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Tubulin Targets in the Pathobiology and Therapy of Glioblastoma Multiforme. I. Class III β-Tubulin

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Glioblastoma multiforme (GBM) is the most common and deadliest form of primary brain cancer in adults. Despite advances in molecular biology and genetics of gliomas currently there is no effective treatment or promising molecularly targeted experimental therapeutic strategies for these tumors. In previous studies we have shown aberrant overexpression of the class III β-tubulin isotype (βIII-tubulin) in GBM and have proposed that this change may reflect perturbations in microtubule dynamics associated with glioma tumorigenesis, tumor progression and malignant transformation into GBM. This minireview focuses on microtubules and tubulin as emerging targets in potential therapy of GBM using a new class of βIII-tubulin-targeted drugs in the light of recent developments concerning the function and potential role of this isotype in clinically aggressive tumor behavior, cancer stem cells, tumor hypoxia and chemoresistance to tubulin binding agents, principally taxanes.

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Gliomas are the most common primary brain tumors of all ages and constitute a genetically and phenotypically heterogeneous group of primary brain tumors arising from glia or their precursors in the central nervous system (CNS). Most gliomas are of astrocytic origin (astrocytomas) although oligodendrogliomas and mixed glial tumors are not uncommon. The clinical behavior of gliomas in general and astrocytomas in particular, is reflected in a four-tier histological grade system (grades I–IV) according to an ascending scale of malignancy. Glioblastoma multiforme (GBM) (grade IV) is the most prevalent and deadliest form of glioma and brain cancer. Two forms of GBM are recognized, primary or de novo and secondary, which follows an evolutionary progression from grade II through grade IV lesions (von Deimling et al., 1993; Watanabe et al., 1996). Despite a plethora of scientific publications reflecting advances in the molecular biology and genetics of brain tumors during that past decade, no significant strides have been made in the treatment of GBM. Currently, GBM is not responsive to conventional surgical, radiotherapeutic and/or chemotherapeutic interventions and as such, it carries a gloomy prognosis.

The authors of this review advocate a novel approach to GBM therapy, which focuses on microtubule dynamics and tubulin targets. During the past decade, we have demonstrated that GBMs exhibit significant changes in their microtubule cytoskeleton, including aberrant expression of the class III β-tubulin isotype (βIII-tubulin) and γ-tubulin, which are associated with the emergence of highly malignant (anaplastic) tumor phenotypes (Katsetos et al., 2001, 2002, 2003a,b, 2006, 2007).

This review is devoted to aberrant expression of β-tubulin isotypes in cancer with special emphasis on βIII-tubulin in GBM, one of the most aggressive and chemorefractory forms of brain cancer. Based on evidence derived from large clinical studies on a host of epithelial solid tumors exhibiting similar patterns of aberrant βIII-tubulin expression, we propose that this isotype may be exploited as a major target focus for GBM treatment using new microtubule acting compounds.

Abbreviations: βIII-tubulin, class III β-tubulin; GFAP, glial fibrillary acidic protein, GBM, glioblastoma multiforme; MTOCs, microtubule-organizing centers; NSCLC, non-small-cell lung cancer; TBA, tubulin binding agent(s).

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Microtubules and Tubulins

Microtubules are highly dynamic cytoskeletal components that are essential for many cellular functions in eukaryotes such as intracellular organization, ordered vesicle transport and cell division. The basic building blocks of microtubules are heterodimers of globular α- and β-tubulin subunits, each of which consists of multiple isotypes differing in amino acid sequence and encoded by different genes (Ludueña, 1998). Tubulins are arranged in a head-to-tail fashion to form 13 protofilaments that constitute cylindrical microtubules with outer diameter around 25 nm. Microtubules are thus, inherently polar with α-tubulin at one end of the polymer, the (−) end, and β-tubulin at the (+) end.

Microtubule ends also differ in their assembly and disassembly characteristics. Tubulin subunits are added to the fast growing (+) ends of microtubules and are preferentially lost from the (−) ends. Microtubules coexist in growing and shrinking populations and this dynamic instability is a general property of microtubules (Mitchison and Kirschner, 1984). Microtubules can self-assemble in vitro from high concentrations of tubulin heterodimers in the presence of GTP, but such microtubules are randomly organized. Yet, microtubules in cells are precisely organized. This is because microtubules are anchored in microtubule-organizing centers (MTOCs) and these organelles assist in microtubule nucleation (Doxsey, 2001). Associated with MTOCs is γ-tubulin, another member from tubulin superfamily that is essential for nucleation of microtubules (Katsetos et al., see Part II).

β-Tubulin Isotypes

α- and β-Tubulin subunits are encoded by multiple genes. Mammalian β-tubulin exists as seven isotypes, termed βI, βII, βIII, βIVa, βIVb, βV, and βVI, each a separate gene product synthesized without alternative splicing (Ludueña, 1998; Ludueña and Banerjee, 2008a). The β-isotypes differ primarily within the C-terminal 15–20 amino acids, a region of the protein that lies on the exterior of the microtubule and is the putative binding sites for microtubule-associated proteins (MAPs) (Downing and Nogales, 1998). In vertebrates, some β-tubulin isotypes are ubiquitously expressed whereas others are mainly expressed in certain cells and tissues while being tightly regulated in others. The expression and cellular distribution of βI is essentially ubiquitous. The distribution of βIV is also widespread among tissues and cell types but is especially prominent in axonemes (cilia and flagella) (Jensen-Smith et al., 2003). βII is particularly abundant in brain, peripheral nerves and muscles but is also expressed to a lesser degree in other tissues. The class II β-tubulin isotype has also been described in tumor cell nuclei in various epithelial cancer types (Yeh and Ludueña, 2004). βIII occurs largely in neurons and testis and in low amounts in a very small number of tissues. βVI is restricted to hematopoietic tissues and the distribution of βV is still largely unknown. Antibodies directed against isotype-defining sequences have been generated (Lee et al., 1990a,b; Dráberová et al., 1998; Katsetos et al., 2000; Ludueña and Banerjee, 2008a). Individual tubulin isotypes contribute to differences in microtubule dynamics and binding of anti-mitotic drugs (Jordan and Kamath, 2007).

High-resolution isoelectric focusing, that separate polypeptides according to their net charge, have revealed that β-tubulin from brain can be resolved into 13 spots, far more than is expected from the number of isotypes that are expressed (Linhartová et al., 1992). This reflects extensive posttranslational modification of β-tubulins. Phosphorylation (Alexander et al., 1991), polyglutamylation (Alexander et al., 1991), polyglycylation (Mary et al., 1994), and glycosylation (Cicchillitti et al., 2008) have been identified in β-tubulins.

Compared to other β-tubulin isotypes, βIII-tubulin possesses certain distinctive properties, which may account for its unique function(s) (Ludueña and Banerjee, 2008a,b). In this regard, unlike the βI, βII, and βIV isotypes, βIII-tubulin lacks the widely conserved and oxidation-sensitive residue cys239, which in this case is replaced by ser239 (Joe et al., 2008; Ludueña and Banerjee, 2008a,b). It has been hypothesized that the absence of cys239 may allow αβIII-tubulin dimers to assemble in the presence of free radicals (Ludueña and Banerjee, 2008a). In addition, the βIII isotype contains an uncommon cys124 residue, in contrast to the other β-tubulin isotypes, which share a ser/ala124 residue (Joe et al., 2008). The remarkable phylogenetic conservation of βIII-tubulin across vertebrate species denotes that cys124 and ser239 may have functional roles (Joe et al., 2008). Unlike the βII- and βIV-tubulin isotypes βIII-tubulin is phosphorylated at a serine in the C-terminus (Khan and Ludueña, 1996; Ludueña and Banerjee, 2008b). Also, unlike in other β-tubulin isotypes, in the βIII isotype, the presence of threonine residue Thr(429) strongly favors microtubule assembly (Joe et al., 2009). And finally, notwithstanding its restricted and highly selective (predominantly neuronal) cell type distribution in normal organs and tissues, the βIII isotype is widely, albeit differentially, expressed in a broad range of human tumors of neuronal and non-neuronal origin (Katsetos et al., 2003a,b).

Expression of βIII-Tubulin in Normal Tissues

The differential expression and cellular distribution of βIII-tubulin has been extensively studied in the context of human fetal development (Katsetos et al., 2003a,b). In normal developing and mature organs and tissues, βIII-tubulin is highly expressed in both the central and peripheral nervous systems (CNS and PNS). As exemplified in cerebellar, retinal and sympathoadrenal neurogenesis in humans the distribution of βIII-tubulin is, for the most part, neuron-associated, conforming to distinct temporospatial gradients traceable to regional neuroepithelia of origin (Katsetos et al., 1991, 1993, 1998). However, recent studies have shown that βIII-tubulin expression is *not* neuron-specific in an ontological context as transient expression of this protein is also present in glial fibrillary acidic protein (GFAP)- and nestin-expressing fetal astrocytes in vitro (Fig. 1) and in germinal matrix cells of the subventricular zones of the mid-gestational human telencephalon, which comprise putative restricted glial precursor cells or bipotential neural stem cells (Dráberová et al., 2008). This is in keeping with previous reports indicating that βIII-tubulin is expressed, albeit transiently, in Kulchitsky neuroendocrine cells of the fetal respiratory epithelium during the canalicular stage of human pulmonary development (Katsetos et al., 2000). This temporally restricted, potentially non-neuronal expression of βIII-tubulin in developing human fetal cells and tissues may have important theoretical and practical implications in the elucidation of cellular phenotypes, particularly in the identification of presumptive neurons derived from stem cells (Dráberová et al., 2008). In adult tissues, the distribution of βIII-tubulin is predominantly, but not exclusively, neuronal (Dráberová et al., 1998, 2008; Katsetos et al., 2003a,b) for its unequivocal expression has been reported in human melanocytes, which are cells of neuroectodermal origin (Akasaka et al., 2009), spermatozoa (Pěknicová et al., 2001), and follicular lymphoid cells (Lee et al., 2005).

Differential Expression of βIII-Tubulin in Neuronal Versus Non-Neuronal Tumors

Whereas all known classes of β-tubulin isotypes are expressed in human cancers, the βIII isotype exhibits distinctive patterns

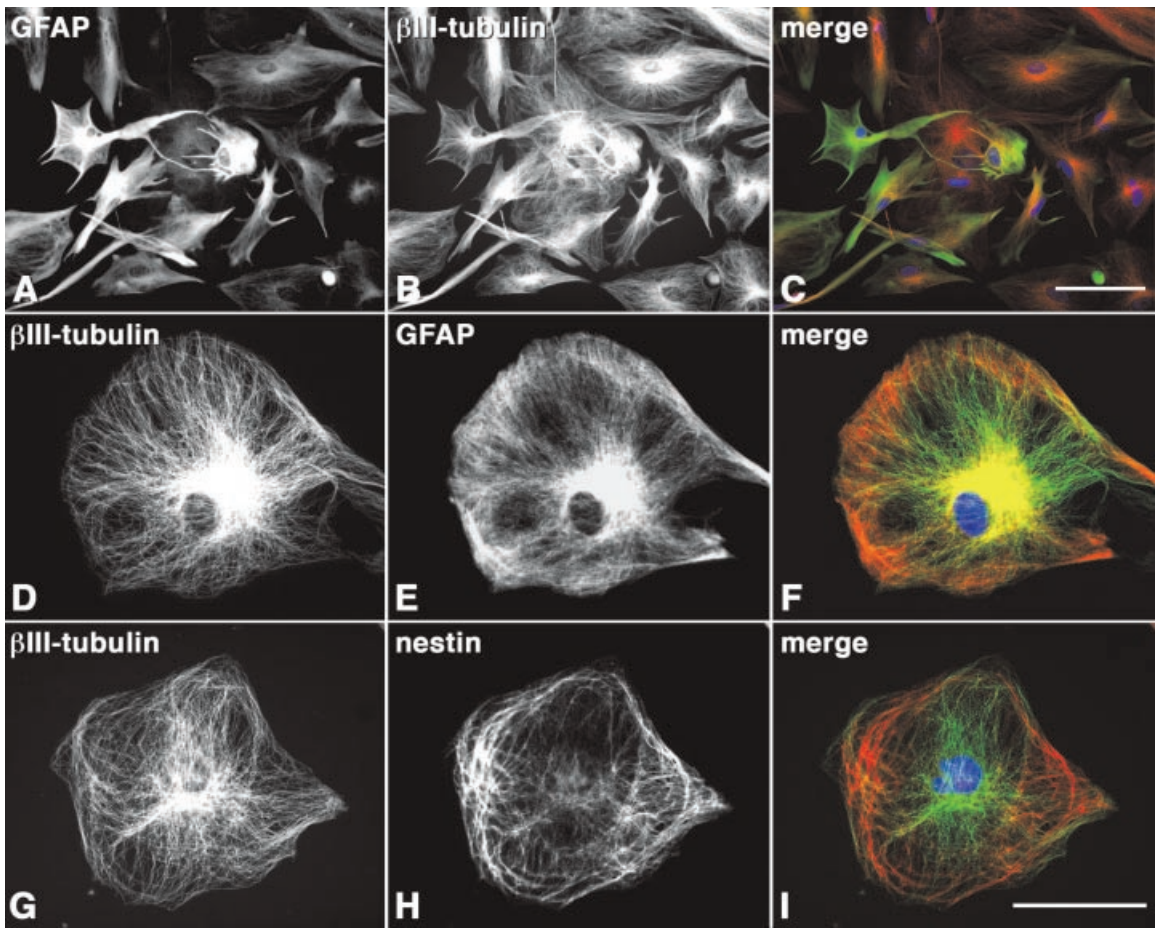

Fig. 1. Distribution of βIII-tubulin, glial fibrillary acidic protein (GFAP) and nestin in primary cultures of human fetal astrocytes maintained as monolayers for 7 days in vitro. While GFAP is an intermediate filament protein, which in the central nervous system is specific for cells of the glial lineage, nestin is a stem cell-associated intermediate filament protein. A–C: Double-labeled cells for GFAP (A, green) and βIII-tubulin (B, red). Superposition is shown in part C. D–F: Double-labeled cell for βIII-tubulin (D; green) and GFAP (E; red) at a higher magnification. Superposition is shown in part F. Note overlapping of βIII-tubulin and GFAP staining in paranuclear cytoplasmic region wherefrom microtubules and glial intermediate filaments emanate (F, yellow). G–I: Double-labeled cell for βIII-tubulin (G; green) and nestin (H; red). Superposition is shown in part I. DAPI (blue) labels nuclei in parts C,F,I. Scale bars 100 μm (A–C) and 50 μm. Reproduced with permission from the Journal of Neuropathology and Experimental Neurology (Dráberová et al., 2008), Copyright © 2008 American Association of Neuropathologists, Inc.

of expression and distribution, which, in part, reflect the narrow distribution of this protein in non-transformed cells and tissues (Dráberová et al., 1998; Katsetos et al., 2003a,b). Two divergent patterns of expression are recognized in neuronal versus non-neuronal neoplasms. In the former, such as cerebellar medulloblastomas, retinoblastomas, central and peripheral (sympathoadrenal) neuroblastomas and pheochromocytomas, the expression of βIII-tubulin in tumor cells is constitutive, associated with morphological changes of neuronal differentiation, such as elaboration of neuritc cell processes (neuritogenesis) and decreased cell proliferation (Katsetos et al., 2003a,b,c). In contrast, the expression of βIII-tubulin in non-neuronal tumors, such as common epithelial tumors of the lung, including small cell- and non-small cell lung carcinomas (NSCLC) (Katsetos et al., 2000; Dumontet et al., 2005; Sève et al., 2005a,b, 2007a), in adenocarcinomas of the ovary (Ferrandina et al., 2006; Ohishi et al., 2007), breast (Bernard-Marty et al., 2002; Hasegawa et al., 2003; Paradiso et al., 2005; Tommasi et al., 2007), stomach (Urano et al., 2006) and prostate, as well as in gliomas of the CNS (Katsetos et al., 2001, 2002, 2003b, 2007), is associated with a trend towards an higher histological grade of malignancy and poor prognosis.

Thus, βIII-tubulin expression in neuronal tumors is constitutive and differentiation-dependent, while in non-neuronal tumors it is either aberrant and/or represents "dedifferentiation" associated with anaplastic transformation and acquisition of progenitor- or stem cell-like phenotypic properties (Katsetos et al., 2003a,b; Dráberová et al., 2008). Interestingly, a recent study has shown that dedifferentiation and tumor progression in malignant melanomas, which are tumors of neuroectodermal origin basally expressing class III β-tubulin, is associated with loss of this protein and acquisition of a paclitaxel-resistant phenotype (Akasaka et al., 2009).

βIII-Tubulin Overexpression and Clinical outcomes in Lung and Gynecological Cancers

To date, systematic analysis of βIII-tubulin expression in large clinical trial studies has been conducted in the context of NSCLC and in gynecological cancers. The prevailing view is that the relative expression of βIII-tubulin correlates with clinical outcomes in several common epithelial tumor types, including NSLC and adenocarcinomas of the breast and ovary (Dumontet et al., 2009). Moreover, in NSCLC and ovarian

adenocarcinomas, βIII-tubulin expression has been associated with chemoresistance to taxanes and is an independent predictor of survival (Mozzetti et al., 2005; Sève et al., 2005a; Ferrandina et al., 2006; Sève and Dumontet, 2008). High expression of βIII-tubulin has been found to be associated either with low response rates in patients treated with regimens containing taxanes or vinorelbine or with reduced survival in patients with NSCLC, in mammary, ovarian and gastric adenocarcinomas, as well as in metastatic carcinomas of unknown primary origin (Dumontet et al., 2005; Sève et al., 2005a,b, 2007a,b, 2008; Galmarini et al., 2008; Sève and Dumontet, 2008). Patients with advanced NSCLC receiving paclitaxel whose tumors expressed high levels of βIII-tubulin had a lower response to paclitaxel and shorter survival, whereas this variable was not found to be predictive in patients receiving regimens without TBAs (Sève et al., 2005a). Loss of βIII-tubulin protein and ERCC1 expression were predictors of better survival in patients who underwent platinum-based plus taxane chemotherapy for completely resected NSCLC (Okuda et al., 2008). However, another clinical trial comparing adjuvant chemotherapy to no further therapy in patients with operable NSCLC, demonstrated that greatest benefit from cisplatin/vinorelbine was encountered in patients with increased expression of βIII-tubulin (Sève et al., 2007a). Similarly, a relationship between βIII-tubulin expression and prognosis was not observed in carcinomas of the uterine cervix (Ferrandina et al., 2007) whereas in clear cell carcinomas of the ovary, taxane-based chemotherapy was significantly more effective in patients whose tumors were positive for βIII-tubulin (Aoki et al., 2009). Collectively taken, these results suggest that although βIII-tubulin can serve as both a prognostic and a predictive factor in certain tumor types and clinical-therapeutic settings, its significance as a tumor biomarker is neither universal nor linear and should be cautiously considered within a cellular and clinico-pathological context.

βIII-Tubulin in Brain Tumors: Clinical Considerations and Caveats

In the CNS, the βIII-tubulin isotype is differentially expressed in *neuronal/neuroblastic* versus *glial* tumors. In embryonal neuronal/neuroblastic tumors, such as cerebellar medulloblastomas, cerebral neuroblastomas/supratentorial primitive neuroectodermal tumors, and in the more differentiated central neurocytomas, βIII-tubulin is expressed in tumor cells exhibiting morphological features of neuronal differentiation such as neurite formation and ganglionic maturation (Katsetos et al., 2003a,b,c). Since βIII-tubulin is not normally expressed in nascent, mature glia, its expression in glial tumors (gliomas) is viewed as both ectopic and aberrant (Figs. 2 and 3). It is variably present in all glioma types and grades but when significantly increased, it tends to be associated with a higher histologic tumor grade (Katsetos et al., 2001, 2002, 2003a,b; Mao et al., 2007). The latter is exemplified in GBM, the most common and deadliest form of primary brain cancer in adults (Katsetos et al., 2001, 2007).

That said, the lack of βIII-tubulin specificity within the context of brain tumors is potentially problematic in the miscellaneous group of CNS neuroepithelial tumors with ambiguous or mixed/glio-neuronal differentiation. This includes the subependymal giant cell astrocytomas (associated with tuberous sclerosis) (grade I), dysembryoblastic neuroepithelial tumors (grade I), gangliogliomas (grades I/II) and pleomorphic xanthoastrocytomas (grade II) in which the detection of βIII-tubulin by immunohistochemical staining can neither distinguish a neuronal from a glial tumor phenotype nor can it predict biological malignancy (Katsetos et al., 2003b; Martinez-Diaz et al., 2003). This points to the caveat that βIII-tubulin immunostaining of brain tumors must be interpreted critically in the appropriate histopathologic context (Katsetos et al., 2001, 2002, 2003b; Laggner et al., 2007).

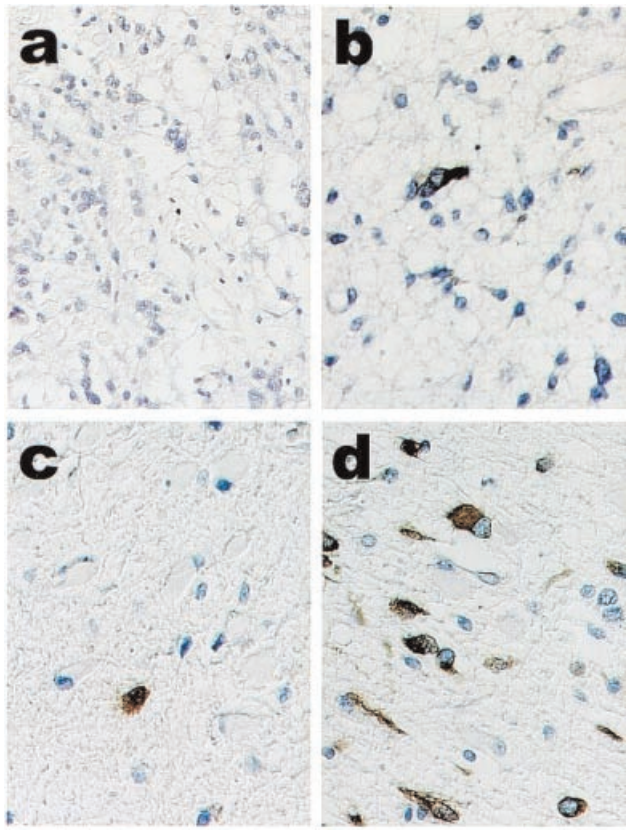

Fig. 2. Cellular distribution of βIII-tubulin in examples of pilocytic astrocytoma (grade I) (a) and diffuse astrocytoma (grade II) (b–d). Part a depicts that βIII-tubulin staining is absent in a low-grade pilocytic astrocytoma of the cerebellum. Parts b–d: feature gradations of βIII-tubulin immunoreactivity in the so-called 'low grade' diffuse astrocytomas. Highly variable and heterogeneous βIII-tubulin staining ranges from rare focal (b,c) to moderate (d). The case depicted in (b) has a labeling index (LI) of 4%, whilst parts c and d are from a different case with a LI of 5%. LI was defined as the percentage of βIII-tubulin (+) cells out of the total number of tumor cells counted in 20 non-overlapping high-power fields (field magnification, 40×). The two adjacent photomicrographs are from different areas of the same tumor, highlighting the intratumoral staining heterogeneity. The bulk of the tumor is βIII-tubulin (−) (c). However, focal areas show clusters of βIII-tubulin (+) fibrillated and gemistocytic astrocytes (d). Note that the βIII-tubulin (+) cells are morphologically indistinguishable from the nearby βIII-tubulin (−) cells. Avidin biotin complex peroxidase with hematoxylin counterstain. Original magnifications 200× (a,b) and 1,000× (c,d). Reprinted with permission from Archives of Pathology & Laboratory Medicine (Katsetos et al., 2001), Copyright © 2001 College of American Pathologists.

βIII-Tubulin Is Expressed According to an Ascending Grade of Malignancy in Diffuse Gliomas

In glial tumors of either astrocytic or oligodendrocytic types (astrocytomas and oligodendrogliomas), increased expression of βIII-tubulin is associated with a correspondingly higher scale of malignancy as determined by histopathology and cell proliferation indices (Katsetos et al., 2001, 2002). As compared to low-grade astrocytic tumors (grades I and II) (Fig. 2) the cellular distribution of βIII-tubulin is significantly increased in high-grade (highly malignant) tumors, notably the anaplastic astrocytomas (grade III) and GBM (grade IV) (Fig. 3) (Katsetos

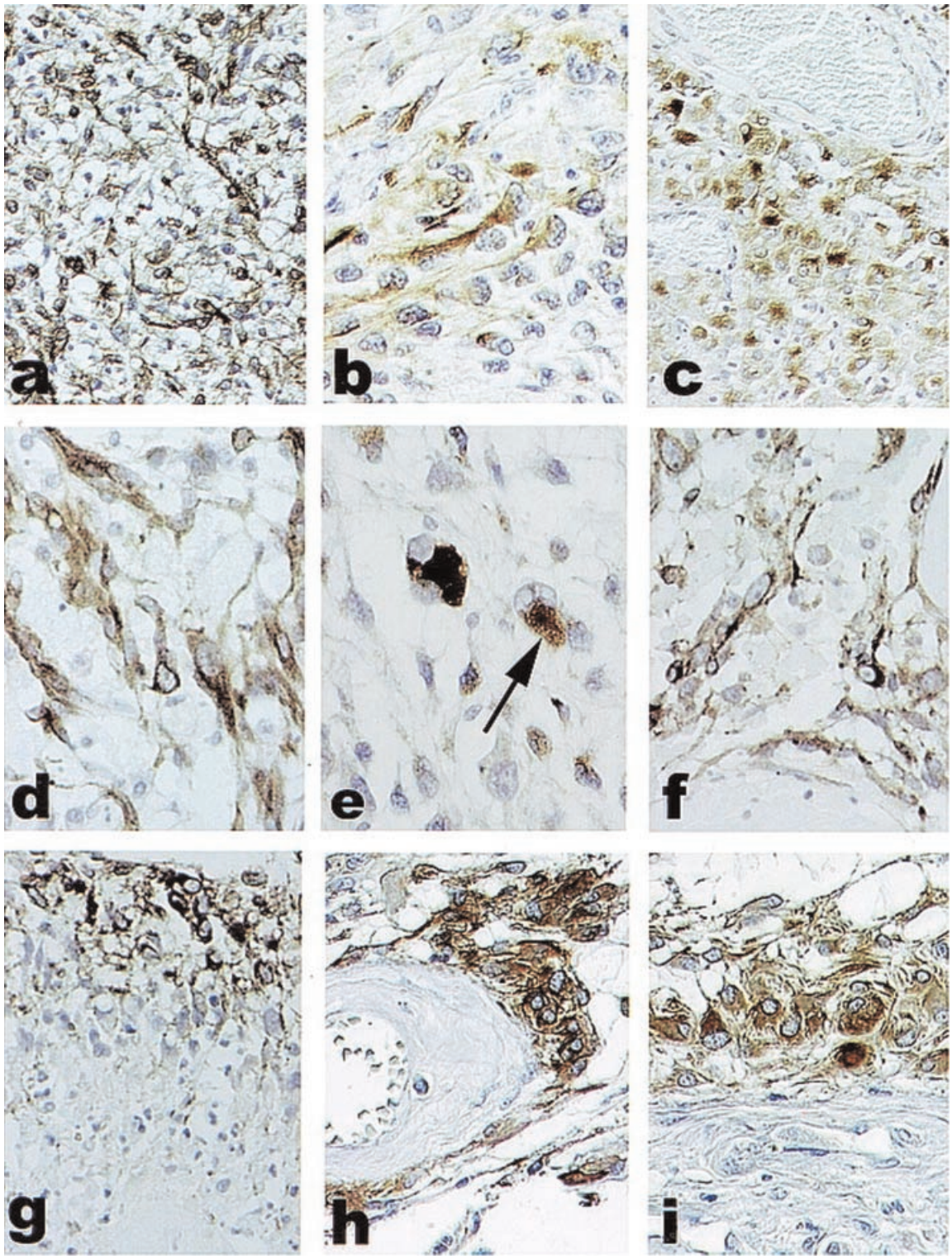

Fig. 3. Cellular distribution of βIII-tubulin in examples of high-grade astrocytomas: anaplastic astrocytoma (grade III) (parts a,b) and glioblastoma multiforme (grade IV) (parts c–i). There is widespread βIII-tubulin immunoreactivity in tumor cells showing a variety of morphologic appearances. Parts a–f: Feature overt astroglial phenotypes with diffuse cytoplasmic localization distributed both in the perikaryal cytoplasm and in fibrillated glial cell processes. In part e the arrow depicts granular βIII-tubulin localization in the cytoplasm of a tumor cell whilst part f features large "ganglioid" βIII-tubulin (+) astroglial phenotypes with prominent nucleoli and thick fibrillated processes. Parts c,h,i: Sheets or clusters of perivascular βIII-tubulin (+) tumor cells. Note that tumor blood vessels in areas of angiogenesis are βIII-tubulin (−). Part g depicts an aggregate of small anaplastic βIII-tubulin (+) cells resembling "primitive" glioblasts in the vicinity bordering (ischemic) tumor necrosis ("palisading necrosis"). Avidin biotin complex peroxidase with hematoxylin counterstain. Original magnifications 200× (a,c,f,g), 400× (b,d), and 1,000× (e,h,i). Reprinted with permission from Archives of Pathology & Laboratory Medicine (Katsetos et al., 2001), Copyright © 2001 College of American Pathologists.

et al., 2001). Specifically, within the context of diffuse astrocytic gliomas (grades II–IV) a statistically significant difference in βIII-tubulin labeling indices has been demonstrated between "low-grade" (grade II) and "high-grade" (grades III and IV) tumors but not between grade III and IV tumors (Katsetos et al., 2001, 2007). Moreover, a highly significant, histological grade-dependent, relationship exists between βIII-tubulin and Ki-67 antigen (marker of cell proliferation) labeling indices (Katsetos et al., 2001).

In an immunohistochemical study on 378 brain tumors using 37 antibodies and tissue microarray (TMA) technology, βIII-tubulin was one of six marker proteins to show significant differences between high-grade and low-grade gliomas (Ikota et al., 2006).

The overall behavior of diffuse astrocytomas may be viewed as a biologic continuum arising as low-grade, well-differentiated tumors (grade II) and evolving into high-grade malignancies (GBMs) (grade IV) through successive, but hitherto molecularly undefinable steps (Katsetos et al., 2003a,b). In this regard, certain tumors in the grade II group have a tendency to undergo a more rapid malignant (anaplastic) transformation, whereas others remain indolent for many years. We postulate that βIII-tubulin expressing cells in these tumors may represent subclones of more aggressive cells portending malignant change.

βIII-Tubulin is overexpressed in GBMs (Katsetos et al., 2001, 2007; Martinez-Diaz et al., 2003) and in glioblastoma cell lines (Lopes et al., 1992; Katsetos et al., 2007). In the T98G human glioblastoma line, βIII-tubulin is co-expressed but differentially compartmentalized with γ-tubulin. In GBM cells these two proteins form complexes denoting a possible functional interaction (Katsetos et al., 2007, Part II).

The histological architecture of GBMs lends certain clues as to the possible morphologic correlates of increased βIII-tubulin expression. Two such features are significant in this regard; (a) poorly differentiated, anaplastic cells reminiscent of glial precursor cells or bipotential neural stem cells and (b) tumor ischemic necrosis.

βIII-Tubulin Expression and Cancer Stem Cells in GBM?

The role of cancer stem cells (CSC) has been previously addressed in GBMs (Visted et al., 2003; Louis, 2006; Gilbertson and Rich, 2007; Altaner, 2008; Das et al., 2008; Eyler and Rich, 2008; Johannessen et al., 2008; Walton et al., 2009). There is an increasing body of evidence indicating that astrocytic gliomas may be derived from intermediate gliotypic neural stem cells (Walton et al., 2009). Undifferentiated cancer cells derived from GBM and glial fibrillary acidic protein (GFAP)+ normal neural progenitors maintained in culture share a "multilineage" antigenic phenotype (CD44+/microtubule associated protein-2+/GFAP+/vimentin+/βIII-tubulin+/fibronectin+) (Rieske et al., 2009). It should be noted that, GFAP+/nestin+/βIII-tubulin+ cells in GBMs resemble normal human fetal astrocytes in vitro (Rieske et al., 2007; Dráberová et al., 2008) and of glial precursor or neural stem cells of the human telencephalic subventricular zones (Dráberová et al., 2008) (Fig. 1). Thus, the expression of βIII-tubulin in GBM may identify tumor cells with cancer stem cell properties, which is in keeping with the tumorigenic characteristics of progenitor cells typified by a "multilineage antigenic phenotype" and continued proliferation in the absence of a complex cellular regulatory environment (Walton et al., 2009).

Relationship of βIII-Tubulin Overexpression to Hypoxia and Angiogenesis in Glioblastomas

Progression-associated genetic alterations in malignant gliomas are common among different tumor types, targeting growth promoting and cell cycle control pathways and resulting in focal hypoxia, necrosis, and angiogenesis (Louis, 2006). Solid cancers in general and GBMs in particular comprise hypoxic cells that are resistant to radiotherapy and chemotherapy. Two histopathological hallmarks of GBMs are the presence of tumor necrosis ("palisading necrosis") and complex vascular abnormalities. The former is characterized by areas of ischemic necrosis owing to vascular pathology (vaso-occlusive phenomena/thrombosis), hypoxia and profuse angiogenesis (microvascular proliferation) and is, in part, hypoxia-inducible factor (HIF-1)-mediated (Fischer et al., 2005; Rong et al., 2006).

The increased expression of βIII-tubulin in tumor cells in GBMs bordering geographic areas of ischemic necrosis ("palisading necrosis") (Fig. 3g) points to a possible relationship between βIII-tubulin expression and tumor hypoxia and oxidative stress. Interestingly, tumor blood vessels in areas of neo-angiogenesis in GBM do not express βIII-tubulin (Fig. 3c,h,i) (Katsetos et al., 2001).

There is evidence of significant protein nitration in human gliomas, especially in GBMs. Nitric oxide upregulation in malignant gliomas may relate to neoplastic transformation, tumor neovascularization, induction of apoptosis, or free radical damage (Lam-Himlin et al., 2006). Proteomic analysis has identified α-tubulin as a target of protein nitration and peptide mass fingerprinting has shown that tubulin is nitrated at αTyr224 in surgically resected GBM specimens but is unmodified in low-grade (grade I) tumor samples or in non-neoplastic brain tissue (Fiore et al., 2006).

Paclitaxel resistance in GBM has been linked to the expression of members of the anti-apoptotic Bcl-2 family through hypoxia induced phosphorylation of Bad, thus protecting hypoxic cells from paclitaxel-induced apoptosis (Merighi et al., 2007). Given the well-known factors of tumor angiogenesis and hypoxia in GBMs, we hypothesize that the increased expression of βIII-tubulin in these tumors, particularly around areas of palisading necrosis (Fig. 3g), may be, in part, hypoxia-induced or be an adaptive response to conditions of increased oxidative stress and free radical production.

Ludueña and Banerjee (2008a) have observed that the few normal tissues that express βIII-tubulin also are rich in free radicals and reactive oxygen species and have formulated the hypothesis that one of the functions of βIII-tubulin is to protect microtubules from the deleterious effects of free radicals. Luduena's hypothesis that lack of cys239 may allow αβIII to assemble in the presence of free radicals may account, in part, for the presence of βIII in tumors, which are very rich in free radicals. It has been shown that silencing βIII-tubulin expression in cancer cells increases the cell's susceptibility not only to compounds that target tubulin but also to antioxidants. Along these lines, it has been suggested that βIII-tubulin may serve as a survival factor rescuing tumor cells from cell death signals triggered by diverse classes of DNA-targeting chemotherapeutic agents, such as cisplatin, doxorubicin, and etoposide (Gan et al., 2007). Moreover, it has recently been reported that in the platinum- and paclitaxel-sensitive human ovarian cancer cell line A2780, hypoxia is a strong inducer of βIII-tubulin expression, which is mediated by HIF-1a through methylation of the 3' enhancer of the βIII isotype (Raspaglio et al., 2008). βIII-Tubulin is also involved in adaptation to oxidative stress and glucose deprivation, suggesting that the βIII isotype may constitute a survival factor capable of directly contributing to drug resistance (Cicchillitti et al., 2008).

βIII-Tubulin in Cancer and Its Relationship to Resistance to Taxanes

Tubulin binding agents (TBAs), such as taxanes and epothilones, block mitosis and cell proliferation by targeting the dynamics of

the microtubule cytoskeleton (Dumontet et al., 2009). TBAs suppress microtubule dynamics by binding to the β-tubulin subunit of α/β-tubulin, inducing mitotic arrest and apoptosis. The taxanes are widely used compounds in the treatment of many cancer types. Paclitaxel binds to β-tubulin, causing microtubule polymerization that blocks mitosis by kinetic stabilization of spindle microtubules (Jordan et al., 1993). The clinical efficacy of these compounds is significantly confounded by primary or acquired chemoresistance (Dumontet et al., 2009). Mechanisms of taxol resistance encompass those that involve microtubule proteins, such as β-tubulin mutations, β-tubulin isotype selection and post-translational modifications, and those operating at the level of regulatory proteins (Orr et al., 2003). Three mechanisms have emerged in this regard, (a) overexpression of the MDR-1 gene (Horwitz et al., 1986), (b) point mutations of β-tubulin at the paclitaxel binding site (Giannakakou et al., 1997), and (c) selective overexpression of class I, III, and IVa β-tubulin isotypes (Kavallaris et al., 1997). Whereas, all three mechanisms are potentially important, the evidence in support of MDR-1 gene overexpression and β-tubulin point mutation in the clinical setting of solid cancers is either tenuous or controversial (Berrieman et al., 2004; Dumontet et al., 2009).

Ludueña was the first to demonstrate in functional studies that the presence of βIII-tubulin inhibits paclitaxel-induced β-tubulin polymerization (Lu and Ludueña, 1993) whilst Kavallaris was the first to discover that paclitaxel-resistant ovarian cancer cells overexpress class I, III, and IVa β-tubulin isotypes (Kavallaris et al., 1997). Subsequent studies have shown that βIII-tubulin overexpression in epithelial tumors represents a major mechanism of drug resistance to microtubule interacting agents such as taxanes and Vinca alkaloids (Kavallaris et al., 1999; Kamath et al., 2005; Mozzetti et al., 2005; Ferlini et al., 2007). Upregulation of βIII-tubulin has been implicated in clinical resistance in NSCLC, ovarian and breast tumors treated in combination with a tubulin-binding and DNA-damaging agents (Gan et al., 2007). In this regard, βIII-tubulin status may have important implications for improving the targeting and treatment of drug-refractory NSCLC (Gan et al., 2007) and other solid tumors. Collectively taken, data indicating overexpression of βIII-tubulin in advanced stage epithelial cancers in patients treated with taxane regimens, suggest that βIII-tubulin overexpression may constitute a common mechanism by which taxane resistance develops in patients with advanced solid tumors of epithelial origin (Mozzetti et al., 2005; Sève et al., 2005b; Dumontet et al., 2009).

Recent studies performed in ovarian cancer cell lines have revealed that βIII-tubulin exists in two posttranslationally modified forms, notably a higher molecular weight glycosylated and phosphorylated form, which is associated with the microtubule cytoskeleton, and a lower molecular weight form, which has a distinct mitochondrial compartmentalization (Cicchillitti et al., 2008). Moreover, the levels of βIII-tubulin phosphorylation and glycosylation were associated with the chemoresistant tumor phenotypes (Cicchillitti et al., 2008). The latter suggests that posttranslational modifications of βIII-tubulin may play an important role in tumor progression and chemoresistance. It has recently been suggested that the observed isotype difference in paclitaxel binding may be a kinetic effect arising from the isotype difference at residue serine 275 which in the βIII and βVI isotypes is replaced by alanine (Freedman et al., 2009). Because βIII- and βV-tubulin may be expressed in a complementary pattern at the protein level in ovarian cancer cells, the dual expression of these two β-tubulin isotypes should be systematically determined in the context of tumor responses to drugs targeting microtubules (Verdier-Pinard et al., 2005).

Exposure of the human glioblastoma cell line T98G to taxol led to the formation of βIII-tubulin labeled microtubule bundles while prominent micropunctate and diffuse γ-tubulin staining was unchanged (Katsetos et al., 2007). However, the effect of taxanes on βIII-tubulin enriched microtubules in glioma cells is—to our knowledge—unknown and warrants further investigation.

Dumontet et al. (2009) have recently suggested that the significant antitumor activity afforded by ixabepilone, a novel analogue of epothilone B, in taxane-resistant tumors, may be related to its preferential suppression of the dynamic instability of αβIII-microtubules in cells expressing high levels of βIII-tubulin. However, the use of ixabepilone to target CNS tumors may be confounded by the fact that the drug does not cross the blood–brain barrier (Lee et al., 2008). Even though there is disruption of the blood–brain barrier in GBM (Schneider et al., 2004; Ishihara et al., 2008), the former still remains an important issue concerning drug delivery in the therapy of brain tumors (Fortin et al., 2005). Consequently, instead of ixabepilone, yet another epothilone compound, patupilone, could be even more promising in this regard as it targets specifically βIII-tubulin (Mozzetti et al., 2008) while it is able to cross the blood–brain barrier since its activity is fully P-gp independent (O'Reilly et al., 2008). In addition to the epothilones, ixapepilone and patupilone, recent studies have shown that taccalonolides, a class of structurally and mechanistically distinct microtubule-stabilizing agents (Risinger et al., 2008) and tasidotin, an analogue of dolastatin 15 (Bai et al., 2009), have been shown to evade taxane resistance mechanisms. Whilst βIII-tubulin is probably not a unique determinant of chemoresistance, direct targeting of this protein may enhance the efficacy of TBAs by ultimately overcoming one of the key factors in the development of drug resistance (Ferlini et al., 2005, 2007; Magnani et al., 2006).

Future Directions

Given the overexpression of βIII-tubulin in GBMs, it is unclear whether expression of this protein enhances or diminishes chemotherapeutic efficacy in GBMs. This provides the rationale for the performance of pre-clinical studies focusing on the effect of novel chemotherapeutic compounds, including but not limited to epothilones and taccalonolides on GBM cells in vitro and on βIII-tubulin overexpressing human GBM xenografts. In addition, large clinical randomized studies are necessary to determine the prognostic or predictive value of βIII-tubulin in the context of different glioma types and grades as well as treatment settings similar to extracranial solid tumors.

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