Overexpression of γ-tubulin in non-small cell lung cancer

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Summary. We and others have previously shown that increased expression and altered compartmentalization of γ-tubulin may contribute to tumorigenesis and tumor progression (J. Cell Physiol. 2009;223:519-529; Cancer Biol. Ther. 2010;9:66-76). Here we have determined by immunohistochemistry the localization and cellular distribution of γ-tubulin in clinical tissue samples from 109 non-small cell lung cancer (NSCLC) cases. The expression and distribution of γ-tubulin protein and transcripts was also determined in the NSCLC tumor cell lines NCI-H460 (HTB-177) and NCI-H69 (HTB-119) by immunocytochemistry, quantitative immunoblotting and reverse transcription quantitative real-time PCR (RT-qPCR). Polyclonal and monoclonal anti-peptide antibodies recognizing epitopes in the C- or N-terminal domains of γ-tubulins and human gene-specific primers for γ-tubulins 1 (TUBG1) and 2 (TUBG2) were used. In non-neoplastic cells of the airway epithelium in situ, γ-tubulin exhibited predominantly apical surface and pericentriolar localizations. In contrast, markedly increased, albeit heterogeneous and variously prominent γ-tubulin immunoreactivity was detected in clinical tumor specimens and in the NCI-H460 and NCI-H69 cell lines, where tumor cells exhibited overlapping multi-punctate and diffuse patterns of localization. Co-expression of γ-tubulin and Ki-67 (MIB-1) was detected in a population of proliferating tumor cells. A statistically significant increase of γ-tubulin expression was found in Stage III compared to lesser stage tumors (p<0.001 v. Stages I/II) regardless of histological subtype or grade. By quantitative immunoblotting NCI-H460 and NCI-H69 cells expressed higher levels of γ-tubulin protein compared to small airway epithelial cells (SAEC). In both tumor cell lines increase in TUBG1 and TUBG2 transcripts was detected by RT-qPCR. Our results reveal for the first time an increased expression of γ-tubulin in lung cancer.

Key words: Gamma-tubulin, Microtubules, Non-small cell lung cancer, NSCLC, Adenocarcinoma, Squamous cell carcinoma

Introduction

Lung cancer is a major public health issue and a formidable therapeutic challenge. Most lung tumors are of epithelial origin and belong to the category of non-small cell lung cancer (NSCLC), which is responsible for 80 % of all lung cancers. According to the World Health Organization (WHO), NSCLC encompasses three main histologic types, adenocarcinoma (AC), squamous cell carcinoma (SCC), and the less common large cell carcinoma (LCC) (Travis et al., 2004). Recent trends indicate that tumors of the NSCLC category are generally treated according to similar strategies without the need for further distinction among histologic types.
(Hirsch et al., 2008). Others contend that a histopathologic distinction between SCC and AC remains relevant in dictating chemotherapeutic strategies in advanced NSCLC (Selvaggi and Scagliotti, 2009). At initial diagnosis, more than half of NSCLCs correspond to advanced clinical stage and patients with advanced NSCLC are candidates for systemic chemotherapy (Sève and Dumontet, 2005).

The role of tubulin in NSCLC has been the focus of previous translational research (Katsetos et al., 2000; Dumontet et al., 2005; Sève et al., 2005a,b, 2007, 2010). To date, large clinical trial studies have focused solely on βIII-tubulin and its roles in tumor prognosis and taxane chemoresistance (Dumontet et al., 2005; Sève et al., 2005a,b, 2007, 2010). The elucidation of microtubule abnormalities in lung cancer and the development of new therapeutic approaches would be further enhanced by the characterization of altered expression of tubulin species involved in microtubule nucleation, which may also potentially serve as clinical biomarkers and molecular targets for cancer therapy.

γ-Tubulin is the principal cytoskeletal constituent of the pericentriolar material of centrosomes, the cell’s microtubule organizing centers (MTOCs), where it plays a central role in microtubule nucleation and in the regulation of cell cycle progression (Zhou et al., 2002). Experimental depletion of γ-tubulin leads to a depletion of microtubules and to growth arrest. In mammalian cells, two γ-tubulin genes TUBG1 and TUBG2 exist, encoding two closely related isotypes (Wise et al., 2000). TUBG1 is ubiquitously expressed, whereas TUBG2 has been found mainly in the brain (Yuba-Kubo et al., 2005; Vinopal et al., 2012).

Although γ-tubulin is localized on MTOCs, a larger amount of γ-tubulin is in soluble form (Moudjou et al., 1996; Sulimenko et al., 2002). γ-Tubulin appears in two main complexes: the large γ-tubulin ring complex (γTuRC) (Moritz et al., 1995; Zheng et al., 1995) and the γ-tubulin small complex (γ-TuSC) (Moritz et al., 1998). The existence of γTuRCs correlates with the ability of centrosomes to nucleate microtubules (Schnackenberg and Palazzo, 2001). Additionally, γ-tubulin is associated with cellular membranes (Chabin-Brion et al., 2001; Dryková et al., 2003; Bugnard et al., 2005; Sulimenko et al., 2006) where it can give rise to non-centrosomal microtubule nucleation (Chabin-Brion et al., 2001; Efimov et al., 2007; Macurek et al., 2008). Nucleus-specific functions of γ-tubulin were recently reported (Hofejší et al., 2012; Hőög et al., 2011).

The role of γ-tubulin in microtubule nucleation and microtubule nucleation-independent functions in non-transformed cells, as well as the altered expression, distribution and subcellular sorting of this protein in cancer cells have been reviewed (Katsetos et al., 2009). We have previously shown overexpression and ectopic compartmentalization of γ-tubulin in malignant brain tumors and have suggested that this can be one of the putative mechanisms of tumorigenesis and tumor progression (Katsetos et al., 2006, 2007, 2009; Caracciolo et al., 2010). Moreover, recent studies have shown increased expression and ectopic soluble distribution of γ-tubulin with decoupling/ dissociation from the centrosomes in aggressive breast cancer cell lines (Cho et al., 2010).

To get a broader insight into the expression of γ-tubulin in lung tumors, we have analyzed the expression and cellular distribution of this protein in a large cohort of NSCLC pathological specimens with comparison to the expression of γ-tubulin protein and mRNA in the human NSCLC cell lines NCI-H460 and NCI-H69.

Materials and methods

Cells

Human lung cell carcinoma cell lines NCI-H460 (Catalog. No. ATCC-HTB-177) and NCI-H69 (Catalog No. ATCC-HTB-119) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10 % fetal bovine serum, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Cells were grown at 37°C in 5 % CO₂ in air and passed every 2 or 3 days. In the case of adherent H460 cells 0.25 % trypsin/0.01% EDTA in PBS, pH 7.5 was used. Proliferating non-immortalized, non-transformed human small airway epithelial cells (SAEC) were obtained from Lonza (Cologne, Germany), and were maintained in supplemented Small Airway Epithelial Cell Basal Medium according to the manufacturer’s directions.

Antibodies

The following anti-peptide antibodies against human γ-tubulin were used: mouse monoclonal antibodies TU-30 and TU-32 to the sequence 434-449 (Nováková et al., 1996); mAb GTU-88 to the sequence 38-53 (Sigma, T6657); rabbit antibodies to sequences 433-451 (DQ-19; Sigma, T3195) and 38-53 (Sigma, T5192). The monoclonal antibody supernatants TU-30 and TU-32 were stored in freeze-dried state in the presence of trehalose (Dráber et al., 1995). Monoclonal antibody GTU-88 and polyclonal antibody (Sigma, T 5192) were raised against peptide sequence (38-53) which is identical both in human γ-tubulin 1 and human γ-tubulin 2. Monoclonal antibodies TU-30, TU-32 and polyclonal antibody DQ-19 were raised against peptides that differ only in one amino acid when sequences of human γ-tubulin 1 and γ-tubulin 2 were compared. A rabbit antibody to actin was purchased from Sigma (A2066) and Cy3-conjugated anti-mouse antibody from Jackson Immunoresearch Laboratories (West Grove, PA). Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit antibodies were obtained from Promega (Madison, WI).

Reverse transcription quantitative real-time PCR (RT-qPCR analysis)

Total RNA from cultured cells was isolated by the
were used as 10 times diluted supernatants, while monoclonal antibody GTU-88 and polyclonal antibody to actin were diluted 1:10,000 and 1:3,000 respectively. Bound primary antibodies were detected after incubation of the blots with HRP-conjugated secondary antibodies diluted 1:10,000. HRP signal was detected with chemiluminescence reagents (Pierce, Rockford, IL) and quantified using LAS 3000 imaging system (Fujifilm, Tokyo, Japan) and AIDA (ver. 4) software (Raytest, Straubenhardt, Germany). Statistical analysis was performed with the Student’s two-tailed unpaired t-test using Microsoft Excel.

**Immunofluorescence on cells in culture**

Immunofluorescence microscopy on methanol-fixed cells was performed as described previously (Dráberová and Dráber, 1993). TU-30 antibody was used as undiluted supernatant. Cy3-conjugated anti-mouse antibodies was diluted 1:500. The preparations were mounted in MOWIOL 4-88 (Calbiochem, San Diego, CA) and examined with an Olympus A70 Provis microscope. Conjugate alone did not give any detectable immunoreactivity.

**Clinical tumor samples**

Formaldehyde-fixed, paraffin-embedded biopsy/resection samples of NSCLC (n=109) were collected retrospectively from “KAT” and “Sismanoglion” General Hospitals, Athens, Greece. Ninety-three patients were male (86.32 %) the mean being 63 years (range 40 to 81 years). The lung tumors were classified histologically according to the WHO criteria as follows: AC (n=56), SCC (n=49), adenosquamous carcinoma (n=3) and LCC (n=1). Among ACs, 52 were classified as “not otherwise specified,” 2 as bronchioloalveolar, 1 as clear cell, and 1 as fetal. Furthermore, the degree of differentiation was evaluated, distinguishing between well differentiated (n=12), moderately differentiated (n=41) or poorly differentiated tumors (n=56) (grades I through III respectively). From the total number of cases examined, clinical staging information was available in 63 cases as follows: Stage I (n=29), Stage II (n=11) and Stage III (n=23). Control tissues included 10 age-matched non-neoplastic lung biopsy specimens, 5 showing varying degrees of interstitial fibrosis and 5 with interstitial pneumonitis. Histologic preparations were evaluated independently by four pathologists, (NM, EM, MC, CDK), who were unaware of the original pathological diagnosis. In the case of disagreement between observers, the diagnosis was assigned by consensus.

**Immunohistochemistry on clinical tumor samples**

Immunohistochemistry was performed according to the avidin biotin complex (ABC) peroxidase method using Rabbit and Mouse IgG ABC Elite® detection kits (Vector Labs, Burlingame, CA) as previously described.
(Katsetos et al., 2006, 2007). Prior to the performance of the immunohistochemical procedure, 5 μm thick histological sections from paraffin-embedded tissue blocks were subjected to non-enzymatic antigen unmasking in 0.01 M sodium citrate buffer (pH 6.0) for 10 minutes in a microwave at medium power. Both rabbit polyclonal antibodies to γ-tubulin (Sigma T5192, T3195) were used at 1:500 dilution, whereas anti-γ-tubulin monoclonal antibodies GTU-88 and TU-32 were used at a 1:300 dilution, or as undiluted supernatants, respectively. Negative controls included omission of primary antibody and substitution with nonspecific mouse IgG1 and IgG2a, which were used as immunoglobulin class-specific controls (corresponding to the immunoglobulin subclasses of the primary antibodies employed in this study) (Becton Dickinson, Franklin Lakes, NJ). Preparations using non-conjugated isotype matched control monoclonal antibodies did not show any non-specific binding of the secondary rabbit anti-mouse IgG1 and IgG2a antibodies. For the evaluation of immunoperoxidase staining, a Nikon Eclipse E400 brightfield microscope equipped with a digital Leica camera (model BFC 280) was used.

Cell counting

Manual cell counting of labeled tumor cells was performed by three observers independently (NM, EM, CDK). Cell counting and statistical analysis were performed with all antibodies employed. The cases were grouped according to histological subtype, grade and stage. To assess the fraction of immunolabeled tumor cells in each specimen, the labeling index (LI), defined as the percentage (%) of γ-tubulin-labeled cells out of the total number of tumor cells counted in each case and for each antibody, was determined as previously described (Katsetos et al., 2001). Between 258 and 805 tumor cells were evaluated per case, in 10 non-overlapping high-power (40x) fields, and LIs were generated per each specimen. The criteria for the identification of a γ-tubulin-positive cell were the detection of 3 or more punctate/dot-like immunoreactive signals and/or robust diffuse staining in the cytoplasm of individual tumor cells as previously described (Katsetos et al., 2006). The level of interobserver agreement was quantitated using generalized kappa and pairwise kappa statistics (Fleiss, 1981; Landis and Koch, 1997). Interobserver agreement was substantial (k=0.65). In the case of disagreement (+/- 10 % in the recording of the LI between the two observers) the immunohistochemical preparations were re-reviewed at a multi-headed microscope in order to achieve consensus. For purposes of LI recording, the consensus opinion was considered as conclusive. For statistical analysis SPSS software was used (SPSS for Windows, version 13, IBM SPSS, Chicago, IL). All p-values were two-tailed and 5% was chosen as the level of statistical significance. Correlation of γ-tubulin with baseline characteristics was performed using Mann-Whitney, Kruskal-Wallis and χ² tests. A p value of less than 0.05 was considered as statistically significant. Because of the small number of adenosquamous and LCC included in this study, these cases were excluded from the statistical analysis.

Immunofluorescence on clinical tumor samples

Deparaffinized histological sections from selected surgically excised NSCLC tissue specimens were utilized for immunofluorescence microscopy. Prior to the performance of the immunological staining, the tissue sections were subjected to non-enzymatic antigen unmasking in 0.01 M sodium citrate buffer (pH 6.0) for 10 min in a microwave at medium power. The slides were then rinsed with PBS and incubated for 1 hour in blocking solution (6% BSA in PBS). The mouse monoclonal antibody to Ki-67 (clone MIB-1) (sc-101861) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at 1:100 dilution in PBS containing 1% BSA. The rabbit polyclonal antibody to γ-tubulin (DQ-19) was used at 1:500 dilution in PBS containing 1% BSA. Secondary fluorochrome conjugated antibodies which included goat anti-mouse AlexaFluor 488 (green) and goat anti-rabbit AlexaFluor 594 (red) (Invitrogen, Carlsbad, CA) were diluted 1:600 with PBS containing 1% BSA. Double staining was performed in two sequential sessions of immunofluorescence staining. At the end of the staining the slides were incubated for 15 minutes in a freshly made PBS solution containing 1mg/ml of sodium borohydride (Sigma) to decrease the autofluorescence, and subsequently they were rinsed extensively with PBS. 4′,6-diamidino-2-phenylindole (DAPI) was used to label cell nuclei. Slides were coverslipped with Vectashield mounting medium (Vector Labs, Burlington, CA) and visualized with an Olympus IX81 deconvolution fluorescence microscope.

Results

Immunohistochemical localization in clinical tumor samples

In non-neoplastic cells of the ciliated airway epithelium, γ-tubulin exhibited predominantly apical surface (Fig. 1a-c) and pericentriolar/centrosomal localizations (Fig. 1d - thick arrow) and to a lesser extent a soluble distribution (Fig. 1b,c). Apical surface labeling was localized to the base of the cilia in the form of a linear appearance corresponding to the basal bodies (Fig. 1a,b - curved arrow, 1c - thick arrows). In contrast, the cilia themselves were uniformly γ-tubulin-negative (Fig. 1a,b - straight arrow, 1c).

Overall, a variously prominent, albeit distinctly heterogeneous γ-tubulin distribution was detected in all NSCLC specimens (Figs. 2,3). Varying degrees of γ-tubulin labeling were detected in all histological types (SCCs and ACs) and tumor grades (I-III). In SCCs, γ-tubulin labeling was noted focally either among individual tumor cells (Fig. 2a - straight arrow) or in...
clusters of neoplastic cells within tumor sheets (Fig. 2b). Occasional tumor cells exhibited staining in the periphery of the cytoplasm (Fig. 2c - straight arrow). Strong cytoplasmic staining was noted in large, pleomorphic cells, surrounding areas of necrosis, including in tadpole-shaped tumor cells (Fig. 2d-f), some with γ-tubulin-positive nuclear pseudoinclusions (Fig. 2f - thick arrow). γ-Tubulin immunoreactive cells displayed an overall tendency for the periphery of tumor sheets and for perivascular areas (not shown). Spindle cells were less immunoreactive as compared to large pleomorphic cells (not shown).

In ACs, there was variable γ-tubulin staining in glandular (Fig. 3a-c, 3e-short arrows) and papillary areas (Fig. 3d) with a trend for increased immunoreactivity in less differentiated areas (Fig. 3e- straight arrows, 3f-i). Strong γ-tubulin labeling was observed in small clusters of invasive poorly differentiated tumor cells (Fig. 3g - straight arrows). However, in some tumors, well-differentiated glandular areas exhibited robust and diffuse cytoplasmic staining (Fig. 3a-c). In gland-forming tumor cells, localization was diffuse but in some tumor cells was more pronounced in the apical surface portion of the cytoplasm (Fig. 3c - short arrow) recapitulating the superficial apical localization of this protein in normal ciliated bronchial epithelium localized to the base of the cilia (Fig. 1a-d). In 2 well-differentiated papillary ACs, apical protrusions in columnar cells of tumor glands, resembling cilia, were γ-tubulin negative (Fig. 3c and 3e-short arrows). In both SCCs and ACs immunoreactivity for γ-tubulin was heterogeneous and, where present, it was characterized by overlapping multi-punctate and diffuse staining patterns (Figs. 2, 3e,f,h - thick arrow, and 3i). Identical immunoreactivity profiles were obtained with the different polyclonal and monoclonal anti-γ-tubulin antibodies employed.

The mean LIs for SCCs versus ACs relative to histological grade are tabulated on Table 1 and the median LIs of combined NSCLCs relative to stage are shown in box plot form on Figure 4. Grade-for-grade, a trend toward increased immunoreactivity was observed.
in grade 3 as compared to grade 1 tumors, regardless of histological subtype, which, however, was not statistically significant. Conversely, a statistically significant difference between expression of γ-tubulin and clinical stage -- regardless of histological subtype or tumor grade-- was found, with the highest mean LI detected in Stage III tumors (p<0.001 vs. Stage I and Stage II) (Fig. 4).

Co-expression of γ-tubulin and Ki-67 (MIB-1) was detected in populations of non-proliferating (Fig. 5a-c) and proliferating tumor cells (Fig. 5d-f) from all histological types and grades of NSCLC. The localization of γ-tubulin in tumor cells was predominantly cytoplasmic (diffuse and punctate patterns) (Fig. 5a,c) in contrast to the localization of Ki-67, which was predominantly nuclear (Fig. 5b,e). Nuclear co-localization of γ-tubulin and Ki-67 (MIB-1) was also detected in a subpopulation of proliferating tumor cells (Fig. 5d-f).

Expression and cellular localization of γ-tubulin in H460 and H69 versus SAEC

Immunofluorescence microscopy revealed more prominent γ-tubulin staining in cytoplasm and MTOCs in H460 cells as compared to SAEC (Fig. 6A, panels “b” and “a” respectively). It is well established that anti-γ-tubulin antibodies intensively label dots in paranuclear/juxta-nuclear positions, which correspond to MTOCs (Nováková et al., 1996), as confirmed also by staining with anti-pericentrin antibody (Katsetos et al., 2006). It was difficult to compare γ-tubulin staining in H69 with that in SAEC, as H69 cells are substantially

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<th>Table 1. γ- Tubulin labeling index versus histologic type and grade in NSCLC</th>
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LI: Labeling index, defined as the percentage (%) of γ-tubulin-labeled cells out of the total number of tumor cells counted in each case and for each antibody, as previously described (Katsetos et al., 2006). SCC: Squamous cell carcinoma

Fig. 2. Immunolocalization of γ-tubulin in squamous cell carcinomas (SCCs) using rabbit polyclonal antibody (Sigma, T5192) (a-c) and mouse monoclonal antibody GTU-88 (Sigma, T6657) (d-f) both of which are specific for the sequence 38-53 of γ-tubulin. c is a higher magnification of panel a. ABC peroxidase with hematoxylin counterstaining. Scale bars: a, 100 µm; b, 50 µm; d-f, 20 µm.
smaller and grow in clusters in suspension. However, when H69 cells were attached to poly-L-lysine coated coverslips, prominent γ-tubulin staining in cytoplasm and MTOC was observed (not shown). Immunoblot analysis with monoclonal antibody TU-32 revealed differential staining of blots prepared from total cell lysates of SAEC, H460 and H69 cells (Fig. 6B). A representative example of immunoblot staining and densitometric quantification of immunostaining is presented in adjacent panels in Figure 6B revealing that H460 and H69 cells expressed substantially higher levels of γ-tubulin protein as compared to SAEC. Similar differential staining of blots was observed with the other monoclonal antibodies (TU-30, GTU-88), as well as with both polyclonal antibodies (Sigma, T3195, T5192) to γ-tubulin (not shown).

By RT-qPCR, γ-tubulin transcripts for TUBG1 and TUBG2 genes were detected in SAEC, H460, and H69 cells. However, transcripts for both TUBG1 and TUBG2 genes were more abundant in H460 and H69 cells when compared with SAEC (Fig. 7). Altogether the data clearly indicate, both at the protein and mRNA levels, that γ-tubulins are overexpressed in NSCLC cell lines as compared to SAEC.

**Discussion**

Expression and cellular distribution of γ-tubulin in NSCLC cells

In the present study we have produced novel evidence for an increased γ-tubulin expression in surgically excised NSCLC specimens and in the NCI-H460 and NCI-H69 NSCLC cell lines. This is, to our
knowledge, the first study to demonstrate increased expression of γ-tubulin in lung cancer.

Diploid cells contain one or two juxtanuclear centrosomes typified by pericentriolar staining for γ-tubulin (Katsetos et al., 2006). This pattern is faithfully reproduced in cells of the human respiratory epithelium wherein in addition to pericentriolar γ-tubulin staining there is evidence of localization in apical adluminal cytoplasmic region near the ciliated brush border. This localization corresponds to putative basal bodies at the base of the cilia in multiciliated epithelia of the bovine trachea and oviduct (Muresan et al., 1993), retinal photoreceptors (Muresan et al., 1993) and in mouse cochlear epithelial cells (Mogensen et al., 1997).

Although NSCLC cells recapitulate -in part- this apical surface and pericentriolar patterns of distribution, they also exhibit variously prominent, diffuse cytoplasmic γ-tubulin staining indicating that this protein, which is normally associated with basal bodies and centrosomes in ciliated epithelia, undergoes altered compartmentalization in tumors where it is either incorporated into insoluble aggregates, associated with membraneous components, or becomes part of an increased soluble pool in the cytoplasm of tumor cells. Cho and coworkers have shown that aggressive breast cancer cell lines with metastatic potential exhibit a

![Box plot of γ-tubulin labeling indices versus stage (p<0.001). Boxes correspond to the values between the 25th and 75th percentiles [Q1-Q3]; the dark line in the middle is the median value; upper and lower whiskers to the highest and lower values of distribution; values in circles are at least 1.5 greater than the 75th percentile; and value in asterisk is at least 3.0 greater than the 75th percentile.](image)

**Fig. 4.** Box plot of γ-tubulin labeling indices versus stage (p<0.001). Boxes correspond to the values between the 25th and 75th percentiles [Q1-Q3]; the dark line in the middle is the median value; upper and lower whiskers to the highest and lower values of distribution; values in circles are at least 1.5 greater than the 75th percentile; and value in asterisk is at least 3.0 greater than the 75th percentile.

![Double labeling of γ-tubulin and Ki-67 (MIB-1) in clinical tumor samples as determined by immunofluorescence microscopy. Note localization of γ-tubulin (a and d, red) and Ki-67 (MIB-1) (b and e, green) in tumor cells. a-c depict distinct localizations of γ-tubulin and Ki-67 (MIB-1) in populations of non-proliferating and proliferating tumor cells respectively. Conversely, d-f illustrate a pattern of (predominantly cytoplasmic) γ-tubulin and (nuclear) Ki-67 (MIB-1) co-expression in individual tumor cells (representative field from a case of poorly differentiated adenocarcinoma, Grade 3, Stage III). Also, note co-localization of both antigens in the nuclei of a subpopulation of poorly differentiated tumor cells. Scale bar: 50 μm.](image)

**Fig. 5.** Double labeling of γ-tubulin and Ki-67 (MIB-1) in clinical tumor samples as determined by immunofluorescence microscopy. Note localization of γ-tubulin (a and d, red) and Ki-67 (MIB-1) (b and e, green) in tumor cells. a-c depict distinct localizations of γ-tubulin and Ki-67 (MIB-1) in populations of non-proliferating and proliferating tumor cells respectively. Conversely, d-f illustrate a pattern of (predominantly cytoplasmic) γ-tubulin and (nuclear) Ki-67 (MIB-1) co-expression in individual tumor cells (representative field from a case of poorly differentiated adenocarcinoma, Grade 3, Stage III). Also, note co-localization of both antigens in the nuclei of a subpopulation of poorly differentiated tumor cells. Scale bar: 50 μm.
diffuse soluble, and more dispersive, subcellular localization of γ-tubulin as compared to non-invasive cell lines in which the localization of this protein is largely centrosomal (Cho et al., 2010). In addition to its traditional role in microtubule nucleation at the pericentriolar material of centrosomes, γ-tubulin can be found in cytosolic non-centrosomal nucleating-sites (Chabin-Brion et al., 2001; Efimov et al., 2007; Macurek et al., 2008) as well as in nuclei/nucleoli (Hořejší et al., 2012), as was also demonstrated in NSCLC cells in the present study.

Translational studies on γ-tubulin overexpression in cancer

Centrosome abnormalities in cancer are characterized by increased, or otherwise altered, expression of centrosomal proteins, including γ-tubulin and pericentrin (Pihan et al., 1998, 2001; Sato et al., 1999; Kuo et al., 2000; Setoguchi et al., 2001; Katsetos et al., 2006, 2007, 2009). We and others have previously shown γ-tubulin overexpression in glioblastomas (Katsetos et al., 2006, 2007, 2009; Loh et al., 2010) and medulloblastomas (Caracciolo et al., 2010). The present study has revealed a statistically significant increase of γ-tubulin expression in Stage III as compared to lesser stage tumors without statistically significant differences in the expression of this protein according to histological subtype or tumor grade. This suggests that γ-tubulin may serve potentially as a prognostic/predictive biomarker in the setting of NSCLC. However, the latter requires future validation in a larger cohort of cases with survival data. A recent study has shown that γ-tubulin may be promising marker of recurrence of SCC of the larynx (Syed et al., 2009). Interestingly, increased γ-tubulin immunostaining in laryngeal cancer has been found to be

Fig. 6. Comparison of γ-tubulin expression in non-transformed small airway epithelial cells (SAEC) and NSCLC cell lines (H460 and H69). A. Immunofluorescence staining of SAEC (a) and H460 cells (b) with monoclonal antibody TU-30 to γ-tubulin. Images were collected and processed in exactly the same manner. Fixation with methanol. Scale bar: 20 µm. B. Immunoblot analysis of whole cell extracts from SAEC, H460, and H69 cells. Blots were immunostained with monoclonal antibody TU-32 to γ-tubulin and polyclonal antibody to actin. Densitometric quantification of immunoblot is shown on the right. Data are presented as the mean fold change ± SD obtained from three independent experiments with triplicate samples. Mean values different from SAEC levels are indicated: ***, p<0.001.
γ-tubulin in NSCLC

Fig. 7. Transcription of genes for γ-tubulin 1 (TUBG1) and γ-tubulin 2 (TUBG2) in lung cell carcinoma cell lines H460 and H69 relative to the level in non-transformed small airway epithelial cells (SAEC). Data are presented as the mean fold change ± SD obtained from two independent experiments with triplicate samples. Mean values different from SAEC levels are indicated: **, p<0.005.

Comparison to the multiple genes encoding for α and β-tubulin isotypes (Banerjee et al., 2008), there are only two functional genes in mammalian cells (TUBG1, TUBG2) that code very similar γ-tubulin species (Wise et al., 2000; Yuba-Kubo et al., 2005). TUBG1 is ubiquitously expressed, whereas TUBG2 is expressed mainly in the brain (Yuba-Kubo et al., 2005). Consistent with this differential tissue distribution pattern between the two functional γ-tubulin genes, in the present study we have observed an increase both in TUBG1 and TUBG2 by RT-qPCR. Conversely, molecular profiling has revealed overexpression of TUBG2 in prostate cancer cells (Li et al., 2005) and in glioblastoma cell lines (Katsetos et al., 2010). In thyroid carcinomas (Montero-Conde et al., 2008), gliomas (Rickman et al., 2001), pediatric pilocytic astrocytomas (Potter et al., 2008), and in medulloblastomas (Caracciolo et al., 2010) an increase in TUBG1 was detected. Moreover, changes in the expression of both TUBG1 and TUBG2 genes have been reported in breast cancer cells (Orsetti et al., 2004).

Conclusion

In summary, our results show for the first time in the context of lung cancer that NSCLC cells from surgically excised tumor samples are accompanied by highly prominent expression and ectopic cellular distribution of γ-tubulin, as compared to non-neoplastic cells of the respiratory epithelium. However, the significance of this overexpression and altered compartmentalization remains to be determined in future functional studies focusing on the role of this protein in ectopic microtubule nucleation, and cell cycle progression.

Given the well-established overexpression of βIII-tubulin in NSCLCs (Katsetos et al., 2000; Sève and Dumontet, 2005; Sève et al., 2010), it is unclear whether increased co-expression of βIII-tubulin/γ-tubulin enhances or diminishes chemotherapeutic efficacy in NSCLC subsets. This provides the rationale for the performance of further studies focusing on the effect of novel tubulin binding agents on γ-tubulin-overexpressing or silenced human NSCLC cells in vitro or in xenografts. In addition, large clinical randomized studies are necessary to further elucidate the prognostic or predictive value of γ-tubulin in the context of different clinical stages, histological types, tumor grades, and treatment settings.

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