immunohistochemistry for OLIG2 and PDGFRα was performed using DAKO Doublestain Envision Kit (#K1395, DakoCytomation). Double label immunohistochemistry and in situ hybridization were performed as previously described (17).

Immunohistochemical staining for OLIG2 in human tumors was independently scored by 3 pathologists (K.L.L., D.N.L., and M.R.K.) with respect to the overall percentage of tumor cells that exhibited nuclear positivity (0 = no cells positive; 1 = 1% to 10%; 2 = 11% to 50%; 3 = 51% to 90%; and 4 = 91% to 100%) within the entire tumor. The average score represents the overall average of the reviewer assigned tumor class averages.

Microarray Gene Expression Analysis

Affymetrix GeneChip (U95Av2, Affymetrix, Santa Clara, CA) microarray data was generated and analyzed as previously described (23). Only tumors that met criteria for reproducible or ''classic'' histology (14 glioblastomas and 7 anaplastic oligodendrogliomas) were included in the analysis. *OLIG2* expression was analyzed as represented by probe set 40624_at. A standard 2-tailed *F*-test was used to evaluate the significance of the difference between variances of *OLIG2* levels in the 2 tumor classes (25).

RESULTS

Antibody DF308 Specifically Recognizes OLIG2 Protein in Ventral Neural Progenitor Cells and Human Gliomas

To obtain a reagent for identification of OLIG2 protein, we generated and purified a rabbit polyclonal antibody (DF308) raised against a GST-OLIG2 fusion protein. Western blot analysis demonstrated that the

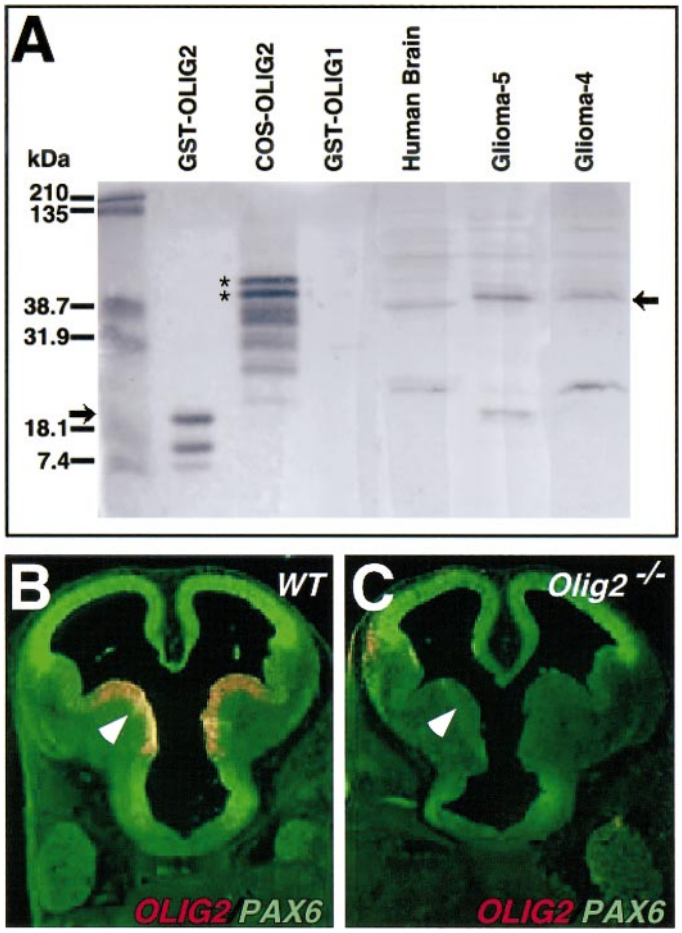

Fig. 1. OLIG2 antibody (DF308) specifically recognizes OLIG2 in normal mouse and human tissue as well as human gliomas. **A:** Western blot analysis shows that DF308 recognizes bacterially expressed GST-OLIG2 fusion protein (left arrow, 20 kDa), full-length mouse OLIG2 protein overexpressed in COS-1 cells (38 and 40 kDa, asterisks), but not GST-OLIG1 fusion protein (20 kDa). Extracts of normal adult human neocortex contained moderate levels of OLIG2 (right arrow, ~38 kDa). Two adult human oligodendroglioma samples (Glioma 5-anaplastic oligodendroglioma, and Glioma 4-oligodendroglioma) also contained OLIG2 of normal size. Additional bands in lane 2 likely represent breakdown products of the GST-fusion protein. Two dominant bands in COS cells presumably result from 2 identified potential initiating methionine residues. Additional smaller products recognized in COS-1 cells and human tumors likely represent proteolytic degradation products. **B, C:** Immunohistochemistry performed on E13.5 wild type and *Olig2*$^{-/-}$ mouse brain shows OLIG2 expression (red-orange) in wild type ventral forebrain (**B,** arrowhead) but not *Olig2*$^{-/-}$ mice (**C**). The dorsal cortical progenitor marker PAX6 (green) is unaffected.

antibody DF308 can recognize the bacterially expressed GST-OLIG2 fusion protein as a band of predicted molecular weight ~20 kDa and full-length mouse OLIG2 overexpressed in transfected COS-1 cells as a ~38-kDa product (Fig. 1A). The antibody is specific for OLIG2 and does not recognize a GST-OLIG1 fusion protein containing the corresponding amino terminal region of OLIG1 and no signal is detected in OLIG2$^{-/-}$ mice by immunohistochemistry (IHC) (Fig. 1B, C). Analysis of normal human brain tissue detects a 38-kDa protein corresponding to full length endogenous OLIG2. In addition, analysis of protein extracts from 2 human oligodendrogliomas demonstrates the presence of OLIG2 protein of apparently normal molecular weight. These findings suggest that DF308 specifically recognizes human and mouse OLIG2 proteins in normal brain tissue and human oligodendrogliomas.

OLIG2 Expression Is Restricted to Neural Progenitor Cells and Oligodendrocytes of the Mouse and Human Nervous System

To validate OLIG2 as a restricted lineage marker of neural progenitor cells and oligodendroglial cells in the developing and adult CNS, we performed immunohistochemistry on a range of mouse and human tissues using antibody DF308. DF308 readily recognizes human and mouse OLIG2 as a nuclear protein in formalin-fixed, paraffin-embedded samples as well as fixed-frozen tissues (Fig. 2). Analysis of developing mouse and human tissues demonstrates that OLIG2 is predominantly expressed in regionally restricted sub-populations of ventral neural progenitor cells that are fated to give rise to oligodendrocytes and specific neuronal subtypes in the spinal cord and brain (Fig. 2). Comparison of mouse (12.5 days post coitus or E12.5) and human (10 weeks estimated gestational age [EGA]) embryonic neural tube shows that OLIG2 protein is highly expressed in ventricular zone (VZ) and subventricular zone (SVZ) progenitor cells within the oligodendrocyte/motor neuron progenitor domain (pMN) consistent with previous results in the mouse (Fig. 2A, D) (26). In the brain, OLIG2 is expressed in VZ/SVZ progenitor cells of the ganglionic eminences (medial and caudal) and diencephalon, which are fated to give rise to oligodendrocytes and GABAergic interneurons of the mouse and human brain (Fig. 2B, E) (27, 28).

Outside the progenitor zones, OLIG2 expression was maintained in migrating and differentiating oligodendrocyte precursor cells identified by co-expression of PDGFRα in developing human fetal white matter at 22 weeks EGA (Fig. 2G). In addition, OLIG2 expression in the adult was maintained in premyelinating and myelinating oligodendrocytes which co-expressed 2′, 3′-cyclic nucleotide-3′-phosphodiesterase (CNPase) protein (Fig. 2H), proteolipid protein RNA (Fig. 2I), and myelin basic protein (data not shown). Although OLIG2 is expressed in undifferentiated progenitor cells that can give rise to neurons, its expression is reportedly not maintained in the neuronal progeny of these cells (29). Indeed, OLIG2 appeared to be rapidly downregulated upon neuronal specification in mouse and humans as TuJ1, an early neuronal marker, and OLIG2 were not co-localized in the

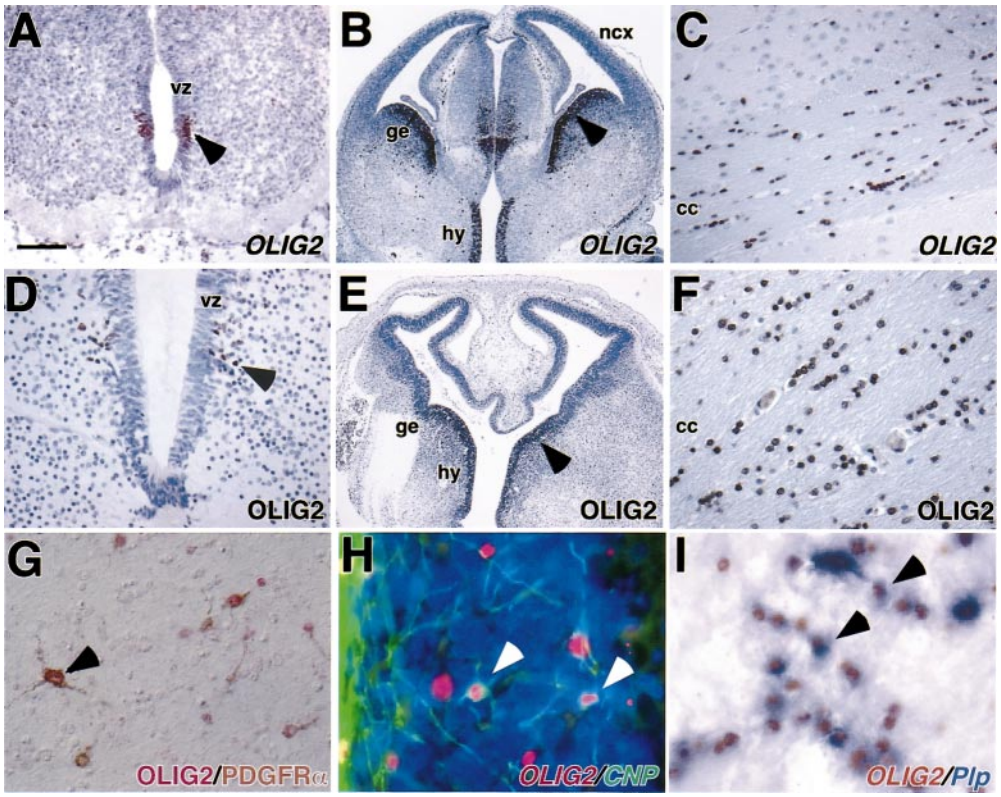

Fig. 2. OLIG2 is expressed in human and mouse ventral neural progenitor cells and oligodendroglia. Immunohistochemistry for OLIG2 in mouse (**A,** E12.5) and human (**D,** 10 weeks estimated gestational age [EGA]) ventral neural tube shows strong, specific, nuclear OLIG2 in the VZ/SVZ of the motor neuron/oligodendroglial progenitor domain (pMN) (arrowheads). Staining of mouse (**B,** E13.5) and human (**E,** 10 weeks EGA) brain shows OLIG2 expression in the VZ/SVZ of the ganglionic eminences and hypothalamus (arrowheads), which give rise to oligodendroglia and cortical interneurons. Adult mouse **(C)** and human **(F)** corpus callosum contain numerous OLIG2-positive nuclei with the morphology of mature oligodendrocytes. Double label IHC **(G, H)** and combined IHC/ISH **(I)** demonstrates expression of OLIG2 (red) in nuclei of PDGFRα (brown) expressing oligodendroglial progenitors (**G,** arrowhead), immature premyelinating and myelinating CNPase (**H,** arrowheads) and proteolipid protein (Plp) (**I,** arrowheads)-positive oligodendrocytes. **A, D,** transverse sections; **B, E,** coronal sections; **G,** 22 weeks EGA human fetal white matter; **H,** P60 days mouse cerebellum; **I,** P60 days mouse brain. Abbreviations: ge, ganglionic eminence; ncx, neocortex; cc, corpus callosum; hy, hypothalamus; vz, ventricular zone. Scale bar = 100 μm.

embryonic brain and neural tube (human-E10 weeks, mouse- E12.5 and E18.5) (D. Rowitch, K. Ligon, data not shown). In addition, OLIG2 was never detected in mature GFAP-positive astrocytes or NeuN-positive neurons in the adult mouse or human CNS by double immunohistochemical analysis (data not shown). In summary, expression of OLIG2 protein suggests that it is a robust and specific lineage marker of ventral neural progenitor cells and oligodendroglial cells in both the mouse and human CNS.

OLIG2 Is a Universal Marker of Diffuse Gliomas

Having established the utility and lineage specificity of the DF308 reagent in humans, we next sought to determine in a comprehensive and detailed manner the expression of OLIG2 in human brain tumors. We performed immunohistochemical analysis of OLIG2 in 180 intracranial human tumors, including primary brain tumors, metastatic tumors, and non-neural intracranial neoplasms (Table). Analysis of primary brain tumors showed that OLIG2 expression is most abundant in oligodendroglial tumors (Fig. 3). We detected OLIG2 in 100% of oligodendrogliomas and anaplastic oligodendrogliomas (n = 32) (Fig. 3K–N; Table) and these tumors were consistently and uniformly composed of a high percentage of OLIG2-positive cells (i.e. 85%–96%).

In contrast to previous studies using RNA ISH, OLIG2 was also strongly and consistently expressed at the protein level in all types of astrocytomas (grade I–IV) (n = 45) (Fig. 3A–J). Pilocytic astrocytomas contained numerous OLIG2-positive cells within dense fibrillary as well as loose ''oligodendroglial'' areas (Fig. 3A, B). Staining of diffuse low-grade astrocytomas (grade II) and anaplastic astrocytomas (grade III) showed that the majority of tumor cells were OLIG2-positive and highlighted numerous large irregular OLIG2-positive tumor nuclei (Fig. 3C–F). Analysis of glioblastomas revealed that these tumors were consistently composed of a high percentage of OLIG2-positive cells (Fig. 3G–J) (avg. 50%–

TABLE
OLIG2 Expression in Human Tumors

	Avg. Score	Pos/total (% pos)
Glial Neoplasms		95/98 (97%)
Oligodendroglioma	3.4	32/32 (100%)
Oligodendrogliomas (WHO Grade II)	3.3	19/19
Anaplastic oligodendrogliomas (WHO Grade III)	3.5	13/13
Astrocytoma	2.4	45/45 (100%)
Pilocytic astrocytoma (WHO Grade I)	2.5	4/4
Diffuse astrocytoma (WHO Grade II)	2.6	11/11
Anaplastic astrocytoma (WHO Grade III)	2.4	9/9
Glioblastoma (WHO Grade IV)	2.2	21/21
Mixed Gliomas	2.2	10/10 (100%)
Oligoastrocytoma (WHO Grade II)	2.2	3/3
Anaplastic oligoastrocytoma (WHO Grade III)	2.1	7/7
Ependymoma	1.1	8/11 (73%)
Ependymoma (WHO Grade II-III)	1.1	8/11
Non-Neural Neoplasms	0.0	0/50 (0%)
Meningioma (WHO Grade I)	0.0	0/21
Metastatic tumors	0.0	0/24
Lymphoma	0.0	0/5
Neuronal Neoplasms		
Medulloblastoma (WHO Grade IV)	1.0	6/8 (75%)
Central neurocytoma (WHO Grade II)	2.3	6/7 (86%)
DNET (WHO Grade I)	3.4	3/3 (100%)
Embryonal Neoplasms		
PNET (WHO Grade IV)	1.0	5/14 (36%)

OLIG2 expression in human tumors. Average score was determined as described in Materials and Methods. The number of positive tumors (score of 1+ or higher by all reviewers) is reported over the total number of tumors in each class along with the percentage of tumors that were positive in each class. Tumors with only focal positivity were still scored as being positive and equivalent for purposes of reporting. Metastatic tumors examined included carcinoma of the lung (n = 13), breast (n = 3), kidney (n = 1), uterus (n = 2), ovary (n = 1), gastrointestinal tract (n = 1), neuroendocrine tumor of the lung (n = 1), and melanoma (n = 2).

70% of positive cells) but they exhibited increased variability (Fig. 3H, J) and heterogeneity in OLIG2 expression when compared to oligodendroglial tumors (Fig. 3H, J, N).

To independently corroborate these surprising observations, we looked at *OLIG2* RNA levels by Affymetrix oligonucleotide microarray analysis of histologically ''classic'' anaplastic oligodendrogliomas (CO, n = 7) and ''classic'' glioblastomas (CG, n = 14) from a previously characterized data set (23). The average *OLIG2* levels in COs was ~3.8 fold higher than in CGs and was statistically significant (Fig. 4). The *OLIG2* expression variation within CGs as a class was significantly different from the variation in the COs (*F*-test of variances, p value = 0.0000020). In fact, the *OLIG2* range of COs was 16.5% of and subsumed within the *OLIG2* expression range of the CGs. These results confirm the immunohistochemical data and demonstrate that the spectrum of *OLIG2* expression levels for glioblastomas is wider than in anaplastic oligodendrogliomas.

Further analysis of immunohistochemistry for OLIG2 in oligoastrocytomas showed that this tumor class is remarkably similar in staining pattern and levels to that seen in ''pure'' astrocytomas (Fig. 3O–Q), but markedly different from pure oligodendroglial tumors. Interestingly, OLIG2 expression in oligoastrocytomas was detected in cells with oligodendroglial morphology as well as those with an astrocytic appearance (Fig. 3Q, arrowheads) indicating that the morphologic features of oligodendroglial differentiation did not segregate with OLIG2 expression.

In contrast to the robust expression seen in diffuse gliomas, we found low to moderate levels of OLIG2 expression in ependymomas, which are a non-diffuse glial tumor (Table; Fig. 3R–U). OLIG2 was expressed in 8/11 cases and when present its expression was typically restricted to a rare subpopulation of cells evenly distributed throughout the tumor, a pattern never observed in diffuse gliomas (Fig. 3U).

Together, these findings indicate that abundant OLIG2 expression is a universal characteristic of all classes of diffuse gliomas and highlight significant heterogeneity in astrocytomas and oligoastrocytomas with respect to OLIG2 expression.

OLIG2 Expression Is Inconsistent in Non-Glial Tumors

Consistent expression of OLIG2 in astrocytomas and oligodendrogliomas suggested that it might be a useful

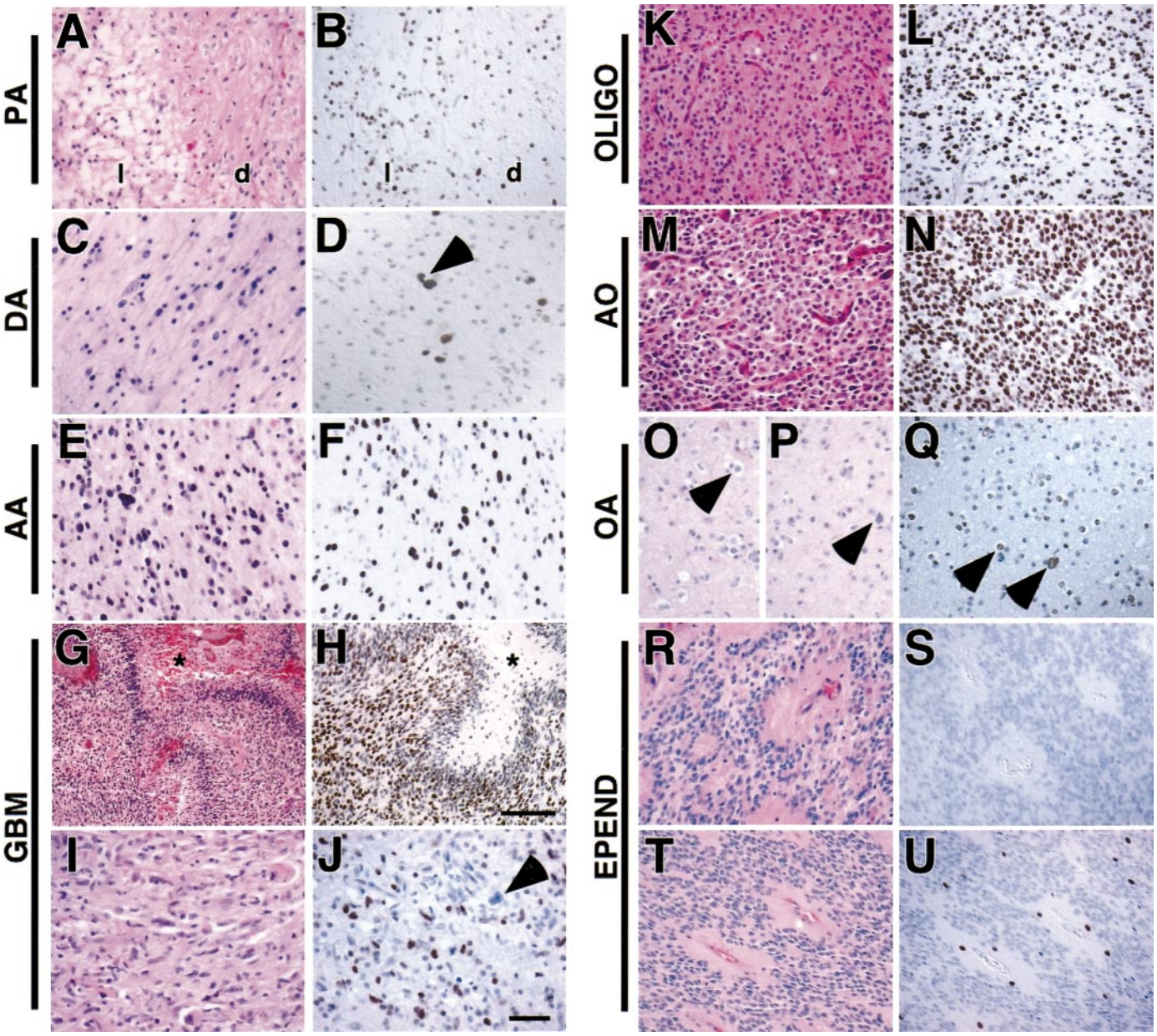

Fig. 3. OLIG2 is expressed in gliomas. Immunohistochemical analysis of OLIG2 shows that astrocytomas **(A–J)**, oligodendrogliomas **(K–N)**, and oligoastrocytomas **(O–Q)** contain a large number of OLIG2-positive cells. OLIG2 staining was most abundant and homogeneous in oligodendrogliomas (greater than 85% of tumor cells positive) **(L, N)**. Astrocytomas were also consistently positive including pilocytic **(A, B)**, diffuse **(C, D)**, and anaplastic astrocytomas **(E, F)**, and glioblastomas **(G–J)**. Tumor cells were positive in both dense fibrillary (d) and loose areas (l) of pilocytic astrocytomas **(A, B)**. Tumor cells with large convoluted OLIG2-positive nuclei were numerous in diffuse astrocytoma (DA) and anaplastic astrocytoma (AA) tumors (**D,** arrowhead). OLIG2 in glioblastoma (GBM) was noted in areas of viable tumor but not in areas of pseudopalisading necrosis (**G, H,** asterisks). Prominent numbers of intermingled OLIG2-negative tumor cells were noted in GBM (**J,** arrowhead) and other astrocytomas suggesting significant intratumoral heterogeneity. In oligoastrocytomas, cells with oligodendroglial features of round nuclei and perinuclear halos (**O,** arrowhead) and cells with astrocytic features of increased pleomorphism and atypia (**P,** arrowhead) were both OLIG2-positive (**Q,** arrowheads). In contrast, the non-diffuse glioma, ependymoma, showed inconsistent OLIG2 expression with most tumors having no OLIG2 **(R, S)** or a rare, evenly distributed subpopulation of positive cells **(T, U)**. Scale bars: H = 100 μm; J = 50 μm.

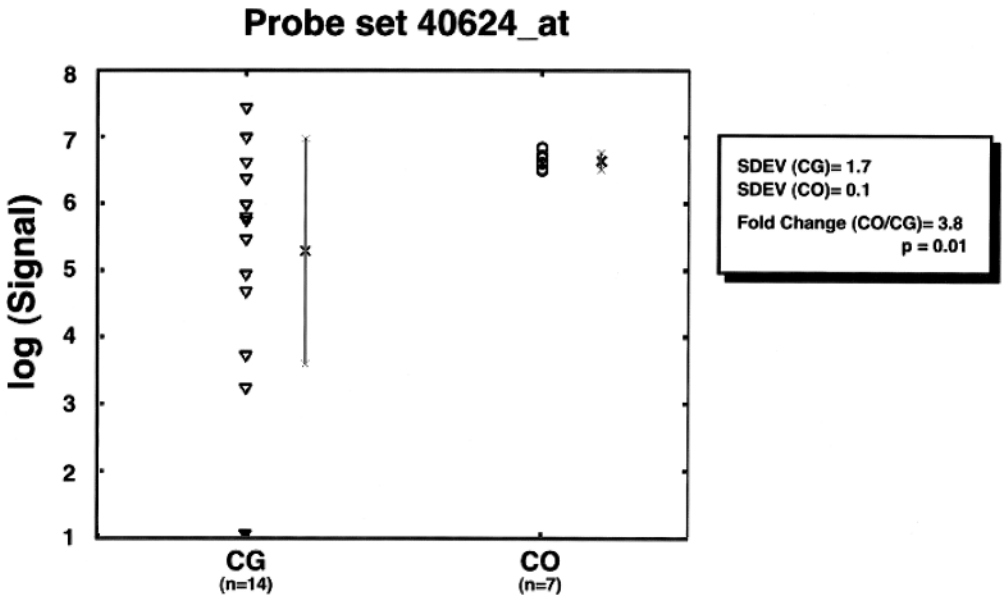

Fig. 4. Oligonucleotide microarray analysis of Olig2 in classic gliomas. *OLIG2* RNA expression was on average 3.8-fold higher in classic anaplastic oligodendrogliomas (CO, n = 7) than in classic glioblastomas (CG, n = 14) (*t*-test, p value = 0.01) for probe set 40624_at. Variance of the signal for CGs was significantly greater than that seen in COs (F-test, p value = 0.0000020). The y-axis is log signal for *OLIG2*. Average log signal for CG is 5.29 and for CO is 6.64. The standard deviation of the log (SDEV, vertical red bars) for CG is 1.68 and for CO is 0.12.

tool to differentiate diffuse gliomas from other types of primary brain tumors. We therefore performed immunohistochemical analysis of OLIG2 on non-glial classes of primary brain tumors (Table). Analysis of poorly differentiated (primitive neuroectodermal tumors) and neuronal tumors (medulloblastomas and central neurocytomas) demonstrated variable or inconsistent expression within each class. Analysis of 14 primitive neuroectodermal tumors (PNET) of the CNS showed that OLIG2 was not expressed in the vast majority of tumor cells (Fig. 5A, B). However, in a subset of tumors (5/14) we observed focal to diffuse OLIG2 positivity (data not shown). Medulloblastoma, a primitive tumor derived from cerebellar neuronal progenitor cells, was also predominantly composed of OLIG2-negative cells, but 6/8 tumors contained focal areas with rare unevenly distributed OLIG2-positive cells (Fig. 5C, D). No medulloblastomas exhibited the abundant OLIG2 positivity seen in diffuse gliomas. Analysis of the neuronal tumor central neurocytoma showed highly variable expression of OLIG2 with tumors having either no expression (1/7), a rare subpopulation of OLIG2-positive cells (3/7), or strong diffuse positivity similar to oligodendrogliomas (3/7) (Fig. 5E–H). These staining patterns did not appear to correlate with a particular histologic or immunohistochemical phenotype and all were confirmed to have neuronal characteristics by IHC or electron microscopy.

In summary, most non-glial primary tumors of the nervous system showed inconsistent but significant OLIG2 expression, with some samples exhibiting regions of strong staining, similar to diffuse gliomas.

OLIG2 Expression Is Restricted to Neuroectodermally Derived Tumors

Normally, OLIG2 expression in the developing embryo is restricted to the neuroectoderm. To determine if this specificity is preserved across the diverse spectrum of human tumors we performed immunohistochemistry on non-neural or metastatic intracranial tumors (Table). Analysis of low-grade meningiomas showed no evidence of OLIG2 staining in any of the cases examined (0/21) (Fig. 6A, B). Similarly, OLIG2 was not detected in any metastatic tumors (0/24) (e.g. carcinomas from the lung, gastrointestinal tract, genitourinary tract, breast, or kidney), in metastatic melanoma (Fig. 6C, D), or in primary CNS lymphomas (0/5) (Fig. 6E, F). Overall, OLIG2 expression is restricted to tumors of neuroectodermal origin and may have clinical utility in their distinction from non-neuroectodermal tumors.

DISCUSSION

OLIG2 Protein Expression in Normal Mouse and Human Development

Our findings demonstrate that human OLIG2 protein expression in developing and adult tissues is analogous to that previously described in other vertebrates (26, 29–33), indicating general conservation of OLIG2 regulation in human ventral neural progenitor cell specification and

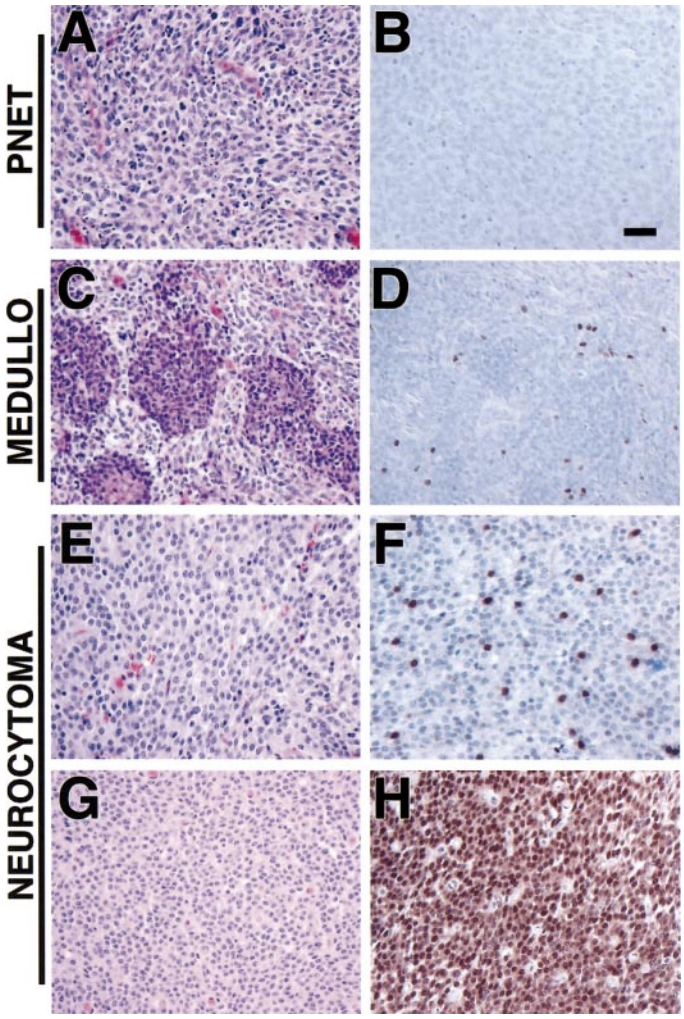

Fig. 5. OLIG2 expression is inconsistent in non-glial tumors. Immunohistochemistry for OLIG2 in non-glial tumors shows that primitive neuroectodermal tumor cells (PNETs) were predominantly OLIG2-negative **(A, B)**, but subsets of tumors contained scattered OLIG2-positive cells or focal diffuse positivity (not shown). Medulloblastomas also were principally OLIG2-negative but some cases contained focal areas with cells expressing OLIG2 **(C, D)**. Central neurocytomas showed highly variable expression with subsets of tumors exhibiting no OLIG2 expression (not shown), a rare evenly distributed subpopulation of OLIG2-positive cells **(E, F)**, and strong diffuse positivity similar to oligodendrogliomas **(G, H)**. Scale bar = 50 μm.

oligodendrocyte development. Our studies also provide additional confirmation of the specificity of OLIG2 expression in progenitor cells and oligodendroglia during development. We have not personally detected OLIG2 in mature neurons or astrocytes of the mouse or human brain and spinal cord by RNA or protein analyses (data not shown). This is in accordance with most reports and fate mapping data in the mouse except for the finding of expression in neurons of the olfactory epithelium (26). In light of this, we believe that OLIG2 can serve as an excellent general-purpose biological marker for identification of progenitor cells or oligodendroglia in developing or adult tissues, analogous to the use of NeuN as a general neuronal marker.

OLIG2 as a Marker of CNS Tumors

From a technical standpoint, OLIG2 is a robust marker for facile detection of diffuse gliomas by virtue of its 1) abundant expression in the majority of tumor cells, 2) nuclear location, 3) preservation in standard paraffin embedded clinical material, and 4) restricted expression within the neuroectoderm and neuroectodermal tumors. These features suggest that OLIG2 may be a helpful marker for positive identification or effective labeling of diffuse glioma tumor cells as well as the non-diffuse glioma, pilocytic astrocytoma. However, our data also clearly show that OLIG2 expression is not specific or exclusive to oligodendrogliomas, diffuse gliomas, or even gliomas as a general class. Very significant differences in pattern and levels of OLIG2 expression were seen when comparing diffuse glial tumors to neuronal tumors, primitive neuroectodermal tumors, and other non-glial tumors by both IHC and microarray analysis. However, analysis of individual tumors within these classes and of enigmatic tumors such as dysembryoplastic neuroepithelial tumors (DNET) (Table) demonstrated expression levels that were indistinguishable from those seen in individual diffuse gliomas. This fact clearly precludes reliance on the presence or absence of OLIG2 expression alone for the specific diagnosis of glial class tumors and indicates that OLIG2 is more likely to be useful when interpreted in context with other markers and the histologic pattern of expression.

Within the category of diffuse gliomas, OLIG2 expression reveals two important points. First, while roughly 30% of individual astrocytomas had high levels of expression similar to oligodendrogliomas, overall the two tumor classes exhibited statistically significant differences in OLIG2 expression ranges by both IHC and oligonucleotide microarray analysis. From a histopathologic perspective, this most likely reflects the high levels of heterogeneity seen within and between individual astrocytomas compared to the homogeneous staining typical of oligodendrogliomas. Additional heterogeneity at the level of expression within individual cells might also be present and is suggested by previous studies (20). Although we found OLIG2 expressed in all diffuse gliomas analyzed by IHC, several of the microarray samples contained extremely low relative levels of *OLIG2*, raising the possibility that rare astrocytic tumors may express negligible levels of OLIG2.

Second, we find that OLIG2, by itself, is not sufficiently reliable to diagnostically distinguish oligodendrogliomas from astrocytomas due to the heterogeneity seen in astrocytomas as a class. Thus, our findings are in contrast to

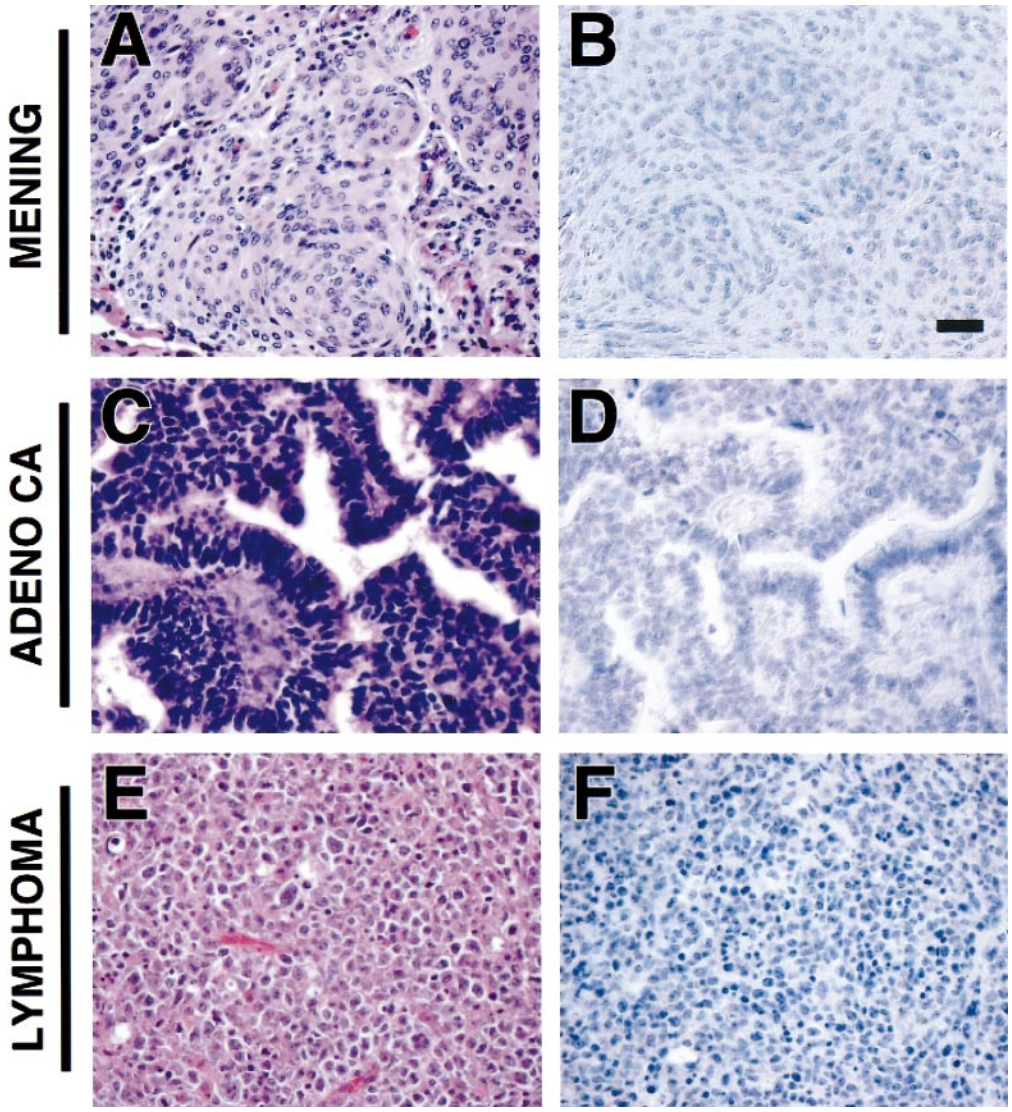

Fig. 6. OLIG2 is not expressed in non-neuroectodermally derived tumors. Immunohistochemical analysis of a representative meningioma **(A, B)**, metastatic adenocarcinoma to the brain **(C, D)** and primary CNS large B-cell lymphoma **(E, F)** demonstrates no OLIG2 expression in these classes of tumors. Adjacent areas of reactive brain contained a near normal number of OLIG2-positive oligodendrocytes and acted as an internal control in most specimens (not shown). Scale bar = 50 μm.

previously published reports that suggest that high levels of OLIG2 alone might distinguish oligodendrogliomas from other diffuse gliomas in a diagnostic setting. The low levels of OLIG2 previously reported for astrocytic tumors could be explained by 1) a higher sensitivity of our antibody versus the probes or antibodies used in other studies, or 2) the small numerical sample size in the context of highly variable expression of OLIG2 in glioblastomas as demonstrated by our group and others (20).

We do find that OLIG2 is very effective at highlighting tumor heterogeneity and may therefore have a diagnostic role in distinguishing ''classic,'' homogeneously staining, oligodendroglial lesions that have been found to have more defined clinical outcomes from more heterogeneous astrocytomas. Additionally, *OLIG2* will be an invaluable marker for validation and investigation of mouse glioma tumor models, which appear to be capable of recapitulating the range differences in OLIG2 expression between astrocytomas and oligodendrogliomas (34) (K.L. Ligon, E.C. Holland, and D.H. Rowitch, unpublished observations).

Significantly, we observed no obvious differences in OLIG2 expression in ''pure'' astrocytomas as compared to mixed oligoastrocytomas and thus application of this prototypical oligodendroglial lineage marker was not helpful in making the difficult histologic distinction of ''pure'' astrocytomas from those with mixed oligodendroglial and astrocytic appearances (5). Further studies of global gene expression in these and other tumors should help to determine whether the presence of OLIG2 in

glioma cells is most indicative of a mature oligodendroglial character or more likely of a less-differentiated progenitor cell nature. Future analysis of additional markers of the oligodendroglial lineage should help to determine the potential lineage implications and biologic meaning of Olig2 in tumors classified as ''pure'' astrocytomas.

OLIG2 and Brain Tumor Origins

While recent advances in the understanding of medulloblastoma have shown that the likely cell of origin is a neural progenitor cell (35), the leading hypotheses for the cell-of-origin for diffuse gliomas in the adult human brain have been wide-ranging and divergent (5, 10, 11). Proposed cells of origin have included 1) dedifferentiated glia (36), 2) proliferative multipotent adult neural progenitor cells located within the adult subventricular zone (10), 3) glial-restricted precursor cells (GRP) (37), and 4) oligodendroglial progenitor cells (12). The cell of origin for many other brain tumors such as DNET or central neurocytoma, both of which have morphological similarities to oligodendrocytes, is even less well understood. Our data provide additional support to the growing body of evidence for the progenitor-like nature of gliomagenic cells. It is possible that expression of OLIG2 could be aberrant (e.g. resulting from genomic instability or rearrangements) without lineage implications for gliomagenesis (38). However, we think this unlikely given the large number of other developmentally appropriate neural progenitor markers identified in these tumors (12, 13, 39–45).

The variable and heterogeneous expression of an oligodendroglial lineage marker such as OLIG2 in astrocytic, neuronal, and primitive lineage tumors also favors the hypothesis that some brain tumors may arise from true multipotent neural progenitor cells. The absent or inconsistent expression of OLIG2 in certain classes of these tumors might reflect the ability of such progenitor cells to choose certain cell fates in response to environmental conditions within the brain, certain growth factors, patient age, or different initiating genetic mutations. Recent discovery of stem-like cells within human brain tumors certainly supports this possibility and suggests exciting new avenues for future investigation (46–48). Olig2 expression in tumors currently classified as neuronal, such as DNET or central neurocytoma, suggests divergent differentiation within these tumors, and the study of additional markers of oligodendroglial differentiation is needed to confirm a glial component. In addition, the variable expression of Olig2 within certain classes of tumors (i.e. central neurocytoma and ependymoma) suggests the possibility that Olig2 may reveal new tumor subtypes whose clinical significance would require further study.

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