β-Tubulin Isotype Classes II and V Expression Patterns in Nonsmall Cell Lung Carcinomas

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Previous studies suggest that β-tubulin isotype protein levels could be useful as indicators of nonsmall cell lung cancer (NSCLC) aggressiveness. However, measurement of protein amounts in tissue samples by staining techniques is semiquantitative at best. Since technologies for measuring mRNA levels have become more efficient and quantitative, we wanted to determine whether β-tubulin message levels may be useful as biomarkers. Quantitative real-time RT-PCR was used to measure the seven classes of β-tubulin isotypes, stathmin and MAP4 mRNA levels in 64 NSCLC and 12 normal lung tissue samples. We found significantly higher fractions of β-tubulin classes II and V mRNA compared to the other isotypes in all lung tumor samples (P < 0.05). In addition, the ratio of β-tubulin classes II/V mRNA was significantly higher in NSCLCs compared to normal lung tissues (P < 0.001). The data suggest that the ratio of β-tubulin classes II and V mRNA could be useful as a biomarker for NSCLC tumor differentiation and/or NSCLC aggressiveness. Furthermore, the ratio of MAP4 to stathmin mRNA was found to be higher in diseased lung tissues compared to normal lung tissues, suggesting this ratio might also be used as a clinically relevant biomarker for NSCLCs. Cell Motil. Cytoskeleton 65: 675–685, 2008.

Key words: tubulin isotypes; nonsmall cell lung cancer; beta II tubulin; beta V tubulin; microtubules

INTRODUCTION

Lung cancer is the leading cause of cancer deaths in the United States [Seer Incidence and US Mortality Statistics, 1975–2004 http://seer.cancer.gov/canques]. Lung tumor heterogeneity hinders efforts to determine prognosis and select appropriate treatment regimens. Unfortunately, there are currently no consistently reliable biomarkers that can be used as prognostic indicators [Brundage et al., 2002]. Typically, lung tumors are classified into two major categories based on morphology and behavior: small cell lung cancer (SCLC) and nonsmall cell lung cancer (NSCLC). Small cell lung cancers (SCLCs) are generally more aggressive than NSCLCs and often display a neuroendocrine phenotype [Zochbauer-Muller et al., 2002]. The NSCLCs are a...
diverse class of tumors (squamous cell, adenocarcinomas and large cell undifferentiated) with some aggressive subsets characterized by neuroendocrine features. Neuroendocrine features can be identified by tissue staining using specific markers such as neuron-specific enolase, chromogranin A, synaptophysin and neurofilament protein. Although these markers are used to identify potentially aggressive tumors, they can not discriminate between high and low grade neuroendocrine tumors. In addition for NSCLCs there are no specific and reliable markers for more aggressive and poorly differentiated squamous cell carcinomas or adenocarcinomas that lack the neuroendocrine phenotype. Thus, biomarkers for aggressive tumors are clearly needed.

In a study of 88 primary and metastatic lung tumors using immunohistochemical staining, β-tubulin class III protein was identified as a biomarker for high grade aggressive tumors (small cell lung carcinomas, adenocarcinomas and neuroendocrine type large cell carcinomas) [Katsetos et al., 2000]. This tubulin isotype was not found in non-neoplastic lung biopsies, pneumonectomy, lobectomy or transbronchial biopsy specimens. It was also absent from airway mucosa, submucosal glands, myoepithelial cells, alveolar pneumocytes and macrophages, hyaline cartilage, fibroblasts, endothelial cells, smooth muscle cells, pericytes and lymphoid cells. In contrast, high levels of β-tubulin class III staining were found in all the small cell lung carcinomas (SCLCs) examined and in large cell neuroendocrine lung tumors. Lower levels of β-tubulin class III were found in NSCLC adenocarcinomas and staining was absent from squamous cell lung carcinomas (SQCC), suggesting that this biomarker might be useful for distinguishing SCLC and SQCC. This work suggested that β-tubulin class III may be a biomarker for aggressive NSCLCs; although other tubulin isotype distributions were not measured. The TUJ1 monoclonal antibody for β-tubulin class III used by Katsetos et al. [2000] is one of the most stable and specific tubulin antibodies commercially available. There are no commercially available monoclonal antibodies for β-tubulin classes V and VI or that differentiate β-tubulin classes IVa from IVb. Therefore studies comparing all of the β-tubulin isoforms found in normal lung tissue or lung tumors by immunostaining are not currently feasible.

A more recent clinical investigation of biomarkers, including tubulin isotypes, distinguished those that predict prognosis for patients with NSCLCs from those that predict drug response. In a study of 91 NSCLCs, low levels of β-tubulin class III protein measured by immunostaining correlated with a better response to paclitaxel, an antimitotic agent often used to treat advanced stage NSCLCs [Seve et al., 2005]. Only β-tubulin classes I, II and III were evaluated. In contrast to Katsetos et al. [2000], the β-tubulin class III protein level was not found to be a prognostic indicator for patients who had not received paclitaxel.

In addition to β-tubulin isotype amounts, altered levels of proteins that interact with tubulin or microtubules, such as stathmin or MAP4, could affect tumor response to antimotics such as paclitaxel. Stathmin belongs to a family of proteins that destabilize microtubules in mitotic spindles. During interphase, stathmin regulates microtubule depolymerization. High levels of stathmin have been found in many tumors, and it has been proposed that the effect of stathmin is to increase the proliferative activity of tissues [Mistry and Atweh, 2002]. MAP4 is a microtubule-associated protein expressed constitutively and known to stabilize microtubules. Decreased levels of MAP4 are associated with decreased sensitivity to paclitaxel and increased sensitivity to other antimiotics such as vinblastine [Wahl et al, 1996; Zhang et al., 1999]. Measurement of the expression of mRNA for proteins that interact with tubulin and microtubules (e.g., stathmin and MAP4) could lead to the identification of biomarker patterns useful as indicators of tumor response to chemotherapy.

The study presented here is the first quantitative comparison of mRNA levels for all seven β-tubulin isoforms, stathmin and MAP4 in NSCLCs. This work included 64 NSCLC samples and 12 normal lung samples in an attempt to describe patterns of mRNA β-tubulin isoforms or microtubule-associated proteins that might serve as biomarkers for tumor differentiation and/or aggressiveness.

MATERIALS AND METHODS

Collection of Lung Tissues

Lung tissue samples and pathology reports for each tissue were obtained from the Cooperative Human Tissue Network (CHTN, National Cancer Institute). Sixty-four (64) primary nonsmall cell lung tumor samples and 12 “normal” lung tissue samples were collected prior to drug treatment and quick-frozen in liquid nitrogen within 1 h of surgical removal. Normal tissues were collected from a section of the lung at a distance from the primary tumor at the same time as surgical resection of the primary tumor. The absence of tumor cells in these normal samples was verified by histopathological examination. The normal lung tissues were from a subset of the 64 patients with primary tumors. Table I describes the samples included in this study as well as the manner in which these samples were subdivided for analysis.

Sample Preparation

Tissues were homogenized in a dounce homogenizer on ice in 1 ml of lysis buffer (25 mM MES, 1 mM
MgSO₄, 2 mM EGTA, 0.1 mM GTP, 0.1% Triton X-100 with a protease inhibitor cocktail [Complete Protease Inhibitor Tablets, Roche, Indianapolis, IN]). 100 μl of tissue homogenate were processed for total RNA extraction using the TRIzol reagent (Invitrogen, Carlsbad, CA) and the RNeasy kit (Qiagen, Valencia, CA) to obtain total RNA for qRT-PCR experiments. After DNase treatment of the total RNA, poly dT was used in reverse transcription reactions to generate cDNA. The concentration of total RNA for each sample was determined by A₂₆₀ measurements. The remainder of the tissue homogenate was made 1:1 with SDS sample buffer and frozen for Western blotting.

Quantitative Real Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Primers were designed for each β-tubulin isotype (classes I, II, III, IVa, IVb, V and VI) [Dozier et al., 2003; Hiser et al., 2006] and for MAP4 and stathmin (Table II). Although β-tubulin isotype class II is considered a single isotype class based upon the carboxyl terminal protein sequence, there are two genes for this isotype (NIH Genbank map elements: TUBB2A; NM_001069 and TUBB2B; NM_178012.3). The primers used in this study recognize the gene product for TUBB2B. Two-step qRT-PCR was used to measure the amount of mRNA for each β-tubulin isotype using protocols described previously [Dozier et al., 2003; Hiser et al., 2006]. To obtain the control cDNA template of a known quantity for the generation of standard curves, product from PCR reactions was isolated from agarose gels with the CONCERT™ Rapid Gel Extraction kit (Marligen Biosciences, Inc., Ijamsville, MD). The use of a standard curve eliminates the need to correct for primer efficiencies. The concentration of purified cDNA was determined by analyzing serial dilutions using a bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). For the standard curve, 10-fold serial dilutions are done to obtain a minimum of five samples and these are plated in triplicate. Each experiment included unknown samples in triplicate run together with the standard curve and a quality control sample. We used SYBR Green I (Invitrogen, Carlsbad, CA) as the detection method. Experiments were repeated at least twice. The data were normalized as the β-tubulin isotype mRNA copy number in the unknown sample to 1 μg of total RNA. The percentage of each β-tubulin isotype was determined by totaling the mRNA copy number/μg of total RNA for all isotypes in a sample and then dividing the copy number/μg total RNA for each isotype by the total. The ratios of copy numbers/μg of total RNA or ratios of percentages therefore are identical.

Western Blotting

Western blots were used to evaluate the presence of β-tubulin isotype classes II, III and V proteins. The antibodies used in this study were mouse monoclonal 7B9 anti-β-tubulin class II, monoclonal TUJ1 anti-β-tubulin class III and rabbit polyclonal anti-β-tubulin class V. TUJ1 and 7B9 antibodies have been previously characterized [Lee et al., 1990; Lobert et al., 1998]. The anti-β-tubulin class V polyclonal antibody was characterized in this study using maltose binding protein (MBP) β-tubulin fusion proteins as described in Hiser et al. [2006]. Fusion proteins and dot blots were used to determine the specificity and optimal solution conditions for the anti-β-tubulin class V antibody. Tissue samples expressing high and low mRNA levels of β-tubulin classes II and V were chosen for Western blotting. Immunoblotting with anti-actin mouse monoclonal antibody (Chemicon International, Inc., Temecula, CA) was

<table>
<thead>
<tr>
<th>TABLE I. NSCLC and Normal Lung Tissue Samples</th>
<th>Race</th>
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<tr>
<td>NSCLC samples</td>
<td></td>
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<tr>
<td>Number of samples</td>
<td>20</td>
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<tr>
<td>Age range</td>
<td>43–79</td>
</tr>
<tr>
<td>Tissue differentiation</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>9</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>4</td>
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<tr>
<td>Well differentiated</td>
<td>3</td>
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<tr>
<td>Not determined</td>
<td>5</td>
</tr>
<tr>
<td>Histological classification</td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>8</td>
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<tr>
<td>Adenocarcinoma</td>
<td>6</td>
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<tr>
<td>Large cell carcinoma with</td>
<td>1</td>
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<tr>
<td>neuroendocrine features</td>
<td>5</td>
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<tr>
<td>Normal lung tissues</td>
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<tr>
<td>Number of samples</td>
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</tr>
<tr>
<td>Age range</td>
<td>75–76</td>
</tr>
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aBM, African American males; WM, White males.

<table>
<thead>
<tr>
<th>TABLE II. Primers for qRT-PCR (MAP4 and Stathmin)</th>
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<tr>
<td>Human stathmin</td>
</tr>
<tr>
<td>Forward primer: 5′-GGTGAGCGAGGACTTTCCTTTCCCTCAGTC-3′ (34 bp)</td>
</tr>
<tr>
<td>Reverse primer: 5′-TTCTCTGCTCTGCTTTAAAGCAGGCACGCAGTGC-3′ (33 bp)</td>
</tr>
<tr>
<td>Human MAP4</td>
</tr>
<tr>
<td>Forward primer: 5′-CCCTTTCTCTAGGTCCTGCTTTTCCTTTGAGGTGAGG-3′ (32 bp)</td>
</tr>
<tr>
<td>Reverse primer: 5′-CTCGCTCCCTCACAAGCTTTTGGCACGCAGAGA-3′ (30 bp)</td>
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used to establish that the total protein loaded in each lane was comparable.

**Statistical Analysis**

For statistical comparisons of mRNA amounts in tissue samples, Student’s t test or one-way ANOVA with Tukey’s Multiple Comparison post test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

**RESULTS**

**β-Tubulin Classes II and V mRNA Predominate in NSCLC Tissues**

The amounts of β-tubulin isoform mRNA classes I, II, III, IVa, IVb, V, and VI were measured by qRT-PCR. This is the first quantitative comparison of mRNA for all seven β-tubulin isoforms in NSCLCs. The sum of the mRNA amounts for all seven isoforms was considered to be the total tubulin for each sample, and the percentage (fraction) of each isoform was calculated (Fig. 1). Data analysis by one-way ANOVA demonstrated that the fractions of β-tubulin classes II and V were significantly higher than the other β-tubulin isoforms in diseased tissues at \( P < 0.05 \). Together they comprise over 65% of all β-tubulin mRNA in NSCLC tissues. When these data were compared with β-tubulin isoform fractions in a small sample of normal lung tissues (\( n = 12 \)), interesting differences were noted. We found that normal lung tissues had on average about 3-fold fewer copies of total β-tubulin mRNA/μg of total RNA compared to diseased lung tissue. For paired normal and tumor lung tissues, we found the mean copies of β-tubulin mRNA/μg of total RNA for normal samples was \( 3.0 (\pm 2.3) \times 10^5 \) and for tumors from the same patient \( 1.0 (\pm 0.003) \times 10^6 \) [all tumors \( 3.3 (\pm 0.05) \times 10^6 \)]. The β-tubulin isoform class distribution in normal lung tissues was as follows: βI 6% ± 0.4%, βII 2.5% ± 0.2%, βIII 36% ± 2%, βIVb 8% ± 0.8%, βV 13% ± 1%, βVI 34.5% ± 2%. The normal lung tissues did not have measurable amounts of β-tubulin class IVa mRNA. Data analysis by one-way ANOVA demonstrated that the predominant isoforms in this small sample of normal lung tissues, β-tubulin classes III and VI, were present in significantly larger amounts than the other β-tubulin isoforms \( (P < 0.001) \). There was no statistically significant difference in β-tubulin isoform classes I, II, IVb or V. Student’s t test demonstrated that the fractions of β-tubulin isoform classes II and V were significantly smaller in normal lung tissues compared to NSCLCs \( (P < 0.05) \).

**β-Tubulin Classes II and V mRNA Fractions Follow Similar Trends by Level of Differentiation and Histopathology in NSCLCs**

NSCLC samples were grouped according to tissue differentiation as determined in the pathology report: poorly-differentiated \( (n = 28) \), moderately differentiated \( (n = 19) \), and well-differentiated \( (n = 7) \). Only data from samples that were classified as one of these three types \( (n = 54) \) were used in this analysis. We observed a trend in the average mRNA fraction of β-tubulin class II mRNA: well-differentiated < moderately-differentiated < poorly-differentiated; in contrast, the mRNA fraction of β-tubulin class V mRNA followed the reverse pattern: well-differentiated > moderately-differentiated > poorly-differentiated (Fig. 2). Statistical analysis did not demonstrate significance, possibly due to the small \( n \) for each class.
β-Tubulin Isotypes in NSCLCs

Fig. 3. Fraction of β-tubulin isotypes in NSCLCs grouped by histopathology. The bar graphs represent the averages and error bars represent the standard error of the mean. The numbers of samples in each group were: large cell carcinoma with neuroendocrine (NE) features n = 4, adenocarcinoma n = 16, and squamous cell carcinoma n = 28.

...not shown). When the β-tubulin II/V ratio data were grouped by level of differentiation a trend was found: the value decreased from poorly- to moderately- to well-differentiated tissues and the ratio in well-differentiated tissues approaches that of the normal lung tissues (ANOVA, P = 0.17) (Fig. 4B). When the data were grouped by histopathology, we found that the trend for the ratio of β-tubulin classes II/V approached statistical significance at P < 0.05: large cell carcinomas with neuroendocrine features > adenocarcinomas > squamous cell carcinoma > normal lung tissues (ANOVA, P = 0.06) (Fig. 4C). The fact that these ratios are determined for each patient before averaging must contribute to the increased statistical significance of these data compared to the aggregate data for each isotype alone, suggesting potential utility of the β-tubulin class II/V ratio as a biomarker for aggressive lung tumors.

β-Tubulin Classes II and V Proteins are Present in NSCLCs

The presence of protein for β-tubulin isotype classes II and V was evaluated by Western blotting of three samples that showed high and low mRNA levels for β-tubulin classes II and V. The specificity of anti-β-tubulin class II antibody, 7B9, has been previously characterized [Lobert et al., 1998]. In this study we used a newly developed polyclonal antibody raised against the carboxy-terminal peptide for β-tubulin class V. Dot blots with maltose binding protein (MBP) β-tubulin isotype fusion proteins representing peptides from the carboxy terminus of all seven β-tubulin isotype classes were used to characterize the specificity of this antibody and to establish the optimal working dilution. The use of MBP tubulin isotype fusion proteins in Western blotting is described in our previous work [Hiser et al., 2006]. The optimal dilution was found to be 1:30,000 (~0.1 μg/ml) (Fig. 5A). Both β-tubulin isotype classes II and V proteins were found in the three tissue samples that were examined. There was no apparent difference in protein expression of β-tubulin isotype class II, but the different levels of β-tubulin isotype class V protein were consistent with the different levels of mRNA (Fig. 5B). The findings for β-tubulin isotype class II protein were not surprising since primers used in our qRT-PCR measurements for this class recognized the mRNA product of the TUBB2B gene only, while the antibody recognizes the products of TUBB2A and TUBB2B. Tubulin class V protein has not previously been measured in lung tumor tissues. Note that we divided our total tissue lysate into fractions to use for total RNA extraction and for semiquantitative protein measurements by Western blotting. Tissue samples are inherently heterogeneous and we wanted to...
minimize differences in mRNA and protein quantities that might be due to different cell types within a section of a sample. Therefore the semiquantitative Westerns show a reasonable estimate of the protein in the sample used for mRNA quantitation.

MAP4 to Stathmin Ratio is Significantly Higher in NSCLCs Compared to Normal Lung Tissues

Stathmin and MAP4 are microtubule associated proteins important in cell division and proliferation [Andersen, 2000], and therefore changes in these proteins may have implications for tumorigenesis. In addition, alterations in levels of stathmin and MAP4 can change the sensitivity of cells to antimitotic drugs commonly used in chemotherapy protocols for NSCLC [Zhang et al., 1999; Alli et al., 2002]. MAP4 and stathmin have opposing effects on microtubules; MAP4 stabilizes and stathmin destabilizes polymer formation. qRT-PCR was used to measure the copy number of MAP4 and stathmin mRNA in normal lung tissue (*n* = 12) and NSCLCs (*n* = 64). Analogous to the results that we found with tubulin message, we found significantly more MAP4 and stathmin in NSCLCs compared to normal lung tissues (Student's *t*-test, *P* < 0.0001). The mean mRNA copy number/µg total RNA of MAP4 in normal lung tissues was 7.2 (±2.8) × 10^4 and in NSCLCs it was 8.8 (±1.3) × 10^5; for stathmin in normal lung tissues, it was 2.8 (±0.4) × 10^5 and in NSCLCs 9.4 (±1.6) × 10^5. In addition, when the ratios of MAP4 to stathmin mRNA copies/µg of total RNA for each patient sample were averaged, we found the ratio was significantly higher in NSCLCs than in normal lung tissues (Fig. 6). This reflects a trend toward increasing microtubule stabilizing factors in NSCLCs compared to normal lung tissues.

DISCUSSION

β-Tubulin Isotypes in Tumor Cells and Tissues: Association with Tumor Formation and Aggressiveness

This study describing β-tubulin, MAP4 and stathmin mRNA in NSCLC tissues was undertaken to search for biomarker patterns useful for determining prognosis. Specific and reliable markers to distinguish NSCLCs are needed, as are reliable predictors of response to chemotherapy that could reduce the morbidity associated with ineffective chemotherapy. This is the first study of NSCLCs comparing all seven β-tubulin isotype mRNA fractions. Thus the findings regarding β-tubulin classes II and V levels are novel and further exploration of these isotypes as prognostic biomarkers is warranted.

Recent work suggested a role for β-tubulin class III protein as a biomarker for NSCLC aggressiveness and patient prognosis, indicating a dysregulated, poorly-differentiated tumor type [Katsetos et al., 2000]. This was in contrast to normal lung tissues that did not have measurable β-tubulin class III protein. However, the only β-tubulin isotype evaluated in that study was β-tubulin class III. Since β-tubulin class III is a member of the family of β-tubulins, comprised of at least seven gene products that are grouped into classes based upon their carboxy-terminal amino acid sequences, a compari-
of avian erythrocytes and mammalian nucleated immature erythrocytes and platelets), as well as in hematopoietic tissues (in mouse spleen and liver) [Murphy and Wallis, 1983; Wang et al., 1986]. In that work it was noted that lung tissue of young mice also expressed β-tubulin class VI [Wang et al., 1986].

Increased levels of β-tubulin class III protein could affect the tumor response to chemotherapy. The properties of β-tubulin classes III, V and VI are reported to render microtubules more dynamic than β-tubulin classes I, II, IVa and IVb [Luduena, 1993; Cabral, 2008]. Some investigators have speculated that alterations in microtubule dynamics due to varying levels of β-tubulin isotypes may contribute to differential responses to antimitotic agents that interact with tubulin heterodimers or microtubules [Hari et al., 2003; Bhattacharya and Cabral, 2004]. This should affect patient outcomes. Recent work indicates that changes in the abundance of β-tubulin isotypes confer differential drug responses perhaps as a result of changes in drug binding affinity and/or microtubule dynamics [Yang and Cabral, 2007]. For example, in a recent study of the effect of increased levels of β-tubulin class IVa in CHO cells on the response to paclitaxel, small increases of this isotype enhanced drug sensitivity [Yang and Cabral, 2007].

Note that in the work of Cabral and colleagues, the amount of only one isotype is changed and therefore, may not reflect global intracellular changes in real physiological systems. Two recent studies identified β-tubulin class III protein as a predictor of NSCLC response to antimitotic drugs [Dumontet et al., 2005; Seve et al., 2005]; although, it may not be a general prognostic indicator [Seve et al., 2005]. Note that in both studies, only β-tubulin isotype classes I, II and III protein were evaluated. While all three isotypes were identi-
We quantitatively measured $\beta$-tubulin isotype mRNA levels in 76 lung tissue samples (64 NSCLCs and 12 normal lung tissues). Normal lung tissues were obtained from a subset of the 64 patients diagnosed with NSCLC and the pathology reports identified the tissues as exhibiting normal histological characteristics. The $\beta$-tubulin isotype pattern was clearly different when normal and NSCLCs were compared; $\beta$-tubulin classes II and V mRNA predominate in NSCLCs and $\beta$-tubulin classes III and VI mRNA predominate in normal lung tissues. This finding was unexpected since Katsetos, et al. [2000] did not find appreciable amounts of $\beta$-tubulin class III protein in normal lung tissues. However, they did not attempt to compare $\beta$-tubulin class III levels with other isotype levels. It is important to note that these data are presented as fractions of each isotype, rather than as copy number. We found that normal lung tissues tend to have on average 3-fold fewer copies of $\beta$-tubulin mRNA/µg of total RNA compared to diseased tissue. More specifically, we found 3.5-fold fewer copies of $\beta$-tubulin class III mRNA in normal tissues compared to NSCLCs [normal 8.0 (±1.4) × 10^5 and NSCLCs 2.8 (±0.6) × 10^5 mRNA copies/µg total RNA]. Although we found relatively high levels of $\beta$-tubulin class III mRNA in normal tissues when analyzed as a percentage of the total tubulin mRNA, the amounts were small compared to the $\beta$-tubulin class II mRNA copy number in NSCLCs [2.0 (±1.3) × 10^6 mRNA copies/µg of total RNA]. Immunostaining for protein is dependent upon the sensitivity of the antibody and the amount of protein present. In the study by Katsetos, et al. [2000], the $\beta$-tubulin class III protein in normal lung tissues was clearly below the level of detection, but this result does not necessarily contradict our qRT-PCR data. The fraction of $\beta$-tubulin class III may be highest in normal lung tissues but the protein signal may not be detectable by immunostaining. Because of the high amounts of $\beta$-tubulin class III mRNA in our normal lung tissue samples, we used Western blotting to determine whether $\beta$-tubulin class III protein was present in selected normal and tumor tissues and found detectable amounts in these samples (data not shown). We also found measurable amounts of $\beta$-tubulin classes II and V protein in NSCLC tissues by selective Western blotting (Fig. 5B); however it was not feasible to do these measurements for all 76 tissue samples.

$\beta$-Tubulin class VI has been reported to be present in significant amounts in blood cells and hematopoietic cells [Murphy and Wallis, 1983; Wang et al., 1986]. The elevated mRNA levels for $\beta$-tubulin class VI in our small sample of normal lung tissues could be due to relatively higher amounts of blood cells in these heterogeneous tissues. We also note that although tumors can be highly vascular, there are sections of tumors that are not penetrated by the developing blood vessels. We measured $\beta$-tubulin isotype mRNA levels in whole blood ($\beta$-tubulin isotypes I, II, III, V and VI) and found that $\beta$-tubulin isotypes I and VI predominated [mean copies mRNA/µg total RNA: 3.3 (±1.9) × 10^6; 2.5 (±0.4) × 10^6; 1.0 (±0.4) × 10^6, respectively]. Class V was in the lowest abundance [mean copies mRNA/µg total RNA: 2.4 (±0.4) × 10^6]. Class III was on average: 2.4 (±0.3) × 10^5 copies mRNA/µg total RNA. Thus it is not clear that the presence of whole blood is the reason for our findings in normal lung tissue. This observation warrants further exploration; however, there is no commercially available antibody to confirm the presence of $\beta$-tubulin class VI protein in these tissues.

In a recent study of twelve human cancer cell lines [Hiser et al., 2006], we found that $\beta$-tubulin class I mRNA predominated and, in several cell lines, significant amounts of $\beta$-tubulin class V mRNA were also found. High levels of $\beta$-tubulin class I have been found in many immortalized cell lines and for that reason this class has been presumed to predominate in all tissues. However, there are few quantitative reports comparing all $\beta$-tubulin isotype classes in human tissues [Dozier et al., 2003] and this is the first comparison in NSCLCs. In our previous study of human cancer cell lines [Hiser et al., 2006], high fractions of $\beta$-tubulin classes I and V mRNA were measured in two NSCLC lung cancer cell lines (A549 and HOP 18). In that study, we also measured protein levels for $\beta$-tubulin isotype classes I, II, III, and IVa plus IVb by quantitative Western blotting. For $\beta$-tubulin isotype classes I and III there was good agreement between the isotype mRNA fractions and protein fractions. For $\beta$-tubulin isotype classes IVa + IVb, there was consistently more protein than mRNA. Thus when quantitative measurements of both mRNA and protein are done, we expect that $\beta$-tubulin mRNA and protein isotype fractions are likely to show reasonable agreement. As noted above, immunostaining of tissues does not yield quantitative data and therefore it is difficult to compare the results of the current qRT-PCR study with previous studies that utilized immunohistochemical staining. Thus, because of the quantitative limitations of immunostaining for proteins and the difficulty of isolating sufficient protein from tissue samples for quantitative Western blotting, the measurements of mRNA levels in the work presented here were done to establish whether $\beta$-tubulin mRNA levels might be more useful as biomarkers for NSCLC aggressiveness and/or level of differentiation.
The Ratio of β-Tubulin Class II to Class V as a Biomarker for NSCLCs

The trends in the β-tubulin class II to V mRNA copy number ratios suggest that β-tubulin isotype patterns should be further explored as biomarkers of NSCLC aggressiveness and/or level of differentiation. We found that this ratio is significantly higher in NSCLCs compared to normal lung tissues (unpaired Student’s t test, \( P < 0.001 \)). We also found the ratio was higher in poorly-differentiated NSCLCs compared to normal lung tissues. A trend was found showing that the ratio for moderately-differentiated tumors tends to fall in between that of normal and poorly-differentiated tissues (ANOVA, \( P = 0.17 \)). Similarly, the β-tubulin class II to V ratio in samples grouped by pathology is highest in large cell carcinomas with neuroendocrine features and lowest in both squamous cell carcinomas and normal lung tissues (ANOVA, \( P = 0.06 \)). These results suggest an exciting possibility for the use of the ratio of β-tubulin class II to V mRNA as a marker for tumor aggressiveness and/or level of differentiation. In fact, analysis of qRT-PCR data from a previous report from our laboratory measuring β-tubulin isotypes in normal and tumor breast tissues [Dozier et al., 2003], shows the same trend for a higher ratio of β-tubulin class II to V mRNA in tumor tissue compared to normal breast tissue (normal, \( n = 6, 0.14 \pm 0.07 \); tumor, \( n = 7, 1.13 \pm 0.74 \); Student’s t test, \( P = 0.10 \)). This biomarker would be a valuable resource for tissues that cannot be easily classified using common histopathological microscopic or staining criteria.

The mechanistic reasons for the differences in the higher fractions of β-tubulin classes II and V in tumor tissues compared to normal lung tissues remain to be explored. It is possible that an increase in the amount of one isotype in these tissues must be compensated by an increase in another. Since β-tubulin classes II and V have contrasting properties, where β-tubulin class V is more dynamic than β-tubulin class II [Cabral, 2008], it is compelling to speculate that the dysregulation of tumor cells that causes an increase in one isotype (e.g. β-tubulin class II or V), leads to an increase in another with complementary properties to meet the intracellular requirement for microtubule dynamics. Thus our measurements of high fractions of β-tubulin classes II and V may be evidence of intracellular changes needed to maintain cell viability. The higher ratios of β-tubulin classes II to V in the poorly-differentiated or large cell (with neuroendocrine features) NSCLCs suggests that these compensatory changes are associated with dysregulation of microtubule dynamics in the disease state. Alternatively, these patterns may also reflect alterations in genomic stability that cause overexpression of many proteins in an unregulated manner [Soucek et al., 2006]. Regardless of the mechanism, our study suggests that ratios of β-tubulin class II to V mRNA may be a biomarker for aggressive NSCLCs.

Altered Expression of MAP4 and Stathmin in Normal Lung Tissue and NSCLCs

In this study we also found a statistically significant difference in the ratio of MAP4 to stathmin when normal and diseased lung tissues were compared. In diseased tissues, the copy number of MAP4 mRNA was nearly equal to that of stathmin mRNA; however normal lung tissues showed significantly higher copy numbers of stathmin compared to MAP4 mRNA. MAP4 and stathmin play important roles in the regulation of microtubule dynamics, essential for cell division and tissue proliferation; MAP4 stabilizes microtubules and stathmin induces depolymerization [Andersen, 2000]. Some studies suggest that MAP4 and/or stathmin may be good targets for combination chemotherapy to improve patient outcomes when treated with antimitotic agents aimed at disrupting microtubule dynamics [Bhat and Setaluri, 2007]. The implications of the different ratios of MAP4 to stathmin for tumor development and/or aggressiveness are unclear. In breast carcinomas, high levels of stathmin were associated with a subset of cancers classified as grade III with a high proliferative index, suggesting these were aggressive tumors [Curmi et al., 2000]. In contrast, in another study of peripheral blood samples from 51 patients with NSCLC, lower MAP4/stathmin ratios were associated with response to the antimitotic vinorelbine that destabilizes microtubules [Galan et al., 2007]. In the NSCLCs examined in our study, there was no trend observed for the MAP4/stathmin ratio within either of the groupings (pathological description or level of differentiation) and therefore, no clear association with prognosis. However, as suggested by the study by Galan et al. [2007], a subset of NSCLCs with low MAP4/stathmin ratios may respond more readily to antimitotic therapy with vinorelbine. Decreased levels of MAP4 in another study were implicated in decreased sensitivity to paclitaxel [Zhang et al., 1999], an antimitotic that stabilizes microtubules and is commonly used to treat advanced-stage NSCLCs. Our data showing that normal lung tissues have relatively lower amounts of MAP4 mRNA compared to diseased tissues suggest that normal lung tissues may be less sensitive to paclitaxel. Differential sensitivity to paclitaxel of NSCLCs compared to normal lung tissues may, in part, be explained by a difference in the MAP4/stathmin ratio. It should be noted that stathmin and MAP4 activities are regulated by phosphorylation [Vandre et al., 1991; Curmi et al., 1999]. Thus, to better understand the role of the MAP4/stathmin ratio, the phosphorylation status of stathmin and/or MAP4 in...
NSCLCs, as well as levels of kinase or phosphatase activity need to be evaluated. Nonetheless, the observed difference between diseased and normal lung tissues suggests that the MAP4/stathmin mRNA ratio may have potential as a biomarker for aggressive NSCLCs.

Properties of microtubules that are critical for cell division, cell movement and response to antimitotic chemotherapy agents are thought to be regulated by the tubulin isotype composition and microtubule interacting proteins (MIPs). The work presented here demonstrates the utility of methods to quantitatively measure tubulin and MIP mRNA amounts in human tissue samples. We designed highly specific primers and were able to quantify message from all seven β-tubulin genes, as well as mRNA for the gene products known to regulate microtubule dynamics and stability, MAP4 and stathmin. Inherent in analysis of data from whole tissue samples is the problem of heterogeneity of cell types, including contamination of samples with whole blood components. This can lead to bias in the data collected and result in unwarranted conclusions. While it is clear that no single biomarker can point conclusively to a prognosis, it is possible that patterns of biomarkers such as those presented here can together be used as reliable indicators of potential outcomes or help in the selection of appropriate treatments.

REFERENCES


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