Cell Motility and the Cytoskeleton 63:41–52 (2006)

Comparison of β-Tubulin mRNA and Protein Levels in 12 Human Cancer Cell Lines

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Antimitotic drugs are chemotherapeutic agents that bind tubulin and microtubules. Resistance to these drugs is a major clinical problem. One hypothesis is that the cellular composition of tubulin isotypes may predict the sensitivity of a tumor to antimitotics. Reliable and sensitive methods for measuring tubulin isotype levels in cells and tissues are needed to address this hypothesis. Quantitative measurements of tubulin isotypes have frequently relied upon inferring protein amounts from mRNA levels. To determine whether this approach is justified, protein and mRNA levels of β-tubulin isotypes from 12 human cancer cell lines were measured. This work focused on only β-tubulin isotypes because we had readily available monoclonal antibodies for quantitative immunoblots. The percentage of β-tubulin isotype classes I, II, III, and IVa + IVb mRNA and protein were compared. For β-tubulin class I that comprises >50% of the β-tubulin protein in 10 of the 12 cell lines, there was good agreement between mRNA and protein percentages. Agreement between mRNA and protein was also found for β-tubulin class III. For β-tubulin classes IVa + IVb, we observed higher protein levels compared to mRNA levels. β-Tubulin class II protein was found in only four cell lines and in very low abundance. We conclude that quantitative Western blotting is a reliable method for measuring tubulin isotype levels in human cancer cell lines. Inferring protein amounts from mRNA levels should be done with caution, since the correspondence is not one-to-one for all tubulin isotypes. Cell Motil. Cytoskeleton 63:41–52, 2006. © 2005 Wiley-Liss, Inc.

Key words: tubulin isotypes; microtubules; tubulin; β-tubulin

INTRODUCTION

Quantitative measurement of drug target proteins is essential for developing chemotherapeutic agents and understanding mechanisms that underlie drug resistance. Quantitative (real-time) reverse transcription polymerase chain reaction (qRT-PCR) is an established method for reliably measuring mRNA levels and may be useful for describing alterations in drug targets if protein levels can be inferred from mRNA levels. However, for many proteins, this is not a valid approach [Gygi et al., 1999; Nicoletti et al., 2001]. To study changes in drug target levels that may be associated with drug resistance to anti-

Abbreviations used: MBP, maltose binding protein; MDR, multidrug resistance; NCI, National Cancer Institute.

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Received 17 August 2005; accepted 17 November 2005

Published online 16 December 2005 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cm.20109

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mitotic drugs, we wanted to determine whether mRNA levels could be used to accurately predict protein levels.

The ability of anticancer agents to destroy tumors is severely hampered by drug resistance that occurs initially or develops after prolonged or repeated exposure of cells and tissues to these drugs. In advanced-stage metastatic cancers, where treatment options are few, this problem is devastating. Important drugs used to treat solid and hematological tumors are antimitotic agents: taxanes (paclitaxel or docetaxel) and vinca alkaloids (vinblastine, vincristine, vinorelbine or vinflunine). Antimitotic drugs bind tubulin, a major protein in microtubules, and halt cell division at metaphase. Their effectiveness is thought to result from alterations of microtubule dynamics in mitotic spindles, thus preventing spindle assembly or interrupting the movement of sister chromatids toward the spindle poles [Dhamodharan et al., 1995; Yvon et al., 1999].

The drug receptor of antimitotic agents, tubulin, is a structurally heterogeneous 100,000 dalton αβ heterodimer. Six or seven genes encode α-tubulin isotypes, and seven genes encode β-tubulin isotypes [Sullivan and Cleveland, 1986; Sullivan, 1988]. Antimitotics interact with β-tubulin, which consists of seven isotype classes distinguished by the last 15–20 amino acids at the carboxyl termini [reviewed in Correia and Lobert, 2001].

We present here a quantitative comparison of β-tubulin isotype mRNA and protein levels (β-tubulin classes I, II, III, and IVa + IVb) in 12 human cancer cell lines. qRT-PCR was used to measure mRNA levels of all seven classes of β-tubulin isotypes. Quantitative immunoblotting was done to measure β-tubulin isotype protein levels. β-Tubulin classes I and IVa + IVb proteins were the most abundant. The mRNA and protein levels of β-tubulin class I were found to be in good agreement. β-Tubulin classes IVa + IVb made up a larger fraction of the total protein than of the total mRNA for 11 of the 12 cell lines. β-Tubulin class III protein was found at low levels that corresponded to mRNA levels, although the relationship was not one-to-one.

MATERIALS AND METHODS

Cell Culture

Twelve cell lines were selected on the basis of their relative sensitivities to vinblastine and paclitaxel, as reported by the National Cancer Institute (NCI) Developmental Therapeutics Program Human Tumor Cell Line Screen (http://dtp.nci.nih.gov/docs/cancer/cancer_data.html). Pairs or groups of cells with reported drug sensitivities that differed significantly were selected. Only one (HCT-15) is reported to have relatively high levels of *p*-glycoprotein/MDR1, a membrane-bound pump known to extrude drugs such as paclitaxel and vinblastine from cells [Alvarez et al., 1995; http://dtp.nci.nih.gov/docs/compare/cellmdr.html]. Cell lines were purchased from American Type Culture Collection (Manassas, VA), except for HOP 18, which was kindly provided by NCI-Frederick Cancer Division of Cancer Treatment and Diagnosis Tumor/Cell Line Repository (Frederick, MD). Cells were cultured according to the suppliers' recommendations, with the exception of MDA-MB-231 and Malme-3M, which were grown in RPMI-1640 rather than Leibovitz's L-15 medium.

Determination of IC_{50} Values

We determined IC_{50} values (concentration at which cell proliferation is reduced by 50%) for each cell line using two independent methods: cell counting and an assay for mitochondrial respiration. Duplicate 12-well plates (5000 cells/well) were set up for each cell counting experiment, and two to four independent experiments were done for each drug (vinblastine or paclitaxel) and cell line. Cells were incubated for 24 h to permit adherence and then exposed to serial dilutions (between one log unit above and below the predicted IC_{50} value) of vinblastine or paclitaxel for 48 h, released using trypsin, and counted in a hemocytometer using trypan blue to exclude nonviable cells. For control wells, an appropriate amount of PBS or dimethyl sulfoxide for vinblastine and paclitaxel, respectively, was added. Data were plotted using Origin 7.0 (OriginLab, Northampton, MA) as cell number vs. drug concentration. Data were fit to first-order exponential decay, and IC_{50} values were calculated.

The cell line sensitivities to vinblastine and paclitaxel were also determined using an assay for mitochondrial respiration. Cells were plated in 96-well microtiter plates at 8000 cells/well (10,000 cells/well for Malme-3M). Duplicate plates constituted each experiment; and two to four independent experiments were done for each drug (vinblastine or paclitaxel) and cell line. Cells were incubated for 24 h, and then the medium was replaced with fresh medium containing appropriate concentrations of drug or solvent. After 72 h, the medium was removed from the wells and 100 μl of CellTiter 96® AQ_{ueous} One solution (Promega, Madison, WI) diluted 1:5 with medium was added. This solution utilizes 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, inner salt) in a colorometric reaction that is a linear measure of mitochondrial respiration. The plates were incubated 2 h, and absorbance at 490 nm was read using a microplate reader. Absorbance values were corrected by subtracting baseline values obtained from wells with no cells. IC_{50} values were determined as described for the cell counting assay.

Quantitative Reverse Transcription Polymerase Chain Reaction

Two-step qRT-PCR was used to determine the amount of mRNA present for each β-tubulin isotype (classes I, II, III, IVa, IVb, V, and VI) in all 12 cell lines. Cells were cultured as described earlier, total RNA was extracted, and qRT-PCR experiments were carried out using primers and protocols described previously [Dozier et al., 2003]. Briefly, qRT-PCR was performed on triplicate samples from at least two independent cell preps using SYBR® Green I (Invitrogen, Carlsbad, CA) as the detection method. The amount of β-tubulin isotype mRNA in a known amount of total RNA, determined by A_{260} measurements, was calculated using standard curves.

Quantitative Immunoblots

We developed a quantitative immunoblot assay for tubulin that is sensitive and highly reproducible. Cell lysates were prepared from 10–20 million cells by douncing on ice in 1 ml PBS with a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets, Roche, Indianapolis, IN). Lysates were spun briefly in a microcentrifuge to remove membranes and mixed 1:1 with SDS sample buffer prior to electrophoresis. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes, incubated with primary antibody (1–2 mg/ml) at 1:2,500–1:10,000 dilutions overnight at 4°C, and developed using a horseradish peroxidase-conjugated secondary antibody. We used monoclonal antibodies that recognize individual tubulin isotypes: SAP4G5, anti-β-tubulin class I (Sigma-Aldrich, St. Louis, MO); 7B9, anti-β-tubulin class II; TUJ1, anti-β-tubulin class III; and 10A2, anti-β-tubulin classes IVa + IVb. The reactivity and specificity of SAP4G5, 7B9, and TUJ1 have been previously described [Lee et al., 1990; Lobert et al., 1995, 1998; Roach et al., 1998]. The monoclonal antibody 10A2 was raised against the carboxyl terminal peptide of β-tubulin class IVa. It was found to be specific for both β-tubulin classes IVa and IVb (Fig. 1). Blots were reacted with chemiluminescent reagents (SuperSignal® West Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL) and exposed to X-ray film. Densities for the known and unknown sample bands on the films were obtained using a Molecular Dynamics densitometer with ImageQuant Software (Amersham, Piscataway, NJ).

Known amounts of pig brain tubulin were used as standards on all blots. Prior work demonstrated that microtubule associated protein-free phosphocellulose-purified tubulin preps from pig brain is 3% β-tubulin class I, 55% β-tubulin class II, 29% β-tubulin class III, and 13% β-tubulin class IV [Banerjee et al., 1988; Dozier et al., 2003]. The quantities of tubulin isotypes in unknown samples were calculated from linear fits of standard curves from each Western blot done in triplicate and for at least two lysates prepared from independently grown cell cultures. It was critical to establish the linearity of response for each antibody and to determine that unknown samples were within the linear range. Extrapolation to values outside the standard curve can result in significant error.

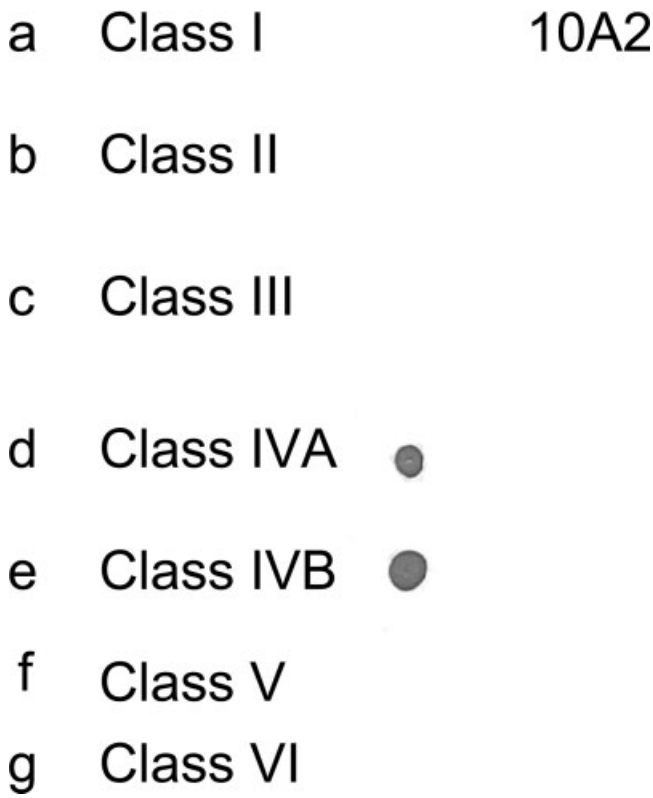

Fig. 1. Dot blots of β-tubulin isotype fusion proteins. β-Tubulin fusion proteins were transferred to PVDF filters and reacted with anti-β-tubulin class IVa + IVb monoclonal antibody 10A2. Fusion protein reactions shown are for β-tubulin class I (Panel a), class II (Panel b), class III (Panel c), class IVa (Panel d), class IVb (Panel e), class V (Panel f), and class VI (Panel g). Only β-tubulin fusion proteins constructed with class IVa or IVb carboxyl terminal peptides were found to react.

Calibration of Antibody Reactivity With β-Tubulin Fusion Proteins

The tubulin protein measurements in the work presented here were done on lysates from human cell lines. Because human and pig brain tubulins may interact differentially with mouse monoclonal antibodies, we constructed fusion proteins with human β-tubulin isotype peptides to compare and calibrate the antibody reactivity. Maltose binding protein (MBP) β-tubulin fusion proteins were constructed for each of the seven human β-

TABLE I. Oligonucleotide Primers and β-Tubulin Carboxyl Terminal Sequences for β-Tubulin Fusion Proteins

Class	Forward primer sequence 5′–3′	Reverse primer sequence 5′–3′	Fragment (bp)	Cloned as	Carboxyl terminal sequences
I	aaa gaa ttc gat gcc acc gca gaa gag gag gag gat ttc	aaa aag ctt cta ccc act acc ttc tac cat t	485	EcoRI/HindIII	DATAEEEEDFGEEAEEEA
II	aaa gaa ttc gac gcc acg gcc gac gaa caa ggg	aaa agc tta caa acg ttt atg tga ttt tag	325	EcoRI/HindIII	DATADEQGEFEEEEGEDEA
III	aaa gaa ttc gac gcc acg gcc gag gaa gag g	aaa aag ctt aag ggt atc tga cag caa taga	388	EcoRI/HindIII	DATAEEEGEMYEDDEEESEAQGPK
IVa	aaa gaa ttc gac gcc acg gcc gag gag g	aaa aag ctt ggg tta aag ata aat tag gg	279	EcoRI/HindIII	DATAEEGEFEEEAEEEVA
IVb	aaa gaa ttc gat gcc aca gcc gag gag ga	aaa tct aga atg aaa atg ctt taa tgg	225	EcoRI/Xba I	DATAEEEGEFEEEAEEEVA
V	aaa gaa ttc gat gcc acc gcc aat gac g	aaa tct aga gaa gac aca cgt tta gta tt	519	EcoRI/Xba I	DATANDGEEAFEDEEEEIDG
VI	aaa gaa ttc gat gcc aaa gca gtt cta ga	aaa aag ctt ggc aaa cac ttt gaa aca aag gga g	388	EcoRI/HindIII	DAKAVLEEDEEVTEEAMEPEDKGH

tubulin isotypes (Table I). DNA fragments corresponding to the coding region of the extreme C-terminus of all seven human β-tubulin isotypes were obtained by PCR amplification from either cDNA or genomic DNA, using the primer sets indicated in Table I. The fragments were cloned in-frame (sites indicated in Table I) into a modified version of the pMBP-His-Parallel vector [Sheffield et al., 1999] (a gift from O. Karginova, Department of Biology and Z. Derewenda, Department of Physiology and Biophysics, University of Virginia), resulting in the generation of fusion proteins whose N-terminus was the MBP, and whose C-terminus was the tubulin isotype indicated, separated by a 6X His tag. Nucleotide sequences across the relevant portions of the tubulin coding regions were verified using the Big Dye method on an ABI 310 DNA sequencer. The fusion proteins were expressed in an *Escherichia coli* DH5α background with induction by 0.5 mM isopropyl-β-D-thiogalactopyranoside, and subsequently purified by metal chelate chromatography on nickel nitrilotriacetic resin (Qiagen). Purity was assessed by SDS-PAGE.

The fusion proteins were loaded in known amounts onto SDS-PAGE along with known quantities of pig brain tubulin. The fusion protein concentrations were determined from the optical density at 278 nm, using extinction coefficients calculated from the amino acid sequence: fusion-β-tubulin class I, $\varepsilon_{278} = 1.493$ l/g cm; fusion-β-tubulin class II, $\varepsilon_{278} = 1.489$ l/g cm; fusion-β-tubulin class III, $\varepsilon_{278} = 1.500$ l/g cm; fusion-β-tubulin class IVa, $\varepsilon_{278} = 1.493$ l/g cm; fusion-β-tubulin class IVb, $\varepsilon_{278} = 1.489$ l/g cm. The concentration of pig brain tubulin was also determined spectrophotometrically ($\varepsilon_{278} = 1.2$ l/g cm) [Detrich III and Williams Jr., 1978]. Gels were transferred to PVDF membranes for immunoblotting and the reactivity of the fusion proteins and pig brain were compared using densitometry. Correction factors and standard errors were calculated for each antibody (Table II) from triplicate Western blots by dividing the known amount of β-tubulin fusion protein loaded on

TABLE II. Corrections for β-Tubulin Isotype Data Determined Using Pig Brain Standards

β- Tubulin class	Correction factor ± SE[a]
I	0.64 ± 0.12
II	0.61 ± 0.17
III	0.27 ± 0.047
IVa + IVb	1.31 ± 0.31

[a]These correction factors were used to calculate the actual amount of β-tubulin in human cell lines: $\beta\text{-tubulin}_{human}$ = correction factor × $\beta\text{-tubulin}_{pig\ brain}$. Correction factors were calculated as the actual amount (μg) of β-tubulin fusion protein loaded on the gel for Western blotting divided by the amount of β-tubulin predicted using pig brain tubulin for the standard curve. The standard error (SE) from triplicate blots is indicated.

TABLE III. β-Tubulin Isotype Protein and mRNA Levels

Cell line	βI	βII	βIII	βIVa	βIVb	βV
Percentage tubulin isotype mRNA						
Colon cancer						
COLO 205	85.7	0.6	2.6	0	11.1	0
HCT-15	65.0	0.8	3.4	0	5.1	25.8
Breast cancer						
BT-549	74.9	0.1	16.0	0	2.3	6.7
T-47D	76.2	0.7	0.7	0.1	6.4	16.4
MCF-7	78.1	0.2	14.1	0.1	4.3	3.2
MDA-MB-231	78.3	0.2	2.4	0	15.8	13.2
Lung cancer						
A549	57.8	2.3	6.7	0.5	4.2	28.5
HOP 18	38.9	6.6	14.8	0	9.7	30.0
Melanoma						
Malme-3M	57.7	13.5	8.0	4.1	1.6	15.2
SK-MEL-2	51.2	1.1	3.5	0.9	3.1	40.2
Ovarian cancer						
OVCAR-3	42.6	5.2	5.2	0.7	9.4	36.8
SK-OV-3	55.9	0	1.6	0.1	4.3	38.1

Cell line	βI	βII	βIII	βIVa + βIVb
Percentage tubulin isotype protein				
Colon cancer				
COLO 205	36.0	0	0	64.0
HCT-15	61.8	0	0.2	38.0
Breast cancer				
BT-549	65.9	0.6	6.4	27.1
T-47D	85.7	0	0	14.3
MCF-7	39.1	0	2.5	58.4
MDA-MB-231	76.1	0	0	23.9
Lung cancer				
A549	71.9	0	1.6	26.5
HOP 18	63.2	1.5	5.0	30.3
Melanoma				
Malme-3M	84.4	3.8	5.1	7.0
SK-MEL-2	73.1	1.4	1.5	24.0
Ovarian cancer				
OVCAR-3	97.0	0	0	3.1
SK-OV-3	85.6	0	0.8	13.6

the gel by the amount of β-tubulin isotype estimated from the pig brain standard curve. These factors were used to correct the data obtained using pig brain standard curves on the Western blots. The percentages of tubulin isotype proteins shown in Table III and Fig. 3b were corrected using the correction factors in Table II.

RESULTS

β-Tubulin mRNA Expression

We used qRT-PCR to measure the amounts of β-tubulin isotype mRNA classes I, II, III, IVa, IVb, V, and VI for each of the 12 cell lines (Fig. 2). The mRNA levels for each isotype, with the exception of class I, differed by more than 10-fold between the 12 cell lines; however, the total tubulin mRNA levels were found to be fairly constant, differing by less than 10-fold across all 12 cell lines. This is consistent with the previous suggestion that total tubulin expression levels are regulated to fulfill the function of the cytoskeleton [Cabral and Barlow, 1991; Wang and Cabral, 2005].

Figure 3a and Table III present the isotype mRNA data as percentages of total β-tubulin mRNA for each cell line. As reported for other cell lines [Nicoletti et al., 2001], it is clear that β-tubulin class I mRNA comprises more than 50% of the β-tubulin mRNA for all but two of the cell lines examined. As expected, the hematopoietic-specific β-tubulin class VI mRNA was either absent or 4–6 orders of magnitude less prevalent (Fig. 2) than β-tubulin class I. The neuron-specific isotype, β-tubulin class III, was expressed at relatively high levels (>10% of total mRNA) in 3 of the 12 cell lines, including 2 of the 4 breast cancer cell lines. In fact, β-tubulin class III mRNA constituted more than 1% of the tubulin message in all of the cell lines except T-47D, a surprising result considering that β-tubulin class III is neuron specific. In addition, several cell lines expressed very low but measurable levels of the ''neuron-specific'' β-tubulin class IVa. Relatively high levels of β-tubulin class V mRNA (>25% of the total β-tubulin mRNA) were detected in half of the cell lines. This raises the intriguing possibility that β-tubulin class V is highly expressed in human cells, something that has not been measurable until recently due to the absence of a commercially available β-tubulin class V antibody. Overall, the mRNA levels of β-tubulin class IVb were comparable to those of β-tubulin class III, although one or the other was significantly more abundant in some cell lines. In general, β-tubulin class II mRNA was less abundant than β-tubulin classes I, III, IVb, and V messages.

Because alterations in tubulin isotype levels have been implicated in resistance to antimitotic agents [Kavallaris et al., 1997, 1999; Ranganathan et al., 1998; Banerjee, 2002], we measured IC_{50} levels for all 12 cell lines used in this study (Table IV). No correlations between IC_{50} values and tubulin isotype mRNA levels were found (data not shown). However, these cell lines represent several tumor types and the contribution of tubulin isotypes to drug sensitivity could be masked by many factors.

β-Tubulin Protein Levels

Mouse monoclonal antibodies specific for β-tubulin classes I, II, III, and IVa + IVb were utilized in quantitative Western blotting of whole cell lysates from 12 human cell lines. We found that pig brain tubulin is a plentiful and reliable source of tubulin for standard curves on Western blots. However, because pig brain tubulin and human cell lysates may react differently with the monoclonal antibodies, we constructed fusion proteins with the human tubulin carboxyl terminal peptides and compared

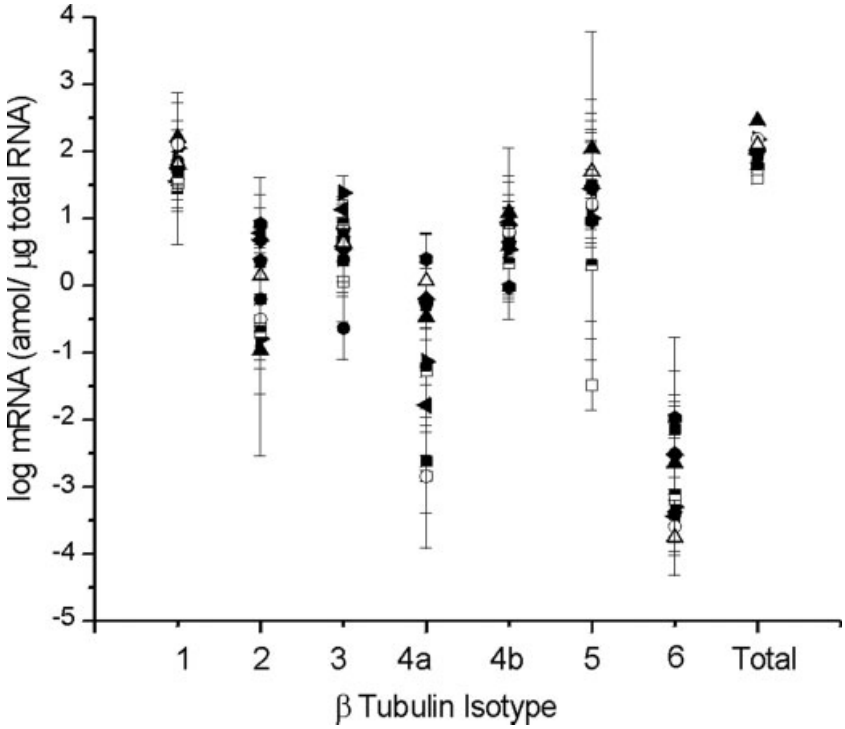

Fig. 2. β-Tubulin isotype mRNA for twelve cell lines. Mean data from qRT-PCR of two or more cell preps are shown. Data from β-tubulin isotype classes I (1), II (2), III (3), IVa (4a), IVb (4b), V (5), and VI (6) were summed to calculate the total β-tubulin mRNA in each cell line.

their reactivities on Western blots with that of pig brain tubulin. The largest difference in reactivity was found for β-tubulin class III (Table II). The reaction of TUJ1 monoclonal antibody with pig brain standard overestimates the actual amount of human β-tubulin class III fusion protein by 3.7 (±0.6)-fold. β-Tubulin classes I and II are overestimated by about 1.6 (±0.4)-fold using the pig brain standards, and β-tubulin classes IVa + IVb are underestimated by about 0.8 (±0.2)-fold. We adjusted all tubulin measurements, using the correction factors in Table II, to estimate actual tubulin protein levels in human cell lines. An alternate method of standardization is to construct standard curves with the β-tubulin fusion proteins. The advantage of the quantitative immunoblot method presented here is that it can be used as a rigorous tool for studying tubulin levels in whole cell or tissue lysates without requiring fusion protein standards.

We used quantitative Western blotting of whole cell lysates to measure β-tubulin isotype protein levels for classes I, II, III, and IVa + IVb (Fig. 3b and Table III). Significant levels of β-tubulin classes I and IVa + IVb were found in all 12 cell lines, ranging from 36.0–97.0% for β-tubulin class I and from 3.1–64.0% for β-tubulin classes IVa + IVb. β-Tubulin class I comprised >50% of the total β-tubulin measured in 10 of the 12 cell lines. β-Tubulin classes IVa + IVb comprised >50% of the total β-tubulin in breast cancer (MCF-7) and colon cancer (COLO 205) cells. β-Tubulin class III comprised more than 1% of the total β-tubulin protein (1.5–6.4%) in breast cancer cell lines, BT-549 and MCF-7; lung cancer cells, A549 and HOP 18; and melanoma cells, Malme-3M and SK-MEL-2. β-Tubulin class II was found at low but measurable levels (0.6–3.8%) in only four cell lines—breast cancer (BT-549), lung cancer (HOP 18) and melanoma (SK-MEL-2 and Malme-3M) cell lines.

Comparison of mRNA and Protein

β-Tubulin mRNA and protein levels for isotype classes I, II, III, and IVa + IVb are compared in Fig. 4. β-Tubulin classes V and VI mRNA data were not included in this analysis because we did not have corresponding protein data. Thus, the percentages of β-tubulin isotype mRNA in Fig. 4 were calculated assuming that the total tubulin was comprised of only β-tubulin isotype classes I, II, III, and IVa + IVb. β-Tubulin classes I and IVa + IVb are present in greatest abundance for both mRNA and protein. Only β-tubulin class I data suggest a near one-to-one correspondence for mRNA and protein. For β-tubulin class I, 10 of the 12 cell lines have both mRNA and protein levels >50%. For β-tubulin class IVa + IVb, there is consistently more protein than mRNA in 10 of the 12 cell lines. β-Tubulin class II protein is found in only four cell lines; therefore, no general conclusions regarding the relationship between mRNA and protein levels can be made. β-Tubulin class III protein is found in eight cell lines. The mRNA and protein amounts for β-tubulin class III correspond (Fig.

4, Panel c insert), although there is consistently more mRNA than protein.

DISCUSSION

The role of β-tubulin isotype levels in determining cellular responses to antimitotic agents has been controversial for more than a decade. Most studies have relied upon measurements of intracellular tubulin mRNA levels to infer protein levels. However, mRNA and protein levels are not correlated for many human and yeast proteins, especially those that are regulated by posttranscriptional processes [Anderson and Seilhamer, 1997; Gygi et al., 1999]. Thus, it is important to establish whether there is a correlation between tubulin mRNA and protein levels so as to better understand mechanisms that may underlie drug resistance.

We present here a reliable method for quantifying β-tubulin protein levels in whole cell lysates. We demonstrated that tubulin isotype fusion proteins or pig brain tubulin calibrated with the fusion proteins can be used to create a standard curve on Western blots. We find this to be an acceptable method for protein quantification for the following reasons: (1) The monoclonal antibodies used in this work are highly specific as demonstrated by blots with either purified tubulin isotypes or tubulin isotype fusion proteins [Lee et al., 1990; Lobert et al., 1995, 1998; Roach et al., 1998] (Fig. 1). (2) The data are highly reproducible. We present here data collected from independent cell protein extractions and at least three Western blots for each whole cell lysate. The standard error for each measurement was propagated from the biological (individual protein preparations) and technical (individual Western blots) replicates and is represented as the error bars in Fig. 3b. For the two most abundant isotypes measured, β-tubulin classes I and IVa + IVb, the average error was 14.6% and 23.3%, respectively. (3) There is good agreement between the mRNA and protein levels for β-tubulin isotype classes I and III (although for β-tubulin class III the correspondence is not one-to-one). The agreement in isotype percentages by two independent methods supports the findings and the method used for protein quantification.

The higher β-tubulin class IVa + IVb protein levels compared to mRNA levels suggest possible differential regulatory mechanisms for this isotype compared to β-tubulin classes I and III. Fine tuning of tubulin levels during the cell cycle occurs via autoregulation. This process alters mRNA stability and requires both an essential sequence on polysomal β-tubulin mRNA and an aminoterminal Met-Arg-Glu-Ile on the nascent tubulin polypeptide [Pachter et al., 1987; Cleveland, 1988; Yen et al., 1988]. Furthermore, α-tubulin mRNA levels remain high even when protein synthesis is repressed [Gonzalez-Garay and Cabral, 1996]. In fact, α-tubulin synthesis in Chinese hamster ovary (CHO) cells appears to play a role in regulating β-tubulin protein levels. This phenomenon of tubulin posttranscriptional autoregulation was demonstrated in a wide variety of vertebrate and invertebrate cell types. Intracellular tubulin levels are also transcriptionally regulated [Cleveland, 1989]. It is possible that individual isotypes may be differentially regulated while maintaining free tubulin subunits at levels necessary for different phases of the cell cycle. Our data demonstrate relatively constant amounts of total tubulin message in spite of variations in individual isotype mRNA levels (Fig. 2). Because of the relatively low levels of β-tubulin classes IVa + IVb mRNA compared to protein, our data suggest that differential methods for regulating β-tubulin classes (I or III vs. IVa + IVb) are likely. Alternatively, pairing of specific α- and β-tubulins may lead to differential stability of β-tubulins [Hoyle et al., 2001].

β-Tubulin class III has been studied extensively for its potential role in resistance to antimitotic agents. For example, paclitaxel-resistant lung cancer cells and ovarian tumors were shown to have increased levels of tubulin isotypes, particularly β-tubulin classes III and IVa mRNA levels [Kavallaris et al., 1997]. Furthermore, when paclitaxel-resistant lung cancer cells were treated with antisense oligonucleotides for β-tubulin class III, the drug resistance was partially reversed, coincident with reduced protein levels of β-tubulin class III [Kavallaris et al., 1999]. In other work, β-tubulin classes III and IVa transcripts were shown to increase in paclitaxel-resistant prostate cancer cells [Ranganathan et al., 1998]. Combined isoelectric focusing and mass spectrometry were used to measure tubulin isotypes in four cell lines [Verdier-Pinard et al., 2003]. This report demonstrated an increase in β-tubulin class III and associated this with paclitaxel resistance in lung cancer cells. In the most convincing study, when cells were transfected with β-tubulin class III, weak resistance to paclitaxel was found and this resistance was associated with decreased microtubule stability [Hari et al., 2002]. Thus, there is considerable interest in determining whether functional β-tubulin class III protein is involved in mechanisms that cause resistance to antimitotic agents. Because qRT-PCR is a commonly used reliable method for quantitation of mRNA levels, it is potentially an important method for better understanding the role of β-tubulin isotypes in drug resistance. To draw conclusions from mRNA measurements, it is essential to know whether transcript levels correlate with protein levels. In the work described here, β-tubulin class III mRNA and protein levels are correlated but do not demonstrate one-to-one correspondence. β-Tubulin class III mRNA may be useful for comparing relative amounts of this isotype across cell and tissue samples; however, quantitatively inferring β-tubulin protein

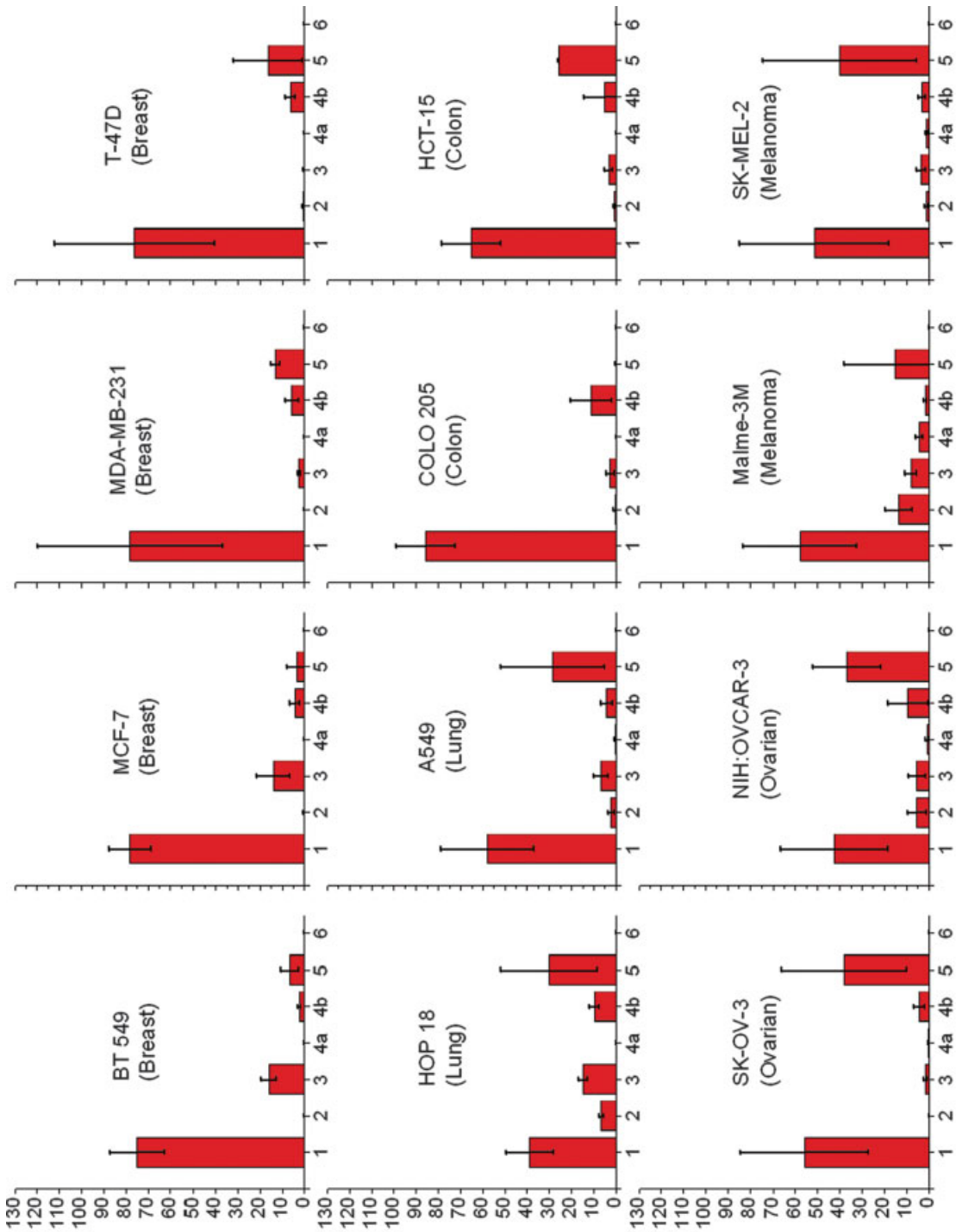

Fig. 3. β-Tubulin isotype profiles for 12 cell lines. The data in Table III are plotted as bars representing the percent contribution of each β-tubulin isotype mRNA (**a**) or protein (**b**) to the total tubulin in each cell line. The sum of the values for all six β-tubulin isotype classes was considered as the total tubulin for the mRNA percentage calculations. The sum of the β-tubulin isotype classes I, II, III, and IVa + IVb was considered to be the total tubulin for the protein analysis. Error bars represent standard deviation from multiple experiments, as described in the Materials and Methods.

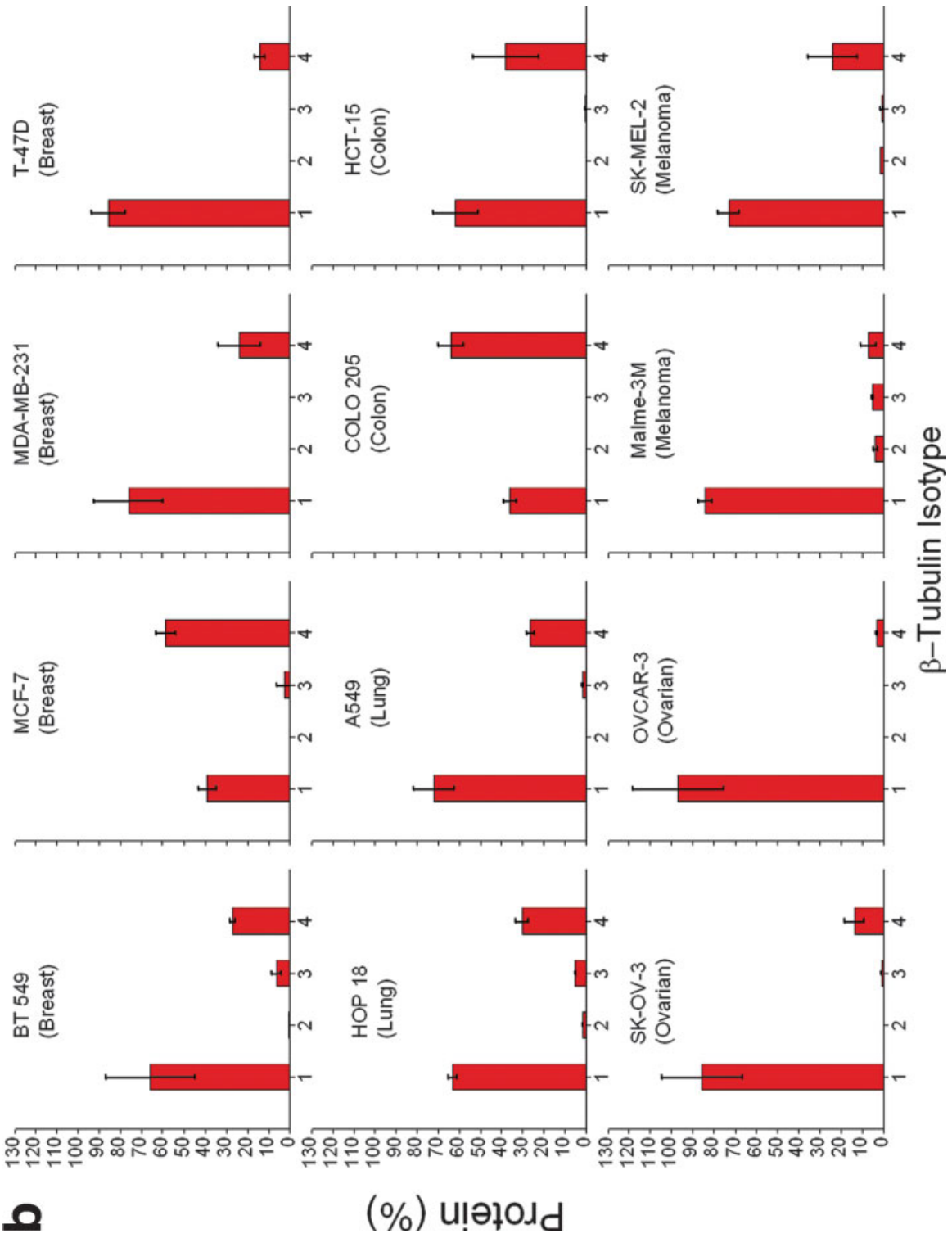

Figure 3. (Continued)

TABLE IV. Drug Sensitivities of Human Cancer Cell Lines to Paclitaxel and Vinblastine as Measured by Two Different Assays

Cancer cell line	Drug	IC_{50} (nM)		IC_{50} (nM) Mean	Fold difference[b]
		Cell count	MTS assay[a]		
Colon					
COLO-205	Paclitaxel	29.2	26.1	27.7	6.0
HCT-15	Paclitaxel	6.4	2.8	4.6	
COLO-205	Vinblastine	0.6	0.7	0.7	
HCT-15	Vinblastine	31.1	24.9	28.0	40
Breast					
BT-549	Paclitaxel	4.3	5.5	4.9	4.9 (MCF7), 12.3 (MDA), 3.3 (T-47D)
MCF-7	Paclitaxel	1.0	1.0	1.0	2.5 (MDA)
MDA-MB-231	Paclitaxel	0.3	0.5	0.4	
T-47D	Paclitaxel	0.8	2.3	1.5	1.5 (MCF7), 3.8 (MDA)
BT-549	Vinblastine	0.9	1.3	1.1	2.8 (MCF7)
MCF-7	Vinblastine	0.4	0.4	0.4	
MDA-MB-231	Vinblastine	2.2	0.9	1.6	1.5 (BT549), 4.0 (MCF7)
T-47D	Vinblastine	5.3	13.5	9.3	8.5 (BT549), 23.3 (MCF7), 5.8 (MDA)
Lung					
A-549	Paclitaxel	2.9	2.5	2.7	3.9
HOP-18	Paclitaxel	0.6	0.8	0.7	
A-549	Vinblastine	2.1	1.2	1.7	3.4
HOP-18	Vinblastine	0.7	0.4	0.5	
Melanoma					
Malme-3M	Paclitaxel	4.2	4.6	4.4	1.1
SK-MEL-2	Paclitaxel	3.7	4.1	3.9	
Malme-3M	Vinblastine	0.3	1.1	0.7	
SK-MEL-2	Vinblastine	0.6	1.3	0.9	1.3
Ovarian					
OVCAR-3	Paclitaxel	2.1	0.8	1.5	
SK-OV-3	Paclitaxel	2.0	4.6	3.3	2.2
OVCAR-3	Vinblastine	3.7	1.3	2.5	1.6
SK-OV-3	Vinblastine	2.3	0.9	1.6	

[a]MTS is 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, a component of CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay.
[b]Determined by dividing the larger mean IC_{50} by the smaller for each pair of cell lines. For the breast cancer cells, the cell line compared is given in parentheses.

levels from mRNA levels within a cell or tissue type should be done with caution.

The work of Bhattacharya and Cabral [2004] suggests a minimum threshold level for β-tubulin class V that alters mitotic spindle function. They found that modest increases in β-tubulin class V, to levels of 15% or more of the total β-tubulin, may increase microtubule dynamics and decrease microtubule polymer levels. This was shown to be associated with weak resistance to paclitaxel. We found threshold levels of β-tubulin class V mRNA (>15%) in several cell lines: colon cancer, HCT-15; breast cancer, T-47D; lung cancer, A549 and HOP-18; melanoma, Malme-3M and SK-MEL-2; ovarian, OVCAR-3 and SK-OV-3. We found no correlation between IC_{50} values for paclitaxel or vinblastine and percentage β-tubulin class V mRNA (data not shown). While the relationship between mRNA and protein levels for this isotype remains to be determined, one difference between the work reported here and that of Bhattacharya and Cabral [2004] is that they carefully established that only one variable is altered in the β-tubulin class V overexpressing CHO cells. In our work, many variables differ among the 12 cell lines and some of them may compensate for changes in β-tubulin class V levels. Preliminary examination of one of the breast cancer cell lines, BT-549, by mass spectrometry revealed the presence of β-tubulin class V (data not shown). Therefore, we expect to find β-tubulin class V protein in some or all of the twelve cell lines. We have recently developed a monoclonal antibody to β-tubulin class V, which should permit quantitative measurement of protein levels and provide data regarding the contribution of β-tubulin class V to drug resistance.

CONCLUSIONS

From our comparison of β-tubulin isotype mRNA and protein levels on 12 human cancer cell lines, we conclude that quantitative Western blotting, utilizing human

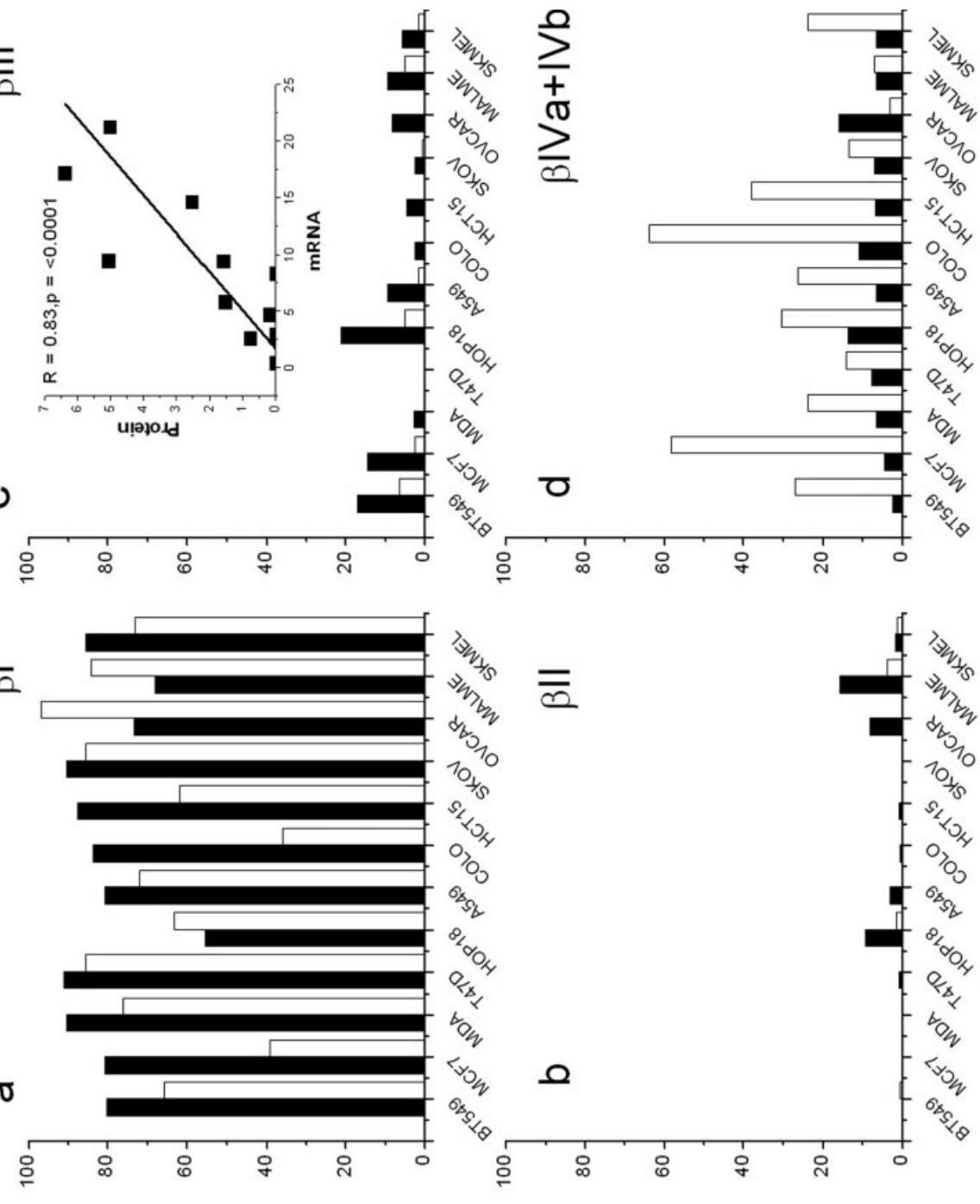

Fig. 4. Comparison of β-tubulin isotype protein levels with mRNA levels. Only β-tubulin isotype classes I (βI), II (βII), III (βIII), and IV (βIVa + IVb) were considered when calculating the total mRNA and protein levels. Solid bars represent mean mRNA and open bars represent mean protein values. Panel a: βI, Panel b: βII, Panel c: βIII, and Panel d: βIVa + IVb. Insert in Panel c shows a plot of mRNA vs. protein data for β-tubulin class III fit by linear regression. The straight line represents the best fit of the data. The correlation coefficient, R (Pearson's product-moment) for the fit and the P-value relative to a horizontal line (no correlation) are shown.

β-tubulin fusion proteins or pig brain tubulin with appropriate correction factors, is a reliable method for quantifying β-tubulin isotypes in human cancer cell lines. We found a good correspondence between β-tubulin mRNA and protein levels for classes I and III. For β-tubulin classes IVa + IVb, the higher levels of protein compared to mRNA suggest potential differential regulatory mechanisms for β-tubulin isotypes.

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