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Localization of the Neuronal Class III β-Tubulin in Oligodendrogliomas: Comparison with Ki-67 Proliferative Index and 1p/19q Status

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Abstract. The class III β-tubulin isotype (βIII) is widely regarded as a neuronal marker in development and neoplasia. Whereas the expression of βIII in neuronal/neuroblastic tumors is differentiation-dependent, the aberrant expression of this cytoskeletal protein in astrocytomas is associated with an ascending gradient of malignancy. To test the generality of this observation we have compared the immunoreactivity (IR) profiles of the βIII isotype with the Ki-67 nuclear antigen proliferative index in 41 archival, surgically excised oligodendrogliomas (32 classical [WHO grade II] and 9 anaplastic [WHO grade III]). Seventeen of 41 tumors were examined by quantitative microsatellite analysis for loss of 1p and/or 19q. Minimal deletion regions were defined on 1p (D1S468, D1S214) and 19q (D19S408, D19S867). Three of 10 classical oligodendrogliomas had combined 1p/19q loss, while 2 exhibited loss of either 1p or 19q. Three of 7 anaplastic tumors had combined 1p/19q loss. βIII IR was present in all tumors, but was significantly greater in the anaplastic (median labeling index [MLI] 61%, interquartile range [IQR] 55%–64%) as compared with the classical variants (MLI, 19%, IQR, 11–36%) ($p < 0.0001$). A highly significant relationship was found to exist between βIII and Ki-67 LIs (βIII, $p < 0.0001$ and Ki-67, $p < 0.0001$, $r = 0.809$). βIII localization delineated hitherto understated unipolar or bipolar tumor phenotypes with growth cones and leading cell processes resembling migrating oligodendrocyte progenitor cells. Codistribution of βIII and GFAP IR was present in ''gliofibrillary'' tumor areas. Synaptophysin IR was detected in rare tumor cells (mean LI, 0.7%), and only in 4/41 samples (10%), denoting a lack of relationship between βIII and synaptophysin expression. No significant differences in βIII LIs were observed in tumors with 1p and/or 19q loss as compared to those with 1p/19q intact status. Increased βIII IR in oligodendrogliomas is associated with an ascending degree of malignancy and thus is a potentially useful tumor marker. However, the significance of high βIII LIs in low-grade oligodendrogliomas with respect to prognostic and predictive value requires further evaluation. Class III β-tubulin expression in oligodendrogliomas should not be construed as *a priori* evidence of divergent neuronal differentiation.

Key Words: 1p/19q; Anaplastic oligodendroglioma; Class III β-tubulin isotype; Ki-67; Oligodendroglioma; Tumor marker; Quantitative microsatellite analysis.

INTRODUCTION

Oligodendrogliomas are common primary neuroepithelial tumors of the central nervous system (CNS) with an overall unpredictable biological behavior (1–3). Their incidence varies considerably among different studies, although recently oligodendrogliomas have been considered to be more common than previously thought (1, 3, 4–6). The latest WHO classification (3) separates oligodendrogliomas into 2 groups, the low-grade or ''classical'' (WHO grade II) and the high-grade or ''anaplastic'' (WHO grade III). Whereas the histogenesis and differentiation potential of these tumors remain uncertain, the prevailing view is that they constitute a nosologically and biologically distinctive category of glioma. Currently, the pathological diagnosis of these tumors relies on the recognition of salient histopathological features (2, 5–8). Despite a morphological resemblance to oligodendrocytes, tumor cells in oligodendrogliomas do not generally express oligodendrocyte lineage markers (9, 10), but may express (together with other glial tumors) oligodendrocyte progenitor cell (OPC) antigens (11–13). Frequently, subpopulations of tumor cells, also referred to as ''gliofibrillary oligodendrocytes,'' express glial fibrillary acidic protein (GFAP) (3, 7, 9, 14, 15). Because a reliable marker for neoplastic oligodendroglial cells is not yet available, the histological recognition of this tumor is often confounded by diagnostic uncertainties (8).

A significant percentage of oligodendrogliomas exhibits combined loss of 1p and 19q, which is considered to

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be a strong predictor of a favorable response to chemotherapy and a better prognosis, particularly in anaplastic tumors (16–21). The efficacy of the procarbazine, lomustine (CCNU), and vincristine sulfate (PCV) regimen, as well as other cytotoxic drugs in patients with anaplastic oligodendrogliomas exhibiting 1p/19q loss, place oligodendrogliomas amongst the most chemosensitive human solid tumors (18, 20, 22–24). Yet, it is well recognized that even patients with an initially good response to chemotherapy will ultimately relapse (25). Also, despite recent advances in drug therapies and molecular genetic typing, the long-term outcome in patients with low-grade oligodendrogliomas remains unpredictable (22, 24–27). In this respect, 1p/19q loss does not differentiate between classical and anaplastic tumors (21), whereas the chemoresponsiveness of WHO grade II oligodendrogliomas is less clear-cut (25, 27). The contribution of the MIB-1/Ki-67 proliferative index notwithstanding, there are no reliable prognostic markers in the main group of classical oligodendrogliomas (3, 8).

The class III β-tubulin isotype (βIII) is one of 7 β-tubulin isotypes expressed in mammals (28). Its expression is contemporaneous with the earliest phase of neural differentiation and is neuron-associated (29–31). We and others have previously shown that this structural protein is expressed in neuronal/neuroblastic tumors, such as cerebellar medulloblastomas (32–34), retinoblastomas (35), central neurocytomas (36), sympathoadrenal neuroblastomas (37), and adrenal pheochromocytomas (38), as well as in neuronal/neuroblastic tumor cell lines (39, 40). Presently, βIII is widely used as a neuronal marker in developmental neurobiology and tumor neuropathology. However, there are only a few studies to date addressing trends in βIII expression in transformed non-neuronal cell types at various stages of neoplastic development, including tumor progression and/or anaplastic transformation. We have recently shown that βIII is also expressed in non-neuronal tumors whose non-transformed, mature ''cell-of-origin'' counterparts do not normally express this isotype (41, 42). This ''aberrant'' expression of βIII may underlie alterations in the isotype composition of β-tubulin in tumor subclones, which may predict the direction of tumor behavior and chemoresponsiveness to microtubule-acting compounds, including taxol agents (43).

Whereas the expression of βIII-tubulin in CNS neuronal/neuroblastic tumors is differentiation-dependent, its localization in common astrocytic gliomas follows a gradient of high-grade malignancy (anaplasia or ''dedifferentiation'') (42). To determine whether the same holds true for other common glial tumors of the CNS, we have examined the immunoreactivity (IR) profile of βIII in human oligodendrogliomas. We now demonstrate that βIII-tubulin is present both in low- and high-grade oligodendrogliomas, but its expression is significantly increased in the anaplastic variant bearing a strong relationship with the Ki-67 proliferative index, but no relationship to 1p/19q status.

MATERIALS AND METHODS

Tissue Samples

Forty-one cases of oligodendroglial tumors, obtained during a 10-yr period (1989–1999), were retrieved from the files of the Department of Pathology, Haukeland Hospital, Bergen, Norway and the Department of Histopathology and Morbid Anatomy, The Royal London Hospital, Whitechapel, London, England. All tumors were supratentorial and were diagnosed according to conventional histopathological criteria based on the World Health Organization (WHO) Histological Typing of Tumours of the Central Nervous System (3, 6) as low-grade or classical oligodendrogliomas (WHO grade II) (n = 32, age range, 26–75 yr, median age, 45 yr) and high-grade or anaplastic oligodendrogliomas (WHO grade III) (n = 9, age range, 39–68 yr, median age, 51 yr). All specimens were derived either from wide tumor resections or debulking. Stereotaxic biopsy specimens were not included in this study. Of the 41 tumors, 40 were primary excisions upon initial clinical presentation and 1 anaplastic oligodendroglioma was from a patient who had received radiotherapy and PCV chemotherapy after excision of a WHO grade II oligodendroglioma ~4 yr prior to recurrence. Both the original and recurrent tumors were available in the present study. Normal human CNS tissues from different ages (fetal to adult), obtained at autopsy, as well as 3 surgically excised central neurocytomas (neuronal tumors known to express the neuronal proteins of interest in a differentiation-dependent manner [36]), served as controls.

Tissue Fixation

All specimens were fixed in 10% neutral buffered formalin by immersion and processed conventionally for histology and immunohistochemistry. Five- to 6-μm-thick sections were stained with hematoxylin and eosin (H&E) for histologic evaluation and the remainder of the serial unstained sections were used for immunohistochemistry.

Antibodies

Two anti-class III β-tubulin antibodies (gifts of Dr. Anthony Frankfurter, University of Virginia, Charlottesville) were used: 1) A mouse monoclonal antibody TuJ1 (IgG2a) and 2) an affinity purified rabbit antiserum specific for the same epitope as that recognized by monoclonal antibody TuJ1. Both antibodies were used at a dilution of 1:500. The staining pattern produced by the anti-βIII antiserum is identical to that produced by TuJ1 (31, 41, 42). The production, purification, and characterization of these antibodies have been described previously (29, 41). All tumors examined in this study were also stained in parallel with commercially obtained mouse monoclonal antibodies to glial fibrillary acidic protein (GFAP; clone 6F2; IgG1 k; Dako, Santa Barbara, CA; dilution 1:100), synaptophysin (clone SVP-38; IgG1; Chemicon, Temecula, CA; dilution 1:100) and Ki-67 nuclear antigen (clone NC-MM1; IgG2a; Novocastra, Newcastle-upon-Tyne, England, UK; dilution 1:200).

Immunohistochemistry

Immunoperoxidase studies were performed on deparaffinized sections of surgical and postmortem specimens according to the avidin-biotin complex (ABC) peroxidase method as previously described (41), using rabbit IgG and mouse IgG ABC Elite Vectastain® kit (Vector Laboratories, Burlingame, CA) for the polyclonal and monoclonal antibodies respectively. For TuJ1 or the anti-βIII antiserum, no antigen retrieval was performed because no differences have been detected with respect to the distribution of immunoreactivity of either antibody following a microwave-based method of antigen retrieval (41). Negative controls included normal rabbit immunoglobulin G (IgG), nonspecific mouse ascites fluid (Becton-Dickinson, Franklin Lakes, NJ), or unrelated primary antibodies.

DNA Preparations

Seventeen oligodendrogliomas (10 classical and 7 anaplastic) were obtained as routinely processed, paraffin-embedded samples from clinical material at the Department of Pathology, Haukeland Hospital, Bergen, Norway. For each case, histological assessment of the tumor tissue to be used was performed by a neuropathologist (KA). If at least 90% of the area of the tissue was interpreted as tumor, the sections were directly cut from the block into an Eppendorf tube for DNA isolation. If the proportion of the tumor was <90%, 10 to 20 sections were cut on glass slides and the tumor tissue was hand-dissected from the non-neoplastic brain tissue. Tumor DNA was prepared from paraffin-embedded tissues by digesting deparaffinized tumor sections for 3 to 5 days with proteinase K at 55°C (0.5 mg/ml in 100 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 8.0, 25 mmol/L, ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulfate) followed by a phenol: chloroform:isoamyl extraction and ethanol precipitation. Concentrations were determined on the ABI 7700 so that each DNA, when amplified with the reference locus, reached a threshold cycle equivalent to a 5-ng control DNA.

Quantitative Microsatellite Analysis

Quantitative microsatellite analysis (QuMA) was performed as described previously (21). Briefly, all primer sets were used to perform amplifications in triplicate on the ABI 7700 instrument (Applied Biosystems, Foster City, CA). The probe for the detection of amplified product was a 21-bp oligomer complementary to the microsatellite SA repeat [5′6-carboxy fluorescein (FAM)-TGTGTGTGTGTGTGTGTGTGT 3′6-carboxy tetramethyl rhodamine (Integrated DNA Technologies, Coralville, IA), rendering it capable of hybridizing to microsatellite loci spread throughout the human genome. Thus, the flanking primers could be continuously changed while the probe remained constant (21). Copy number is determined from the PCR cycle number (Ct) at which DNAs reach a threshold amount of fluorescence above background. Reactions (50 μl) were performed in 1× PCR buffer A (Perkin Elmer, Foster City, CA), 2.5 mmol/L $MgCl_2$, 0.4 μmol/L each primer, 200 μmol/L each dNTP, 60 nmol/L probe, and 1.25 U AmpliTaq Gold. Cycling parameters were as follows: 95°C for 12 min × 1 cycle, (95°C for 20 s, 55°C for 20 s, 72°C for 45 s) × 40 cycles. A master mix of the components including the equivalent of 0.5 ng of genomic DNA per well (10 μl at 0.5 ng/μl) was made and aliquoted into a 96-well optical plate. Ten μl of the primer sets were subsequently added to designated wells.

For these experiments, 4 primer sets were chosen for individual loci on chromosomes 1p (D1S468, D1S214) AND 19q (D19S408, D19S867) together with appropriate reference pool primers as previously described (21). Calculations of Ct values and copy numbers were also carried out, as previously described (21). Copy numbers of equal or <1.58 were considered as losses, whereas those >2.53 were considered to be gains.

Primer sets used for QuMA are as follows:

Primer	Forward	Reverse
1p		
D1S468	TAAAATATTAGGTCAAACCATG	ATGGCTGCATATAATGTTG
D1S214	CCGAATGACAAGGTGAGGACT	AATGTTGTTTCCAAAGTGGC
19q		
D19S408	AGCTCTATGGGGTGGTGCC	GCCTCTTAGAGTTTTGGGAG
D19S867	CAATGAAAATGCTTTGTAAAAC	CCTTCAGAGGTGACCAG

Analysis and Statistical Methods

Histological preparations were evaluated by a panel of 5 neuropathologists (CDK, LDV, JFG, KA, and SJM). Histologic classification was made according to homogeneous criteria (6) by having specimens evaluated independently among 4 members of the panel. In case of disagreement, histological typing was assigned by consensus at conference. Manual cell counting of labeled tumor cells was performed independently by 2 observers (CDK and LDV). Twenty non-overlapping high-power fields (field magnification, ×40) were evaluated and the percentage of labeled tumor cells was calculated for each specimen and for each antibody (anti-βIII and anti-Ki-67). Total cells counted per specimen ranged from 681 to 1,384 (median, 1,044). Interobserver agreement for the evaluation of immunohistochemical staining was within 15% (k = 0.82) (42).

Cases were grouped into classical oligodendrogliomas (WHO grade II) and anaplastic oligodendrogliomas (WHO grade III) based on well-defined morphological criteria (6). To assess the fraction of immunolabeled cells in specimens from each patient case, the labeling index (LI), defined as the percentage of βIII-tubulin+ (labeled) or Ki-67+ cells out of the total number of tumor cells counted, was determined. In the case of Ki-67, labeled cells in tumor blood vessels/microvascular proliferation were excluded from the cell counts. The median LI (MLI) and interquartile range (IQR) of LIs were determined for the set of cases comprising each histological grade using the UNIVARIATE procedure of the SAS package (SAS Institute, Cary, NC). The IQR is delimited by the 25th and 75th population percentiles. The statistical significance of differences in LIs between groups was examined non-parametrically using the Wilcoxon rank sum test. These analyses were carried out using the NONPAR1WAY procedure of the SAS package (SAS Institute).

To investigate the existence of a relationship between βIII and Ki-67 LIs according to an ascending scale of malignancy, linear regression analysis was performed between the WHO tumor grades and the LIs for each marker. Loss of individual 1p or 19q markers were recoded as ''positive'' (equal or <1.58 copy number) and ''negative'' (>1.58 copy number) using a

		1p		19q		
		1p36				
		D1s468	D1s214	D19s408	D19s867	
cM		6.2	16.9	67.8	81.2	
636	O	1.86	0.85	1.53	1.85	1p/19q intact
6500	O	2.62	1.01	1.43	1.25	19q loss
15210	O	1.58	0.98	1.32	1.65	1p loss
7217	O	1.09	1.16	1.13	1.41	1p/19q loss
19574	O	2.99	1.09	2.50	2.11	1p/19q intact
12147	O	2.12	1.97	1.95	2.30	1p/19q intact
27233	O	1.00	0.89	0.86	1.51	1p/19q loss
4411	O	1.27	1.49	1.24	1.35	1p/19q loss
19019	O	1.40	1.79	1.26	2.57	1p/19q intact
9872	O	1.45	2.73	1.70	2.08	1p/19q intact
3363	AO	1.87	1.83	1.00	3.05	1p/19q intact
34637	AO	1.22	1.04	0.90	1.16	1p/19q loss
458	AO	1.43	1.89	1.15	2.30	1p/19q intact
23947	AO	1.95	1.70	1.60	2.03	1p/19q intact
845	AO	1.21	1.64	1.23	1.79	1p/19q intact
21238	AO	1.25	1.05	1.00	1.07	1p/19q loss
17911	AO	1.46	1.15	0.92	1.43	1p/19q loss

Fig. 1. Copy number of loci in tumors as determined by QuMA. Seventeen cases of oligodendroglioma have been investigated for the deletion of two 1p and two 19q loci. Rows represent individual cases. Copy numbers for genomic loci (position indicated in cM) have been arranged in columns. Histopathological diagnosis is designated as ''O'' for classical or low-grade oligodendroglioma (WHO grade II) and ''AO'' for anaplastic or high-grade oligodendroglioma (WHO grade III). Gain or loss is assigned to a copy number. For these data, a calculated copy number of equal or <1.58 was scored as a loss, whereas >2.53 was scored as a gain. Calculated numbers are as indicated in the boxes: black boxes represent losses, white boxes intact status (normal), and light gray boxes indicate gains.

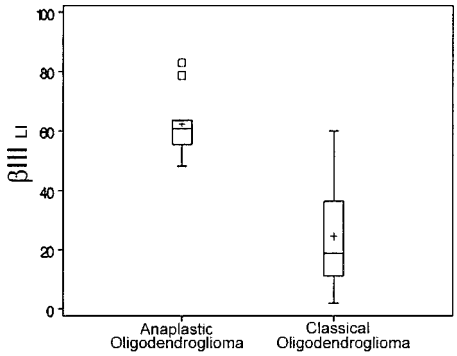

Fig. 2. Box plots of the distribution of class III β-tubulin (βIII) labeling indices (LIs) in patients with anaplastic oligodendrogliomas (WHO grade III) and classical oligodendrogliomas (WHO grade II). The lower and upper bounds of the box represent the 25th and 75th population percentiles respectively. Whiskers extend from the ends of the boxes as far as the data extend up to a distance of at most 1.5 interquartile ranges (represented by the distance between the top and the bottom of the box). Data points more extreme than this are shown as individual small squares. The center horizontal line within the box represents the median of the distribution and the central plus sign (+) the mean of the distribution. Despite some overlap of LIs between the 2 groups, βIII immunoreactivity is significantly greater in the high-grade oligodendrogliomas ($p < 0.0001$)

threshold value of 1.58, and were thence compared with diagnosis. The Fisher exact test was performed to investigate any significant association between loss of 1p/19q markers with diagnosis of classical versus anaplastic oligodendrogliomas using contingency tables. Mann-Whitney *U*-test comparisons of the copy numbers for 1p/19q markers between low-grade versus high-grade oligodendrogliomas were also carried out.

Logistic regression models were used for predicting diagnosis based upon Ki-67 LI, βIII LI, or the loss of 1p/19q markers. Multivariate models were also used to assess if a variable (i.e. βIII LI or loss of 1p/19q markers) significantly improved upon the predictive accuracy of the Ki-67 LI. Spearman non-parametric correlation analysis was carried out between βIII LI, Ki-67 LI, and the two 1p (D1s468, D1s214) and two 19q (D19s408, D19s867) markers.

RESULTS

Determination of 1p/19q Status by Quantitative Microsatellite Analysis (QuMA)

Copy numbers at 2 loci on each 1p and 19q were determined in 17/41 tumors (10 classical and 7 anaplastic) and the cumulative data are presented in Figure 1. Loss of 1p and 19q is defined by copy number equal or <1.58 in 2 different loci of each chromosome. Calculated copy number at locus corresponds to 2 primers (markers), i.e. D1s468 and D1s214 for 1p, and D19s408 and D19s867 for 19q. Loss of 1p and/or 19q denotes combined loss of both markers (i.e. values equal or <1.58 per marker) on each chromosome. QuMA detected combined 1p/19q loss in oligodendroglial tumors of both grades. Five of 10 classical oligodendrogliomas (50%) exhibit genetic abnormalities in the 1p and 19q regions: 3 have combined 1p/19q loss (cases 7217, 27233, and 4411), while losses involving only 1p (case 15210) or 19q (case 6500) are observed in 2 tumors. Three of 7 anaplastic oligodendrogliomas have 1p/19q loss (cases 34637, 21238, and 17971) while 1 anaplastic oligodendroglioma (case 845) has a borderline 1p loss.

Differential Cellular Distribution of βIII in Classical versus Anaplastic Oligodendrogliomas

A comparison of the MLIs for class III β-tubulin (βIII) in classical versus anaplastic oligodendrogliomas is presented in Figure 2. βIII IR is present in all tumors (100%) from both histological grades (WHO grades II and III). No age- or gender-related differences were observed.

The MLI for classical oligodendrogliomas is 19% (IQR, 11%–36%). There is considerable βIII staining heterogeneity among different tumors, which is independent of the degree of cellularity and presence of calcifications or microcysts (LI range, 2%–60%). Variability in the topographic distribution of βIII+ cells is also frequent within individual tumor samples (intratumoral staining

heterogeneity). βIII IR is typically multifocal and is detected either in scattered isolated tumor cells, or in clusters of cells exhibiting the so-called typical oligodendroglial-like morphological features, i.e. uniform cell density, cytologic monotony, and round nuclei surrounded by a clear halo (imparting a honeycombed/vacuolated appearance) (Fig. 3a–d). βIII+ cells are morphologically indistinguishable from the predominantly βIII− cells (Fig. 3a–d). Strong βIII localization is present in the thin cytoplasmic rim around the nuclei of apolar round-shaped oligodendroglial-like cells (Fig. 3a–d) and in ''gliofibrillary oligodendrocytes'' exhibiting GFAP IR in immediately adjacent sections (Fig. 3e, f). Furthermore, βIII staining delineates unipolar or asymmetrically bipolar tumor cells with growth cones and leading cell processes resembling migratory OPCs (Figs. 3g, 4a, b). Robust βIII IR is also consistently observed in poorly fibrillated cells with plump perinuclear cytoplasm and eccentric nuclei compatible with ''microgemistocytes'' (Fig. 4c, d).

βIII staining is diffuse within the individual tumor cells and is distributed equally in both the perinuclear cytoplasm and in the short, poorly ramified unipolar or bipolar cell processes (Figs. 3a–d, g, 4a, b). The patterns of immunohistochemical localization are predominantly diffuse or filamentous (Figs. 3d, 4a, b) and less frequently are finely granular (Fig. 4d). Occasional cells exhibit a narrow, annular perinuclear rim of prominent filamentous-like IR, surrounded by a diffuse, albeit less prominent, localization in the residual cytoplasm (Fig. 4b).

βIII+ tumor cells are frequently detected in areas of cortical infiltration (Fig. 3h–j). An increased number of βIII+ tumor cells is consistently present in foci of subpial infiltration (Fig. 3h). This is associated with an increased Ki-67 labeling in these cells (not shown). Also, βIII IR is present in areas of leptomeningeal spread whereby tumor cells acquire spindle morphology (Fig. 3k, l).

Compared with the classical oligodendrogliomas (MLI, 19%, IQR, 11%–36%), the cellular distribution of βIII IR is substantially greater in anaplastic oligodendrogliomas (MLI, 61%, IQR, 55%–64%) ($p < 0.0001$). However, in a number of cases there is an overlap of βIII LIs between classical and anaplastic oligodendrogliomas (Fig. 2). In high-grade oligodendrogliomas there is a greater density of βIII+ tumor cells that also exhibit a variety of morphological appearances. Widespread, variably intense βIII staining is present in aggregates of small anaplastic cells resembling ''primitive'' glioblasts forming lobules (Fig. 5a–d), in larger angulated cells with microgemistocytic features and hyperchromatic nuclei (not shown), or in sheets of densely packed, apolar or unipolar cells with growth cones or short processes (Fig. 5e, f).

Neither is βIII-tubulin staining detected in the banal blood vessels of oligodendrogliomas (''chicken-wire'' pattern) (Figs. 3a, b, e, 4a), nor in the large caliber blood vessels with endothelial hypertrophy of anaplastic oligodendrogliomas (Fig. 5e).

In the normal human CNS from different ages, βIII localization is solely neuron-specific (not shown). Expected βIII staining is present in pre-existing gray matter neurons (Fig. 3i, j) and white matter axons in areas of tumor infiltration (Fig. 4).

Comparison between βIII LI, Ki-67 LI and 1p/19q Status

A comparison of the LIs between βIII and Ki-67 in classical versus anaplastic oligodendrogliomas is presented in a scatter plot graph (Fig. 6). The Ki-67 MLIs for classical oligodendrogliomas (WHO grade II) versus anaplastic oligodendrogliomas (WHO grade III) are 11% (IQR, 7%–18%) and 46% (IQR, 34%–51%) respectively ($p < 0.0001$). Linear regression analysis reveals the existence of a highly significant relationship between βIII and Ki-67 according to an ascending scale of malignancy (βIII, $p < 0.0001$, Ki-67, $p < 0.0001$).

That βIII IR in oligodendrogliomas is increased according to an ascending gradient of malignancy is exemplified in 2 metachronous lesions from the same patient presented in this series (cases 9872 and 3363). The patient, a 56-yr-old male, was aged 52 during the first operation. The original tumor (case 9872) had a βIII LI of 13% and a Ki-67 LI of 2.7%. The recurrent tumor (case 3363), ~4 yr after the first excision and following radiotherapy (52 Gy) and chemotherapy (PCV regimen), was consistent with anaplastic oligodendroglioma (βIII LI, 48.3%, Ki-67 LI, 43.2%).

βIII-tubulin IR is localized in all tumor specimens irrespective of 1p/19q status. Figure 4a–d illustrates βIII IR profiles in 2 representative cases (27233 and 4411) both exhibiting combined 1p/19q loss. No statistically significant differences in βIII LIs were found between subsets of oligodendrogliomas with or without 1p or 19q loss. Similarly, no statistically significant relationship was found between βIII LIs and copy number equal or <1.58 in individual loci, i.e. 1p distal markers D1s468 and D1s214 and the 19q markers D19s408 and D19s867. Moreover, when these markers were recoded as to ''loss'' (equal or <1.58 copy number) and ''non-loss'' (>1.58 copy number) using a threshold value of 1.58 and compared with histopathological diagnosis using contingency tables, none of them showed any significant association with the diagnosis of classical versus anaplastic oligodendroglioma (Fisher exact test).

In Mann-Whitney *U*-test comparisons of the loss of 1p/19q markers between classical oligodendrogliomas ($n = 10$) and anaplastic oligodendrogliomas ($n = 7$), no significant differences could be discerned for D1s468 ($p = 0.5582$), D1s214 ($p = 0.4350$), or D19s867 ($p = 0.8071$). However, for D19s408, the difference between groups approached statistical significance ($p = 0.0508$). In logistic

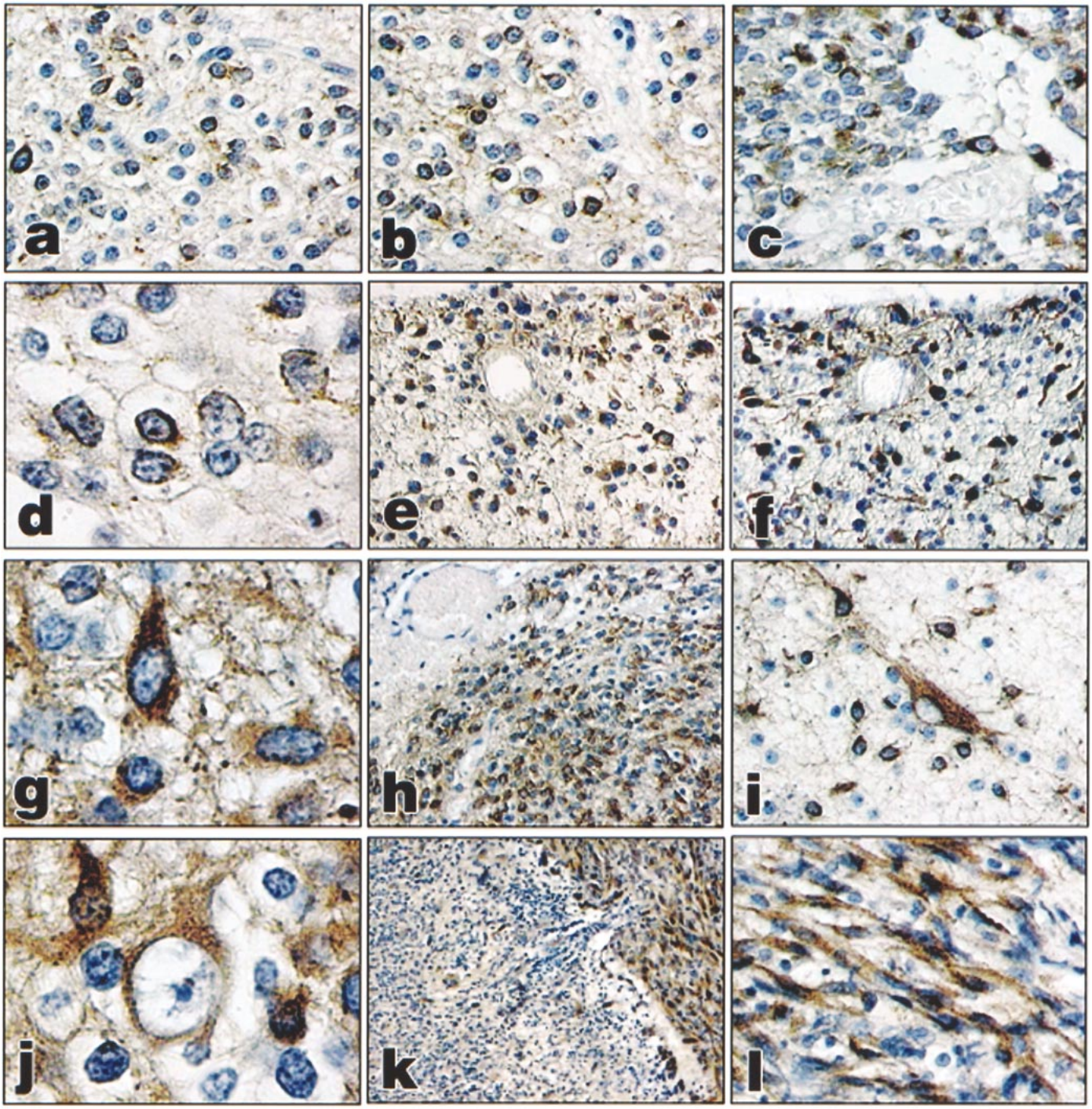

Fig. 3. Panel depicting the cellular distribution of βIII in classical oligodendrogliomas (WHO grade II). βIII IR in tumor cells showing a variety of morphological appearances. a–d: βIII localization in the thin cytoplasmic rim around the nuclei of apolar, uniform, round-shaped oligodendroglial-like cells surrounded by a clear halo (honeycombed/ vacuolated architecture). Panel (d) shows mucinous change. βIII+ and βIII− cells are morphologically indistinguishable. e, f: ''Gliofibrillary'' areas in oligodendroglioma exhibiting βIII (e) and GFAP IR (f) in immediately adjacent sections. g: βIII staining in unipolar tumor cells with growth cone-like process. h–j: βIII+ tumor cells in areas of cortical infiltration. h: Focus of subpial infiltration exhibiting an increased number of βIII+ tumor cells. i, j: Infiltrating βIII+ tumor cells in relation to βIII+ pre-existing cortical neurons (''perineuronal satellitosis''). k, l: βIII IR in an area of leptomeningeal spread (panel k, right upper corner) where tumor cells acquire spindle morphology (i). ABC peroxidase with hematoxylin counterstain.

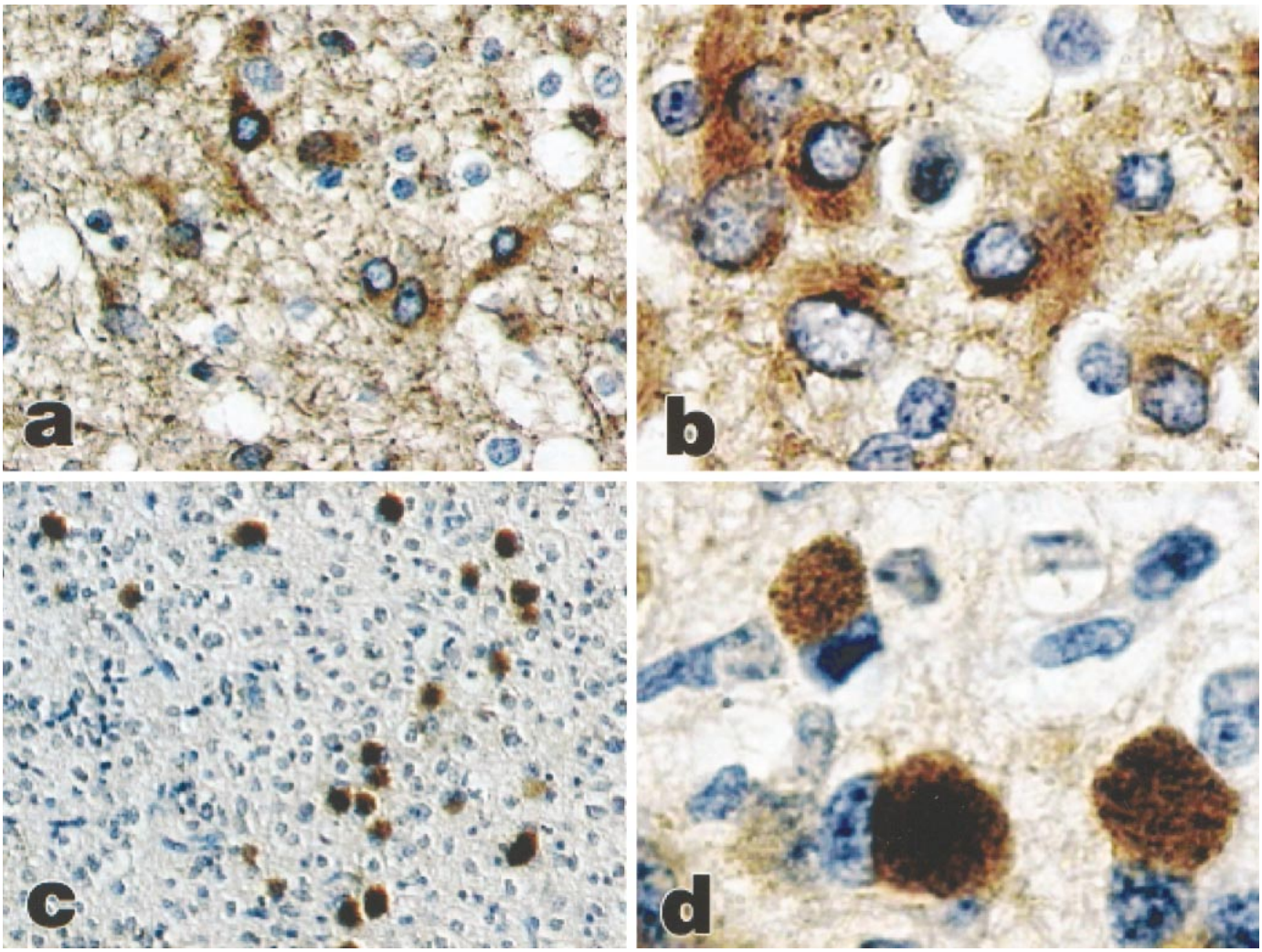

Fig. 4. Panel depicting βIII IR in 2 representative cases of classical oligodendroglioma characterized by 1p/19q loss. a: βIII staining highlights the common unipolar morphology of tumor cells. Note βIII IR in indigenous axons. b: Accentuated rim-like, perinuclear localization of βIII in a cluster of neoplastic cells. Note less intense staining in the residual cytoplasm. c, d: Robust βIII IR in poorly fibrillated cells with plump perinuclear cytoplasm and eccentric nuclei, compatible with ''microgemistocytes.'' Note finely granular/particulate localization in (d). ABC peroxidase with hematoxylin counterstain.

TABLE
Correlations between β III LI, Ki-67 LI, and 1p/19q Markers Using Spearman Non-Parametric Correlation Analysis

Relationship	r-value	p-value
Ki-67 vs βIII	0.82	<0.0001
Ki-67 vs Prm19s408	−0.49	0.0480
Prm1s468 vs Prm19s408	0.71	0.0015
Prm1s214 vs Prm19s867	0.62	0.0077

regression models for predicting diagnosis based upon Ki-67 LI, βIII LI, or the loss of 1p/19q markers, the Ki-67 LI was the best predictor variable, whereas in multivariate models no other variable (i.e. βIII LI or loss of 1p/19q markers) significantly improved upon the predictive accuracy of the Ki-67 LI. The significant correlations between βIII LI, Ki-67 LI, and the two 1p (D1s468, D1s214) and two 19q (D19s408, D19s867) markers, using Spearman non-parametric correlation analysis, are shown in the Table.

βIII and Synaptophysin Immunoreactivity in Oligodendrogliomas versus Central Neurocytomas

Synaptophysin IR is limited to rare tumor cells in 2 classical and 2 anaplastic oligodendrogliomas of this series (4/41 tumor samples [~10%]). Synaptophysin+ tumor cells have an increased cytoplasm, imparting a ''mini-gemistocytic'' appearance (not shown). Synaptophysin LI values in these 4 tumors range from 0.5% to 1.5% (mean LI, 0.7%). For comparison, the corresponding LIs for βIII range from 28% to 79% (mean LI, 47%). All 4 tumors were examined by QuMA for 1p/19q loss:

2 exhibited combined 1p/19q loss while the remaining 2 had 1p/19q intact status. Hence, in immediately adjacent sections, the overwhelming majority of βIII+/GFAP+ or βIII+/GFAP− tumor cells are synaptophysin-, denoting a dissociation between βIII and synaptophysin expression in oligodendroglial tumors. The distribution of synaptophysin staining in normal CNS is neuron-specific, exhibiting predominantly neuropil (axonal) and perisomatic patterns (not shown). Synaptophysin staining is invariably present in the entrapped or infiltrated neuropil of the gray and white matter, delineating indigenous brain tissue (+) from infiltrating tumor cells (−).

In contrast to oligodendrogliomas, all 3 cases of central neurocytomas (included in this study as reference neuronal tumors) exhibit a diffuse and dense βIII IR, which is consistently associated with widespread synaptophysin IR in immediately adjacent sections, thus effectively highlighting differentiation-dependent areas of neoplastic neuritogenesis (not shown). The low growth potential of these neuronally differentiating phenotypes is also supported by the low Ki-67 LIs detected (<2%).

DISCUSSION

We have found that βIII-tubulin IR is present both in low- and high-grade oligodendrogliomas, with a significantly greater expression in high-grade tumors (WHO grade III). In this regard, the degree of βIII staining in oligodendrogliomas mirrors the relation between histologic malignancy and βIII-tubulin expression in astrocytic gliomas (42). In contrast to the differentiation-dependent, neuronal lineage-associated expression of βIII in neuronal/neuroblastic tumors (32–38), the presence of this neuronal isotype in human astrocytic gliomas is associated with an ascending gradient of malignancy (42). Deregulation of βIII expression in glial tumors may reflect loss of epigenetic control, and thus ''dedifferentiation'' towards immature progenitor-like phenotype(s) marking potentially anaplastic tumor subclones (41, 42).

To further corroborate that βIII is expressed in oligodendrogliomas according to an ascending gradient of malignancy, we explored the eventual relationship between βIII and Ki-67 LIs in these tumors. In addition to clinical (i.e. patient's age, Karnofsky score) and traditional histopathologic parameters distinguishing between classical and anaplastic variants (6), the Ki-67 nuclear antigen is currently the most widely used cell proliferation marker for the prognostic and predictive assessment of oligodendrogliomas because it correlates well with histological malignancy and distinguishes grade II from grade III tumors (8, 44–47). A significant difference in Ki-67 LIs between classical (WHO grade II) and anaplastic oligodendrogliomas (WHO grade III) is also confirmed in the present study.

Previous studies have reported a wide range of LI values without a consensus regarding a specific cut-off point for individual tumors (8, 46, 48). Variability of Ki-67 immunostaining in glial tumors may be due to the use of different Ki-67 equivalent antibodies, manual staining versus the use of automated immunohistostainers, or the use of frozen versus paraffin-embedded sections (42, 49). For example, the NC-MM1 monoclonal antibody yields consistently higher MLIs for each tumor group as compared to the mean LIs reported in previous studies using the monoclonal antibody MIB-1 (44, 45, 47). However, no significant overlap of Ki-67 LIs was detected between anaplastic versus classical oligodendrogliomas in the present study.

Our results indicate that a highly significant grade-dependent relationship exists for both βIII and Ki-67 (βIII, $p < 0.0001$ and Ki67, $p < 0.0001$). Thus, βIII-tubulin is a marker of potential diagnostic significance in the context of malignancy in oligodendrogliomas. The increased expression of βIII in anaplastic as compared to classical oligodendrogliomas is also exemplified in the 2 metachronous lesions from the same patient (cases 9872 and 3363) supporting the assertion that βIII is expressed according to an ascending scale of malignancy in glial tumors.

Alterations in the cellular microtubule proteins including expression differences and/or mutations in the tubulin genes may be among the potential mechanisms of resistance to antimicrotubule agents (50). Taxol alkaloid compounds, such as paclitaxel, are currently being explored as salvage chemotherapeutic agents in gliomas because they appear to potentiate the effects of radiation therapy (51). However, the modest or suboptimal therapeutic response produced by these agents in malignant/recurrent gliomas (52, 53) points to the existence of taxol resistant phenotypes, which are hitherto molecularly undefinable. As the binding sites of taxol and related compounds have been delineated on the β-tubulin subunit, βIII is potentially significant in the context of taxol chemoresistance in gliomas, much like in the tumors of the lung, prostate, and ovary (54, 55). To this end, it remains to be determined whether oligodendroglial tumors with lower βIII LIs may be more amenable to chemotherapy with taxol compounds.

Genetic Alterations

Glioma tumorigenesis embodies the accumulation of multiple genetic alterations, which include chromosomal derangements and gene mutations, causing extensive changes in the expression of proteins involved in the regulation of cell proliferation and signal transduction (3). A relationship between combined loss of 1p and 19q and chemoresponsiveness in a high percentage oligodendrogliomas has emerged (16, 17–21). Combined losses of 1p/19q are statistically significant predictors of chemosensitivity and longer recurrence-free survival after chemotherapy for patients with anaplastic oligodendrogliomas, while allelic loss of chromosome 1p alone is a statistically significant predictor of chemosensitivity (16,

19, 20). Certain anaplastic oligodendrogliomas with chromosome 1p alterations may respond to chemotherapy, but with shorter duration of response and patient survival, whereas tumors lacking 1p loss can be further subdivided into those with TP53 mutations (initially chemosensitive but with rapid recurrence) and those without TP53 mutations (chemoresistant and akin to glioblastomas) (20). 1p/19q deletions occur in both classical and anaplastic oligodendrogliomas (17, 21). This genetic abnormality indicates that putative suppressor gene loci in these 2 regions may be involved in tumorigenesis (21).

In the present study ~45% of all tumor samples examined showed allelic alterations in 1p/19q as compared to the higher figures (50%–100%) previously reported for oligodendrogliomas (17, 21, 56). Minimal deletion regions in this study were defined on 1p (D1S468, D1S214) and 19q (D19S408, D19S867) (21). It should be noted that these were among the 1p/19q loci evaluated by the repertoire of markers used in a recent study (21), but 3/4 markers were different from those described on 19q13.3 (D19S412-D19S596) and 1p (D1S468-D1S1612) in an earlier study (17). It is possible that this, as well as the inherently confounding factor of entrapped normal tissue observed in a number of the tumor samples (that could not be microdissected owing to the infiltrative nature of the neoplastic process) may account for the smaller percentage of allelic loss reported in this series.

The human gene for class III β-tubulin has been mapped to the telomeric region of the long arm of chromosome 16 (within D16S422-qtel on the radiation hybrid map) (http://www.ncbi.nlm.nih.gov/genemap99). Loss of genetic material on chromosome 16q has been noted in gliomas. One study identified 16q loss in 5/28 tumors (57). Interestingly, in none of these 5 cases was there concomitant 1p/19q loss. Both losses and gains of 16q have been reported in astrocytic tumors, especially in children (58). This region is frequently lost in breast cancer and candidate genes in the region include the cyclin-dependent kinase cdk10 (59) and a gene expressed during growth arrest, GAS11 (60). In addition, a provisional nasopharyngeal susceptibility protein named LZ16 (http://www.ncbi.nlm.nih.gov) has been mapped to this region. The relationship of 16q aberrations and βIII-tubulin expression, if any, remains to be determined.

Tumor Phenotype in Oligodendrogliomas in the Context of βIII Ontogeny in the CNS

The class III β-tubulin isotype, also known as ''minor neuronal'' or human β4 (hβ4), differs from the other tubulin isotypes with respect to its amino acid sequence and post-translational modifications, which include phosphorylation (61, 62). βIII appears to be neuron-specific and the earliest lineage-specific marker protein for neurons (29–31). The immunocytochemical localization of βIII in immature neuroepithelial cells, including neural precursors, is widely construed as evidence of neuronal lineage differentiation (29–31, 62–68).

During development, immature oligodendrocytes express transiently several ''neuronal-type'' proteins of the microtubule cytoskeleton, including the microtubule-associated proteins MAP1B/MAP5 (69, 70), MAP2c (71, 72) and τ (72, 73). The cytoskeleton of oligodendrocytes, particularly the developing oligodendrocytes that elaborate filopodia-like processes, is rich in tubulin, τ, MAP1B/MAP5, and MAP2 (69–75). In this regard, MAP2 has recently been shown to be expressed in oligodendrogliomas (76).

Tubulin in differentiated oligodendrocytes is associated with soluble, cytoskeletal and membrane fractions (including myelin) where it exhibits significant microheterogeneity (77). The abundance of β-tubulin in normal oligodendrocytes notwithstanding, recent studies have failed to report the presence of the βIII isotype in these glial cell types (78, 79).

Whilst committed oligodendrocytes in the developing, or the mature, human CNS are βIII−, it is unclear whether human OPCs express transiently βIII. Weickert and colleagues (68) assert that subpopulations of immature neuroepithelial cells co-expressing the polysialated form (PSA)-NCAM and βIII in the anterior subventricular zone of human infants are presumptive neurons. This interpretation is consistent with most published reports to date, attesting to an early neuronal specificity of βIII in the mammalian CNS. By the same token, Murray and Dubois-Dalcq (80) have shown that >90% of cells in expanded neural spheres derived from human embryonic brain are OPCs expressing PSA-NCAM, whereas many outwardly migrating cells express also the neuronal microtubule proteins, βIII-tubulin and MAP2. Whether subpopulations of these neural sphere-derived βIII+ cells represent OPCs requires further elucidation. Yachnis and colleagues have suggested that oligodendrocyte-like cells (OLC) in the human amygdaloid region are presumptive neuroblasts based on βIII and Neu-N staining (81). Yet the latter study reports a disparity in the number of βIII+ OLCs as compared with OLCs labeled with the neuronal marker Neu-N (βIII+ OLCs > Neu-N+ OLCs), suggesting that subpopulations of OLCs may not be necessarily neuronal. It is therefore possible that a fraction of βIII+ cells in the human subventricular zone (or possibly elsewhere) may represent OPCs expressing this neuron-associated cytoskeletal protein in a developmentally defined or restricted fashion.

βIII + Unipolar or Bipolar Tumor Phenotypes in Oligodendrogliomas

In accord with the classical demonstration of tumor cells with silver carbonate-stained polar cytoplasmic expansions (82, 83), the localization of βIII-tubulin unravels hitherto understated morphologic features in subpopulations of tumor phenotypes in oligodendrogliomas: In

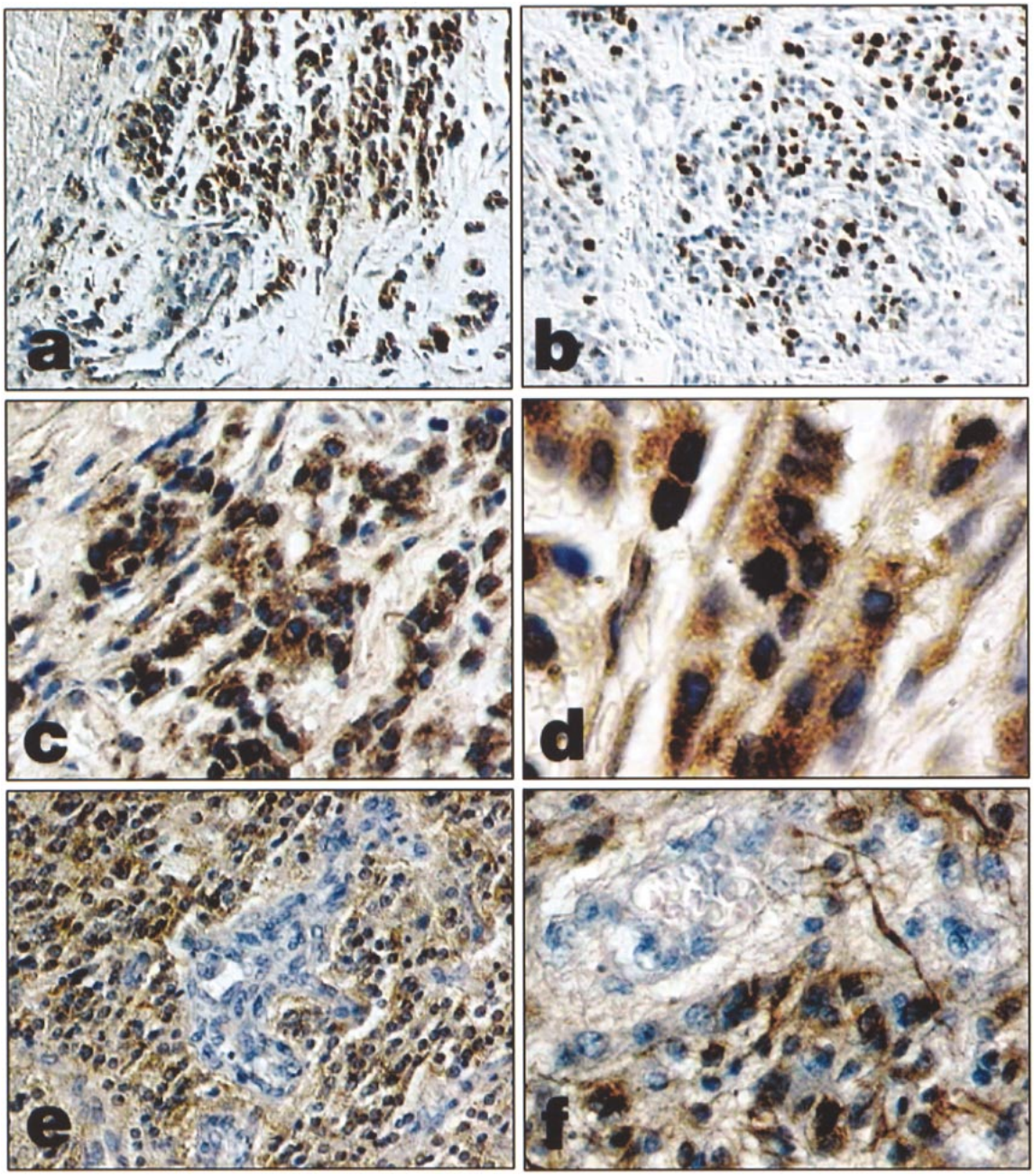

Fig. 5. Panel depicting the cellular distribution of βIII in anaplastic oligodendrogliomas (WHO grade III). a, b: Homologous fields from immediately adjacent sections depicting sheets of anaplastic tumor cells (arranged in lobules) with increased βIII (a) and Ki-67 (b) staining. c–f: Widespread βIII IR in densely packed anaplastic tumor cells. A mitotic cell is present in the center of the photomicrograph (*d*). e, f: The large caliber, hypertrophic, tumor-associated blood vessels are βIII− in contrast to the surrounding sheets of βIII+ tumor cells. Note that certain perivascular βIII+ tumor cells retain a polar morphology (the 2 intensely IR fibers in the right upper corner of the photomicrograph are pre-existing axons). ABC peroxidase with hematoxylin counterstain.

addition to the variable βIII IR in the typical round cells retaining a simple (apolar) morphology, βIII staining delineates both unipolar and asymmetrically bipolar tumor phenotypes bearing growth cones, or 1 or 2 short leading cellular processes. These neoplastic cells are reminiscent of the migrating OPCs in the developing rat CNS or the embryonic chick CNS (84–86). The visualization of simple unipolar or bipolar oligodendroglial cell types is highlighted in the developing rat optic nerve by quisqualate-stimulated cobalt uptake (84). Also, the demarcation of the cytoplasmic contour of tumor cells by βIII IR supports the view that the halo surrounding tumor

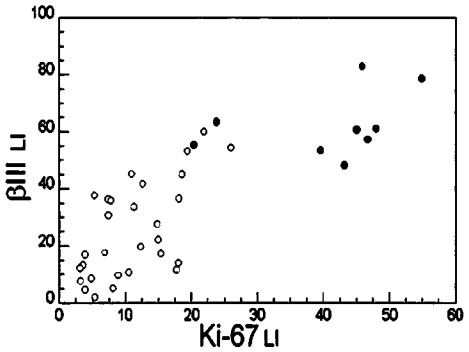

Fig. 6. Scatter plot graph comparing the labeling indices (LIs) for Ki-67 and class III β-tubulin (βIII) in a series of 41 low-grade or classical oligodendrogliomas (WHO grade II) represented by open circles, and high-grade or anaplastic oligodendrogliomas (WHO grade III) depicted by closed circles. Linear regression analysis reveals the existence of a highly significant relationship between βIII and Ki-67 according to an ascending scale of malignancy ($p < 0.0001$).

cells with round nuclei in histological preparations does not represent an artifactually swollen, clear cytoplasm, but rather a vacuolar tissue retraction.

Class III β-Tubulin in Relation to Other Neuronal Markers in Oligodendrogliomas

The detection of neuronal antigens in subpopulations of tumor cells in certain oligodendrogliomas, together with the demonstration of neuron-like physiological properties at the cellular level, have previously raised the conjecture of a neuronal nature for these tumors (46, 87–90). To a large extent, this reflects the widely held belief that the presence of a neuronal protein in a neoplastic cell denotes neuronal differentiation. Among the most extensively studied neuronal proteins in oligodendrogliomas are synaptophysin (46, 88, 90) and neurofilament protein (89, 90). The IR profiles of these neuronal proteins in oligodendrogliomas differ considerably amongst published data, whereas no relationship to tumor grade or biologic behavior have been reported in tumors expressing synaptophysin or neurofilament protein (46,88–90). Interestingly, synaptophysin and other SNARE complex proteins have been detected in non-transformed oligodendroglial cells in vitro (91).

The question of ''divergent'' neuronal differentiation in oligodendrogliomas should be viewed circumspectly in light of the aberrant expression of neuron-associated proteins, including βIII, in astrocytic gliomas (42). The present study demonstrates that in contrast to βIII, there is a paucity of synaptophysin staining in oligodendrogliomas. Furthermore, unlike synaptophysin and neurofilament protein (46, 89, 90), a statistically significant relationship exists between βIII expression, histological grade, and Ki-67 LIs in these neoplasms. The dissociation between βIII and synaptophysin expression in oligodendrogliomas is contrary to the differentiation-dependent co-expression of these 2 proteins in neuronal/neuroblastic tumors (32, 34, 35, 42). In this regard, synaptophysin IR distinguishes the extraventricular neuronal neoplasms with neurocytoma features from the oligodendrogliomas with which they are frequently confounded on morphological grounds (92). Taken together, the above observations have potential implications for the diagnosis of putative mixed glioneuronal tumors, insofar as the detection of βIII in tumor cells is not tantamount to neuronal differentiation in the absence of synaptophysin staining (42).

Collectively, our findings underscore the fact that tumor phenotypes do not always follow developmental principles and that their interpretation should be based on a critical assessment of tumor morphology in conjunction with other well-characterized cellular and molecular markers. As such, βIII staining should not be construed by itself as *a priori* evidence of ''divergent'' neuronal differentiation in neuroepithelial tumors that are otherwise phenotypically glial. Because βIII is expressed in neoplastic, but not in normal oligodendrocytes, the elucidation of mechanisms responsible for the altered expression of this isotype may provide important insights into the evolution and tumor progression in oligodendrogliomas. Additionally, given its widespread distribution in tumor cells, βIII emerges as a potential molecular target for novel therapeutics aiming at isotype-specific tubulin binding.

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