

# Analysis of Tubulin Isotypes and Mutations from Taxol-Resistant Cells by Combined Isoelectrofocusing and Mass Spectrometry<sup>†</sup>

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**ABSTRACT:** Six human  $\alpha$ -tubulin and seven human  $\beta$ -tubulin isotypes, each of which can undergo posttranslational modifications, have been detected by the reverse transcriptase–polymerase chain reaction. This repertoire of tubulin isotypes plays a role in development and in the building of specialized microtubule-based structures. In cell lines, the relationship between resistance to microtubule-interacting drugs and altered tubulin isotype expression profiles is often established by quantitation of cDNA and/or Western blot analysis. Tubulin mutations in major isotypes are detected by sequencing cDNA, but more analysis of expression of tubulin mutations at the protein level, to assess their role in drug resistance, is needed. We utilized a Taxol-based purification and high-resolution isoelectrofocusing combined with a mass spectrometry-based analysis of tubulin. This approach has allowed the separation and relative quantitation of tubulin isotypes having a difference in isoelectric point values of 0.01, without the need for two-dimensional gel electrophoresis. The specificity of tubulin isotype antibodies also has been established. In cell lines resistant to microtubule-stabilizing drugs that express heterozygous tubulin mutations, the relative amount of mutant tubulin expression has been determined. In these cell lines, the absence of  $\beta$ II- and  $\beta$ IVa-tubulin has been demonstrated, and an increased level of expression of  $\beta$ III-tubulin in resistant cells has been confirmed, indicating that this tubulin isotype is a unique marker of resistance.

Studies on the microtubule cytoskeleton reveal a spectrum of activities from structural to mechanical to signal transduction processes (1). Mitosis is a microtubule-dependent process, and inhibition of mitotic spindle function represents the mode of action of major anticancer drugs, i.e., the taxanes and vinca alkaloids. Microtubules are built upon the assembly of  $\alpha/\beta$ -tubulin heterodimers. In human tissues, the expression of six  $\alpha$ - and seven  $\beta$ -tubulin isotypes has been detected (Table 1). Additionally, tubulin undergoes posttranslational modifications, and complex patterns of tubulin isoform expression can be observed, especially in the central nervous system (2).

The existence of multiple tubulin species raises the question of the physiological relevance of such diversity (3). The greatest sequence divergence among tubulin isotypes resides in their C-termini (Table 1), sites that are exposed at the surface of microtubules and interact with microtubule-associated proteins and undergo posttranslational modifica-

tions. Specific tubulin isotypes are constituents of specialized microtubules (2). Moreover, alterations in tubulin isotype expression profiles and tubulin mutations have been reported in cell lines resistant to antimetabolic agents (4–8).

The expression of a given tubulin isotype in a cell line or tissue is usually detected by quantification and sequencing of RT-PCR<sup>1</sup> products. Using this method, mutations in tubulin have been detected in cell lines resistant to Taxol and the epothilones (5–8). Despite the convenience of this approach, it does not give direct access to the corresponding protein expression levels. This is particularly relevant to  $\beta$ -tubulin mRNA because it appears to be autoregulated by the level of the free  $\beta$ -tubulin pool in cells (9). Similarly, heterozygous transcriptional expression of a tubulin mutation does not indicate that both wild-type and mutant tubulin proteins are expressed and, if so, at what ratio. Antibodies directed against the C-terminus of each tubulin isotype and against some of their posttranslational modifications provide useful tools for evaluating their respective levels of expression. However, possible cross-reactivity of these antibodies against different tubulin isotypes may introduce some quantitation bias, because of the lack of resolution of the different tubulin isoforms by SDS–PAGE.

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<sup>1</sup> Abbreviations: RT-PCR, reverse transcriptase–polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DTT, dithiothreitol; IPG, immobilized pH gradient; IEF, isoelectric focusing; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; LC–MS, liquid chromatography coupled to mass spectrometry; 2D, two-dimensional; pI, isoelectric point.

Table 1: Human Tubulin Isootypes

Isotype (accession #)	Human gene	C-terminus sequence <sup>a</sup>	Tissue expression	pI <sup>b</sup>
<b>α-TUBULINS</b>				
<b>1</b> (I77403)	TUBA1/k-α1	MAALEKDYEEVGVDSEGESEEGEEY	Widely expressed	4.94
<b>1</b> (CAA25855)	TUBA3/b-α1	MAALEKDYEEVGVSVEGESEEGEEY	Mainly in brain	5.02
<b>3</b> (Q13748)	TUBA2	MAALEKDYEEVGVDSEGESEEGEEY	Testis-specific	4.98
<b>4</b> (A25873)	TUBA4	MAALEKDYEEVGIDSYEDDEGESE	Brain, muscle	4.95
<b>6</b> (Q9BQE3)	TUBA6	MAALEKDYEEVGADSDGDEGESEY	Widely expressed	4.96
<b>8</b> (Q9NY65)	TUBA8	MAALEKDYEEVGTDSESESEGESE	Heart, muscle, testis	4.94
<b>β-TUBULINS</b>				
<b>I</b> (AAD33873)	HM40/TUBB	YQDATAEEEEDFGEEAEEEE	Constitutive	4.78
<b>II</b> (AAH01352)	Hβ9/TUBB2	YQDATADEQGEFEEEGEDEA	Major neuronal, lung	4.78
<b>III</b> (AAH00748)	Hβ4/TUBB4	YQDATAEEEGEMYEDDEESEAQGPK	Minor neuronal, testis	4.83
<b>IVa<sup>c</sup></b> (P04350) (NP_006078)	Hβ5/TUBB5	YQDATAEEQGEFEEEAEEVVA	Brain specific	4.81
		YQDATAEEGFEFEFAEEVVA		4.78
<b>IVb</b> (P05217)	Hβ2	YQDATAEEEGEFEEEAEEVVA	Major testis	4.79
<b>V</b> (NP_115914)	5-beta/Beta V	YQDATANDGEFAFEDEEEEIDG	Uterine adenocarcinoma	4.77
<b>VI</b> (NP_110400)	Hβ1/TUBB1	FQDAKAVLEEDDEEVTEEAEMEPEDKGH	Blood	5.05

<sup>a</sup> Amino acids differing from isotype 1 (Kα1) for α-tubulins or from isotype I (HM40/TUBB) for β-tubulins are highlighted in black. <sup>b</sup> The isoelectric points were calculated on the basis of the tubulin primary sequences found in the NCBI protein database (accession numbers given in first column) using the ExPaSy Compute pI/MW tool. <sup>c</sup> Two βIVa-tubulin sequences with distinct C-termini were found in the NCBI protein database. The top C-terminus sequence was found in human brain, and the bottom sequence was found in a human oligodendrogloma and in mouse brain.

We sought to perform both qualitative and quantitative analysis of tubulin content in cancer cell lines where tubulin represents ~3–4% of the total protein (10–12). Here we present a method based on Taxol-driven polymerization of total soluble tubulin from cell lysates. The resulting Taxol-stabilized microtubule pellets were analyzed by high-resolution isoelectrofocusing on immobilized pH gradient strips. Mass spectrometry analysis of in-gel tryptic digests definitively assigned each major tubulin species to a tubulin isotype class. Western blotting of these strips further established the specificity of tubulin isotype-specific antibodies. This methodology allowed us to evaluate the percentage of each tubulin isotype in cancer cell lines and to detect the expression of charge-altering mutations in tubulin from both Taxol- and epothilone-resistant cell lines. Additionally, the apparent absence of detectable βII- and βIVa-tubulin protein in cell lines where these isotypes were detected at the mRNA level demonstrated that RT-PCR-based analysis of tubulin isotype expression has to be interpreted cautiously. The increase in the level of βIII-tubulin expression in cell lines resistant to microtubule-interacting drugs may represent a significant tubulin isotype-related marker.

## MATERIALS AND METHODS

**Chemicals.** Taxol was obtained from the Drug Development Branch of the National Cancer Institute (Bethesda, MD), dissolved in sterile dimethyl sulfoxide, and stored at –20 °C. Trypsin was obtained from Promega (Madison, WI).

All other chemicals were obtained from Sigma (St. Louis, MO) except where noted.

**Cell Culture.** The human A549 lung, MDA-MB-231 breast, HeLa cervical carcinoma, and CA46 Burkitt lymphoma cell lines were maintained as described previously (10, 13, 14).

**Isolation of Tubulin from Cell Lines.** Subconfluent cultures of cells from seven to ten 100 mm dishes were washed once with PBS and scraped in 1 mL of PBS. For CA46 cells, 250 mL of suspension culture at a cell density of 10<sup>6</sup> cells/mL was centrifuged and the cell pellets were washed with PBS. Pellets of 0.3–0.4 mL of packed cells were obtained. One and one-half volumes of MME buffer [0.1 M 2-(N-morpholino)ethanesulfonic acid (pH 6.9), 1 mM MgCl<sub>2</sub>, and 1 mM EGTA] was used to resuspend the cell pellets. The resulting cell suspensions were frozen in liquid nitrogen. Upon use, cell suspensions were rapidly thawed, and 1/10 volume of a 10-fold stock solution of protease inhibitor cocktail (Boehringer Mannheim) in MME buffer and 1 mM DTT were added. Cell suspensions were sonicated with a microtip probe (Ultrasonics) seven times for 30 s with 30 s rest intervals on melted ice. Cell lysates were centrifuged at 120000g (Beckman TLA100.3 rotor) for 1 h at 4 °C. Cytosolic supernatants (SI) were transferred to new tubes, and the DNA and cell debris pellets (PI) were discarded.

Taxol-stabilized microtubule pellets were isolated by following the method of Vallee (15). In brief, cytosolic supernatants were incubated at 37 °C in the presence of 10

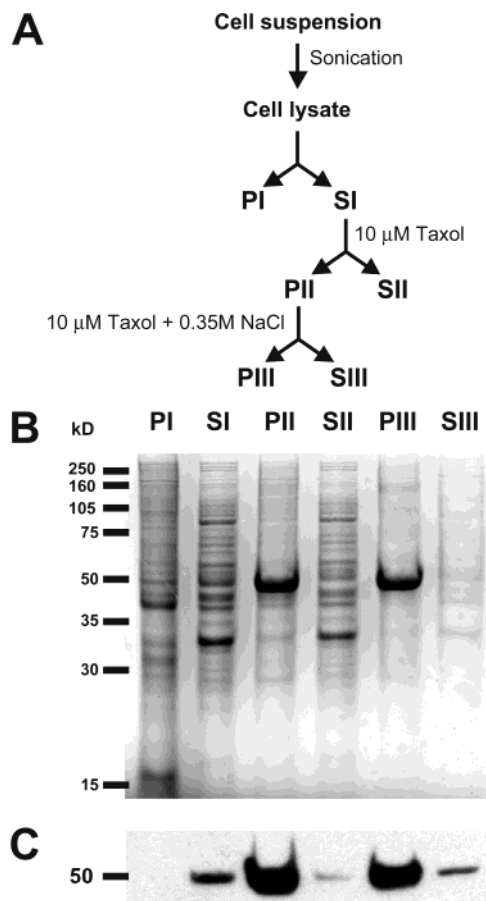


FIGURE 1: Taxol-based purification of tubulin from A549 cells. (A) Tubulin purification scheme (see Materials and Methods for details): PI, DNA and cell debris pellet; SI, cytosolic extract; PII, Taxol-stabilized microtubule pellet; SII, supernatant II; PIII, washed Taxol-stabilized microtubule pellet; SIII, supernatant III. (B) The different fractions were analyzed by SDS-PAGE (10% acrylamide gel, MW markers indicated on the left), and the gel was stained with Coomassie blue. (C) After SDS-PAGE of the same fractions, proteins were transferred to a nitrocellulose membrane that was probed with a pan  $\alpha$ -tubulin antibody.

$\mu$ M Taxol and 1 mM GTP for 20 min. Reaction mixtures were layered on a 0.1 mL cushion containing 5% sucrose in MME, 10  $\mu$ M Taxol, and 1 mM GTP. Samples were centrifuged at 80000g (Beckman TLA100.3 rotor) for 30 min at 37 °C. Microtubule pellets (PII) were washed and resuspended in 0.1 mL of MME buffer containing 0.35 M NaCl and 10  $\mu$ M Taxol. After centrifugation at 80000g for 30 min at 37 °C, microtubule pellets (PIII) were frozen on dry ice and kept at -70 °C until they were used. The Taxol-based purification of tubulin from A549 cells was monitored by SDS-PAGE and Western blot analyses (Figure 1) using either DM1A, a pan  $\alpha$ -tubulin antibody, or DM1B, a pan  $\beta$ -tubulin antibody (Biogenex, San Ramon, CA), and pure bovine brain tubulin as a standard (Cytoskeleton Inc., Denver, CO).

**Isoelectric Focusing.** Microtubule pellets (containing approximately 100–200  $\mu$ g of protein) were resuspended in 350  $\mu$ L of solubilization buffer {7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, 0.5% Triton X-100, 0.5% ampholyte-containing buffer (pH 4.5–5.5), 20 mM DTT, and bromophenol blue}, loaded onto 18 cm IPG strips at pH 4.5–5.5 (Amersham Pharmacia Biotech), and run on an IPGphor IEF system for

a total of 70 000 Vh. IPG strips were then stored at -20 °C. Prior to use, IPG strips were incubated in equilibration buffer [125 mM Tris-HCl (pH 6.8), 5%  $\beta$ -mercaptoethanol, and 1% SDS] for 2  $\times$  20 min each, and then incubated in IPG strip transfer buffer [25 mM Tris-HCl (pH 8.3) and 192 mM glycine] for 20 min.

**Coomassie Blue Staining.** IPG strips were fixed in a 50% methanol/20% trichloroacetic acid mixture for 30 min, stained with 0.1% R250 Coomassie blue in a 50% methanol/10% acetic acid mixture, destained in a 35% methanol/10% acetic acid mixture, incubated for 30 min in 2% glycerol, layered on filter paper with the plastic backing facing the paper, covered with a piece of transparent plastic sheet, and air-dried prior to scanning. IPG strips were scanned and bands quantitated using Image Quant (Amersham).

**Western Blotting of IPG Strips.** IPG strips were electrotransferred onto nitrocellulose membranes with the gel facing the membrane (without removing the plastic backing from the strip) and transferred for approximately 20 h at 100 mA. Only a partial transfer of proteins was achieved, and after being transferred, strips were directly fixed and stained with Coomassie blue stain (see above) so that the blots and gel could be aligned.

The  $\beta$ -tubulin isotype content was examined using a panel of mouse monoclonal antibodies directed against the C-terminus of  $\beta$ I-,  $\beta$ II-,  $\beta$ III-, and  $\beta$ IV-tubulins originally produced by R. F. Ludueña and co-workers. The anti- $\beta$ I-tubulin antibody (generous gift from R. F. Ludueña) was used at a 1:10000 dilution; the anti- $\beta$ II-,  $\beta$ III-, and  $\beta$ IV-tubulin antibodies (Biogenex) were used at a 1:250 dilution, and a goat anti-mouse HRP-linked IgG was used as the secondary antibody (1:2000 dilution, Transduction Laboratories, San Jose, CA).

Membranes were probed for  $\alpha$ -tubulin isotypes with an anti-K $\alpha$ 1-tubulin rabbit polyclonal antibody (SRa1) at a 1:20000 dilution or an anti- $\alpha$ 6-tubulin rabbit polyclonal antibody (SRa6) at a 1:5000 dilution. A donkey anti-rabbit HRP-linked IgG was used as the secondary antibody (1:2000 dilution, Amersham). Antibodies SRa1 and SRa6 were prepared using synthetic C-terminus peptides GVDSVEG-EGEE and GADSADGEDEG as antigens, respectively. A Cys residue was added at the N-terminus of these peptides for their conjugation to maleimide-activated keyhole limpet (Pierce, Rockford, IL). The conjugated peptides were injected into rabbits, and bleeds were analyzed by an enzyme-linked immunosorbent assay using the same K $\alpha$ 1- and  $\alpha$ 6-tubulin C-terminus peptides conjugated to maleimide-activated bovine serum albumin (Pierce). All blots were visualized using an enhanced chemiluminescence detection system (Amersham).

The only major non-tubulin proteins in the salt-extracted microtubule pellets (PIII) were  $\gamma$ - and  $\beta$ -actin, whose pIs are more basic than those of the tubulins. Blots were probed with a monoclonal anti-actin antibody (1:1000 dilution, clone AC-40, Sigma), allowing precise alignment of the different blots and the corresponding transferred Coomassie blue-stained gel. Membranes were first probed with a tubulin isotype-specific antibody and then with the anti-actin antibody, and finally, they were stripped before another tubulin isotype-specific antibody was used.

**IPG Strip in-Gel Trypsin Digestion.** IPG strips were stained with imidazole and zinc salts according to the



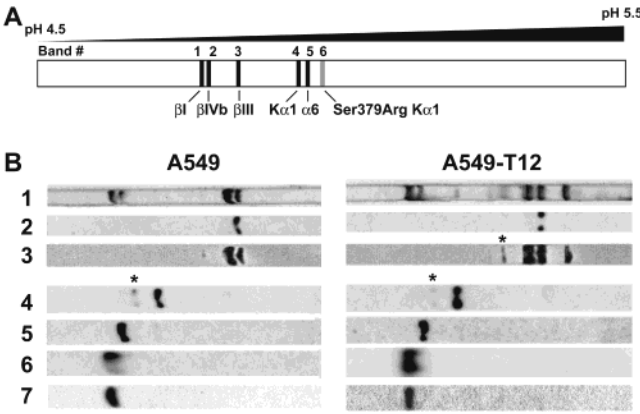
procedure of Castellanos-Serra et al. (16). Tubulin binds zinc, and positively stained tubulin bands appeared. The bands were cut with the plastic backing using scissors, washed with 1 mL of water ( $2 \times 5$  min,  $1 \times 10$  min), and dehydrated in 90% aqueous acetonitrile (0.5 mL, 2 min). After centrifugal evaporation of residual acetonitrile, 25  $\mu$ L of 12.5 ng/ $\mu$ L trypsin in 20 mM ammonium bicarbonate buffer (pH 8.3) containing 80 mM glycine was added to each tube. Trypsin digestion was performed at 37 °C overnight with constant shaking. The supernatant was desalted on a Millipore C18 ZipTip, and the tryptic peptides were eluted from the ZipTip with 4.0  $\mu$ L of a 50% acetonitrile/H<sub>2</sub>O mixture containing 0.1% trifluoroacetic acid.

**Protein Identification.** One microliter of a tryptic peptide mixture was mixed with 1  $\mu$ L of a saturated  $\alpha$ -cyano-4-hydroxycinnamic acid solution in a 50% acetonitrile solution containing 0.1% TFA, and 1  $\mu$ L of the resulting solution was deposited on a clean mass spectrometer probe surface. Mass spectra were recorded in the positive or negative mode on a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a 2.0 m flight tube and a 337 nm nitrogen laser. Protein identification was accomplished through a database search (Swiss-Prot and NCBI) using MS-Fit and ProFound programs (*Homo sapiens/Mus musculus*, mass measurement error within 1 Da, partially oxidized methionine, average and/or monoisotopic masses, and two maximum miscleavages). The isoelectric point for each tubulin was calculated using the Compute MW/pI tool from the ExPaSy Web site.

# RESULTS

**Taxol-Based Purification of Tubulin from Cell Lines.** Taxol microtubule pellets were isolated from cell lines using the method of Vallee (15). A representative preparation of tubulin from A549 cells is presented in Figure 1. There was no detectable tubulin in the first pellet (PI) containing cell debris and DNA, indicating that essentially all tubulin in the cell lysate (SI) was present in a soluble form. Tubulin polymerization by Taxol was quantitative as 98% of the tubulin present in SI was recovered in the Taxol-stabilized microtubule pellet (PII). The Taxol-stabilized microtubule pellet was washed with NaCl to release microtubule-associated proteins (MAPs). The resulting pellet (PIII) was highly enriched in tubulin, but a significant presence of tubulin was observed in SIII. Nevertheless, this loss from PIII was random regarding tubulin isotypes (data not shown) and most likely represented denatured tubulin. Therefore, the final microtubule pellet, PIII, was representative of cellular tubulin composition. We estimated that ~0.3 mg of tubulin was obtained from each preparation. Tubulin represented ~2% of total protein and approximately 5% of total soluble proteins in A549 cells.

**Separation of Tubulin Isotypes by Isoelectric Focusing.** IPG plates (pH 4.5–5.4) and IPG strips (pH 4–7) have been previously used to separate tubulin isoforms (17–19), but as indicated in Table 1, differences in pI values between two isotypes can be as small as 0.01. Only narrow-range IPG gels (pH 4.5–5.5) can resolve tubulin isotypes with such small differences in pI values. The length of the IPG gel and the shape of the pH gradient are also factors that increase the maximum separation of proteins. Therefore, we used 18



**FIGURE 2:** IEF-based analysis of tubulin isotypes in A549 and A549-T12 cells. (A) Schematic representation of an IPG strip (pH 4.5–5.5). Predicted positions of tubulin isotypes present in A549 cells are depicted as vertical black bands. A gray band indicates the predicted position of the mutant  $\alpha$ 1-tubulin present in A549-T12 cells. Bands were numbered from the most acidic to the most basic pI value. (B) Tubulin isotypes present in Taxol-stabilized microtubule pellets from A549 (left) or A549-T12 (right) were separated by IEF on 18 cm IPG strips and electrotransferred to nitrocellulose membranes: lane 1, IPG strips stained with Coomassie blue after electrotransfer; lane 2, blots probed with anti- $\alpha$ 6-tubulin antibody; lane 3, blots probed with anti- $\alpha$ 1-tubulin antibody; lane 4, blots probed with anti- $\beta$ III-tubulin antibody; lane 5, blots probed with anti- $\beta$ IV-tubulin antibody; lane 6, blots probed with anti- $\beta$ I-tubulin antibody; lane 7, blots probed with anti- $\beta$ II antibody. Asterisks denote minor acidic isoforms (see the Results).

**Table 2:** pI Differences between Tubulins

$\Delta$ pI	calculated	measured <sup>a</sup>
$\alpha$ 6 – $\alpha$ 1	+0.020	+0.017
mutant $\alpha$ 1 – $\alpha$ 1	+0.040	+0.044
$\beta$ III – $\beta$ I	+0.050	+0.055
$\beta$ IVb – $\beta$ I	+0.010	+0.011
glu $\alpha$ 1 – $\alpha$ 1	–0.030	–0.033
glu $\beta$ III – $\beta$ III	–0.030	–0.027

<sup>a</sup> Based on distances between bands (linear pH gradient).

cm IPG strips with a linear pH gradient from 4.5 to 5.5, on which tubulin bands having a pI difference of only 0.01 could be separated from each other by 2 mm. To visualize tubulin isotypes, tubulin in cell extracts was purified and concentrated by Taxol-driven polymerization. This approach allowed us to use Coomassie blue staining of the IPG strips. On the basis of our previous studies in A549 non-small cell lung carcinoma cells (13, 14), we expected to find the following tubulin isotypes from acidic to basic pI:  $\beta$ I-,  $\beta$ IVb-,  $\beta$ III-,  $\alpha$ 1-, and  $\alpha$ 6-tubulin. Lane 1 in Figure 2B shows that we effectively separated four major protein bands contained in Taxol-stabilized microtubules from A549 cells. Two bands were in the pI range expected for most  $\beta$ -tubulins, and two bands were in the pI range for  $\alpha$ -tubulins (Table 1 and Figure 2A). In A549-T12 cells, an A549 Taxol-resistant cell line containing a Ser to Arg substitution at residue 379 in  $\alpha$ 1-tubulin, an additional major band in the pI range for  $\alpha$ -tubulins and a minor band with an intermediate pI between those of  $\alpha$ - and  $\beta$ -tubulins were observed. The differences in pI observed between each band on the IPG strips stained with Coomassie blue matched the calculated pI differences between  $\beta$ I-,  $\beta$ IVb-, and  $\beta$ III-tubulin and between  $\alpha$ 1- and  $\alpha$ 6-tubulin (Table 2). The minor band in A549-T12 matched the expected position for  $\beta$ III-tubulin, and the major ad-

Table 3: Analysis of Tryptic Digests from IEF Gel Bands by MALDI-TOF Mass Spectrometry

IEF gel piece <sup>a</sup>	tubulin isotype-specific peptide <sup>b</sup>	measured mass of peptide (Da)	calculated mass of peptide (Da)	$\Delta$ mass (Da)	protein determination <sup>c</sup>
band 1	47ISVYYNEATGGK <sub>58</sub>	1300.7	1300.6	-0.1	$\beta$ I-tubulin
	283ALTVPQLTQQVFDK <sub>297</sub>	1658.6	1658.9	0.3	55% sequence coverage
	363MAVTFIGNSTAIQELFK <sub>379</sub>	1869.2	1869.0	-0.2	
band 2	63AVLVDLEPGTMDSVR <sub>77</sub>	1600.7	1600.8	0.1	$\beta$ IVb-tubulin
	20FWEVISDEHGIDPTGTYHGSDLQLER <sub>46</sub>	3115.6	3115.4	-0.2	49% sequence coverage
band 3	155VREEYPDR <sub>162</sub>	1062.7	1062.5	-0.2	$\beta$ III-tubulin
	310YLTVATVFR <sub>318</sub>	1068.8	1068.6	-0.2	42% sequence coverage
	47ISVYYNEASSHK <sub>58</sub>	1396.6	1396.7	0.1	
	363MSSTFIGNSTAIQELFK <sub>379</sub>	1872.6	1872.0	-0.6	60% sequence coverage
	217LATPTYGDLNHLVSATMSGVTTSLR <sub>241</sub>	2604.7	2604.3	-0.4	
	217LATPTYGDLNHLVSATMSGVTTSLR <sub>241</sub> (MSO)	2620.5	2620.3	-0.2	
	20FWEVISDEHGIDPSGNYVGSDLQLER <sub>46</sub>	3076.8	3076.4	-0.4	
band 4	340SIQFYDWCPTGFK <sub>352</sub>	1527.5	1527.7	0.2	$\alpha$ 1-tubulin
	281AYHEQLSVADITNACFEPANQMVK <sub>304</sub> (MSO)	2694.0	2694.2	-0.2	60% sequence coverage
	423EDMAALEKDYEEVGVDSEVEGEGEEEGEEY <sub>451</sub> <sup>d</sup>	3235.4	3235.3	-0.1	
band 5	431DYEEVGADSDGEDEGEY <sub>449</sub> <sup>d</sup>	2077.8	2077.7	-0.1	$\alpha$ 6-tubulin
	281AYHEQLTVAEITNACFEPANQMVK <sub>304</sub> (MSO)	2706.5	2706.3	-0.2	56% sequence coverage
	423EDMAALEKDYEEVGADSDGEDEGEY <sub>449</sub> <sup>d</sup>	2964.9	2965.1	0.2	

<sup>a</sup> IEF gel pieces were numbered from the most acidic to the most basic protein band (see Figure 2A). <sup>b</sup> Identified peptides and combinations of peptides specific for a tubulin isotype. MSO represents oxidized methionine. <sup>c</sup> Sequence coverage was calculated on the basis of all the matched tryptic peptides, i.e., tubulin isotype-specific and shared peptides detected. <sup>d</sup> Detection of these peptides was greatly improved in the negative mode.

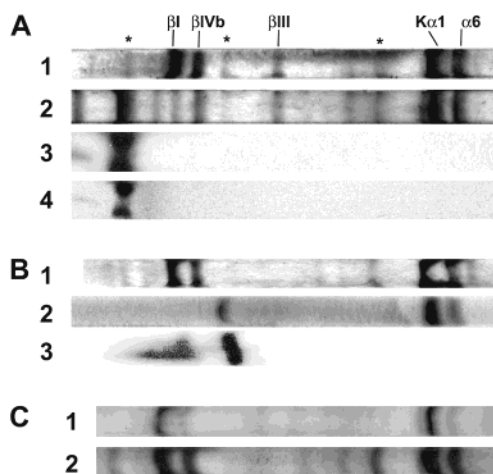
ditional band, with the most basic pI, matched the position of  $\alpha$ 1-tubulin harboring the Ser379Arg mutation (20). The Coomassie blue-stained IPG strips shown in Figure 2 (lane 1) were stained after electrotransfer onto nitrocellulose membranes, indicating that only a small portion of the proteins in each band was effectively transferred.

**Validation of Tubulin Isotype Positions on IPG Strips by MALDI-TOF Mass Spectrometry.** The position of each tubulin band on the IPG strips was definitively assigned by performing in-gel trypsin digestion followed by analysis of the respective tryptic digests by MALDI-TOF MS (Table 3). The major tubulin species were assigned numbers from 1 to 6 from the most to the least acidic tubulin, i.e., from  $\beta$ I-tubulin to the mutant  $\alpha$ 1-tubulin present in A549-T12 microtubules (Figure 2A). After tryptic digestion of each excised band, mass analysis detected several isotype-specific tryptic peptides that confirmed the assignment of each band to an individual tubulin isotype class based on the sequence-derived pI values (see above). For band 6, corresponding to the mutant  $\alpha$ 1-tubulin, the tryptic peptide profile was identical to the wild-type  $\alpha$ 1-tubulin peptide profile, except that the ion with an  $m/z$  ratio of 1808.5, corresponding to the peptide 374–390, was not detected, whereas an additional ion with an  $m/z$  ratio of 1203.5 was present (data not shown). The Ser379Arg replacement in the mutant  $\alpha$ 1-tubulin introduces an additional trypsin cleavage site in the wild-type peptide 374–390 ( $m/z$  ratio of 1808.1), resulting in two new tryptic peptides, peptide 374–379 ( $m/z$  ratio of 691.9) that was not detected and peptide 380–390 ( $m/z$  ratio of 1203.3) that was detected.

**Probing of IEF Western Blots with Tubulin Isotype-Specific Antibodies.** A polyclonal anti- $\alpha$ 6-tubulin antibody recognized one band at the position corresponding to  $\alpha$ 6-tubulin (Figure 2B, lane 2). Our anti- $\alpha$ 1-tubulin antibody was apparently also reacting with the  $\alpha$ 6-tubulin band and labeled the band corresponding to the mutant  $\alpha$ 1-tubulin from A549-T12 (lane 3). A minor  $\alpha$ -tubulin species, having

a pI more acidic than that of  $\alpha$ 1-tubulin, was also labeled with this antibody and corresponded most likely to monoglutamylated  $\alpha$ 1-tubulin (Table 2) (14). The anti- $\beta$ III-, anti- $\beta$ IV-, and anti- $\beta$ I-tubulin antibodies (Figure 2B, lanes 4–6, respectively) were remarkably specific, each of them labeling only the respective band for  $\beta$ III-,  $\beta$ IV-, and  $\beta$ I-tubulin. In the case of  $\beta$ III-tubulin, a second minor band was labeled and could correspond to either monoglutamylated or phosphorylated  $\beta$ III-tubulin (Table 2; see the Discussion). The anti- $\beta$ IV-tubulin antibody labeled one band corresponding to  $\beta$ IVb-tubulin. No labeling occurred at positions expected for either of the two possible  $\beta$ IVa-tubulin isotype sequences (Table 1). As expected, the anti- $\beta$ II-tubulin antibody and the anti- $\beta$ I-tubulin antibody labeled the same band because these two tubulin isotypes have the same pI value (Figure 2B, lane 7). Because of the previously mentioned potential cross-reactivity of this antibody with  $\beta$ I-tubulin (21) and the reported presence of  $\beta$ II-tubulin mRNA in these cells (13), we further examined the reactivity of this antibody (see below). The fact that none of the nine  $\beta$ II-tubulin-specific peptides were found in the tryptic digest of the  $\beta$ I-tubulin band (Table 3) strongly suggested that the anti- $\beta$ II-tubulin antibody was cross-reacting with  $\beta$ I-tubulin.

**Relative Quantitation of Tubulin Isoforms in A549 and A549-T12 Cells.** We evaluated the amount of total  $\alpha$ -tubulin and total  $\beta$ -tubulin by Western blot analysis after complete transfer of tubulin from a SDS-PAGE gel loaded with the different fractions presented in Figure 1. Using a standard curve with bovine brain tubulin and a pan  $\alpha$ - or a pan  $\beta$ -tubulin antibody, we obtained an  $\alpha$ : $\beta$  tubulin ratio of  $1.1 \pm 0.12$ . This ratio is close to the theoretical ratio of 1, considering that microtubules are formed of tubulin  $\alpha$ / $\beta$  heterodimers. However, the same evaluation of total  $\alpha$ -tubulin and total  $\beta$ -tubulin by Western blot analysis, after transferring IEF gels to nitrocellulose, was not possible because, as mentioned above, only a portion of the tubulin contained in each band transferred to the membrane.



**FIGURE 3:** Tubulin isotypes in human cancer cell lines. Tubulin was isolated from parental and drug-resistant cell lines and analyzed by IEF. (A) Lane 1, A549 tubulin isotypes stained with Coomassie blue; lane 2, A549-EpoB40 tubulin isotypes stained with Coomassie blue; lane 3, A549-EpoB40 tubulin isotypes blotted on a nitrocellulose membrane that was probed with anti- $\beta$ I-tubulin antibody; lane 4, A549-EpoB40 tubulin isotypes blotted on a nitrocellulose membrane that was probed with anti- $\beta$ II-tubulin antibody. Asterisks denote minor acidic isoforms. (B) Lane 1, MDA-MB-231 tubulin isotypes stained with Coomassie blue; lane 2, MDA-MB-231-K20T tubulin isotypes stained with Coomassie blue; lane 3, MDA-MB-231-K20T tubulin isotypes blotted on a nitrocellulose membrane that was probed with anti- $\beta$ I-tubulin antibody. (C) CA46 (lane 1) and HeLa (lane 2) tubulin isoforms were stained with Coomassie blue.

Therefore, we stained the IEF gels with Coomassie blue and quantified each tubulin band, and when we summed all the signals from the  $\alpha$ -tubulin bands or all the signals from  $\beta$ -tubulin bands, the resulting ratio of total  $\alpha$ -tubulin to total  $\beta$ -tubulin was greater than 1 ( $1.4 \pm 0.45$  for A549  $1.5 \pm 0.33$  for A549-T12). This ratio was dependent on tubulin concentration, and the ratio decreased to 1.1 when the IEF gels were loaded with a larger amount ( $>200 \mu\text{g}$  of protein) of tubulin (see Figure 3A, lane 1). When Fast green was used as the stain in place of Coomassie blue, the same differential staining between  $\alpha$ - and  $\beta$ -tubulin was observed (data not shown). It would appear that this excess of  $\alpha$ -tubulin over  $\beta$ -tubulin is only apparent and is due to differential staining of  $\alpha$ - and  $\beta$ -tubulin by Coomassie blue. For this reason, on Coomassie blue-stained IEF gels, the relative amount of a particular  $\alpha$ -tubulin or a particular  $\beta$ -tubulin isotype was calculated as a percentage of the total  $\alpha$ -tubulin or total  $\beta$ -tubulin, respectively. From at least three independent measurements,  $\beta$ I- and  $\beta$ IVb-tubulin in A549 microtubules represented  $66 \pm 10.3$  and  $29 \pm 1.2\%$  of total  $\beta$ -tubulin, respectively. In most samples,  $\beta$ III could not be detected by Coomassie blue staining except when a larger amount of protein was loaded on the IEF gel (Figure 3A, lane 1). K $\alpha$ 1- and  $\alpha$ 6-tubulin in A549 represented  $66 \pm 1.4$  and  $34 \pm 1.3\%$  of the total  $\alpha$ -tubulin, respectively. In A549-T12,  $\beta$ I-,  $\beta$ IVb-, and  $\beta$ III-tubulin represented  $56 \pm 5.0$ ,  $38 \pm 4.0$ , and  $6 \pm 4.0\%$  of the total  $\beta$ -tubulin, respectively. K $\alpha$ 1-tubulin,  $\alpha$ 6-tubulin, and the mutant K $\alpha$ 1-tubulin represented  $41 \pm 3.6$ ,  $28 \pm 6.0$ , and  $30 \pm 2.4\%$  of the total  $\alpha$ -tubulin, respectively. These relative percentages of the different tubulin isotypes were similar regardless of the amount of protein loaded on the IEF gel or whether Coomassie blue or Fast green was used as a stain.

**Expression of Tubulin Isotypes and of  $\beta$ I-Tubulin Mutations in Cell Lines.** We have examined the tubulin isotype composition in other cell lines. Some of these cell lines were resistant to Taxol or epothilones and harbored pI-altering mutations in  $\beta$ I-tubulin. The A549-derived epothilone B-resistant cell line, A549-EpoB40, has a  $\beta$ I $\text{Gln292Glu}$  mutation (5), and we observed the expected shift of  $\beta$ I-tubulin to a more acidic pI with no change in the pIs of other tubulins. However, an apparent decrease in the amount of  $\beta$ IVb-tubulin was noted (Figure 3A, lane 2). Western blotting with the anti- $\beta$ I-tubulin antibody confirmed that the  $\beta$ I-tubulin shifted to a more acidic pI and demonstrated that only the mutant  $\beta$ I-tubulin, but not the wild type, was expressed in this cell line (lane 3; see the Discussion). When the anti- $\beta$ II-tubulin antibody was used, the same labeling pattern was obtained as with the anti- $\beta$ I-tubulin antibody. Importantly, there was no labeling at the position corresponding to  $\beta$ II-tubulin or wild-type  $\beta$ I-tubulin, confirming the cross-reactivity of the anti- $\beta$ II-tubulin antibody with  $\beta$ I-tubulin (lane 4). The tubulin expression profile in MDA-MB-231 breast cancer cells was not significantly different from the A549 tubulin profile (Figure 3B, lane 1). The MDA-MB-231.K20T cell line has a  $\beta$ I $\text{Glu198Gly}$  mutation (22), and we observed the expected shift of  $\beta$ I-tubulin to a more basic pI (lane 2). Western blotting with the anti- $\beta$ I-tubulin antibody confirmed that the  $\beta$ I-tubulin shifted to a more basic pI and demonstrated that most of the  $\beta$ I-tubulin consisted of mutant protein with possibly a trace of wild-type protein (lane 3; see the Discussion). The same labeling pattern was observed with the anti- $\beta$ II antibody (data not shown). Moreover, in this resistant cell line,  $\beta$ IVb-tubulin, when observed, appeared only as a faint band by Coomassie blue staining compared to the parental cell line. These observations were in agreement with RT-PCR quantitative results on this cell line.<sup>2</sup> In the Burkitt's lymphoma cell line CA46, we observed only two major tubulins,  $\beta$ I- and K $\alpha$ 1-tubulin, and two minor tubulins,  $\beta$ IVb- and  $\alpha$ 6-tubulin (Figure 3C, lane 1). In HeLa cells, the tubulin profile was very similar to A549 and MDA-MB-231 profiles except that there was an apparent increase in the level of monoglutamylated K $\alpha$ 1 and possibly monoglutamylated  $\beta$ I-tubulin (lane 2).

## DISCUSSION

Alterations in tubulin isotype expression and introduction of point mutations into one or both alleles of specific tubulin genes have been associated with resistance to microtubule-stabilizing agents. RT-PCR analysis, although useful for RNA quantitation and sequencing, does not provide insight into the levels of protein expression. With the advent of proteomic-based approaches, it becomes clear that there can be a discrepancy between mRNA transcripts and corresponding protein levels (23, 24).

The isolation of tubulin from cell extracts using Taxol-driven polymerization coupled to high-resolution IEF enabled us to easily visualize and analyze the different tubulin isoforms present in cancer cell lines. We confirmed the presence of the tubulin isotypes in A549 and MDA-MB-231 cell lines identified previously by MALDI-TOF mass spectrometry analysis of C-terminal tubulin peptides (14).

<sup>2</sup> K. Wiesen, unpublished observations.



Because the C-terminal sequence obtained previously by MS/MS for  $\alpha^*$ -tubulin (14) matches the C-terminal sequence of the recently described human  $\alpha 6$ -tubulin, and because in the study presented here a protein having the pI value predicted for human  $\alpha 6$ -tubulin was detected and confirmed as being  $\alpha 6$ -tubulin by peptide mapping, we consider them to be identical. An increase in the level of  $\beta$ III-tubulin was detected in A549-T12 cells compared to the parental A549 cells, which is in agreement with previous immunofluorescence studies (13). A minor  $\beta$ III-tubulin species that corresponded most likely to monoglutamylated or phosphorylated  $\beta$ III-tubulin was also detected. Only monoglutamylated  $\beta$ I-tubulin, identified previously by MALDI-TOF mass spectrometry analysis of C-terminal tubulin peptides (14), was not readily detected by Western blotting (14). In all the IPG strips stained with Coomassie blue, we generally observed a barely detectable band at the position of monoglutamylated  $\beta$ I-tubulin, whereas a band at the position of monoglutamylated K $\alpha$ 1-tubulin was clearly visible and was detected by the anti-K $\alpha$ 1-tubulin antibody. Non-neuronal polyglutamylases generate these isoforms (25), but the process that limits the glutamylation to only one residue is unclear. The higher percentage of stable microtubules in the brain may favor extensive polyglutamylation, and conversely, the greater dynamicity of cancer cell microtubules (26) may limit polyglutamylase activity. Noticeably, known posttranslational modifications of tubulin are predicted to induce a shift in pI values. This is exemplified with the highly modified brain tubulin that appears as multiple bands on IPG strips and gels (17, 18).

In our previous approach, C-terminal peptides were not separated prior to MALDI-TOF mass spectrometry analysis (14).  $\beta$ III-Tubulin was not detected, and  $\beta$ IVb-tubulin represented a very small amount of C-terminal peptide. In this case, ions for minor tubulin species such as  $\beta$ III-tubulin or  $\beta$ IVb-tubulin could have been suppressed by the presence of the more highly abundant tubulin C-terminal peptides and/or because of differential ionization efficiencies of the various peptides. These technical limitations are overcome in the present isoelectrofocusing/mass spectrometry approach where the total amount of each tubulin isotype is analyzed directly, giving a better appreciation of isotype ratios. Our quantitation of Coomassie blue-stained IEF gels is still only relative. Nevertheless, when LC-MS was used to analyze the same Taxol-stabilized microtubule pellet from A549 and the deconvoluted mass peaks corresponding to each tubulin isotype were integrated, similar relative percentages were obtained.<sup>3</sup> Interestingly, the ratios of K $\alpha$ 1-tubulin to  $\beta$ I-tubulin and  $\alpha 6$ -tubulin to  $\beta$ IVb-tubulin are close to 1 in the cell lines that have been examined so far. In CA46 Burkitt's lymphoma,  $\alpha 6$ - and  $\beta$ IVb-tubulin are both minor species. This trend suggests that there could be a specific pairing of a particular  $\alpha$ -tubulin isotype with a particular  $\beta$ -tubulin isotype, and we plan to investigate the possibility of such  $\alpha/\beta$  subunit sorting.

The most striking difference between our analysis of tubulin isotype expression at the protein level (ref 14 and this study) and previous studies at the mRNA level (13) is the absence of detectable  $\beta$ IVa- and  $\beta$ II-tubulin expression

in both sensitive and resistant cell lines. In A549,  $\beta$ III-,  $\beta$ II-,  $\beta$ IVa-, and  $\beta$ IVb-tubulin mRNA were present at low and similar levels (RT-PCR tubulin isotype: $\beta 2$ -microglobulin ratio of  $\sim 0.15$ ) that were approximately 10 times lower than that of  $\beta$ I-tubulin mRNA (RT-PCR ratio of  $\sim 1.5$ ). A 2-fold increase in  $\beta$ III-,  $\beta$ II-, and  $\beta$ IVa-tubulin mRNA levels was observed in A549-T12 (13). In agreement with the results presented here, both  $\beta$ II- and  $\beta$ IVa-tubulin isoforms could not be detected by MALDI-TOF mass spectrometry of C-terminal peptides derived from total A549 cell extracts (14). The reasons for the discrepancy between tubulin isotype mRNA and protein expression levels are not clear, but such differences have been observed for other proteins (23, 24). Since our data established that tubulin was quantitatively recovered from cells by Taxol-driven polymerization (Figure 1), either there is an unknown bias in the RT-PCR results or there is a still unknown silencing mechanism of  $\beta$ II- and  $\beta$ IVa-tubulin mRNA translation. Increased  $\beta$ III-tubulin expression levels have been repeatedly confirmed at the protein level using a variety of methods in cell lines resistant to Taxol (13, 27–30). Together with the study presented here, these observations strongly suggest that only  $\beta$ III-tubulin levels correlate with sensitivity toward Taxol (13, 27–30) and that the regulation of  $\beta$ -tubulin expression is even more complex than the autoregulatory mechanism described by Cleveland and colleagues (9).

Mutations that produce a change in charge are likely to have a significant effect on protein function and/or drug binding. In the case of tubulin, we and others have obtained such mutations in cell lines selected for resistance to microtubule-stabilizing drugs (5–8). When analyzed by two-dimensional gel electrophoresis, the wide pH range used in the first dimension resulted in a poor resolution of the spots corresponding to wild-type and mutant proteins and prevented quantitation. Like the expression of a particular isotype, the detection of wild-type and mutant mRNA of a specific tubulin does not indicate that it is translated and, if so, at what ratio. Most likely, mutations affect only one allele (8), and it is expected that both tubulin isoforms would be produced in equal amounts. We found that situation in A549-T12, where the percentage of wild-type K $\alpha$ 1-tubulin decreased at the expense of mutant K $\alpha$ 1-tubulin ( $\sim 30\%$  of the total  $\alpha$ -tubulin). But in two cell lines, MDA-MB-231-K20T and A549-EpoB40, the wild-type  $\beta$ I-tubulin mRNA was not detected by RT-PCR (5, 22) and only the mutant protein was detected in these cells. The heterozygous status would need to be checked at the DNA level, and if confirmed, it would imply a silencing mechanism of the wild-type allele (8). If a mutation in tubulin plays a role in resistance to microtubule-interacting agents, the ratio of mutant tubulin to wild-type tubulin in a cell should influence the degree of resistance. In fact, when equal amounts of wild-type and mutant tubulin are expressed, the level of resistance to Taxol tends to be lower than when only the mutant tubulin is expressed (5, 8, 13).

The anti- $\beta$ -tubulin isotype monoclonal antibodies produced and characterized by R. F. Ludueña and co-workers (31–33) represent valuable tools that have helped to unravel the functional significance of the different  $\beta$ -tubulin isoforms. No cross-reactivity of the anti- $\beta$ I-, anti- $\beta$ III-, and anti- $\beta$ IV-tubulin antibodies was observed in this study. This is contrary

<sup>3</sup> P. Verdier-Pinard, manuscript in preparation.

to what has been published for nondenaturing immunoaffinity isolation of  $\alpha/\beta$ -tubulin heterodimers enriched in  $\beta$ I-,  $\beta$ II-,  $\beta$ III-, or  $\beta$ IV-tubulin isotypes (21, 31, 34, 35). Therefore, the presence of contaminating  $\beta$ -tubulin isotypes of a different class in these fractions is more likely due to heterodimer–heterodimer interactions in residual small oligomers than to cross-reactivity of these antibodies with multiple isotypes. However, extensive cross-reactivity of the anti- $\beta$ II-tubulin antibody with  $\beta$ I-tubulin was confirmed. The peptide EGEDEA that was used to generate the anti- $\beta$ II-tubulin antibody is from the C-terminal sequence of chicken  $\beta$ II-tubulin (EEGEDEA in humans; see Table 1) (21). The human  $\beta$ I-tubulin C-terminus contains the sequence GEE-AEEEA, so it is possible that the anti- $\beta$ II-tubulin recognizes the GEE sequence present in the  $\beta$ I-tubulin C-terminus. Recently, Arai et al. (36) described an anti- $\beta$ II-tubulin polyclonal antibody that specifically recognized the EEEE-GED sequence of the human  $\beta$ II-tubulin C-terminus and did not cross-react with C-terminal peptides of other  $\beta$ -tubulin isotypes. The peptide EAEVEA that is common to  $\beta$ IVa and  $\beta$ IVb was used to generate the anti- $\beta$ IV-tubulin antibody (34). Thus, both isotypes, with clearly distinct pIs, can be labeled with this antibody. However, we have observed only the presence of  $\beta$ IVb-tubulin in the cell lines examined so far.

There are fewer  $\alpha$ -tubulin isotype-specific antibodies available (37). A rabbit polyclonal antibody against human  $\alpha$ 6-tubulin, made in our laboratory by S. Rao, did not cross-react with  $\alpha$ 1-tubulin. However, the anti- $\alpha$ 1-tubulin polyclonal antibody cross-reacted with  $\alpha$ 6-tubulin. Further testing of these antibodies on samples containing different  $\alpha$ -tubulin isotypes will permit a more extensive definition of their specificity. Altogether, our approach provides a method for screening the specificity of antibodies directed against tubulin isotypes and establishes that the anti- $\beta$ I-, anti- $\beta$ III-, anti- $\beta$ IV-tubulin antibodies are highly specific.

A similar analysis of tubulin isotypes at the protein level in human tumor biopsies would provide an alternative to RT-PCR for investigating the clinical relevance of differential expression of tubulin isotypes in relation to the responsiveness of patients to microtubule-interacting drugs. Recent clinical data from non-small cell lung cancer patients indicated that both  $\beta$ III-tubulin mRNA levels (38) and  $\beta$ III-tubulin protein levels (39) were higher in tumors that did not respond to taxane-based treatment. Finally, the microtubule cytoskeleton appears to be increasingly involved in several neurodegenerative diseases (40–45), and comparative analysis of tubulin protein expression with normal tissue would provide greater insight into these pathologies.

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