

ORIGINAL ARTICLE

Altered Cellular Distribution and Subcellular Sorting of γ -Tubulin in Diffuse Astrocytic Gliomas and Human Glioblastoma Cell Lines

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Abstract

Centrosome amplification is a pivotal mechanism underlying tumorigenesis but its role in gliomas is underinvestigated. The present study specifically examines the expression and distribution of the centrosome-associated cytoskeletal protein γ -tubulin in 56 primary diffuse astrocytic gliomas (grades II–IV) and in 4 human glioblastoma cell lines (U87MG, U118MG, U138MG, and T98G). Monoclonal anti-peptide antibodies recognizing epitopes in C-terminal or N-terminal domains of the γ -tubulin molecule were used in immunohistochemical, immunofluorescence, and immunoblotting studies. In tumors in adults ($n = 46$), varying degrees of localization were detected in all tumor grades, but immunoreactivity was significantly increased in high-grade anaplastic astrocytomas and glioblastomas multiforme as compared to low-grade diffuse astrocytomas ($p = 0.0001$). A similar trend was noted in diffuse gliomas in children but the sample of cases was too small as to be statistically meaningful. Two overlapping patterns of ectopic cellular localization

were identified in both primary tumors and glioblastoma cell lines: A punctate pattern, in which γ -tubulin was partially co-distributed with pericentrin in the pericentriolar region, and a diffuse pattern, independent of pericentrin staining, denoting a soluble pool of γ -tubulin. Cellular γ -tubulin was detected in both soluble and insoluble (nocodazole-resistant) fractions of glioblastoma cells. Divergent localizations of γ -tubulin and pericentrin suggest a differential distribution of these 2 centrosome-associated proteins in glioblastoma cell lines. Our results indicate that overexpression and ectopic cellular distribution of γ -tubulin in astrocytic gliomas may be significant in the context of centrosome protein amplification and may be linked to tumor progression and anaplastic potential.

Key Words: Anaplastic change, Astrocytoma, Centrosome amplification, Glioblastoma, Glioma, Pericentrin, γ -tubulin.

INTRODUCTION

Tumor progression in diffuse gliomas is an intricate multistep process characterized by accumulation of genetic defects, including an abnormal number of chromosomes, a condition known as aneuploidy. Genomic stability requires proper coordination of DNA replication and centrosome duplication, which is facilitated by factors such as p53, pRb, cyclin E, and cyclin A (1, 2).

The centrosomes are organelles subserving diverse cellular functions. First, they are essential for the process of cytoplasmic division (cytokinesis), ensuring balanced chromosome segregation (1–3). Second, they are involved in the initiation of the S-phase and the regulation of cell cycle progression (4–7). Third, centrosomes play an important role in microtubule nucleation and organization, thus contributing to cell architecture and the acquisition of cell polarity (4). Normal diploid somatic cells contain a single centrosome, which consists of 2 centrioles that are surrounded by an amorphous pericentriolar matrix. The latter comprises a large repertoire of proteins including pericentrin, the cytoskeletal protein γ -tubulin, and numerous key regulators of cell cycle progression (2, 5, 8–12). Pericentrin and γ -tubulin form a protein complex with a lattice-like organization at the

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Dedicated to Professor Peter L. Lantos on the occasion of his retirement as Head of the Department of Neuropathology, Institute of Psychiatry, London, England.

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centrosome (11, 12). The centrosome-associated γ -tubulin plays an important role in the nucleation of microtubule assembly and regulates tubulin synthesis (13). There is evidence that γ -tubulin may also exist in a soluble form and associate with the lateral arms of microtubules (14–16). γ -Tubulin has also been found to bind to membranous components of the cell (17–19).

The nucleation of microtubules occurs from within the pericentriolar material where γ -tubulin ring complexes act as microtubule nucleation templates (20, 21). During normal mitosis, 2 centrosomes ensure the assembly of bipolar spindles and proper chromosomal segregation. Extra copies of centrosomes result in the formation of multipolar spindles resulting in chromosomal missegregation (2, 22, 23).

The cells of many human and animal cancers contain an excess number of abnormal centrosomes (“supernumerary centrosomes”), which contribute to the formation of multipolar spindles that underlie unbalanced chromosome segregation and aneuploidy (1, 2, 22). This process is commonly known as centrosome amplification and involves alterations in diverse centrosome-associated molecules. Although the exact mechanisms of centrosome amplification are unknown, several models for the generation of supernumerary centrosomes and centrosomal dysfunction have been proposed (reviewed in [1, 2, 22, 23]). Overexpression of the centrosomal protein pericentrin in primary prostate epithelial cell transfectants gives rise to cells with tumor-like phenotypic features, suggesting that primary centrosome dysfunction may be pivotal to tumorigenesis (24). Whether abnormalities in centrosomal proteins constitute the primary cause of chromosomal instability or are secondary to cell cycle deregulation, they are, nonetheless, regarded as important correlates of tumorigenesis and anaplastic potential (2). Regardless of their origin, “supernumerary” (or otherwise structurally abnormal or dysfunctional) centrosomes promote malignant change and tumor progression by favoring the development of chromosomal instability and by influencing tumor cell motility and architecture (2).

To date, centrosome abnormalities have been reported in a wide range of predominantly epithelial tumors, including carcinomas of the breast, bile ducts, pancreas, adrenal cortex, colon, prostate, cervix, lung, and head and neck region (22, 25–32). Moreover, centrosome abnormalities have been described in conjunction with genomic instability in osteosarcoma, a malignant mesenchymal neoplasm (33).

With respect to the central nervous system, centrosome amplification has, until now, only been reported in cerebral primitive neuroectodermal tumors exhibiting *TP53* mutations (34). However, the presence of centrosome protein abnormalities among common astrocytic tumors is a hitherto underinvestigated aspect in glioma tumorigenesis.

We have hypothesized that centrosomal abnormalities are associated with malignant (anaplastic) transformation in diffuse astrocytic gliomas by favoring the development of genomic instability (aneuploidy) and also, by exerting influence on aberrant microtubule nucleation and tumor architecture. The present study specifically examines the cellular expression and localization of γ -tubulin, a key structural protein of the pericentriolar material of centrosomes, in the

context of histological tumor grade in primary diffuse astrocytic gliomas and in 4 established human glioblastoma cell lines.

MATERIALS AND METHODS

Tissue Samples

The localization of γ -tubulin was evaluated in 56 surgically resected samples of diffuse astrocytic gliomas from adults ($n = 46$) and children ($n = 10$) representative of the World Health Organization (WHO) histological grades II to IV. The tumor specimens from adult patients included low-grade diffuse astrocytomas/WHO grade II ($n = 20$), anaplastic astrocytomas/WHO grade III ($n = 5$), and glioblastomas multiforme/WHO grade IV ($n = 21$). Pediatric gliomas included examples of anaplastic glioma (WHO grade III) ($n = 2$), and diffuse gliomas (WHO grade II) of the cerebral hemispheric white matter ($n = 2$), thalamus ($n = 3$), brainstem ($n = 2$), and spinal cord ($n = 2$). Sections from formalin-fixed, paraffin-embedded archival tissues were processed for immunohistochemistry. Control tissues included surgical and autopsy tissue samples from cases exhibiting gliosis ($n = 8$). Paraffin sections of normal cerebral hemispheric white matter from term piglet brains (marked by active gliogenesis) were also included ($n = 5$). The archival tissue samples used in this study were derived from the following institutions: Department of Anatomical Pathology and Cytology, University of Patras School of Medicine, Patras, Greece, the Department of Histopathology and Morbid Anatomy, The Royal London Hospital, London, UK, and the Department of Pathology and Laboratory Medicine, St. Christopher’s Hospital for Children, Philadelphia, PA.

Cell Lines

Four well-established human glioblastoma cell lines, T98G, U87MG, U118MG, and U138MG were used in the present study. T98G and U87MG were maintained in 10% FBS in DMEM, U118MG was maintained with Dulbecco’s modified Eagle media containing 4 mM L-glutamine, and U138MG was maintained in Minimum Essential Media containing 2 mM L-glutamate. All cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA).

Antibodies

For immunohistochemistry, immunofluorescence, and immunoblotting, 3 monoclonal anti-peptide antibodies recognizing epitopes in C-terminal or N-terminal domains of γ -tubulin molecule were used. These included GTU-88 (IgG1) generated against the EEFATEGTDRKDVFFY peptide corresponding to the human sequence 38–53 in the N-terminal region of γ -tubulin sequence (Sigma Aldrich, St. Louis, MO; cat. no. T6557) and antibodies TU-31 (IgG2b) and TU-32 (IgG1) generated against EYHAATRP-DYISWGTQ peptide corresponding to the human sequence 434–449 in the C-terminal region of γ -tubulin (35). Tubulin was detected by mouse monoclonal antibody TU-01 (IgG1)

(36, 37) or by affinity purified rabbit antibody TUB against α/β -tubulin dimer (38). Affinity-purified rabbit antibody M8 against pericentrin (39) and a polyclonal antibody against pericentrin (Covance, Berkeley, CA) were used as generic centrosome markers.

Indocarbocyanate (Cy3)-conjugated anti-mouse and anti rabbit antibodies as well as fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody were from Jackson Immunoresearch Laboratories (West Grove, PA). FITC-conjugated anti-mouse antibody was also bought from Vector Laboratories. Anti-mouse antibodies conjugated with horseradish peroxidase were purchased from Promega (Madison, WI). FITC-conjugated anti-mouse (IgG) and Texas red anti-rabbit (IgG) antibodies were purchased from Vector Laboratories (Burlingame, CA).

Preparation of Cell Extracts

For preparation of soluble and detergent-resistant fractions at 37°C, cells on 6-cm Petri dishes were either directly used for the assay, or they were preincubated 6 hours with 0.5 μ g nocodazole/ml to before assay to disrupt microtubule arrays. Attached cells were rinsed twice in microtubule-stabilizing (MSB) buffer (100 mM MES adjusted to pH 6.9 with KOH, 2 mM EGTA, 2 mM MgCl₂) or in MSB buffer containing nocodazole (MSB/nocodazole), and then extracted with 0.5 ml of MSB buffer (37°C) or MSB/nocodazole supplemented with protease inhibitor cocktail ("Complete EDTA-free" tablets) (Roche Molecular Biochemicals, Mannheim, Germany), phosphatase inhibitors (1 mM Na₃V0₄, 1 mM NaF) and 0.2% (v/v) Triton X-100. After one-minute incubation at 37°C, the extract was gently removed, spun down at 5,000 g for 1 minute at 25°C, and one fourth volume of 4x SDS/PAGE-sample buffer was added to the supernatant. The cytoskeletons remaining on the plate were gently rinsed twice with warm MSB buffer containing inhibitors and solubilized with 0.625 ml of sample buffer, prepared by mixing 2x SDS/PAGE-sample buffer with 2x extraction buffer (1:1). Pelleted material obtained after spinning down the extract was combined with the cytoskeletal fraction. Samples were boiled for 5 minutes.

Immunohistochemistry

Prior to immunohistochemistry, tissue sections were subjected to microwave antigen unmasking in Na⁺ EDTA buffer at pH 8.0. Immunohistochemistry was performed according to the avidin biotin complex (ABC) peroxidase method using the Mouse IgG ABC Elite® detection kit (Vector Labs) as previously described (40). Anti- γ -tubulin monoclonal antibody GTU-88 was diluted 1:300 and polyclonal antibody against pericentrin was diluted 1:150.

Histologic and immunostained preparations were evaluated by a panel of 2 neuropathologists (CDK and SM) and one pediatric pathologist (JPD). Because the tissue specimens were obtained from 3 different laboratories, we opted to ensure that histologic classification and tumor grading were made according to homogeneous criteria by having specimens evaluated by different members of the panel. In case of disagreement, histologic typing was assigned by consensus at

conference. Clinicopathological-neuroimaging correlations were carried out whenever feasible.

The minimal criterion for the identification of a positive cell in the context of putative centrosome dysfunction and possible amplification was the detection of 3 or more punctate dot-like immunoreactive signals in the cytoplasm of a single tumor cell. The indirect immunoperoxidase method employed in this study was sufficiently sensitive to detect the gain of one or more punctate signals in excess of 1 or 2 γ -tubulin-positive juxta-nuclear presumptive centrosomes typifying non-transformed diploid cells (29, 31). Tumor cells with a multipunctate staining pattern were admixed in any given case with cells exhibiting a nondescript diffuse pattern of immunoreactivity (see Results). Accordingly, for the purpose of cell counting, immunolabeled tumor cells were considered positive regardless of whether they displayed a predominantly multipunctate or diffuse pattern of γ -tubulin localization.

Manual cell counting of labeled tumor cells was performed independently by 3 observers (CDK, AT, and KY). Cell counting and statistical analysis were carried out only in the adult group of astrocytic gliomas (n = 46). Between 298 to 954 tumor cells were evaluated per case in 20 non-overlapping high-power (40x) fields and a labeling index was determined for each case. Labeling index (LI) was expressed as the percentage (%) of γ -tubulin-labeled cells out of the total number of tumor cells counted in each case. The median labeling index (MLI) and the interquartile range (IQR) - delimited by the 25th and 75th percentiles - were determined for the set of cases in each histological grade using the UNIVARIATE procedure of the SAS package (SAS Institute, Cary, NC). The statistical significance of differences in labeling indices between WHO histological grades were examined with non-parametric statistical techniques using Kruskal-Wallis analysis of variance tests and Wilcoxon rank-sum post-hoc tests. These analyses were carried out using the NONPAR1WAY procedure of the SAS package (SAS Institute) as described previously (40). Because of the small number of pediatric gliomas (n = 10) included in this study, only qualitative assessment was performed in these cases.

Immunofluorescence Microscopy

For immunofluorescence microscopy, cells were plated onto two-chamber glass slides coated with poly-L-lysine (Boehringer-Mannheim) until cultures reached approximately 80% to 85% confluence. After rinsing with PBS, cells were fixed with cold acetone for 3 minutes and rinsed with PBS. Cells were then blocked with 5% normal horse serum for 2 hours at room temperature and primary antibodies were incubated overnight at room temperature. Alternatively, cells grown on coverslips were fixed for 10 minutes in methanol at -20°C followed by 6 minutes in acetone at -20°C, and after rinsing with PBS were incubated with primary antibodies for 45 minutes at room temperature. Affinity purified polyclonal antibody TUB against $\alpha\beta$ -tubulin dimer was diluted 1:10, anti- γ -tubulin monoclonal antibody GTU-88 was diluted 1:500, and anti- γ -tubulin monoclonal antibody TU-31 was

used as undiluted hybridoma supernatant. Polyclonal antibody against pericentrin was diluted 1:250. After rinsing with PBS, Cy3-tagged or FITC-tagged secondary antibodies were incubated for 1 hour at room temperature in the dark. For double label fluorescence the coverslips were incubated simultaneously with the primary antibodies and after washing incubated simultaneously with secondary antibodies. Cy3-conjugated anti-mouse or anti-rabbit antibodies were diluted 1:1000, and FITC-conjugated anti-mouse or anti-rabbit antibodies were diluted 1:200. For double labeling immunofluorescence studies on archival histological sections, we used monoclonal antibody GTU-88 against γ -tubulin, and anti-pericentrin antiserum at the same dilutions as in the methanol-fixed cells from the glioblastoma cell lines, as well as FITC- and Texas red-tagged secondary antibodies both diluted at 1:200. 4',6-diamidino-2-phenylindole (DAPI) was used to label cell nuclei. Finally, after rinsing with PBS, slides were coverslipped using an aqueous based mounting medium (Vector Laboratories). Visualization was carried out with a Nikon Eclipse TE300 inverted fluorescence microscope and images were captured and processed using IPLab Scientific Image Processing software, version 3.5.4 (Scanalytics Inc. Fairfax, VA). Alternatively samples were examined with Olympus A70 Provis microscope. Conjugates alone did not give any detectable staining.

Immunoblots

Whole-cell extracts for SDS-PAGE were prepared by solubilizing washed cells in hot SDS-sample buffer and boiling for 5 minutes. Proteins were separated by 7.5% SDS-PAGE and subsequently transferred onto nitrocellulose membranes. Molecular weight standards were from Sigma. Blocking was achieved by incubating with 3% BSA in TBST (10 mM Tris-HCl adjusted to pH 7.4 with HCl, 150 mM NaCl, 0.05% (v/v) Tween 20) and then incubated overnight with primary antibodies diluted in TBST. The anti-tubulin antibodies TU-01 and TU-32, in the form of spent culture supernatants, were diluted 1:10, whereas the antibody GTU-88 was diluted 1:5,000. The membranes were then washed 3 times with TBST and incubated for 1 hour at room temperature with secondary anti-mouse antibody conjugated with peroxidase diluted 1:10,000 (Promega). After several washes, the binding was detected with chemiluminescence reagents (Pierce, Rockford, IL) in accordance with the manufacturer's directions. Exposed autoradiography films X-Omat AR (Eastman Kodak, Rochester, NY) were evaluated using gel documentation system GDS 7500 (UVP).

RESULTS

Primary Brain Tumors

In non-neoplastic glia from human and piglet brains, including cases of gliosis, discrete γ -tubulin labeling was detected in the form of one or 2 paranuclear dots corresponding to the expected cytoplasmic localization of centrosomes (Fig. 1A, B).

In the series of astrocytic gliomas in adults, varying degrees of γ -tubulin labeling were detected in all histological grades of diffuse astrocytomas (grades II–IV). However, staining was significantly increased in the high-grade anaplastic astrocytomas and glioblastomas (grades III/IV) (MLI: 56.4%; IQR: 41.2%–73.4%) as compared to the low-grade diffuse astrocytomas (grade II) (MLI: 6.4%; IQR: 1.6%–9.1%) ($P = 0.0001$) (Figs. 1C–F, 2A–F).

As demonstrated by both immunohistochemistry in primary tumors (from both adults and children) and FITC-immunofluorescence microscopy in tumor cell lines (see below), the localization of γ -tubulin acquires 2 overlapping staining patterns, notably punctate and diffuse, which merge imperceptibly within individual tumor cells. The punctate pattern was characterized by 3 or more either discrete and/or coalescent, variously sized and abnormally shaped immunoreactive dots that were present throughout the cytoplasm of tumor cells (Figs. 1C–E, 2C). The diffuse staining pattern was evidenced throughout the cytoplasm of tumor cells, including the proximal segments of glial cell processes (Figs. 1F, 2B, D–F). The latter was frequently encountered in conjunction with the multipunctate pattern. Immunolabeled cells were focally and unevenly dispersed within tumor sheets (Fig. 2A, B, D). As a rule, γ -tubulin-positive tumor cells were morphologically indistinguishable from γ -tubulin-negative cells. Staining for γ -tubulin was not associated with distinctive morphology, although there was an overall tendency of immunoreactive tumor cells to be pleomorphic and multinucleated (Figs. 1F, 2B–F). These alterations were considerably less widespread in the low-grade astrocytic gliomas where the localization of aberrant γ -tubulin-positive cytoplasmic dots was generally patchy and less frequent as compared to the high-grade tumors (Fig. 1C–F). Various pronounced γ -tubulin localization was present in the cytoplasm of vascular endothelial cells in areas of tumor angiogenesis (microvascular proliferation) in glioblastomas (Fig. 3A–D).

Glioblastoma Cell Lines

Variably robust fluorescein green (FITC) staining was present in cell cultures of all 4 glioblastoma lines reproducing overlapping punctate and diffuse patterns of γ -tubulin localization (Fig. 4A–F). Cells of the U118MG cell line predominantly exhibited a multi-punctate pattern of localization consisting of irregular, variously sized γ -tubulin aggregates suggestive of abnormal centrosome protein aggregates (Fig. 4A, B), while cells of the other cell lines exhibited predominantly diffuse or mixed patterns of immunoreactivity (Fig. 4C–F). Confluent cytoplasmic mass-like conglomerates were also identified (Fig. 4C, E, F, arrowheads).

By immunoblotting, prominent γ -tubulin protein bands were present in supernatants of glioblastoma cell extracts (Fig. 5). Differences in staining intensity observed with GTU-88 and TU-32 antibodies could reflect concentration and/or affinity of antibodies. No cross-reactivity was observed with GTU-88 and TU-32 on immunoblots.

Extraction of T98G and U87MG cells with 0.2% Triton X-100 in microtubule stabilizing buffer at 37°C for 1 minute showed differences in relative distribution of the proteins between the soluble and insoluble fractions. Results of a

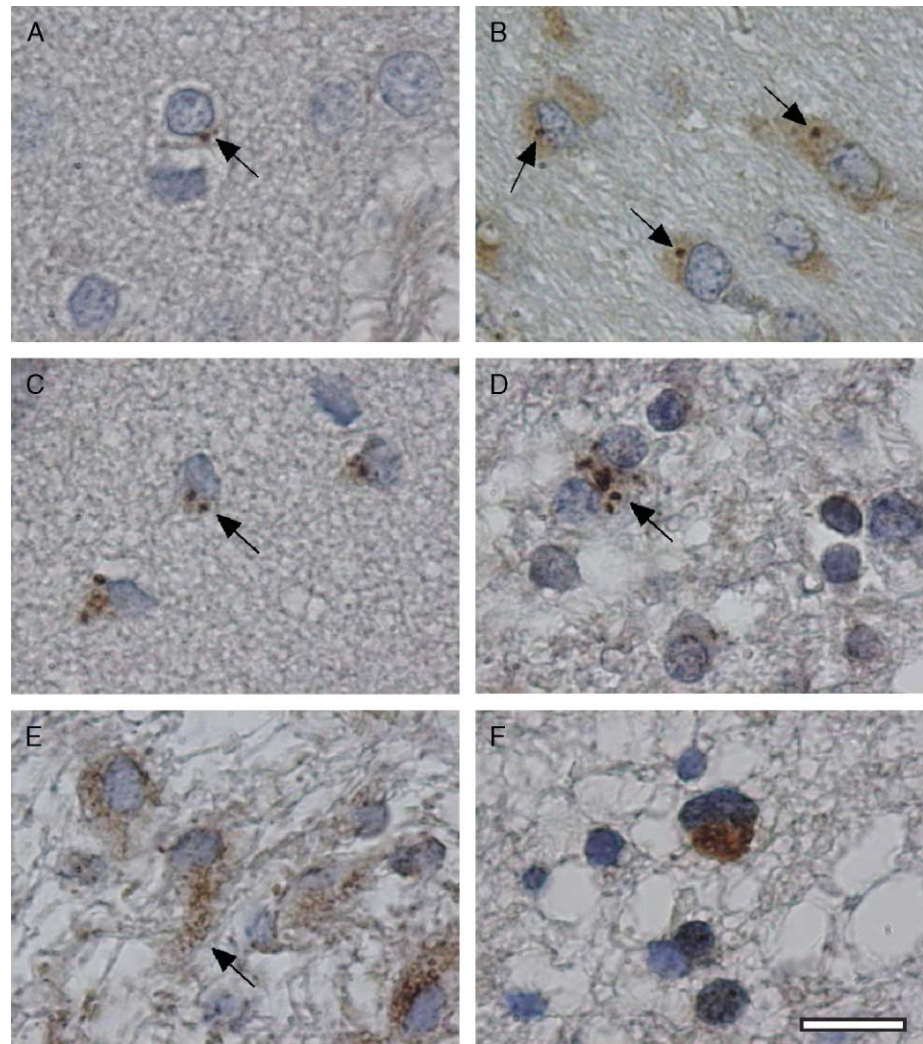


FIGURE 1. Cellular distribution of γ -tubulin in non-neoplastic glia from adult human (A) and newborn piglet (B) brains. Single or double dot-like staining (arrows) is consistent with centrosomal localization. (C–F) Panels depict the cellular distribution of γ -tubulin in diffuse astrocytoma, WHO grade II. Staining is present in a small number of tumor cells (arrows) where it is discrete and variably prominent (C–F). Scattered cells exhibit multiple (>3) punctate, irregular and coalescent cytoplasmic localizations (C–E) merging into a diffuse staining pattern. Avidin biotin complex (ABC) peroxidase with hematoxylin counterstain. Scale bar = (A, B, D–F) 10 μ m; (C) 20 μ m.

typical experiment are shown in Figure 6. γ -Tubulin was present in soluble and cytoskeletal fractions in similar quantities in resting cells and cells preincubated with nocodazole that efficiently disrupted microtubule arrays, which was confirmed by immunofluorescence microscopy (not shown). The same results were obtained with antibodies GTU-88 and TU-32 that are directed against different epitopes in the γ -tubulin molecule. On the other hand, α -tubulin was present in both soluble and cytoskeletal fractions in similar quantities in resting cells only. When microtubules were disrupted, the substantially lower amount of α -tubulin was detected in the insoluble fraction. These data indicate that in these cell lines a substantial portion of cellular γ -tubulin is present in the detergent-resistant fraction.

Differential Distribution of γ -Tubulin and Pericentrin

Staining for α/β -tubulin dimers yielded 2 patterns of localization. The first and most prominent was a robust centripetal distribution that was mainly detected in the pericentriolar region where it attained in some cells the proportions of confluent paranuclear immunoreactive masses

(Fig. 7A). The second staining pattern corresponded to microtubule arrays in the tumor cell bodies and proximal segments of cell processes (Fig. 7A). Double labeling localization revealed that γ -tubulin had a punctate or diffuse, centrifugal localization extending to the periphery of the cytoplasm of T98G glioblastoma cells. However, in some cells, multiple bright γ -tubulin dots were observed from which microtubules were not nucleated (Fig. 7B, C).

By immunofluorescence microscopy performed on glioblastoma cell lines and surgically resected glioblastomas there was a discernible spatial dissociation between these 2 centrosome-associated proteins (Fig. 8A–F). Pericentrin staining was detected for the most part in the paranuclear pericentriolar region (Fig. 8B and E [single pericentrin labeling] and Fig. 8C and F [double labeling for pericentrin and γ -tubulin]). Confluent perinuclear areas of punctate pericentrin staining consistent with supernumerary centrosomes were demonstrated in some cells (Fig. 8E [single pericentrin labeling] and Fig. 8F [double labeling for pericentrin and γ -tubulin]). Although colocalization of γ -tubulin and pericentrin was demonstrated in the pericentriolar region (Fig. 8C, F), the cytoplasmic distribution of γ -tubulin was distinctly more widespread as

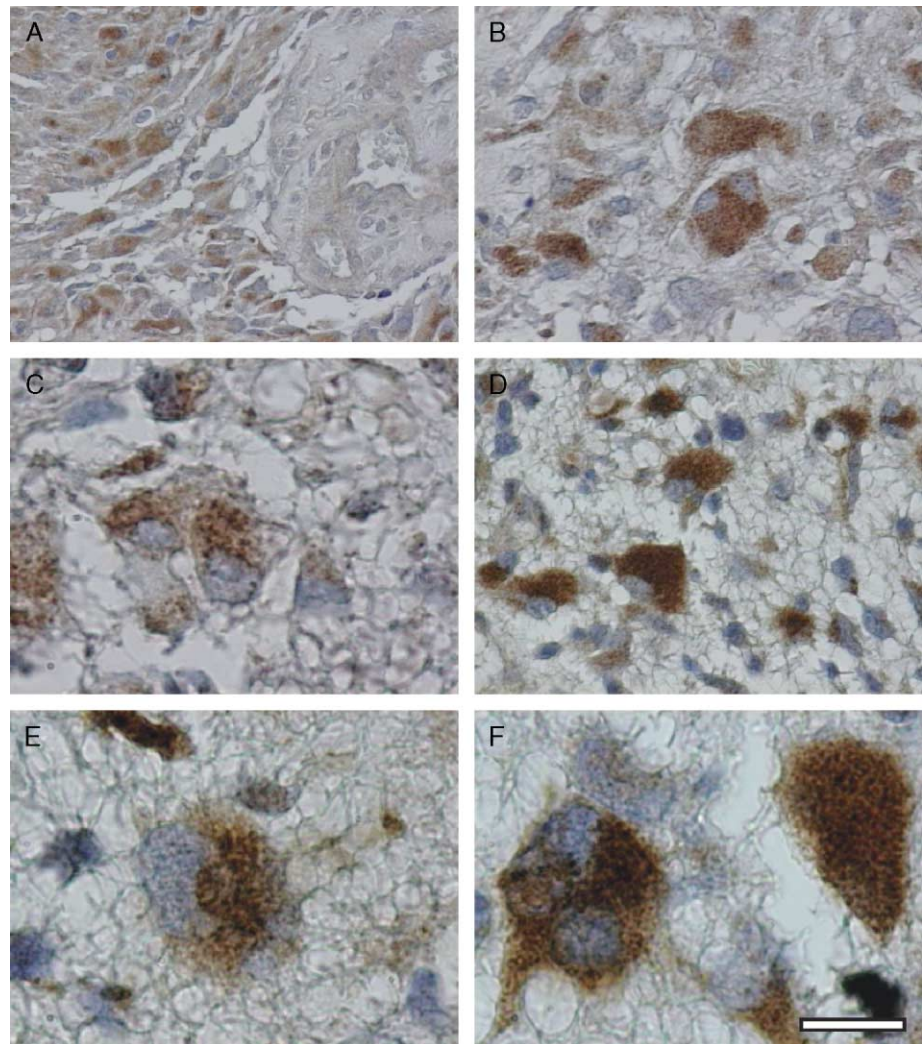


FIGURE 2. Cellular distribution of γ -tubulin in glioblastoma multiforme, WHO grade IV. Robust staining is present in a large number of tumor cells (**A–D**). Pleomorphic and multinucleated tumor cells exhibit innumerable, finely punctate and confluent cytoplasmic localizations merging into a diffuse staining pattern (**B–F**). ABC peroxidase with hematoxylin counterstain. Scale bars = (**A**) 50 μm ; (**B, D**) 20 μm ; (**C, E, F**) 10 μm .

compared to pericentrin (Fig. 8A, C, D, F). The former (γ -tubulin) displayed a confluent punctate or diffuse centrifugal localization extending to the periphery of glioblastoma cells, whereas the latter (pericentrin) was spatially restricted to a relatively narrow, irregular cytoplasmic rim surrounding the tumor cell nuclei (Fig. 8C, F). On the other hand, colocalization of γ -tubulin and pericentrin was demonstrated in scattered neoplastic cells in low-grade diffuse astrocytomas (grade II) (Fig. 9A–C).

DISCUSSION

The identification of a number of molecular alterations in transformed glial cells has provided critical insights into the role of tumor suppressor genes and signaling pathways in the development of gliomas (41–45). Genetic alterations frequently observed in high-grade adult astrocytic gliomas involve either the *p53/MDM2/p14ARF* or the *Rb/CDK4/p16INK4a* tumor suppressor pathways (46). However, there are presently no specific, or clinically useful, cellular or molecular genetic markers that can reliably predict the onset of a tumor's shift to a higher level of malignancy in this

clinico-pathological setting. Although numerical chromosomal abnormalities (present predominantly in high-grade as compared to low-grade tumors) contribute to this process, the patterns of chromosomal instability and involvement of tumor suppressor pathways are both varying and different between the adult and pediatric groups (46, 47). This disparity of cytogenetic findings points to underpinning fundamental defects of cellular systems governing the physical segregation of chromosomes as opposed to the presence of specific primary chromosomal abnormalities per se. It is thought that centrosomes, which integrate control of cell division and genomic stability, cell cycle progression, and cytoskeletal dynamics, may be critically important in this regard (2, 23).

Centrosome Amplification in Cancer

There is persuasive evidence to date pointing to abnormalities in the number of chromosomes (aneuploidy) as a cause rather than an effect of anaplastic transformation (48). The chromosome missegregation in malignant tumors could result from defects in centrosome function (1–3, 22, 23). Consequently, centrosome dysfunction, reflected by abnor-

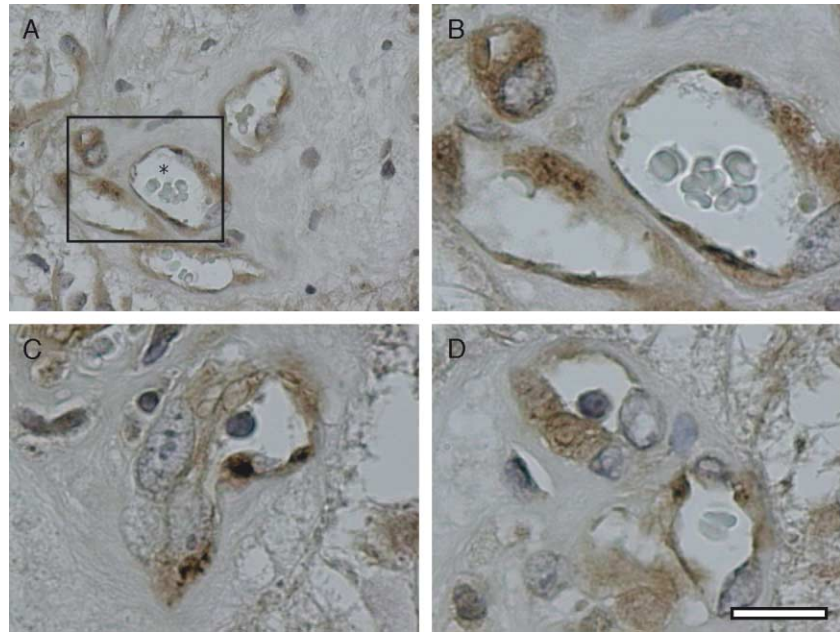


FIGURE 3. Glioblastoma multiforme: Localization of γ -tubulin in endothelial cells of tumor blood vessels exhibiting microvascular proliferation. Note endothelial cell hypertrophy, nuclear atypia, and particulate multipunctate cytoplasmic localization of γ -tubulin. (A) Image depicts vascular lumen; panel (B) is a higher magnification of the frame in (A). Scale bars = (A) 20 μ m; (B–D) 10 μ m.

malities in the expression and sorting of centrosomal proteins, may antecede changes of DNA content. We have focused our attention on γ -tubulin, a key cytoskeletal protein of centro-

somes, in the context of malignancy of astrocytic gliomas and in an effort to elucidate new markers of incipient anaplastic phenotypes in low-grade diffuse astrocytomas.

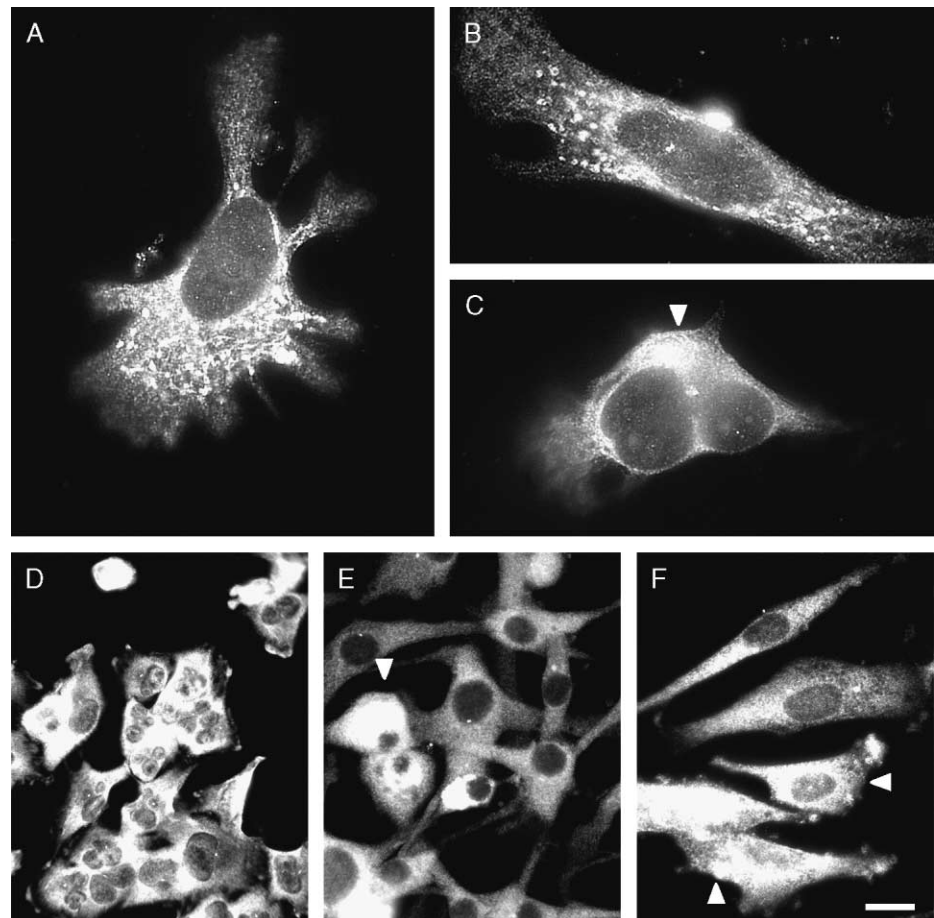


FIGURE 4. Localization of γ -tubulin with monoclonal antibodies GTU-88 (A–C) and TU-31 (D–F) in human glioblastoma cell lines. (A, B) Panels (U118MG line) depict multi-punctate, irregularly shaped and variously sized coalescent cytoplasmic localizations. Panels (C, D) (T98G line), (E) (U87MG line), and (F) (U138MG line) reveal diffuse and confluent localizations in cells exhibiting also single bright paranuclear dots consistent with centrosomes. Note confluent cytoplasmic mass-like aggregates in ([C, E, F], arrowheads) in addition to diffuse, less robust cytoplasmic staining. FITC labels cytoplasmic protein. Scale bar = (A–C) 10 μ m; (D) 28 μ m; (E, F) 19 μ m.

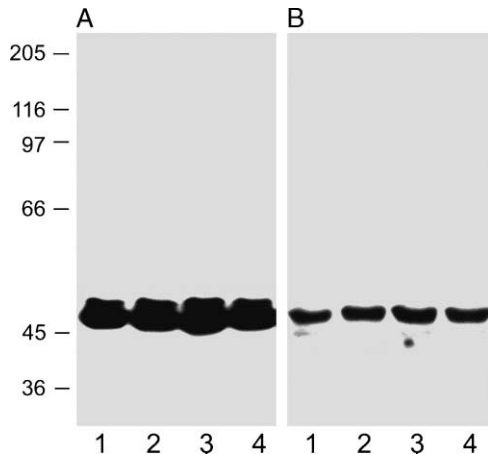


FIGURE 5. Immunoblots with anti- γ -tubulin antibodies GTU-88 (**A**) and TU-32 (**B**) on panel of human glioblastoma cell lines. Lanes 1 to 4 correspond to total lysates of U118MG (lane 1), U138MG (lane 2), U87MG (lane 3), and T98G cells (lane 4). The same amount of protein (10 μ g) was loaded into each lane. Positions of molecular mass markers (in kDa) are indicated on the left.

Amplified centrosomes are accompanied by increased or otherwise altered expression of centrosomal proteins, including γ -tubulin and pericentrin (24, 25, 28, 29, 31). Also, they exhibit increased protein phosphorylation and altered functional properties, such as an increased or faulty microtubule nucleating capacity (reviewed in [2, 3, 22]).

Indirect evidence of centrosome amplification in astrocytomas may be deduced from the increased human polo-like kinase-1 expression in these tumors (49). Increased polo-like kinase expression is also present in 3 human glioblastoma cell lines (U87MG, U118MG, and U138MG) and primary explants from patients with glioblastoma (49). In addition, c-Jun N-terminal kinase (JNK), a stress-activated protein kinase associated with the centrosome (50), is thought to play a significant role in glioma tumorigenesis (51).

Overexpression of γ -Tubulin in Astrocytic Gliomas

To assess the presence of centrosome amplification in glial tumors we evaluated the cellular expression and localization of γ -tubulin in 56 surgically resected diffuse astrocytic gliomas representing WHO grades II through IV, and in 4 human glioblastoma cell lines (U87MG, U118MG, U138MG, and T98G). We demonstrate that neoplastic cells of human astrocytic gliomas exhibit increased expression of γ -tubulin associated with altered patterns of cellular distribution in malignant tumor phenotypes. These alterations occur according to an ascending scale of histological malignancy and are particularly prominent in tumor cell subpopulations of glioblastomas. Our results indicate that γ -tubulin expression is significantly increased in the high-grade, anaplastic astrocytomas, and glioblastomas multiforme (grades III/IV) as compared to low-grade diffuse astrocytomas (grade II). In the context of the findings of the present study, the detection of either multipunctate and/or diffuse γ -tubulin localizations in

tumor cells relates statistically to a high-grade histological lesion if it falls within the interquartile range (IQR) of labeling index (LI) values (42% to 73% or above). At the same time, even LI values within the IQR for low-grade diffuse astrocytomas (2% to 9% or upwards), may be potentially significant inasmuch as they may portend incipient genomic instability in a subpopulation of tumor cells with a predisposition for anaplastic change.

Non-neoplastic glial cells contain one or two paranuclear centrosomes, which are visualized using anti- γ -tubulin, anti-pericentrin and anti- α -tubulin antibodies; in contrast, tumor cells from primary glial tumors and tumor cell lines exhibit two distinctive but overlapping patterns of altered cellular localization of γ -tubulin. These include punctate and diffuse patterns of immunoreactivity. Large amounts of cytoplasmic γ -tubulin exhibiting a predominantly diffuse staining pattern were demonstrated by bright-field immunohistochemistry and immunofluorescence microscopy in glioblastoma cells from primary surgically resected specimens and tumor cell lines. We have confirmed by immunoblotting that diffuse staining may represent monomeric or oligomeric pools of γ -tubulin, which in tumor cells probably exist in a state of dynamic instability, possibly available for rapid recruitment by tumor cells in the context of centrosome

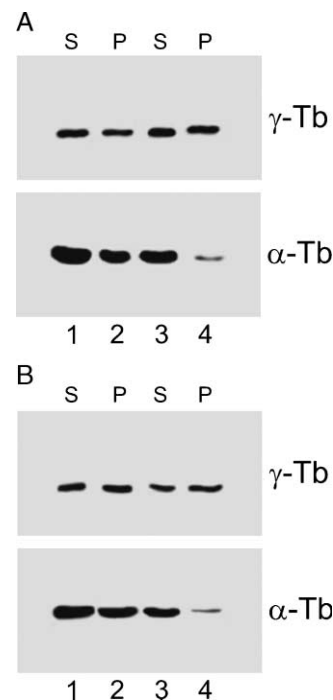


FIGURE 6. Immunoblot analysis of soluble and insoluble fractions from human glioblastoma cell line T98G and U87MG. Samples were prepared from untreated T98G (**A**) and U87MG (**B**) cells (lanes 1 and 2) or from cells pre-treated with nocodazole to disrupt microtubule arrays (lanes 3 and 4). To compare the relative distribution of immunoblotted proteins, pelleted material was resuspended in a volume equal to the corresponding supernatant. Immunostaining with antibody TU-01 against α -tubulin (α -Tb) and antibody TU-32 against γ -tubulin (γ -Tb). S, supernatant; P, pellet.

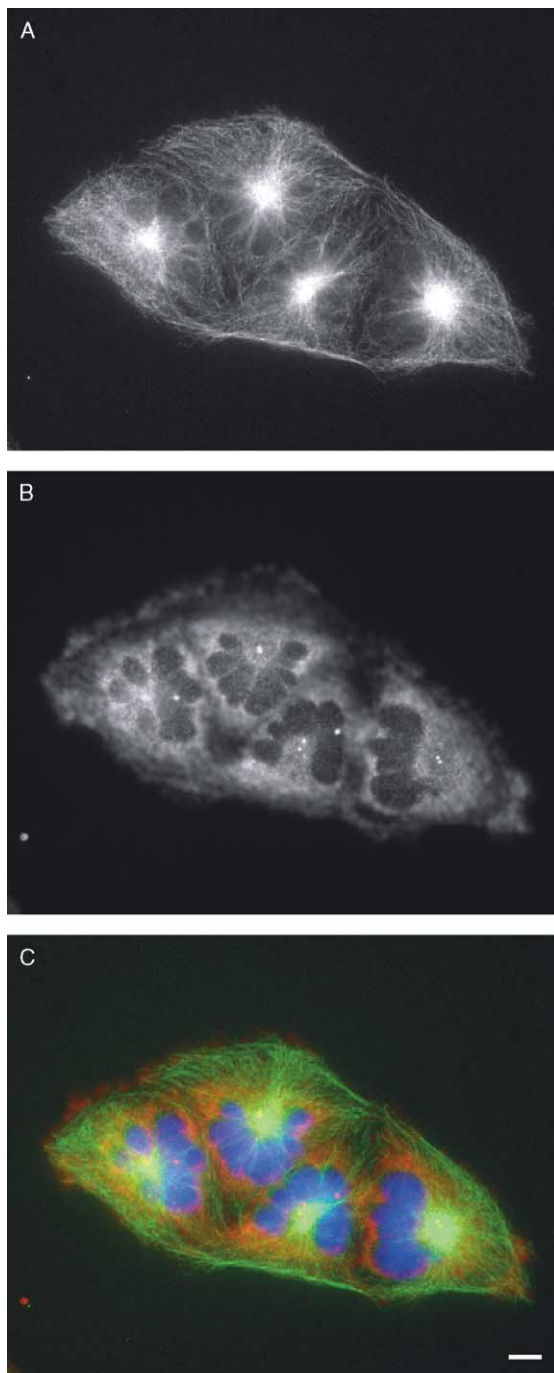


FIGURE 7. Distribution of γ -tubulin and tubulin dimers in the human glioblastoma cell line T98G. Cells were stained by double labeling with polyclonal antibody specific for tubulin dimers (**A**) and monoclonal antibody TU-31 for γ -tubulin (**B**). Superposition of stainings is shown in panel (**C**). Note robust tubulin dimer staining (green) delineating a large paranuclear immunoreactive mass. Staining of microtubule arrays (green) in the tumor cell bodies and proximal segments of cell processes is also detected. Double labeling reveals that the localization of γ -tubulin (red) is spatially distinct and exhibits a diffuse localization involving (in this field) the outer cytoplasmic portions of tumor cells. DAPI (blue) labels multiple nuclei in each cell (**C**). Scale bar = 10 μ m for all panels.

amplification. In addition, we have noted coalescent punctate localizations especially in the U118MG glioblastoma cell line. Although these immunofluorescence localizations do not appear to correspond to intact “supernumerary centrosomes” they are likely to represent abnormal protein assemblies either in the form of “acentriolar bodies,” aberrant accumulations of ectopic pericentriolar material, and/or fragmented centrosomes (2). It is noteworthy that a substantial proportion of cellular γ -tubulin was found in the detergent (nocodazole)-resistant fraction of glioblastoma cell line extracts.

Our findings suggest that centrosome amplification does not necessarily need to be accompanied by structurally “intact” supernumerary centrosomes, but rather it may be characterized by molecular/biochemical alterations in which there is increased (or otherwise altered) expression of proteins of the pericentriolar material, including γ -tubulin.

In the developmental context, soluble γ -tubulin has been demonstrated in nucleated chicken erythrocytes and pig brain cells (19, 20). However, to date, the subcellular sorting of γ -tubulin in cancer cells in general and glioma cells in particular is unknown. Dráberová and co-workers have demonstrated that rat basophilic leukemia cells contain large soluble pools of α/β -tubulin dimers and γ -tubulin, which exist in large complexes with other molecules and are localized around centrosomes (52). These investigators have shown that complexes of soluble γ -tubulin released from activated rat basophilic leukemia cells contain tyrosine-phosphorylated proteins (52). Similarly, in P19 embryonal carcinoma cells undergoing neuronal differentiation, γ -tubulin is phosphorylated and forms complexes with protein tyrosine kinases of the Src family (53).

γ -Tubulin interacts, in addition to α/β -tubulin dimers (16) and pericentrin (11, 12), with a host of other proteins. These include BRCA-1, Ras association domain family protein 1, RAC GTPase activating protein 1, paxillin, phosphatidylinositol 3 kinase (PI3K) regulatory α -subunit, RING finger protein 19 (54), and dynamin-2 (55). Genetic alterations of class IA PI3K subunit genes have been shown to play a role in human glioblastomas (56). In addition, colocalization of γ -tubulin with nerve growth factor has been demonstrated at the centrosomes or the spindle poles throughout the cell cycle in the human glioblastoma cell line U251 MG (57).

The presence of numerous key regulators of cell cycle progression at the centrosome has led to the speculation that the centrosome itself provides an important structural context for coordinating cell cycle regulation (2, 5).

Genes involved in different signal transduction pathways have been implicated in centrosome amplification. These include genes of the p53 pathway (*p53*, *WAF-1*, *Gadd45*, and *Mdm2*) and the DNA-repair pathway (*ATR*, *BRCA-1*, *BRCA-2*, and *XRCC2/3*), as well as genes involved in ubiquitin-related protein degradation (*Tsg101*, *Skp2*, and *RAD6*) and mitosis (*Aurora-A*) (reviewed in (2), (32), (58), (59)).

Deletion or mutational/functional inactivation of p53 leads to centrosome amplification (60). Because *TP53* gene mutations are genotypic hallmarks of “secondary” glioblastomas, which arise as a consequence of malignant change in pre-existing diffuse low-grade astrocytomas (42, 43), we

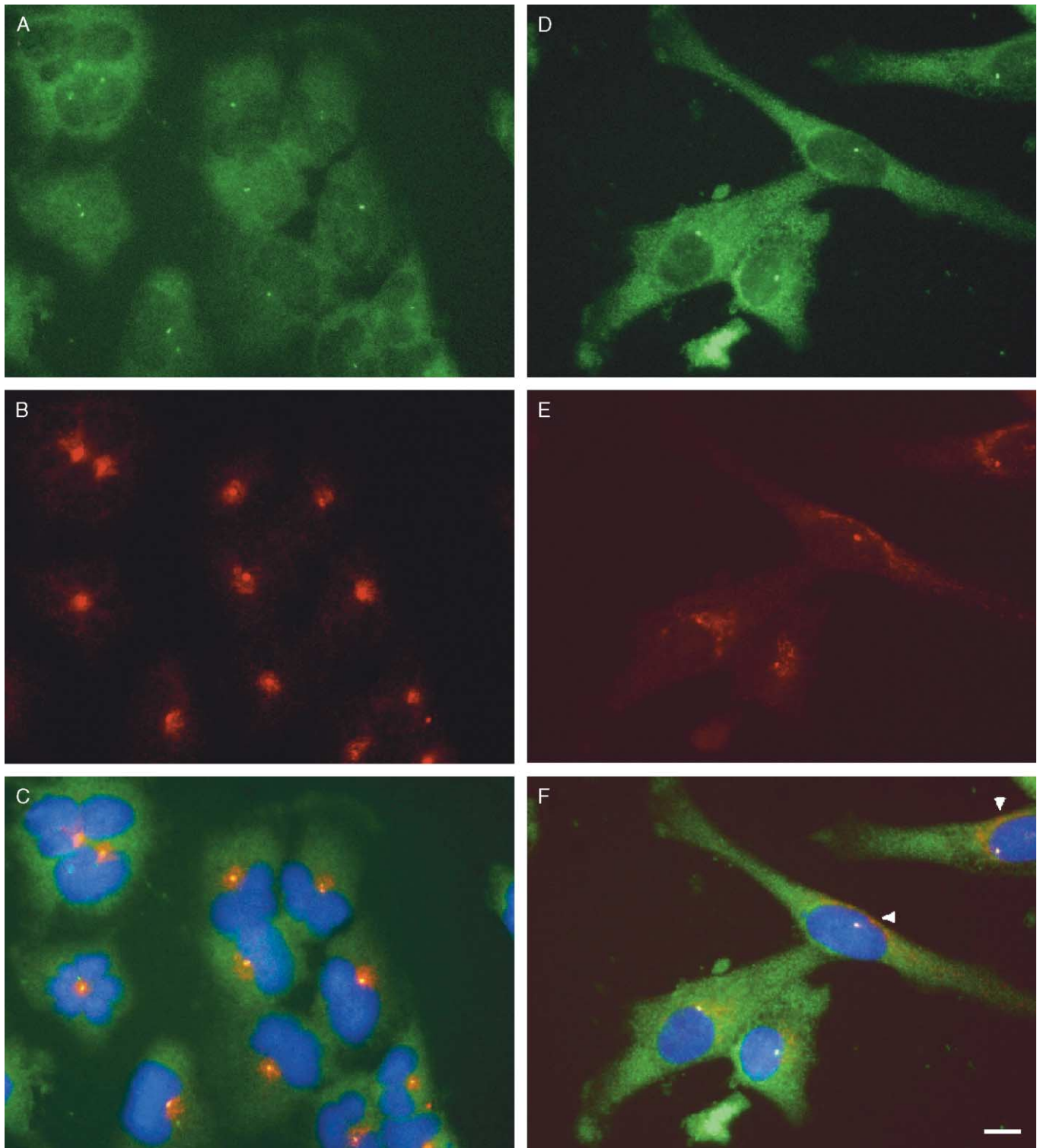


FIGURE 8. Distribution of γ -tubulin and pericentrin in the human glioblastoma cell line T98G (**A–C**) and U118MG (**D–F**). Cells were stained by double labeling with monoclonal antibody TU-31 specific for γ -tubulin (**[A–D]**, green) and polyclonal antibody specific for pericentrin (**[B, E]**, red). Superposition of stainings is shown in panels (**C**) and (**F**). There is spatial dissociation between these 2 centrosome-associated proteins. Pericentrin staining is present in the juxta-nuclear pericentriolar region. Although colocalization of γ -tubulin (green) and pericentrin (red) was demonstrated in normal juxta-nuclear centrosomes (yellow overlay), the distribution of γ -tubulin is more widespread displaying a confluent punctate or diffuse localization extending to the periphery of tumor cells. DAPI (blue) labels nuclei. Scale bar = 10 μ m for all panels.

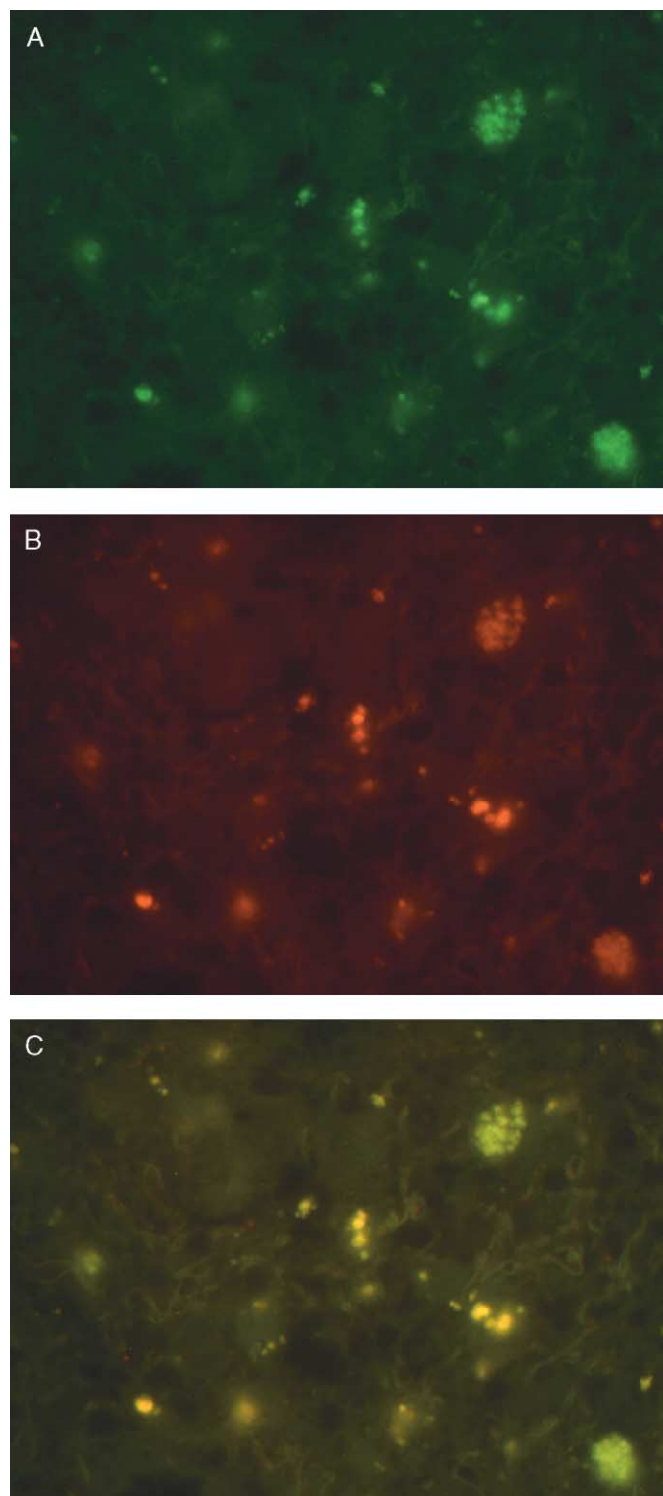


FIGURE 9. Double labeling showing colocalization of γ -tubulin (**A**), green) and pericentrin (**B**), red) in scattered neoplastic cells of a low-grade diffuse astrocytoma of the parietal lobe (grade II). Acquisition of a yellow color in the merged image (**C**) indicates codistribution.

postulate that mutational inactivation of p53 may potentially result in γ -tubulin amplification in these tumors.

17q21: A Region Comprising Genes for γ -Tubulin and Other Proteins Involved in Cancer

The locus of the gene encoding for γ 1- and γ 2-tubulins has been mapped on chromosome 17q21 where it is centromeric to BRCA-1 (61). Like γ -tubulin, the BRCA-1 gene product is expressed in the pericentriolar material of centrosomes (2). BRCA-1 plays a pivotal role in ensuring the faithfulness of centrosome duplication and spindle formation, thus preventing genomic instability (23). During mitosis, BRCA-1 interacts with γ -tubulin in the centrosome (62). It has recently been shown that γ -tubulin constitutes a ubiquitination target of BRCA-1 (63). The number of centrosomes in non-transformed, diploid cells is regulated by BRCA1-dependent ubiquitination of γ -tubulin and inhibition of BRCA1 function leads to amplification and fragmentation of centrosomes (63).

Among other genes mapped in the 17q21 region are those encoding for glial fibrillary acidic protein, keratins 10 and 13, vesicle amine transport protein 1, members 3 and 15 of the wiggless-type MMTV integration site family, homeo-box genes B1–B9, and the growth factor receptor-bound protein 7 (Grb7) (64). The gene encoding for Grb7 (17q21–22q) is upregulated in esophageal cancer (65). 17q21 is the locus for the human granulocyte colony-stimulating factor (G-CSF) gene (66). G-CSF protein is expressed in primary and recurrent astroglial tumors (67, 68). Also, abnormalities in 17q21 have been reported in astrocytic gliomas (69).

Potential Significance of γ -tubulin Abnormalities in Tumor Cell Motility and Architecture

To date, most studies on the relationship of centrosome amplification and cancer have focused on the impact of centrosomal dysfunction on genetic stability. However, considering that centrosomes determine the cell cleavage planes and symmetry of cytoplasmic division, the impact of centrosomal abnormalities on tumor morphology and architecture can provide critical insights into the cytoskeletal changes of malignant and invasive tumor phenotypes (2, 24, 27). Importantly, by controlling the number, polarity, and distribution of microtubules, centrosomes govern and coordinate all microtubule-related functions (2).

Cancer cells, particularly the highly malignant or anaplastic tumor phenotypes, may exhibit aberrant microtubule nucleation resulting in modified microtubule properties as reflected by the altered expression or posttranslational modifications of proteins of the microtubule cytoskeleton, including tubulin isoforms (70). Thus, the microtubule nucleation capacity of aberrant centrosomes may be reduced or enhanced, depending on the identity and modification of the overexpressed components of the pericentriolar material (2). Such altered microtubule organizing centers will affect the synthesis and isotype composition of tubulin, rendering dynamically unstable microtubules, thus contributing to abnormalities of cell shape, polarity, adhesion, and motility, including invasion. Khodjakov and Rieder have shown that

at the onset of mitosis, the centrosome suddenly gains the ability to bind several times the amount of γ -tubulin than during interphase, which does not require microtubules (71). It follows then that mitotically active tumor cells may require increased amounts of γ -tubulin. Moreover, since the recruitment of γ -tubulin ring complexes is a prerequisite for increased microtubule-nucleating activity, in addition to the regulation of tubulin synthesis and cycle progression are (13), the increased expression of γ -tubulin may potentially culminate in aberrant expression of tubulin isoforms in gliomas (Katsetos et al, unpublished observation). In an oligonucleotide microarray analysis of high- versus low-grade gliomas, Rickman and colleagues observed that genes encoding for a number of cytoskeletal and cytoskeleton-associated proteins, including γ -tubulin and β IV-tubulin, were highly expressed in glioblastomas (72).

Interestingly, both diffuse and multipunctate γ -tubulin staining was detected in endothelial cells in areas of microvascular proliferation in glioblastomas. This indicates that centrosome abnormalities may underlie neoplastic neovascularization in gliomas. It has recently been shown that endothelial cells in solid tumors are aneuploid and are associated with multiple centrosomes (73). Studies are underway in our laboratory to elucidate the role of γ -tubulin in glioblastoma angiogenesis.

Our results indicate that ectopic cellular expression of γ -tubulin in diffuse astrocytic gliomas may be significant in the context of centrosome dysfunction/amplification and may be linked to tumor progression where it may potentially serve as a novel marker of anaplastic change. It remains to be determined in a significantly larger cohort of tumor specimens whether centrosomal defects, including derangements in the expression of γ -tubulin (which may precede alterations of genomic stability) may lay the foundation for a new and promising approach to molecular stratification of gliomas.

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