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Human mutations that confer paclitaxel resistance¹

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Abstract

The involvement of tubulin mutations as a cause of clinical drug resistance has been intensely debated in recent years. In the studies described here, we used transfection to test whether β 1-tubulin mutations and polymorphisms found in cancer patients are able to confer resistance to drugs that target microtubules. Three of four mutations (A185T, A248V, R306C, but not G437S) that we tested caused paclitaxel resistance, as indicated by the following observations: 1. Essentially 100% of cells selected in paclitaxel contained transfected mutant tubulin. 2. Paclitaxel resistance could be turned off using tetracycline to turn off transgene expression. 3. Paclitaxel resistance increased as mutant tubulin production increased. All the paclitaxel resistance mutations disrupted microtubule assembly, conferred increased sensitivity to microtubule disruptive drugs, and produced defects in mitosis. The results are consistent with a mechanism in which tubulin mutations alter microtubule stability in a way that counteracts drug action. These studies show that human tumor cells can acquire spontaneous mutations in β 1-tubulin that cause resistance to paclitaxel, and suggest that patients with some polymorphisms in β 1-tubulin may require higher drug concentrations for effective therapy.

Keywords

tubulin; patients; vinblastine; epothilone; colcemid; drug resistance; acquired resistance; clinical resistance; tetracycline regulated expression

Introduction

Microtubules are a major target in cancer chemotherapy. For example, the vinca alkaloids have long been used in chemotherapeutic regimens for the treatment of leukemia, lymphoma, testicular carcinoma, and other malignancies. More recently, paclitaxel has emerged as a powerful drug for treating a number of solid tumors including breast, ovarian, and non-small-cell lung carcinomas. In addition to these well established drugs, a number of new agents that target microtubules are under development and many are already in clinical trials (1). Although microtubule-targeted drugs have proven to be highly effective for treating cancer, the development of drug resistance continues to present challenges to successful outcomes. Cell culture studies have identified several potential mechanisms by which resistance can develop, but to date none of these has conclusively been shown to be a major cause of resistance in patients undergoing therapy (2,3). One resistance mechanism that has received a lot of attention in recent years involves mutations in tubulin (4).

Microtubules assemble from heterodimers of α - and β -tubulin, but each of these proteins is encoded by at least 6-7 genes that are expressed in a tissue specific manner (5,6). Although

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human α -tubulin proteins are highly homologous and differ by only a few amino acids, β -tubulins can differ by as many as 40 or more amino acid residues. The most variable region of β -tubulin involves the extreme C-terminal 15 residues and these sequences have been used to classify β -tubulin proteins into the 7 distinct isotypes: β I, β II, β III, β IVa, β IVb, β V, and β VI (7). Most tissues express varying amounts of at least 3 of these 7 isotypes; thus, microtubule composition is heterogeneous and can differ considerably from one cell type to the next.

β 1-Tubulin is the major isotype found in most mammalian tissues as well as most cultured tumor cell lines. Therefore, it is not surprising that most of the mutations that cause drug resistance in cell culture studies have been found in this isotype (8,9). Given the high incidence of tubulin mutations as a cause of drug resistance in these studies, the question of whether tubulin mutations also play a major role in the development of in vivo resistance to drug treatment has been hotly debated in recent years. An initial report that tubulin mutations were common in patients with non-small-cell lung carcinoma sparked a considerable amount of activity in this area (10). However, it was later found that the “mutations” came from sequencing pseudogenes that were amplified because of poor primer design (11), and a number of subsequent studies found little evidence for tubulin alterations in tumors from patients with a variety of malignancies (12-17). It should be noted, however, that while these latter studies found few tubulin mutations in tumor samples, most of those tumors came from patients who had not been treated with microtubule targeted drugs and thus shed little light on whether tubulin mutations play a role in acquired resistance to drug therapy. Nevertheless, a tubulin mutation and several polymorphisms were reported among these studies but their ability, if any, to confer drug resistance was not explored. To address this issue, we recreated the tubulin alterations as mutations in a β 1-tubulin cDNA that is under the transcriptional control of a tetracycline regulated promoter. We then transfected Chinese hamster ovary (CHO) cells with the cDNA to determine whether the mutations are capable of conferring drug resistance. The changes we tested included an R306C mutation found in 1 out of 62 patients with breast cancer (14), A248V and A185T nonsynonymous SNPs found among 24 leukemia patients (18), and a G437S heterozygous polymorphism found in the tumor of a breast cancer patient who exhibited a partial response to an epothilone B analog (19).

Materials and Methods

Site-Directed Mutagenesis and Transfection

CHO tTA cells that produce a tetracycline regulated transactivator (20) were used for all transfections. The cells were maintained in α MEM (Sigma-Aldrich, St. Louis, MO) containing 5% fetal bovine serum (Gemini), 50 U/ml penicillin, 50 μ g/ml streptomycin, 10 μ g/ml puromycin, and 1 μ g/ml tetracycline (all from Sigma-Aldrich). CHO β 1-tubulin cDNA (GeneBank TM accession no. U08342) was cloned into the tetracycline regulated mammalian expression vector, pTOPneo (20). The β 1-tubulin cDNA also encoded a 9 amino acid hemagglutinin (HA) epitope tag (YPYDVDPYA) at the C-terminus for convenient detection of the protein. Site-directed mutagenesis was carried out with the QuickChange mutagenesis kit (Invitrogen) as recommended by the manufacturer. Stably transfected cells were selected in 2 mg/ml G418 plus 1 μ g/ml tetracycline for 10 d.

Drug Resistance

G418 resistant transfected cells were seeded in duplicate into 6-well dishes containing drug (200 nM paclitaxel or 50 nM colcemid). One of the two drug-containing wells also contained 1 μ g/ml tetracycline. A third well containing 50-fold fewer cells in α MEM plus 1 μ g/ml tetracycline was used to estimate the number of viable cells that were seeded. Cells were grown until visible colonies formed (7-10 d) and were stained with methylene blue as previously

described (21). Finally, the plates were gently rinsed with water to remove excess stain and were then photographed.

Immunofluorescence

Cells were grown on sterile glass coverslips for 1-3 d, rinsed briefly in PBS, pre-extracted with microtubule buffer (MTB) (20 mM Tris-HCl, pH 6.8, 1 mM MgCl₂, 2 mM EGTA, 0.5% NP-40) containing 4 µg/ml paclitaxel for 2-3 min on ice, and fixed in methanol at -20°C for at least 20 min. The fixed cells were then incubated with mouse monoclonal α -tubulin antibody DM1A (1:100, Sigma-Aldrich) and/or affinity purified rabbit polyclonal anti-HA antibody (1:50, Bethyl Laboratories, Montgomery, TX) for 30-60 min at 37°C in a humid chamber. After rinsing in PBS, the cells were incubated with 1:50 dilutions of Alexa 594-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) and/or Alexa 488-conjugated goat anti-rabbit IgG plus 1 µg/ml 4'6-diamidino-2-phenylindole (DAPI). The microtubules were visualized using an Optiphot microscope equipped with epifluorescence and a20X or 60X objective (Nikon Inc., Melville, NY). Images were captured using a Magnafire digital camera (Optronics, Goleta, CA).

Antibodies and Western Blots

Cells grown in 24 well dishes until they were about 70-80% confluent were lysed in 1% SDS. Proteins were precipitated in 5 volumes of acetone, resuspended in 100 µl SDS sample buffer (0.0625 M Tris-HCl, pH6.8, 2.5% SDS, 5% 2-mercaptoethanol, 10% glycerol), separated on a 7.5 % polyacrylamide SDS minigel, and transferred to PROTRAN nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). After blocking the membranes with PBST (PBS with 0.05% Tween 20) containing 3% dry milk, they were incubated 45-60 min with a 1:2,000 dilution of β -tubulin antibody Tub 2.1 (Sigma-Aldrich) and a 1:20,000 dilution of anti-actin antibody C4 (Chemicon International Inc., Temecula, CA). Immunoreactive band were visualized by incubating the membrane 30-45 min in a 1:2,000 dilution of peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich), washing in PBS, incubating with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL), and exposing to X-ray film.

Extent of Cellular Tubulin Polymerization

The extent of tubulin polymerization, defined as the fraction of total cellular tubulin present in sedimentable microtubules, was measured as previously described (22). Cells were grown overnight in triplicate wells of 24-well dishes and lysed in 100 µl of MTB containing 0.14 M NaCl and 4 µg/ml paclitaxel. Lysates were scraped from the wells, transferred into 1.5 ml microcentrifuge tubes, and centrifuged at 12,000 X g for 15 min at 4°C. The supernatants carrying the unpolymerized tubulin were transferred to fresh tubes and the pellets containing the polymerized tubulin were solubilized in SDS sample buffer. Each sample (supernatant and pellet) also received 10 µl of a bacterial lysate expressing a GST-tagged α -tubulin which acted as an internal control for potential losses of sample in subsequent steps. Proteins were precipitated using 5 volumes of acetone and resuspended in 100 µl of SDS sample buffer. Equal volumes of samples were separated on 7.5% polyacrylamide SDS minigel (BIORAD) and transferred to nitrocellulose membranes. The membranes were incubated with antibody DM1A followed by Alexa 647-conjugated goat anti-mouse IgG (Invitrogen). Bands corresponding to α -tubulin and GST- α -tubulin in each fraction were detected by fluorescence using a Storm Imager (Molecular Dynamics, Sunnyvale, CA) and quantified using Image J. The extent of tubulin polymerization was calculated by first normalizing the α -tubulin in the pellet and supernatant fractions to the amount of GST- α -tubulin present in those fractions. The normalized value from each pellet was then divided by the sum of the values from the pellet plus supernatant, and the resultant fraction was multiplied by 100%.

Dose Response and IC50s

Approximately 200-300 cells from each cell line were seeded into each of 6 replicate wells in 24-well dishes with increasing concentrations of drug and incubated until visible colonies appeared (7-10 d). The surviving cells were then stained with 0.5% methylene blue in water, dried, and photographed. To calculate IC50 values, the cell-associated dye was solubilized using 200 μ l of 1% SDS in 50 mM Tris HCl, pH 6.8, 50 μ l was transferred to a 96-well dish, and the absorbance was read at 630 nm using an Emaxmicroplate reader (Molecular Dynamics). The optical density at each drug concentration was normalized to the value with no drug present, and a graph of the normalized optical density versus drug concentration was plotted for each sample using ProFit software (QuantumSoft, Uetikon am See, Switzerland). Data points came from at least 3 experiments run in triplicate, and the IC50 value was calculated from the graph as the concentration of drug that inhibited cellgrowth by 50%.

Results

Mutations in β 1-Tubulin Enable Cells to Form Colonies in Paclitaxel

To test whether β -tubulin mutations or polymorphisms that have been identified in human tumors and normal tissues are able to confer resistance to anti-mitotic drugs, four mutations (A185T, A248V, R306C and G437S) were introduced into HA β 1-tubulin cDNA and transfected into CHO tTA cells that express a tetracycline regulated transactivator (20). Immunofluorescence with an HA tag antibody was used to monitor the transfection efficiency and DAPI was used to stain nuclei. Transfected cells were enriched by selection with G418; tetracycline was also added to inhibit transgene expression and thereby limit any potential toxicity due to mutant HA β 1-tubulin production. Equal numbers of G418 resistant cells were then seeded into two replicate wells of a 6-well dish, both of which contained 200 nM paclitaxel, but only one of which contained tetracycline to inhibit HA β 1-tubulin production. A third well containing 50-fold fewer cells in medium with tetracycline but no paclitaxel was used to estimate the number of cells plated. As shown in Figure 1, cells transfected with wild type HA β 1 cDNA failed to form colonies in paclitaxel. In contrast, cells transfected with HA β 1 containing 3 of the 4 mutations formed numerous colonies. Significantly, these same cells failed to survive in paclitaxel when tetracycline was included to inhibit production of the mutant tubulin. From these results, we conclude that the A185T, A248V, and R306C mutations are all capable of conferring resistance to paclitaxel in a cell culture environment. We carried out a similar experiment to test whether the mutations could confer resistance to colcemid, a drug known to disrupt rather than promote microtubule assembly, but found no survivors in the drug (data not shown).

Paclitaxel Selects For Cells That Express Mutant Tubulin

Our prior experience indicated that only about 50% of cells selected in G418 are positive for expression of the transfected gene (23-25). This number is less than 100% because a break occurs in the circular plasmid during its integration into the genomic DNA and it can sometimes disrupt promoter or coding sequences necessary for expression of the transfected gene. Additionally, the plasmid may occasionally integrate into areas of the genome with low transcriptional activity. On the other hand, we reasoned that if production of the mutant tubulin is required for paclitaxel resistance, then only the cells that express the mutant cDNA should be able to survive selection in paclitaxel. To test this prediction G418 resistant populations of cells transfected with each of the 4 mutant HA β 1-tubulins were compared to the same populations that were subjected to further selection in a normally lethal dose of paclitaxel. As predicted, immunofluorescence analysis using an antibody to the HA tag demonstrated that survivors of the G418 selection were a mixture of HA β 1-tubulin producing and non-producing cells, whereas paclitaxel resistant cells were uniformly positive for mutant HA β 1-tubulin expression (Figure S1, supplementary figures). These results provide further evidence that

production of HA β 1-tubulin containing A185T, A248V, and R306C substitutions is sufficient to confer resistance to paclitaxel. Cells expressing the G437S mutation, however, were again unable to survive in the drug.

Mutant Tubulin Production Correlates With the Degree of Paclitaxel Resistance

To determine how much mutant HA β 1-tubulin production is necessary to confer resistance, G418 resistant cells were reselected in 200 or 300 nM paclitaxel and then analyzed by western blots using an antibody that recognizes both endogenous and transfected β -tubulin. For all 3 mutations that confer drug resistance, cells that survived selection in paclitaxel had a higher ratio of mutant to endogenous β -tubulin than the starting G418 resistant population (Figure 2). Moreover, this ratio was significantly higher for the cells selected in 300 versus 200 nM paclitaxel. It thus appears that the degree of paclitaxel resistance conferred by each of the mutations is proportional to the amount of mutant tubulin that is produced and incorporated into cellular microtubules. The data in Figure 2 also suggest that of the 3 mutations that confer resistance to paclitaxel, A185T is able to do so at lower levels of expression than the other two.

Mutant Tubulin Affects Microtubule Organization and Cell Division

To assess whether production of mutant tubulins could affect microtubule organization, transfected paclitaxel resistant cells were grown in the absence of paclitaxel for 3 days and stained with an anti-HA antibody. Compared to control cells that express wild type HA β 1-tubulin (Figure 3A), cells transfected with HA β 1-tubulin containing A185T, A248V or R306C mutations appeared to have significantly lower microtubule content (Figure 3 B, C, and D); cells transfected with HA β 1_(G437S), on the other hand, appeared essentially normal (Figure 3E). It is also evident that the three mutations that produced clearly visible effects on microtubule density caused the cells to become multinucleated, indicating that all of these cells experienced defects in chromosome segregation and cell division (Figure 3 B, C, and D). In contrast, the cells transfected with HA β 1_(G437S), that did not produce changes in the microtubule network, had normal nuclear size and morphology (Figure 3E).

Microtubule Content Is Reduced in Cells That Produce Mutant Tubulin

A microtubule fractionation procedure (22) was used to quantify changes in microtubule assembly in stable cell lines that produce mutant tubulin. The procedure involved lysing cells in a microtubule stabilizing buffer, separating microtubule polymer from soluble tubulin by centrifugation, and quantifying the amount of tubulin in the supernatant and pellet fractions. As shown in Figure 4, approximately 40% of the total tubulin in cells transfected with wild-type HA β 1-tubulin was found in the pellet (microtubule) fraction. This estimate agrees well with previously published data (20,22,23,25-29). Cells transfected with the same cDNA, but containing A185T, A248V, or R306C mutations on the other hand, had a lower percentage of assembled tubulin. Moreover, the decrease in assembled tubulin was greater for the cells that expressed more of the mutant tubulin and were more paclitaxel resistant. This indicates that increasing the incorporation of mutant tubulin lowers microtubule stability and increases cellular resistance to paclitaxel. In contrast to these results, cells transfected with HA β 1-tubulin containing the G437S mutation that did not confer paclitaxel resistance had a normal percentage of assembled tubulin.

Mutant Tubulin Alters the Sensitivity of Cells to Antimitotic Drugs

Tubulin contains at least 3 topologically distinct drug binding sites: a taxane binding site in the β subunit, a colchicine binding site in the β subunit, and a vinca alkaloid binding site at the interface between successive heterodimers in microtubule protofilaments (30-32). To determine whether the mutations in β -tubulin affect sensitivity to drugs other than paclitaxel, we tested the ability of transfected cells to form colonies in varying doses of paclitaxel,

epothilone A, colcemid, and vinblastine. The dose response curves shown in Figure 5 confirmed that cells transfected with HA β 1-tubulin containing A185T, A248V, or R306C mutations were more resistant to paclitaxel (Figure 5A). Similar results were obtained with epothilone A, another microtubule stabilizing drug that is known to share the paclitaxel binding site (Figure 5B). In contrast, all 3 cell lines were more sensitive to both colcemid (Figure 5C) and vinblastine (Figure 5D), microtubule destabilizing drugs that bind to the colchicine and vinca alkaloid sites respectively. Cells transfected with HA β 1-tubulin containing the G437S mutation, on the other hand, had normal sensitivity to all 4 drugs. A summary of the IC₅₀s for all of the cell lines to each of these drugs is provided in Table 1.

Discussion

The four mutations examined in this study came from published sequence information reported for cancer patients undergoing treatment. These included an R306C mutation found in 1 of 62 patients with breast cancer (14), A248V and A185T polymorphisms each found in 3 of 24 patients with hematologic malignancies (18), and a G437S polymorphism found in 1 of 17 patients with breast cancer (19). An S201C polymorphism was also reported in one of these studies (18), but cysteine is already the normal, conserved amino acid at this position. No clear correlation between these mutations/polymorphisms and patient response was noted in any of the studies. Despite being located at distant sites from one another on β -tubulin, 3 of the 4 amino acid substitutions we tested produced a similar phenotype when introduced as mutations into wild-type CHO cells. All three reduced microtubule polymer levels, conferred resistance to drugs that stabilize microtubules, and conferred increased sensitivity to drugs that destabilize microtubules. Thus, they resemble a large number of mutations we and others have previously characterized in paclitaxel resistant cells; and they likely confer resistance by a mechanism that involves changes in microtubule assembly and stability that counteract the actions of the drugs (2,33). We also tested a C201S mutation, but found no evidence that it is able to confer drug resistance (Bhattacharya and Cabral, data not shown).

As previously argued, changes in drug binding are unlikely to contribute to resistance because decreased drug binding is a recessive phenotype that is rarely encountered in diploid cells. Moreover, decreased drug binding cannot explain the hypersensitivity of mutant cells to microtubule destabilizing agents that bind to different sites, nor can it account for the ability of paclitaxel and related compounds to rescue the growth of mutant cells that have defects in cell division (2). Similarly, changes in tubulin levels or isotype composition resulting from transfection are an unlikely cause of resistance. Wild-type CHO cells have a β -tubulin composition that consists of 70% β 1, 25% β 4b, and 5% β 5 (34,35). All three endogenous isotypes, as well as all transfected isotypes we have tested, coassemble into all microtubules, but only overexpression of β 3 or β 5 is able to confer resistance to paclitaxel (29,36). Although transfection of wild-type HA β 1-tubulin cDNA does not confer drug resistance ((23) and Figure 5), transfection of mutant HA β 1-tubulin clearly does (Figure 1). Therefore, it is unlikely that other changes resulting from transfection such as possible alterations in total tubulin levels or isotype ratios are responsible for resistance. As further evidence for this argument, we previously demonstrated that overexpression of HA β 1-tubulin produces only a small increase in cellular tubulin levels because excess β -tubulin (not in a heterodimer with α -tubulin) is rapidly degraded (20,37). We conclude that mutant HA β 1-tubulin acts by incorporating into microtubules and altering their assembly and stability, a mechanism for which we have direct evidence ((22) and Figure 4).

Consistent with the view that decreased microtubule stability is responsible for resistance to paclitaxel, all 3 mutations that cause resistance are located at sites that could influence subunit interactions. A structural model based on published atomic coordinates (31) indicates that alanine 248 is positioned in a loop that connects helix 7 to helix 8, and is near the intra-dimer

interface where it could influence longitudinal subunit interactions along protofilaments (Figure 6). Alanine 185 is a buried residue in helix 5 near the GTP binding site in β -tubulin and could be influencing inter-dimer interactions along protofilaments. Arginine 306 is found in a highly conserved small helical region of a loop that connects helix 9 and sheet 8. It is a surface-exposed residue that is positioned in a region that could influence lateral interactions between protofilaments. The G437S mutation (not shown in Figure 6) that fails to alter microtubule assembly or confer drug resistance, on the other hand, is located on the outer surface of assembled microtubules (38).

A review of the data from several laboratories clearly implicates mutations in tubulin as a major cause of resistance to paclitaxel and other antimitotic drugs in mammalian cell culture models (8). For example, in a single step selection for resistance to paclitaxel, greater than 90% of the isolated clones had properties consistent with mutations in tubulin, while the remainder had the multidrug resistance (mdr) phenotype (39). For colchicine and vinblastine, on the other hand, the majority of resistant cell lines had the mdr phenotype and only about 15% were tubulin mutants (40). Given the high incidence of tubulin mutations in these and other selections, it was natural to assume that tubulin mutations might also be responsible for chemoresistance in patients.

One of the first studies to test this prediction examined 49 patients with non-small-cell lung cancer. The authors reported that 16 of these patients had mutations in the β 1-tubulin gene and exhibited no objective response to subsequent paclitaxel treatment. Of the remaining 33 patients with no mutations, 13 responded to therapy (10). These results have since been called into question, however, by studies demonstrating that the exon primers used in the earlier study were able to amplify pseudogenes in addition to the authentic β 1-tubulin gene (11). Subsequent work using intron primers to avoid the amplification of pseudogenes have gone on to show that tubulin mutations are exceedingly rare in human tumors and led to the conclusion that β -tubulin mutations are unlikely to be a significant cause of treatment failure in patients (12-17). It should be pointed out, however, that most of these later studies focused on patients who were not pretreated with paclitaxel or other taxanes. Thus, the studies indicate that β 1-tubulin mutations are an unlikely cause of inherent drug resistance, but they do not address the possibility that such mutations might be responsible for acquired resistance during or following treatment. One study did look for tubulin mutations as a possible cause of acquired resistance, but again found no alterations in the β 1-tubulin gene among 30 paclitaxel resistant samples that included tumors from 9 patients with ovarian cancer, 9 cell lines selected for paclitaxel resistance in cell culture, and 12 ovarian tumor xenografts in nude mice (41). These negative results could have been caused in part by methodological problems (discussed below), but the data from this study support the view that tubulin mutations are also not involved in acquired drug resistance.

In contrast to these reports, some recent observations support the idea that tubulin alterations could be playing a role in clinical resistance to microtubule targeted drugs. In one particularly interesting study, human tumor xenografts from 10 children with acute lymphocytic leukemia were established in nude mice and assayed for vincristine sensitivity in cell culture (42). The results indicated that drug sensitivity of the cells in culture correlated with favorable patient outcomes. Moreover, the more resistant cells tended to have higher amounts of microtubule polymer. Comparison of the two xenografts having the highest and lowest amount of polymerized tubulin further showed that the cells with higher microtubule polymer were not only more resistant to vincristine, they were also more sensitive to paclitaxel compared to the cells with lower polymer levels. The relationship between drug resistance and microtubule polymer levels was further supported by the observation that polymer levels again increased in 2 sublines from one of the xenografts that acquired additional drug resistance by treating the animals with vincristine. A second study has indicated that vinorelbine, a vinca alkaloid

that destabilizes microtubules, is particularly effective in patients with metastatic breast carcinoma who have failed anthracycline and taxane treatment (43). Although neither study provided sequence information for tubulin, the phenotypes of increased polymer levels and enhanced paclitaxel sensitivity in vincristine resistant cells, as well as increased vinca alkaloid sensitivity in paclitaxel resistant patients are in agreement with phenotypes reported for drug resistant cells with mutations in tubulin reported here and elsewhere (see references 2,3, and 8 for reviews).

We are of the opinion that if tubulin mutations can cause drug resistance in a cell culture model, they should be capable of producing resistance in clinical settings. Why then has it proved to be so difficult to find evidence for tubulin mutations in human cancer? A number of factors may have contributed to this conundrum. 1) As already mentioned, it is unreasonable to expect a high incidence of tubulin mutations as a cause of inherent drug resistance in untreated patients. Tubulin is a highly conserved and essential protein; most mutations will cause defects in mitosis leading to cell death. Thus, tubulin mutations will rarely be found in unselected tumor cell populations. They are more likely to be found in relapsed patients. 2) Tumor biopsies contain many noncancerous cells that may obscure the presence of a mutation in DNA sequencing experiments. 3) Most cells express multiple tubulin genes and it is well established that mutations in both α - and β -tubulin can cause drug resistance (39). Thus, potential drug resistance mutations may be missed when only the $\beta 1$ -tubulin gene is sequenced.

Despite limitations that may previously have caused under-reporting of tubulin mutations in patients being treated for cancer, we believe that even with improved approaches these mutations will continue to be difficult to find because other mechanisms of resistance may occur much more frequently. Recent studies, for example, have indicated that expression of $\beta 3$ -tubulin, an isoform that is normally restricted to neuronal tissue, correlates well with poor patient response to chemotherapy (44). It has been confirmed that overexpression of $\beta 3$ -tubulin can cause paclitaxel resistance in cell culture models (45), but it should be noted that high levels of expression are needed and that only a marginal degree of resistance is gained (36). Thus, while $\beta 3$ expression may contribute to drug resistance, it is unlikely to be a major cause of resistance. The most likely cause of treatment failure may simply be patient-to-patient variability in pharmacological parameters such as drug distribution, metabolism, and excretion that may limit the amount of drug that is actually delivered to the tumor cells (46). In support of this notion, it has been observed that some patients who fail treatment with paclitaxel remain sensitive to docetaxel, a drug with a similar mechanism of action that binds to the same site on the protein (47). Also, it has been reported that formulations of paclitaxel that alter the drug's pharmacological properties appear to be more effective in treating tumors (48,49). Continued improvements in drug design and protocols for the administration of paclitaxel and other taxanes may ultimately decrease or, perhaps even eliminate pharmacological barriers to drug action. As that happens, we predict that tubulin mutations, such as the ones described here, will emerge as a major cause of acquired drug resistance in patients who relapse after successful therapy with these drugs.

Tubulin polymorphisms, in contrast to tumor specific mutations in tubulin, occur at an appreciable frequency in humans. One of the most common appears to be an L217L polymorphism reported by several groups (11,14,16,41). Non-synonymous changes occur less frequently but are still appreciable. For example, the A248V and A185T polymorphisms examined here were found in 12.5% of the patients in one study (18), while G437S was found in 5.9% of the patients in a second group (19). The fact that 2 of the 3 polymorphisms were shown to be capable of conferring resistance to paclitaxel suggests that these differences in sequence could contribute to variability in patient response to antimetabolic drugs. Although the tumor cells in such patients might be relatively resistant compared to patients without these tubulin alterations, their bone marrow would likely also be relatively resistant allowing higher

doses of chemotherapy to be administered. Thus, the presence of the polymorphisms might not ultimately contribute to the success or failure of chemotherapy, but they might be useful guides to the doses of drug that should be administered for optimum patient response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

The abbreviations used are

DAPI	4'6-diamidino-2-phenylindole
mdr	multidrug resistance
HA	hemagglutinin
CHO	Chinese hamster ovary
MTB	microtubule buffer
MEM	minimal essential medium
Ptx	paclitaxel
GST	glutathione S-transferase
Tet	tetracycline

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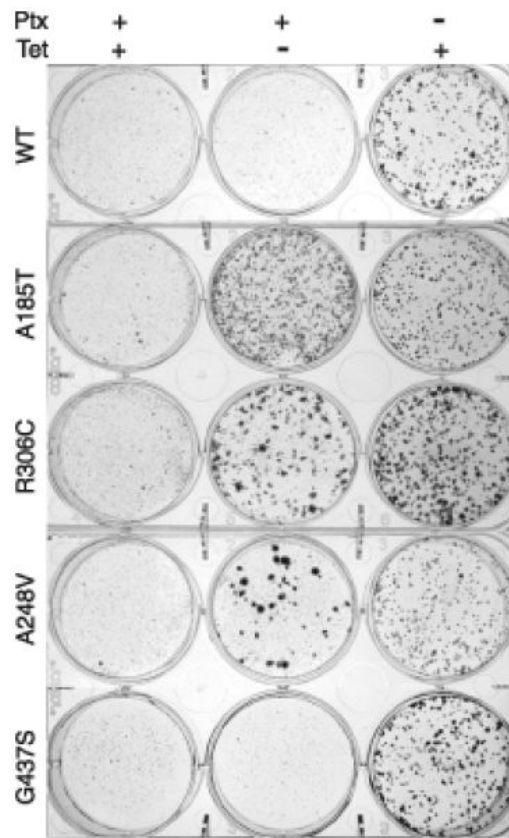


Figure 1.

Survival of transfected cells in paclitaxel. Cells stably expressing wild-type (WT) or mutant HA β 1-tubulin were incubated in 200 nM paclitaxel (Ptx) (left two wells), with or without tetracycline (Tet), and grown until visible colonies appeared. Controls containing 50 times fewer cells and incubated in tetracycline alone are shown in the rightmost wells to estimate the relative number of cells seeded. Colonies were stained with methylene blue and photographed.

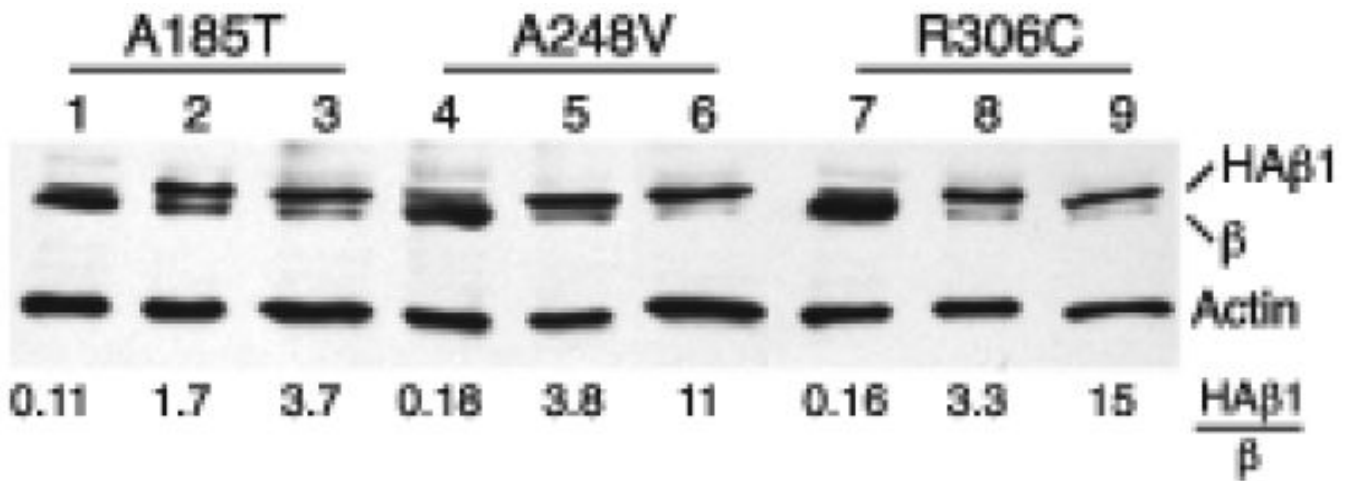


Figure 2.

Production of mutant HAβ1-tubulin in paclitaxel resistant cells. G418 resistant cells (lanes 1, 4, and 7) were grown overnight without tetracycline. Cells selected for resistance to 200 nM (lanes 2, 5, and 8) or 300 nM (lanes 3, 6, and 9) paclitaxel were maintained in selection medium. The cells were lysed in SDS and the proteins were separated on an SDS gel, transferred onto nitrocellulose, and probed with antibodies to β-tubulin and actin. Ratios of the exogenous HAβ1-tubulin to the endogenous β-tubulin (HAβ1/β) for each lane are shown at the bottom of the figure.

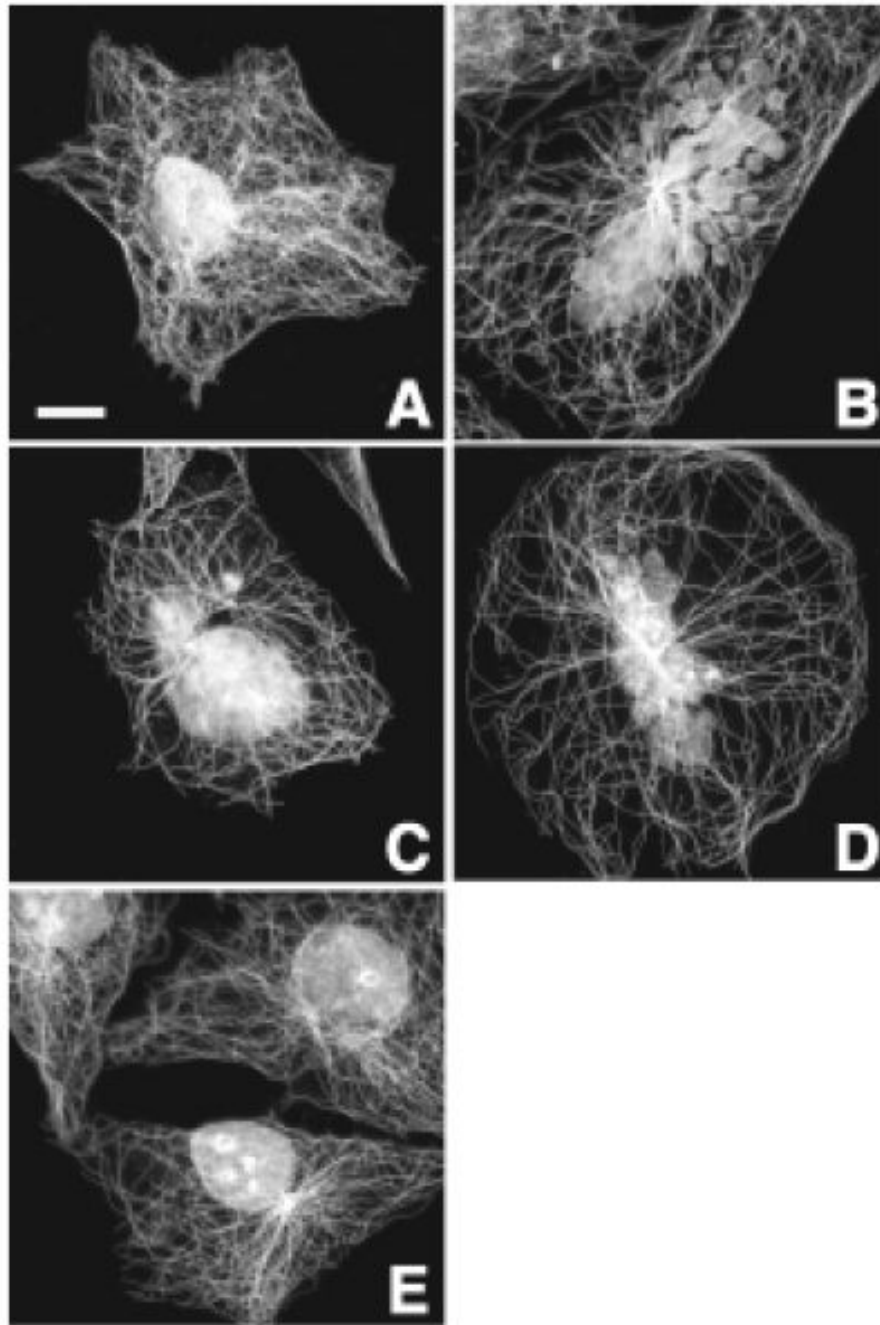


Figure 3. Immunofluorescence microscopy of cells transfected with wild-type and mutant HA β 1-tubulin. Cells transfected with wild-type (WT, panel A) or the four mutant HA β 1-tubulin cDNAs [A185T (B), A248V (C), R306C (D), and G437S (E)] were stained with an antibody to the HA tag and with DAPI, a fluorescent molecule that binds to DNA. Note that the 3 mutant tubulins that confer paclitaxel resistance (A185T, A248V, and R306C) reduce microtubule density and cause problems with chromosome segregation as indicated by the abnormal nuclear morphologies (Fig. 3 B-D). Bar = 10 μ m.

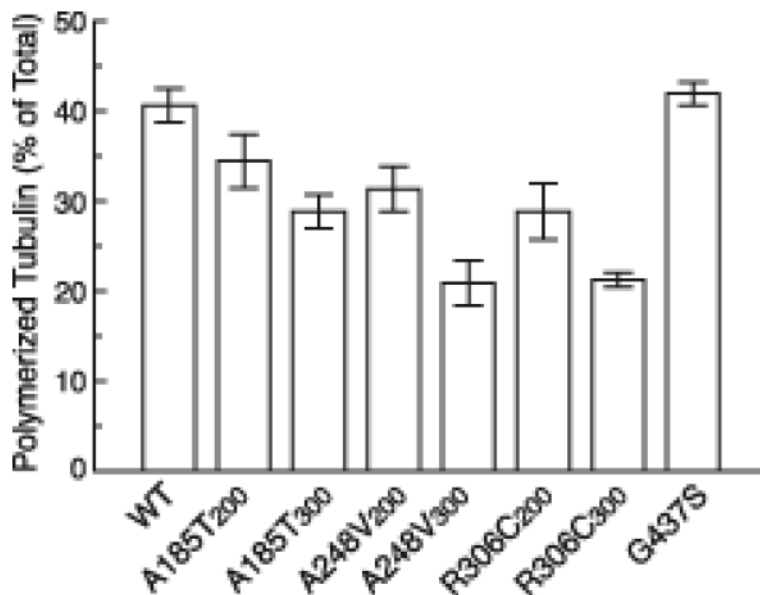


Figure 4. Microtubule content in transfected cell lines. Stable cell lines expressing wild-type or mutant HA β 1-tubulin cDNA were tested for their relative microtubule content. Cell lines transfected with wild-type or G437S HA β 1-tubulin were selected in G418 and screened for clones with high expression of the exogenous tubulin. Cell lines with the other mutations were selected in 200 or 300 nM paclitaxel but tested in the absence of the drug. The data were calculated from three independent experiments with triplicate samples, and represent the percentage of total tubulin found in the microtubule fraction. Error bars indicate the standard deviation.

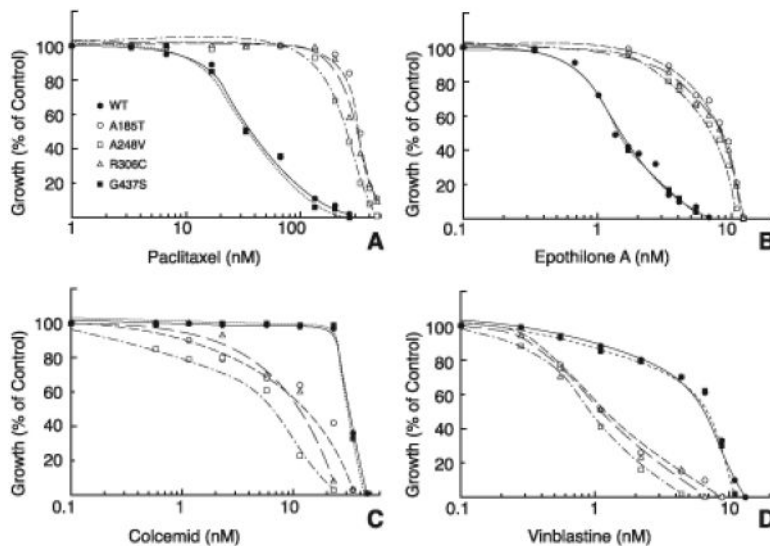


Figure 5.

Sensitivity of transfected cells to drugs that target microtubules. An equal number of cells (100-300) that stably express wild-type or mutant HA β 1-tubulin cDNA were seeded into replicate wells in a 24-well dish with increasing concentrations of paclitaxel (A), etoposide A (B), colcemid (C), or vinblastine (D). The cells were grown until visible colonies appeared (7 days), and were then stained with methylene blue. To quantify the data, dye was extracted with SDS and the absorbance was read at 630 nm. The relative growth was calculated by dividing the absorbance at a given drug concentration by the absorbance in the absence of drug, and the quotient was plotted against the drug concentration. Each data point represents the average of 3 independent experiments. The IC₅₀s and standard deviations are summarized in Table 1. Filled circles and solid line, wild-type; open circles and short-dash line, A185T; open squares and dash-dot line, A248V; open triangles and long-dash line, R306C; filled squares and dotted line, G437S.

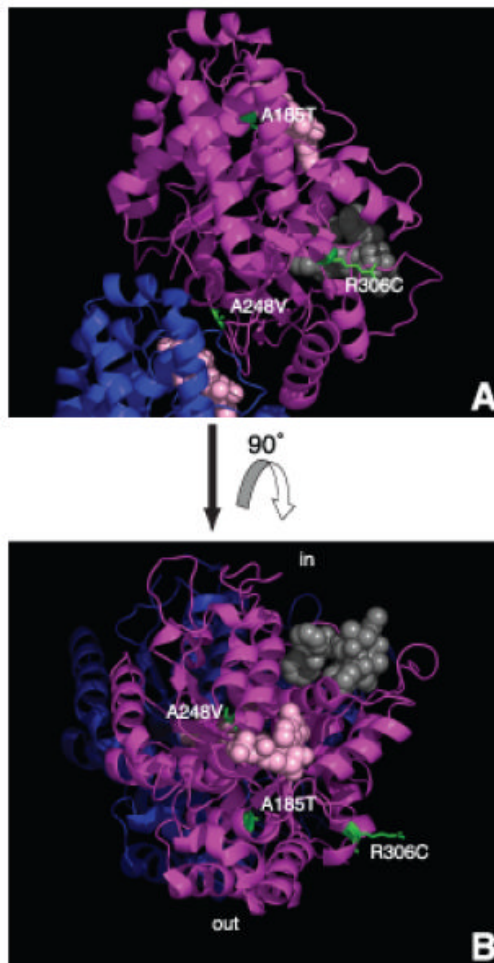


Figure 6. Tubulin structure. The structure was drawn with MacPymol(50) using the published atomic coordinates 1JFF (31). Magenta, β subunit; blue, α subunit; gray spheres, paclitaxel; pink spheres, GDP/GTP, green, mutant residues. G437S could not be shown because it is located in the carboxy terminal tail for which no structure is available. (A) lateral view; (B) axial view with indicated orientation relative to the inside and outside of a microtubule.

Table 1

Sensitivity of stably transfected cell lines to drugs that target microtubules

Cell line	Paclitaxel	Epothilone A	Colcemid	Vinblastine
Wild-type	50 ± 1.4 (1)	1.35 ± 0.02 (1)	31.7 ± 0.11 (1)	8.5 ± 0.16 (1)
A185T	366 ± 6 (7.3)	9.01 ± 0.14 (6.7)	18.9 ± 1.21 (-1.7)	1.7 ± 0.07 (-5)
A248V	282 ± 3 (5.6)	7.54 ± 0.35 (5.6)	7.2 ± 0.09 (-4.4)	1.3 ± 0.03 (-6.5)
R306C	321 ± 4 (6.4)	8.33 ± 0.28 (6.2)	13 ± 0.89 (-2.4)	1.7 ± 0.05 (-5)
G437S	49 ± 4 (1)	1.38 ± 0.04 (1)	31.1 ± 0.29 (1)	8.4 ± 0.05 (1)

Values indicate nanomolar IC50s ± the standard deviation. Numbers in parentheses are the fold-resistance (positive numbers) or fold-increased sensitivity (negative numbers) relative to the cell line transfected with wild-type HAβ1-tubulin.