Expression of β-Tubulin Isotypes in Human Ovarian Carcinoma Xenografts and in a Sub-Panel of Human Cancer Cell Lines from the NCI-Anticancer Drug Screen: Correlation with Sensitivity to Microtubule Active Agents[1]

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ABSTRACT

Paclitaxel resistance has been associated with overexpression of P-glycoprotein and alterations involving tubulin. To investigate the clinical relevance of these *in vitro* resistance mechanisms, we established 12 human ovarian carcinoma xenografts, using samples from patients before the start of therapy or after paclitaxel treatment. These xenografts showed a wide range of sensitivity to paclitaxel, and in 4 of them, very low levels of *multidrug resistance-1* expression were detected. Using quantitative PCR and human specific primers, the expression of five β-tubulin isotypes was determined. HM40 was the predominant, accounting for 84.7–98.7% of all tubulin; expression of the other four isotypes (Hβ9, Hβ4, H5β, and Hβ2) was also detected but at lower levels. No correlation could be demonstrated between isotype expression and paclitaxel sensitivity in these 12 xenografts. A similar pattern of β-tubulin isotype expression was observed in a subset of cell lines from the National Cancer Institute-Anticancer Drug Screen. In these cell lines, however, a significant correlation between increased expression of Hβ4 isotype and resistance to paclitaxel was found. Taken together, these results suggest that altered expression of specific β-tubulin isotypes may not play a significant role in paclitaxel sensitivity *in vivo* and argue against a possible significance in a clinical setting.

INTRODUCTION

Although paclitaxel has shown clinical antitumor activity against different refractory tumors, including breast and ovarian carcinomas (1–4), inherent or acquired clinical drug resistance is still a major obstacle in the treatment of cancer. Resistance to paclitaxel has been associated with overexpression of P-glycoprotein, as part of the MDR[3] phenotype (5). In addition, *in vitro* studies have reported that resistance can also occur in association with altered α- or β-tubulin subunits (6), point mutations in β-tubulin (7), or differential expression of β-tubulin isotypes (8–12). Recent data also suggest that the development of paclitaxel resistance is associated with changes in chemokine/cytokine gene expression (13). Although the α- and β-tubulin subunits exist in multiple isotypic forms encoded by several genes (14), the functional significance of the different isotypes is not yet fully understood. To date, six β-tubulin isotypes have been identified in humans, and based on their COOH-terminal variable region, they have been classified as follows: class I (HM40), class II (Hβ9), class III (Hβ4), class IVa (H5β), class IVb (Hβ2), and class VI (Hβ1; Arabic numerals refer to the human gene, and roman numerals refer to the protein isotype class; Ref. 15).

One crucial aspect of resistance mechanisms observed *in vitro* is whether it is representative of clinical resistance. To investigate the clinical relevance of *in vitro* resistance mechanisms, HOC xenografts derived from patient tumors refractory to paclitaxel were established. In these xenografts, we analyzed tubulin levels and, by RT-PCR, the expression of five β-tubulin isotypes (HM40, Hβ9, Hβ4, H5β, and Hβ2) to determine the β-tubulin isotype expression profile and the correlation of β-tubulin isotype composition with paclitaxel sensitivity. The use of human specific primers that do not recognize contaminating tubulin from normal mouse cells allows discrimination of human from mouse tubulin expression. This discrimination is not possible when one examines samples obtained from patients. The variable contamination of such samples with normal cells

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[3] The abbreviations used are: MDR, multidrug resistance; HOC, human ovarian carcinoma; RT-PCR, reverse transcription-PCR; MST, median survival time; NCI, National Cancer Institute; ECL, enhanced chemiluminescence; %ILS, increment of life span; T/C%, optimal growth inhibition.

makes interpretation of results in such a setting difficult, if not impossible. We also studied expression of β-tubulin isotypes in a sub-panel of human tumor cell lines from the NCI-Anticancer Drug Screen to query whether preferential expression of one or more isotypes occurs in a particular tissue type and to determine the correlation between the isotype expression profile and drug sensitivity. Our results show that total tubulin protein levels were highly variable in the ovarian xenografts. Using quantitative PCR, HM40 was the predominant isotype, with much lower amounts of the other β-tubulin isotypes. Furthermore, a correlation with paclitaxel sensitivity in the HOC xenografts could not be demonstrated. A similar pattern of expression of β-tubulin isotypes was found in a sub-panel of human tumor cell lines from the NCI-Anticancer Drug Screen. In this subset of cell lines, whereas isotype expression was found to be independent of the tissue of origin, a significant correlation between Hβ4 (class III) and sensitivity to paclitaxel was found.

MATERIALS AND METHODS

Animals

Female NCr-*nu/nu* mice were obtained from the animal production colony of the NCI-Frederick Cancer Research and Development Center, Frederick, MD. The mice used were 8–10 weeks of age and had a mean body weight of 23 ± 2 g. Throughout this study, nude mice were housed in filtered-air laminar flow cabinets and manipulated after aseptic procedures. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. No. 116, G.U., Suppl. 40, Feb. 18, 1992; Circolare No. 8, G.U., July, 1994) and international laws (EEC Council Directive 86/609, OJ L 358. 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996).

Establishment of HOC Xenografts

Tumor specimens obtained via laparotomy or paracentesis from patients with ovarian carcinoma were established and maintained in nude mice as described previously (16–19). Briefly, solid tumor samples dissected free of necrotic areas, connective tissue, and blood clots were cut into 2–4-mm fragments and injected s.c. in the flanks of nude mice. Ascites samples were centrifuged, washed, and resuspended in HBSS and injected as suspension in the peritoneal cavity of nude mice. Two HOC xenografts were established from patients with *de novo* adenocarcinoma of the ovary, one HOC was established from a patient who did not respond to carboplatin treatment, and nine HOCs were established from patients with paclitaxel-refractory cancer. Paclitaxel-resistant patients had received one to seven cycles of paclitaxel and had progressive disease during paclitaxel therapy or had tumor recurrence within 6 months of paclitaxel treatment. HOC lines were maintained for serial passages by inoculation of 10^7 tumor cells i.p. or transplantation of tumor fragments s.c. and tested within 10 passages of establishment in nude mice. All specimens from patients and xenograft samples were classified by routine histopathological examination according to the International Federation of Gynecologists and Obstetricians classification for ovarian tumors. The histotype of the tumors growing in nude mice was also compared with the patient's tumor.

Drug Treatment and Efficacy Evaluation

Paclitaxel was provided by Bristol-Myers Squibb (Wallingford, CT) and was formulated in a vehicle containing 50% ethanol and 50% Cremophor EL and additionally diluted with 5% glucose in water. Paclitaxel was administered as three i.v. injections 4 days apart (Q4 × 3) at an optimal dose of 20 mg/kg. Control groups received injections of vehicle alone. Mice were weighed three times a week to evaluate drug-induced toxicity, and body weight changes were recorded.

s.c. Tumor Growth: HOC tumors were implanted s.c. in the flanks of nude mice (5–10 mice/group). Treatments started when tumors reached ~150 ± 50 mg weight. Tumor growth was measured in two dimensions twice a week, and estimates, in grams, of tumor weights were calculated as [length × $(\text{width})^2$/2]. Tumor doubling times and treatment efficacy were calculated as described previously (18). Results are expressed as T/C% defined as: (median tumor weights of treated/median tumor weights of controls) × 100% (20). A T/C% ≤ 50% is considered active (20).

i.p. Tumor Growth: HOC tumors were injected i.p. as a cell suspension containing 1×10^7 cells (eight mice per group). Treatment was started at one-fourth of the MST. The presence of tumor in the peritoneal cavity at the start of therapy was confirmed by cytological analysis of three additional mice per group as described previously (21). Mice were monitored twice a week for presence of tumor in the abdomen, and the survival time was recorded. Treatment efficacy was then calculated as described elsewhere (22). Results are expressed as %ILS: 100 × [(MST of treated group − MST of control group)/MST of control group]. ILS ≥ 40% indicates the treatment is active.

Cell Lines

A subset of cell lines from the NCI-Anticancer Drug Screen Panel were obtained: OVCAR-3 and IGROV1 (ovary), HS 578T and MCF7 (breast), MOLT-4 and RPMI-8226 (leukemia), SF-539 and SF-268 (central nervous system), PC-3 and DU-145 (prostate), SK-MEL-28 and UACC-62 (melanoma), 786–0 and SN12C (kidney), SW620 and KM12 (colon), and NCI-H460 (lung). The 1A9 cell line is a subclone of the HOC cell line, A2780 (23). All cell lines were grown in RPMI 1640 with 10% FCS, 5 mM L-glutamine, and 1% penicillin-streptomycin. A suspension of 1A9 cells ($5–10 \times 10^6$) was injected either i.p. or s.c. into nude mice to obtain the corresponding xenograft for use as a reference.

Immunoblot Analysis of Tubulin

Tumor tissues and cell lines were lysed in ice-cold TNESV buffer [50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 100 mM NaCl, and 1% NP-40] containing the following protease/phosphatase inhibitors: phenylmethylsulfonyl fluoride (1 mM), aprotinin (200 units/ml), sodium fluoride (25 mM), and sodium orthovanadate (1 mM). After a 30-min incubation on ice, samples were centrifuged at 14,000 rpm for 20 min, and the supernatants were collected. Total cellular proteins (50 μg) were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes. Im-

Table 1 Sets of primers for PCR amplification and nucleotide sequencing of M40-β-tubulin-isotype

Primers			Sequences
PCR primers			
Set 1	Forward	(5′–UTR[a])	CTTGCCCCATACATACCTTGA
	Reverse	(748–768)	CTCCGCAAGTTGGCAGTCAAC
Set 2	Forward	(574–594)	TTGGATGAGAATACTGATGAG
	Reverse	(3′–UTR)	GTAAGACGGCTAAGGGAACTG
Sequencing primers			
Set 3	Forward	(19–35)	ATCCAGGCTGGTCAATG
	Reverse	(357–377)	GTGGTACGGAAGGAGGCAGA
Set 4	Forward	(283–301)	TCTGGGGCAGGTAACAACT
	Reverse	(628–648)	ATCTGCTTCCGCACTCTGAAG
Set 5	Forward	(682–702)	CTTGTCTCAGCCACCATGAGT
	Reverse	(1018–1038)	ACTTTGTGGAATGGATCCCC
Set 6	Forward	(985–1008)	CAGATGCTTAACGTGCAGAACAAG
	Reverse	(1247–1267)	CGACCTCGTCTCTGAGTATC

[a] UTR, untranslated region.

munoblotting was performed using monoclonal anti-α (T-9026; Sigma Chemical Co.) or anti-β tubulin (T-4026; Sigma Chemical Co.) antibody, visualized with antimouse horseradish peroxidase-linked secondary antibody and the ECL procedure (Amersham). Comparable loading and efficient transfer were confirmed by immunoblotting with anti-actin antibody (A-2066; Sigma Chemical Co.) or by staining the membrane with Ponceau S red before immunodetection.

RT-PCR Analysis of β-Tubulin Isotypes

Total RNA was isolated from tumor cells harvested from nude mice and from cell lines using the single-step method as described by Chomczynski and Sacchi (24). Quantitative PCR for β-tubulin isotypes was performed as described previously (25). Reverse transcription of total RNA (1 μg), using a specific 3′ primer, was performed for 45 min at 37°C and then at 95°C for 5 min. PCR amplification of the resulting cDNA was performed >30 cycles with serial 2-fold dilutions to ensure that amplification was in the exponential range of the reaction. After an initial denaturation of 4 min at 94°C, each cycle consisted of 60 s at 94°C, 60 s at 55°C, and 2 min at 72°C, with an extension time of 10 min at 72°C. Specific primers for each human β-tubulin isotype, HM40 (class I), Hβ9 (class II), Hβ4 (class III), H5β (class IVa), and Hβ2 (class IVb; Ref. 15), were used as described previously (12). With the exception of Hβ9, the primers span one or two introns, allowing for discrimination between RNA and DNA products. For Hβ9, PCR was performed without reverse transcription, and no product was observed, confirming the absence of DNA contamination. For the determination of *MDR-1* expression, quantitative PCR was also performed using specific primers and reaction conditions as described previously (25). PCR products were resolved on 2% NuSieve/1% agarose gels, stained with ethidium bromide, and subjected to densitometry. β-tubulin isotype and *MDR-1* levels were normalized to the input RNA from each cell line, and their relative expression was determined. The levels of expression of β-tubulin isotypes were also corrected for product size and PCR efficiency, and the total β-tubulin percentage of each isotype was then determined.

Sequence Analysis of β-Tubulin

For PCR amplification and sequencing of M40 isotype of β-tubulin, six overlapping sets of primers were used, as summarized in Table 1. The primers were designed using published sequence data for isotypes of β-tubulin (26). The GenBank accession no. for M40 isotype is J00314. PCR-amplified cDNA was purified with PCR Select-III spin columns and directly sequenced with the Taq DyeDeoxy terminator cycle sequencing kit following the manufacturer's instructions (Applied Biosystems, Inc.). The primers used for sequencing were the same primers used for PCR amplification. The reaction products were purified with Centri-Sep spin purification columns, electrophoresed on 48-cm/4.75% polyacrylamide/urea gels, and analyzed by an automated DNA sequencing system (model 377; Applied Biosystems, Inc.).

Determination of Paclitaxel Levels in Tumor Tissue

Paclitaxel was formulated as detailed above for the efficacy studies. Nude mice were injected with 1A9 cell suspension s.c. or HOC79 cell suspension i.p. and treated with a single i.v. administration of paclitaxel (20 mg/kg) when mice had advanced tumors (15–20 days later). After 1 h, tumors were taken from four animals (each xenograft) and immediately stored at −20°C until analysis. The high-performance liquid chromatography method used to measure paclitaxel levels in tumor tissues was developed by modifying a method originally described by Colombo *et al.* (27).

Statistical Analysis

Correlation analysis between β-tubulin isotype expression and sensitivity to microtubule active agents in the human tumor cell lines was performed using the absolute levels of β-tubulin expression obtained by PCR and the GI50 values (the concentration of agent causing a 50% growth inhibition) obtained from the NCI-Anticancer Drug Screen database. The correlation coefficients used are the Spearman correlation coefficients because our small subset of data (cell lines representing a selected sample from an assumed underlying population) did not follow a normal distribution to use the Pearson method. The same analysis was performed between the absolute levels of β-tubulin

Table 2 Clinical history of patients' derived tumors

Tumor model	Specimen		Histotype	Grade
	Origin[a]	Treatment[b]		
HOC76	Ascites (R)	PAC;[c] Taxol® (3 cycles)	Serous	3
HOC79	Ascites (R)	DDP; DOX + CTX; mitoxantrone + ifosfamide + Taxol® (1 cycle)	Endometrioid	2
HOC84	Ascites (R)	DDP; Taxol® (2 cycles)	Serous	3
HOC94/2	Ascites (R)	PAC, Taxol® (6 cycles)	Serous	2
HOC106	Tumor (R)	PAC, Taxol® (6 cycles)	Serous	3
HOC107	Tumor (R)	PAC, Taxol® (6 cycles)	Serous	3
HOC109	Peritoneal nodule (R)	Carboplatin, Taxol® (7 cycles)	Endometrioid	2
HOC110	Ascites (R)	Carboplatin	Serous	2
HOC119	Tumor (I)	None	Clear cells	3
HOC130	Tumor (I)	None	Serous	3
MNB-PTX1	Ascites (R)	PAC, Taxol® (6 cycles)	Serous	2
MNB-PTX2	Peritoneal nodule (R)	Carboplatin, Taxol® (7 cycles)	Endometrioid	2

[a] Type of specimen from which tumor xenografts were derived; (I), primary tumor; (R), recurrent tumor.
[b] Patients' treatment at time of injection into nude mice; the number of cycles of Taxol®-containing regimen are indicated.
[c] Abbreviations: PAC, cisplatin + doxorubicin + cyclophosphamide; DDP, cisplatin; CTX, cyclophosphamide; DOX, doxorubicin.

Table 3 Growth behavior and drug sensitivity of HOC xenografts transplanted in nude mice

Tumor model	Growth site	Doubling time ± SD (s.c. tumors)	MST (range) (i.p. tumors)	Paclitaxel sensitivity[a]
1A9	i.p./s.c.	4 ± 2	38 (23–51)	++++
HOC76	i.p./s.c.	10.2 ± 1.9	67 (46–169)	+
HOC79	i.p./s.c.	12.5 ± 2.5	71 (38–96)	+
HOC84	s.c.	12 ± 3	n.t.[b]	++++
HOC94/2	s.c.	10 ± 2	n.t.	+++
HOC106	s.c.	12.2 ± 2	n.t.	n.t.
HOC107	s.c.	37.7 ± 10	n.t.	+++
HOC109	s.c.	8.2 ± 2	n.t.	+++
HOC110	i.p.	n.t.	92 (90–108)	+
HOC119	s.c.	7.7 ± 3.6	n.t.	++
HOC130	i.p.	n.t.	50 (36–67)	−
MNB-PTX1	s.c.	8 ± 2	n.t.	−
MNB-PTX2	s.c.	11 ± 2.5	n.t.	+

[a] 20 mg/kg paclitaxel (Q4 × 3, i.v.); treatments started at 1/4 MST for i.p. tumors and at 150 ± 50 g of weight for s.c. tumors. Criteria of antitumor activity: −, inactive (ILS < 40%; T/C > 50%); +, minimal activity (40% < ILS < 75%; T/C < 50%); ++, moderate activity (75% < ILS < 100%; T/C < 40%); +++, good activity (100% < ILS < 150%; T/C < 25%); and ++++, optimal activity (ILS > 150% or complete remissions; T/C < 10%).
[b] n.t., not tested.

isotype expression and sensitivity to paclitaxel in the HOC xenografts.

RESULTS

***In Vivo* Growth Behavior and Drug Sensitivity Profile of HOC Xenografts.** In the course of the study, we attempted to establish continuous xenografts from HOC specimens, and transplantable tumors were obtained in 12 cases, with a total take rate of 20% (see Table 2 for clinical characteristics). In all instances, the histopathological features of the primary tumors were maintained in the xenografts. The HOC xenografts were then studied for their growth behavior and sensitivity to paclitaxel at different passages in nude mice (Table 3). 1A9 ovarian xenograft, used as a reference, was derived from *in vitro* cell culture as described in "Materials and Methods." The data shown are representative of at least two independent experiments, and all studies were performed within a range of five to six consecutive passages in nude mice.

Five xenografts (1A9, HOC76, HOC79, HOC110, and HOC130) were initially established i.p. Three of these also grew s.c. (1A9, HOC76, and HOC79). The other eight HOCs (HOC84, HOC94/2, HOC106, HOC107, HOC109, HOC119, MNB-PTX1, and MNB-PTX2) were directly established s.c. in nude mice, either from cell suspensions or tumor fragments. The doubling time of the s.c. growing tumors and the MST of the i.p. growing tumors varied widely among the xenografts, reflecting their differences in proliferation rate (Table 3).

The activity of paclitaxel on 12 of the 13 xenografts was subsequently investigated (Table 3), and the level of sensitivity was determined according to NCI standards of *in vivo* antitumor activity (see Footnote [a] to Table 3). HOC130 and MNB-PTX1 were insensitive to paclitaxel; HOC76, HOC79, HOC110, and MNB-PTX2 demonstrated minimal response, whereas with 1A9, HOC84, HOC94/2, HOC107, HOC109, and HOC119, paclitaxel activity was moderate to optimal (Table 3).

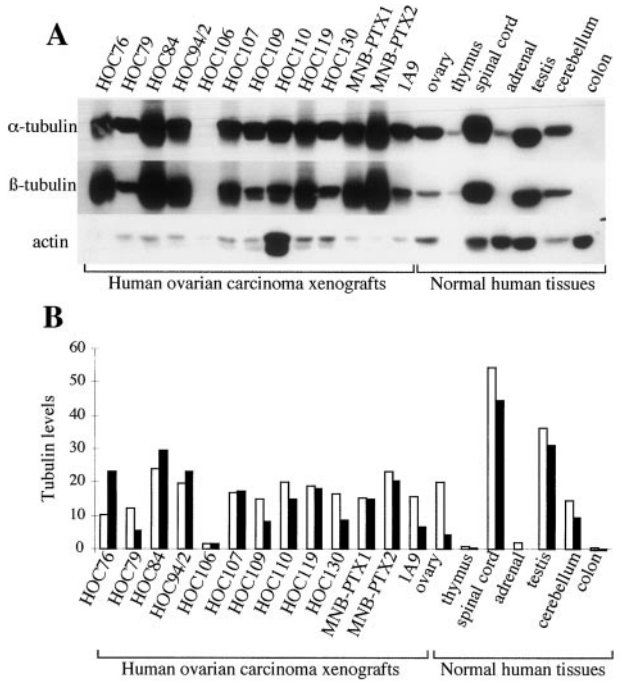


Fig. 1 Immunoblot analysis of α- and β-tubulin in HOC xenografts. In *A*, total cellular protein (50 μg) from HOC xenografts and human normal tissues was loaded onto each lane, transferred to a membrane, and hybridized with α- and β-tubulin monoclonal antibodies and an anti-actin antibody. Visualization was achieved using an antimouse or antirabbit horseradish peroxidase-linked secondary antibody and the ECL procedure. *B*, bar graph representation of the results displayed in *A*. The amounts of α- and β-tubulin were determined by densitometry, and the absolute levels are reported.

Analysis of MDR-1 Expression and Paclitaxel Level in Tissues. RT-PCR was performed to determine *MDR-1* gene expression in the HOC xenografts. Primers specific for human *MDR-1* were used. SW620, a human colon cancer line which expresses low levels of *MDR-1*, was used as the reference and arbitrarily given a value of 10. Expression of *MDR-1* was detected by RT-PCR in only 4 of the 13 ovarian xenografts (data not shown). HOC130 showed the highest level (7.5), whereas HOC76, HOC79, and HOC84 had much lower and probably insignificant levels (range, 0.2–0.5). No significant correlation could be demonstrated between *MDR-1* expression and paclitaxel sensitivity ($P < 0.2$). The ability of tumor cells to accumulate paclitaxel was also determined in two different ovarian xenografts: 1A9 (highly sensitive to paclitaxel) and HOC79 (minimally responsive to paclitaxel). After i.v. administration (1 h), the levels of paclitaxel were similar in both tumors (4.91 ± 0.8 and 4.38 ± 0.6 μg/g for 1A9 and HOC79, respectively). These results argue against the influence of transport mechanisms in paclitaxel sensitivity.

Immunoblot Analysis of α- and β-Tubulin. Protein levels of α- and β-tubulin in the HOC xenografts were determined by immunoblot analysis of total cell lysates using monoclonal antibodies (Fig. 1*A*). Extracts from seven normal human tissues were included for comparison. The data shown are representative of two independent experiments in which total protein extracted from tumors harvested at two different passages in nude mice gave reproducible results. As shown in Fig. 1*A*, the level of tubulin varied widely among the xenografts and the normal tissues; antibodies against either α- or β-tubulin gave comparable results. These levels were independent of the level of sensitivity of the HOC xenografts to paclitaxel. Immunoblotting with an anti-actin antibody found highly variable actin levels. This occurred despite careful determination of protein content and Ponceau S red staining of the membrane showing comparable loading and suggests that in these xenografts, actin is not a reliable control. Consequently, the immunoblots were subjected to densitometry, and the absolute levels of α- and β-tubulin in the xenografts and the normal human tissues were determined (Fig. 1*B*).

Expression of β-Tubulin Isotypes in HOC Xenografts. Expression of five β-tubulin isotypes was examined in the HOC xenografts (Fig. 2) using a quantitative PCR methodology we have described previously and validated (25, 28). At least four 2-fold serial dilutions of input RNA were used for each isotype to ensure that amplification was in the exponential range. Different amounts of input RNA were amplified for different numbers of cycles because isotype levels and PCR efficiencies varied, as discussed below. Fig. 2 depicts the expression results for each xenograft relative to that of 1A9 xenograft, determined from the serial dilutions. The largest relative differences were observed with the Hβ4 isotype, whereas the narrowest range was detected in the HM40 isotype. When sensitivity to paclitaxel was correlated with the expression of each of the five isotypes, no significant correlation could be found, suggesting the absolute level of any of the five isotypes does not affect drug sensitivity.

Determination of PCR Efficiency for β-Tubulin Isotypes. Whereas the results indicated that the absolute level of none of the five β-tubulin isotypes determined drug sensitivity, the possibility remained that the percentage of composition could predict drug response. To accurately determine the percentage of composition of β-tubulin in the HOC xenografts, we first needed to establish the relative efficiencies of the PCR reactions. Because different primers were used for each β-tubulin isotype, we began by performing standard curves for each β-tubulin isotype. As a first step, cDNAs corresponding to the amplified products were isolated so that these could be used as templates in the standard curves. To isolate these, 1 μg of RNA from the 1A9 cells was reverse transcribed and amplified 40 cycles by PCR. The resulting cDNAs were resolved on 1% low melting point agarose gels, purified, and redissolved. After carefully determining the concentration of each template solution and confirming comparability by ethidium bromide staining after electrophoresis, standard curves were generated for each isotype by PCR amplification of different input amounts. The resulting PCR products were then resolved on a 3% agarose gel, stained with ethidium bromide, and quantitated by densitometry (as described in "Materials and Methods"). Because the size of the isotype fragments differed and because ethidium staining is proportional to fragment size, the optical densities (reflecting the amount of PCR product) were normalized by dividing the absorbance measurement by the size of the isotype fragment. This value was then plotted against the input concentration of each isotype, as shown in Fig. 3. This representation allows for

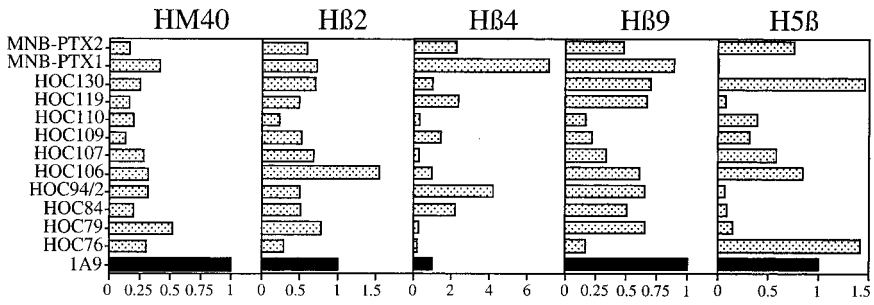


Fig. 2 Expression of β-tubulin isotypes in HOC xenografts. Relative expression of β-tubulin isotypes in HOC xenografts examined by quantitative PCR after reverse transcription. The level of each β-tubulin isotype was normalized to the input RNA from each tumor and compared with the 1A9 xenograft used as a reference, which is arbitrarily given a value of 1.

calculation of PCR efficiency by comparing the slopes of each curve or the PCR product for the same amount of input cDNA for each purified isotype. This analysis showed that the Hβ9 tubulin reaction was the most efficient, followed by Hβ2, HM40, H5β, and Hβ4. Specifically, the Hβ9 reaction was 1.3-fold more efficient than the Hβ2 reactions, 10-fold more efficient than the HM40, and 23.3- and 100-fold more efficient than H5β and Hβ4, respectively. It should be pointed out that it is possible that reaction conditions could have been adjusted to approximate the PCR efficiencies of the various isotypes. However, we found results performed under identical reaction conditions repeatedly reproducible and chose this approach.

Using these results to adjust the actual PCR products measured by densitometry, the relative percentage of each isotype was calculated, as shown in Fig. 4, *A* and *B*. HM40 was the predominant isotype accounting for 84.7–98.7% of β-tubulin. The second most predominant isotype was Hβ4, accounting for 0.5–14.3% of β-tubulin. The other three isotypes (Hβ2, Hβ9, and H5β) comprise 0–1.4% of β-tubulin. When the percentage of expression of each isotype was correlated with paclitaxel sensitivity, again no significant correlation could be demonstrated.

Effect of Xenografting and Serial Passaging of the HOC Xenografts on β-Tubulin Isotype Expression. Because the transplantation of patient specimens into nude mice could influence the β-tubulin isotype expression, we determined the percentage of composition of the five β-tubulin isotypes in two HOC xenografts (HOC106 and HOC110) before and after transplantation into nude mice. We found that the expression of the five β-tubulin isotypes in the patient specimen closely resembles the pattern observed after the tumor line was established as xenograft in nude mice (data not shown) We also examined the possibility that serial passaging of the xenograft from animals could lead to differences in β-tubulin isotype composition. To do this, we analyzed the 1A9 cell line from *in vitro* culture and after three consecutive passages in nude mice. Again, similar results were also found in the *in vitro* and *in vivo* samples (data not shown). Finally, because the class I (HM40) isotype has usually been found to be the predominant isotype expressed in tumor cells, and recent studies have suggested that point mutations in this isotype may affect paclitaxel sensitivity (29), we sequenced the entire class I (HM40) isotype from all 15 HOC samples. This analysis demonstrated that the sequence was wild type in all of the xenografts (data not shown).

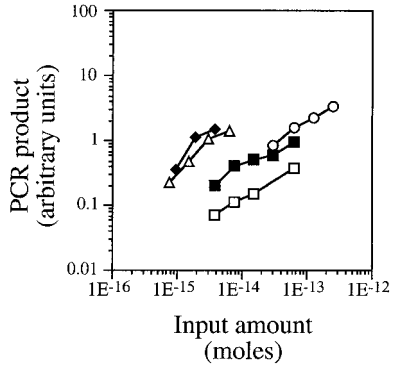


Fig. 3 Comparative PCR efficiency for β-tubulin isotypes. PCR amplification of 1 μg of RNA after reverse transcription was performed using RNA from 1A9 human ovarian cancer cells. The resulting cDNAs were purified, their concentration was determined, and PCR amplification with different input amounts of cDNA of each isotype was then performed. PCR products were resolved in a 3% agarose gel, stained with ethidium bromide, and subjected to densitometry (as described in "Materials and Methods"). Results are expressed as PCR product (absorbance normalized by isotype size) *versus* input amount of cDNA for each isotype. PCR efficiency for the different β-tubulin isotypes was then calculated.

Tubulin Content Analysis and Expression of β-Tubulin Isotypes in a Sub-Panel of the Cell Lines of the NCI-Anticancer Drug Screen. Because in all of the ovarian xenografts, the predominant isotype was HM40, and no apparent correlation emerged between isotype expression and drug sensitivity, we subsequently tried to determine whether there was a greater diversity of isotype expression among different tumor types. For this purpose, 17 cancer cell lines were selected from the 60 cell lines of the NCI-Anticancer Drug Screen panel (2 of each tissue type, with the exception of only 1 lung carcinoma). Immunoblot analysis of cell lysates showed that compared with the ovarian xenografts, much less variability in either α- or β-tubulin levels was found among the cell lines; antibodies against either α- or β-tubulin gave comparable results. Fig. 5*A* shows the results in 1 cell line/tissue type studied. Immunoblotting with anti-actin antibody gave less variable results than for the xenografts, but still Ponceau S red staining of the membrane showed comparable loading. The absolute levels of α- and β-tubulin, determined by performing densitometry on the immunoblots, are reported in Fig. 5*B*. The MOLT-4 leukemia cell line, which grows in suspension, showed the highest levels of both α- and β-tubulin,

A

Xenograft	ß-tubulin isotypes (% composition)				
	HM40	Hß2	Hß4	Hß9	H5ß
1A9	97.6	0.2	0.9	0.4	1.0
HOC76	98.3	0.2	0.6	0.2	0.7
HOC79	98.7	0.3	0.5	0.5	0.0
HOC84	88.9	0.4	9.7	0.9	0.1
HOC94/2	87.3	0.3	11.7	0.7	0.0
HOC106	95.2	0.8	3.0	0.7	0.4
HOC107	97.8	0.4	1.0	0.5	0.3
HOC109	88.4	0.7	10.1	0.6	0.3
HOC110	97.6	0.2	1.6	0.3	0.3
HOC119	85.6	0.5	12.5	1.4	0.1
HOC130	93.9	0.5	3.8	1.1	0.8
MNB-PTX1	84.7	0.3	14.3	0.7	0.0
MNB-PTX2	85.8	0.6	12.0	1.0	0.6

B

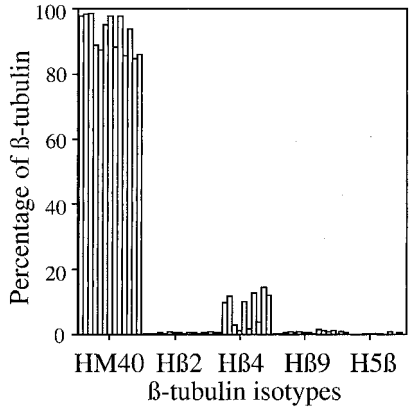


Fig. 4 Percentage of composition of β-tubulin by isotype in HOC xenografts. In *A*, β-tubulin isotype levels were normalized to the input RNA from each tumor and corrected for product size and PCR efficiency. The total β-tubulin percentage of each isotype in all of the HOC xenografts is presented. *B*, bar graph representation of the results displayed in *A*.

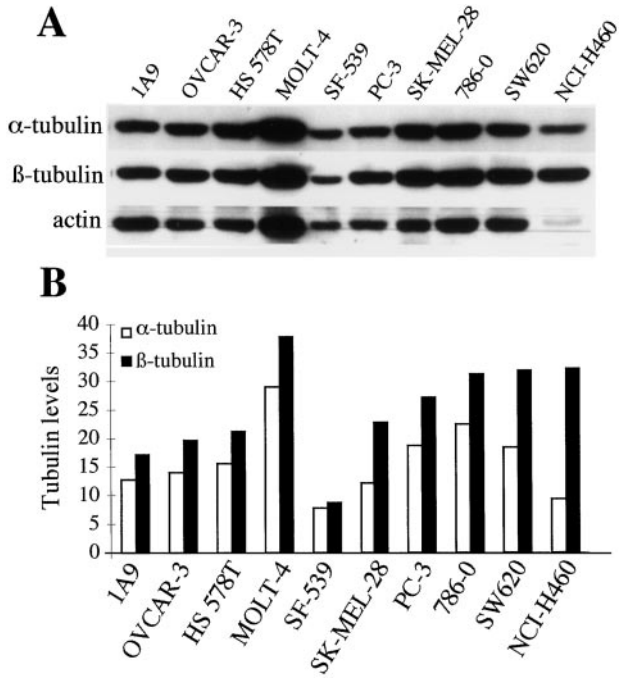


Fig. 5 Immunoblot analysis of α- and β-tubulin in human cancer cell lines from the NCI-Anticancer Drug Screen. In *A*, total cellular protein (50 μg) was loaded onto each lane, transferred to a membrane, and hybridized with α- and β-tubulin monoclonal antibodies and an anti-actin antibody. Visualization was achieved using an antimouse or an antirabbit horseradish peroxidase-linked secondary antibody and the ECL procedure. *B*, bar graph representation of the results displayed in *A*. The amounts of α- and β-tubulin were determined by densitometry, and the absolute levels are reported.

whereas the NCI-H460 lung cell line showed the highest difference in the absolute levels of α- and β-tubulin.

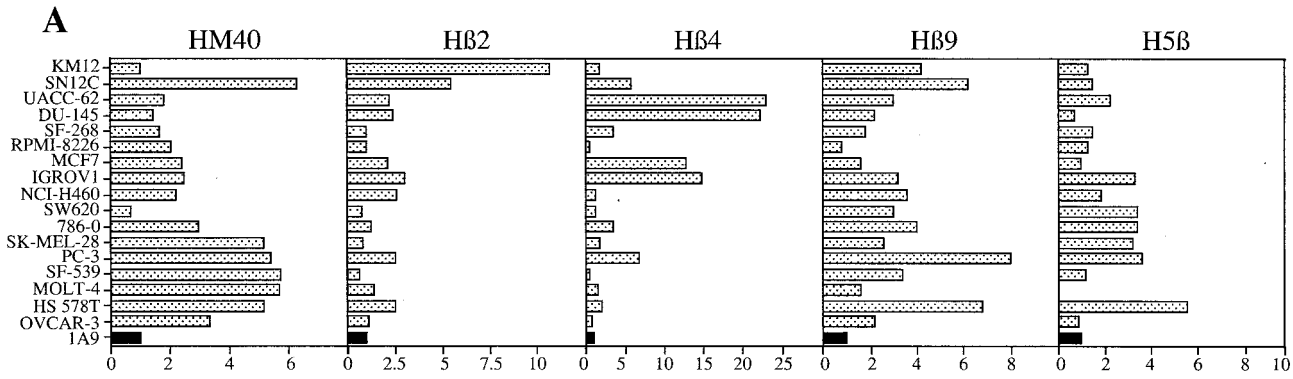
β-tubulin isotype expression in 17 human cancer cell lines is summarized in Fig. 6. The absolute levels of each isotype relative to those of the 1A9 cell line are shown (*A*). When the percentage of composition of each β-tubulin isotype was calculated (*B* and *C*), the pattern of expression observed was similar to the results with the HOC xenografts (Fig. 4, *A* and *B*): HM40 accounted for 76.4–99.7% of the total β-tubulin content followed by Hβ4 (0.2–15%), H5β (0–6.9%), Hβ9 (0.1–1.5%), and Hβ2 (0.001–0.2%).

When absolute levels of expression of the five isotypes were correlated with sensitivity to antimicrotubule agents, including paclitaxel, vincristine, vinblastine, and rhizoxin, a significant correlation ($P < 0.007$) was found between the Hβ4 (class III) isotype and paclitaxel (Table 4). Increased expression of the Hβ4 isotype was found associated with resistance to paclitaxel in the 17 cell lines analyzed. No correlation was observed between the other four isotypes and the same microtubule active agents. It should be noted that the 17 human cancer cell lines were deliberately chosen as a subset with very little or no *MDR-1* expression. This was done because previous studies have shown a high correlation between *MDR-1* expression and paclitaxel sensitivity (28). Consequently, to dissect out the contribution of β-tubulin isotype expression, the *MDR-1* "effect" had to be removed.

DISCUSSION

A variety of mechanisms of drug resistance have been described mainly in *in vitro* cell lines, selected in the presence of the cytotoxic agent under study. For paclitaxel, a variety of resistance mechanisms have been described, including altered drug uptake and/or metabolism and mutations in β-tubulin (5). Whereas these *in vitro* studies have contributed enormously to our understanding of this complex phenomenon, some have questioned the extent to which *in vitro* models represent clinical phenomena.

In an attempt to obtain more clinically relevant models, we set out to establish HOC xenografts using samples obtained from patients before the start of therapy or after paclitaxel treatment. The take rate was in agreement with published data on the establishment of ovarian cancer xenografts in nude mice (30). HOC xenografts were established in nude mice from 2 patients with untreated ovarian cancer, 1 patient resistant to carboplatin, and 9 patients with paclitaxel-refractory cancer.



B

Cell line	Tissue (type)	ß-tubulin isotypes (% composition) HM40	Hß2	Hß4	Hß9	H5ß
1A9	ovarian	87.8	0.06	9	0.4	2.7
OVCAR-3	ovarian	95.5	0.06	3.3	0.3	0.9
HS 578T	breast	93.3	0.06	4.1	0.6	1.9
MOLT-4	leukemia	95.4	0.01	4.4	0.1	0.0
SF-539	CNS	97.6	0.02	1.4	0.3	0.7
PC-3	prostate	87.6	0.07	10	0.5	1.6
SK-MEL-28	melanoma	91.5	0.03	6	0.2	2.2
786-0	renal	86.3	0.05	11.2	0.5	1.8
SW620	colon	76.4	0.12	15	1.5	6.9
NCI-H460	lung	88.8	0.2	7.8	1	2
IGROV1	ovarian	95.8	0.006	3.8	0.2	0.08
MCF7	breast	96.4	0.004	3.4	0.1	0.02
RPMI-8226	leukemia	99.7	0.002	0.2	0.07	0.04
SF-268	CNS	98.9	0.003	0.8	0.2	0.07
DU-145	prostate	94.3	0.007	5.3	0.3	0.04
UACC-62	melanoma	95.8	0.002	3.8	0.2	0.08
SN12C	renal	99.5	0.001	0.3	0.1	0.01
KM12	colon	98.7	0.014	0.5	0.6	0.07

C

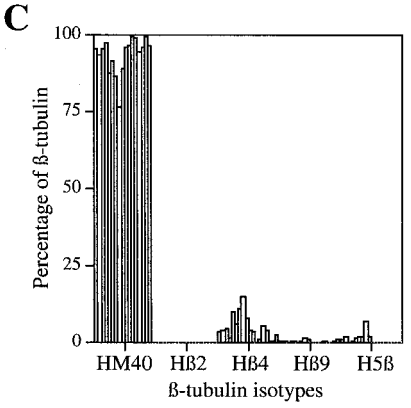


Fig. 6 Expression of β-tubulin isotypes in human cancer cell lines from the NCI-Anticancer Drug Screen. *A*, relative expression of β-tubulin isotypes in the human cancer cell lines examined by quantitative PCR after reverse transcription. The level of each β-tubulin isotype was normalized and compared with the 1A9 cell line as described in Fig. 2. In *B*, β-tubulin isotype levels were normalized to the input RNA from each cell line and corrected for product size and PCR efficiency. The total β-tubulin percentage of each isotype in all of the cell lines is presented. *C*, bar graph representation of the results displayed in *B*.

Table 4 Correlation analysis of Hβ4 isotype *vs.* sensitivity to micrutubule active agents[a]

Agent	Spearman correlation coefficient	*P* (two-sided)
Paclitaxel	0.609	0.007[b]
Vincristine	0.437	0.06
Vinblastine	−0.066	0.4
Rhizoxin	−0.2	0.2

[a] Absolute levels of expression of Hβ4 isotype for the 17 tumor cell lines were correlated with their sensitivity to microtubule active agents (GI50 values).
[b] Statistically significant.

Refractory patients had received one to six cycles of paclitaxel and had either progressed while on therapy or had relapsed within 6 months of paclitaxel treatment. Paclitaxel sensitivity in these ovarian xenografts demonstrated a broad range, from minimal or no sensitivity to moderate/optimal sensitivity.

Expression of *MDR-1* was detected by RT-PCR in only 4 of the 12 ovarian xenografts, and in 3 of these, the level was very low. It is not clear the extent to which such low levels of *MDR-1* contribute to paclitaxel sensitivity. However, extrapolation from *in vitro* data and the observation that one of the *MDR-1* positive xenografts was very sensitive to paclitaxel suggests *MDR-1* expression was not an important variable in these xenografts. Moreover, there was no correlation between *MDR-1* and the number of paclitaxel cycles patients had received. In addition, the sensitive 1A9 and the minimally responsive HOC79 were able to accumulate paclitaxel to the same level, thus excluding the influence of transport mechanisms on the differential sensitivity of the HOC xenografts to paclitaxel.

Having excluded *MDR-1* expression and paclitaxel accumulation as significant variables in the xenografts, we examined the expression of five β-tubulin isotypes. We did this because several studies have reported that altered expression of tubulin isotypes may confer resistance to paclitaxel (8, 10–12), as well as other microtubule agents such as estramustine (31). Initially, levels of α- and β-tubulin were determined by immunoblot

analysis, and a high variability was found in the xenografts and normal tissues. Despite these differences, neither the levels of α- nor β-tubulin correlated with drug sensitivity. Then, using quantitative PCR, the absolute levels of expression, and the percentage of total β-tubulin was determined for the five isotypes. Expression of the class I (HM40) isotype was predominant, accounting for 84.7–98.7% of total β-tubulin expression. Expression of class II (Hβ9), class III (Hβ4), class IVa (H5β), and class IVb (Hβ2) was also detected but at lower levels. However, no correlation could be demonstrated between isotype expression and paclitaxel sensitivity in these 12 HOC xenografts.

Because human tumors are known to be heterogeneous, we also considered the possibility that a selection of a predominant cell population occurred after transplantation of tumors in nude mice. However, our results excluded this possibility. Furthermore, we demonstrated that serial passaging of the same xenograft in the animals did not affect the β-tubulin isotype composition, thus making this system a useful and reliable tool for preclinical investigations.

The results with the HOC xenografts demonstrating a lack of correlation between paclitaxel sensitivity and the expression of β-tubulin isotypes contrast with studies showing increased expression of specific β-tubulin isotypes in clinically derived Taxol-resistant ovarian cancer samples compared with primary untreated tumors (8). These results must also be reconciled with recent *in vitro* evidence, which suggests that the rate of polymerization depends on the isotype used (9, 32, 33). More importantly, they must be harmonized with our own observations in 17 cancer cell lines from the NCI-Anticancer Drug Screen. Similar to previous studies, the results with the 17 cell lines suggest differences in isotype expression confer differential sensitivity. To explain the apparent discrepancy, we considered several possibilities: (*a*) The most straightforward is sample size. We cannot exclude that a correlation could be observed with a larger sample size, although the magnitude of its contribution to paclitaxel resistance could then be questioned, especially because previous studies have reported correlations with smaller sample sizes (8), and in our own experience, the class III (Hβ4) correlation in the cell lines was apparent with as few as 9 cell lines (the number of cell lines originally examined. (*b*) We also entertained the possibility that the apparent discrepancy may be explained in part by the differences in the samples analyzed, *e.g.,* clinical samples obtained from patients will invariably be contaminated with normal cells that may alter the level of each isotype. In fact, the percentage of normal cells may vary over time and depend on the method of procurement. In this respect, the experimental model of human ovarian cancer used in this study provided a unique opportunity to discriminate human from mouse tubulin expression because of the use of human specific primers that do not recognize contaminating normal mouse cells. The nearly uniform pattern observed in the xenografts and the cell lines suggests that in tumors, class I (HM40) is the predominant isotype mRNA, with class III (Hβ4) a distant second. A greater diversity has been observed in normal tissues, and variable contamination could alter the percentages in tumor samples. Yet we were cognizant that whereas this could explain differences in clinical results, it could not explain the results obtained *in vitro* with cell lines and with purified tubulin; although with regard to the *in vitro* polymerization studies, it should be emphasized that these have used a single β-isotype, a somewhat artificial situation when compared with the mixtures found in cells (see below). (*c*) A final possibility, and that which we find most appealing, reconciles the apparent discrepancy by accepting both as correct, with each reflective of the model used. Thus, in the *in vitro* system, differences in isotype composition confer differential sensitivity, but the same does not occur *in vivo*. The latter could be explained if another, more critical "factor" negates the contribution of the isotype composition, *e.g.,* if *MDR-1* expression was important, the level of expression of *MDR-1* would determine intracellular drug concentration and drug sensitivity, in effect obscuring the contribution of isotype composition. Whereas in cells with identical *MDR-1* expression, isotype distribution could be discerned as an important factor, the range of *MDR-1* expression, presumably independent of isotype distribution, would confound any analysis. Indeed, to observe the correlation in the cell lines, we deliberately examined a subset with very low or undetectable *MDR-1* expression, because we knew from studies done before (28) that *MDR-1* expression was the overriding factor in determining paclitaxel sensitivity in the 60 cell lines. *MDR-1* expression is discussed here only as a well understood resistance factor which could confound the interpretation, but in the HOC xenografts, *MDR-1* was excluded as an important factor. However, other mechanisms of resistance, or even more "proximal" factors such as drug distribution/penetration, could be the predominant determinant(s) of paclitaxel sensitivity in the HOC xenografts.

Whereas the *in vivo* results did not discern a correlation between isotype expression and paclitaxel sensitivity, the *in vitro* results found that higher class III (Hβ4) expression correlated with increased paclitaxel resistance. Recent *in vitro* evidence suggests that the rate of polymerization depends on the isotype used (9, 32, 33). In these studies, experiments were performed using microtubules depleted of one β-tubulin isotype (33) or comparing mixtures containing only class II (αβII; Hβ9) with mixtures containing only class III (αβIII; Hβ4; Refs. 9 and 32). The results of these investigations suggest that the dynamics/stability of microtubules and their sensitivity to paclitaxel are dependent on the β-tubulin composition. However, unlike cells, these studies used a homogenous tubulin preparation. In our analysis, the class I (HM40) isotype was the most abundant, and thus we were surprised to find that small increases in the percentage of a minor isotype could significantly influence microtubule behavior and, in turn, sensitivity to tubulin-binding drugs. Specifically, in cells where class I (HM40) expression is >76%, it was surprising to find that an isotype (Hβ4) with a range of expression of 0.3–11.2% had an impact. This may indicate differential roles for the various isotypes in the initiation of polymerization *versus* elongation, or it could reflect a significant discrepancy between mRNA expression and protein levels so that Hβ4 protein levels are substantially higher, and HM40 are significantly lower. This latter issue will only be resolved when reliable isotype-specific antibodies can be used to precisely quantitate protein levels. Theoretically, it could also indicate a preferential binding of paclitaxel to certain isotypes, but evidence in support of this is not available, and indeed, our own observations with acquired mutations conferring resistance argues against this (7).

In summary, our results suggest that β-tubulin isotype composition does not have a significant impact on paclitaxel sensitivity *in vivo* and that in cell culture, a statistically significant correlation can be demonstrated in cell lines without *MDR-1* expression. The observation that a correlation between class III (Hβ4) expression and paclitaxel sensitivity was found *in vitro* but not in a panel of *in vivo* human tumors raises the question of whether altered expression of specific β-tubulin isotypes is a significant mechanism of resistance *in vivo*. Our data argue against its significance and suggest that it is unlikely that measurements of isotype expression will be of value in clinical samples.

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Clinical Cancer Research

Expression of β-Tubulin Isotypes in Human Ovarian Carcinoma Xenografts and in a Sub-Panel of Human Cancer Cell Lines from the NCI-Anticancer Drug Screen: Correlation with Sensitivity to Microtubule Active Agents

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