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RESEARCH PAPER

Random Mutagenesis of β-Tubulin Defines a Set of Dispersed Mutations That Confer Paclitaxel Resistance

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ABSTRACT

Purpose Previous research showed that mutations in β1-tubulin are frequently involved in paclitaxel resistance but the question of whether the mutations are restricted by cell-type specific differences remains obscure.

Methods To circumvent cellular constraints, we randomly mutagenized β-tubulin cDNA, transfected it into CHO cells, and selected for paclitaxel resistance.

Results A total of 26 β1-tubulin mutations scattered throughout the sequence were identified and a randomly chosen subset were confirmed to confer paclitaxel resistance using site-directed mutagenesis of β-tubulin cDNA and transfection into wild-type cells. Immunofluorescence microscopy and biochemical fractionation studies indicated that cells expressing mutant tubulin had decreased microtubule polymer and frequently suffered mitotic defects that led to the formation of large multinucleated cells, suggesting a resistance mechanism that involves destabilization of the microtubule network. Consistent with this conclusion, the mutations were predominantly located in regions that are likely to be involved in lateral or longitudinal subunit interactions. Notably, fourteen of the new mutations overlapped previously reported mutations in drug resistant cells or in patients with developmental brain abnormalities.

Conclusions A random mutagenesis approach allowed isolation of a wider array of drug resistance mutations and demonstrated that similar mutations can cause paclitaxel resistance and human neuronal abnormalities.

KEY WORDS cell division · drug resistance · microtubule assembly · mutant tubulin · neuronal disorders

ABBREVIATIONS

CHO	Chinese hamster ovary
cmd	colcemid
GST	glutathione *S*-transferase
HA	hemagglutinin antigen
ptx	paclitaxel
tet	tetracycline

INTRODUCTION

Microtubules are involved in a variety of important cellular functions such as maintaining cell structure and shape, transporting vesicles, and ensuring the equal partitioning of chromosomes prior to cell division. The filamentous structures are nucleated at centrosomes located near the nuclear membrane and extend toward the plasma membrane by the stepwise addition of αβ tubulin heterodimers. Tubulin is encoded by a multigene family consisting of at least 7 genes each for the α and β subunits found in vertebrates, but most cells express only a subset of these genes depending on their tissue of origin (1,2). For example, Chinese hamster ovary (CHO) cells express 3 isotypes of α-tubulin and 3 isotypes of β-tubulin (3–5). The β-tubulin

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composition of these cells is 70% Class I (hereafter called β1), 25% Class IVb, and 5% Class V (3,5).

Because of their involvement in cell division, microtubules are the target for increasingly important drugs that are used in cancer chemotherapy (6). These drugs interact with tubulin at 4 distinct sites. Most drugs that destabilize microtubules and inhibit their formation bind to the the colchicine site that is located on the β subunit near the intradimer α-β interface (7), but additional drugs such as vinblastine and vincristine bind to a distant vinca alkaloid site that is predominantly on the α subunit near the interdimer αβ-αβ interface (8). In contrast to these destabilizing drugs, a number of agents have been shown to stabilize microtubules and promote their assembly. The prototype for these compounds is paclitaxel which binds to a site on the β subunit that faces the interior of the microtubule and appears to act by strengthening the lateral interactions between protofilaments and/or maintaining tubulin in an assembly competent conformation (9,10). The fourth drug binding site was recently shown to also be on the β subunit at a location close to, but distinct from, the paclitaxel site. This site binds peluroside and laulimalide that, like paclitaxel, act to stabilize microtubules (11–14). Microtubule destabilizing and microtubule stabilizing drugs have both proved to be important agents in cancer treatment. The vinca alkaloids have been used since the 1950's to treat a variety of hematological and solid tumors while paclitaxel and its analog docetaxel have more recently been shown to be effective for treating breast, ovarian, lung and an increasing number of other malignant carcinomas (15).

Despite the success of these agents, many patients suffer a recurrence of their disease. It is not yet known why drug treatment ultimately fails (16,17), but a number of laboratories including our own have identified a small set of mostly non-overlapping mutations in β1-tubulin among cell lines selected for their ability to survive in elevated concentrations of paclitaxel (18–20). The identification of different mutations in different laboratories using diverse cell lines suggested that either there are a vast number of possible mutations that are able to confer drug resistance or that the possible mutations are restricted in a cell-type specific manner. To resolve these questions and enlarge the spectrum of known mutations that can confer resistance, we developed a new approach involving the random mutagenesis of β1-tubulin cDNA followed by transfection and selection of cells for paclitaxel resistance.

MATERIALS AND METHODS

Cells and Materials

KB3-1 human oral adenocarcinoma cells obtained from Dr. Michael Gottesman (NIH) were subcloned and the resultant KB3-1.3 cells were maintained in alpha modified minimum essential medium (αMEM) (Sigma) containing 5% fetal bovine serum (Atlanta Biologicals), 50 U/ml penicillin, and 50 μg/ml streptomycin. Selection of paclitaxel resistant cells was carried out by seeding 5 X 10^5 non-mutagenized cells into 100 mm dishes with complete media containing 3 nM paclitaxel. After 10–14 d, individual surviving colonies were isolated and grown for further analysis. The resultant cell lines were approximately 3-fold resistant to paclitaxel and did not show resistance to drugs that do not interact with tubulin.

CHO tTA puro 6.6a cells (21), transfected with a tetracycline regulated transactivator, were maintained in complete αMEM medium as described above but also contained 10 μg/ml puromycin to retain the transactivator plasmid and 1 μg/ml tetracycline to repress transactivator expression. pTOPHAβ1-tubulin was constructed by cloning CHO β1-tubulin cDNA (GeneBank ™ accession no. U08342) into pTOPneo, a mammalian expression vector that drives transcription in the absence of tetracycline using a minimal CMV promoter preceded by 7 copies of the tet operator (21). The cDNA also encoded a 9 amino acid hemagglutinin antigen (HA) epitope tag (YPYDVPDYA) at the C-terminal end of β1-tubulin for the convenient detection of the protein in transfected cells. Drugs and chemicals were from Sigma-Aldrich unless otherwise stated.

Random Mutagenesis and Selection of Paclitaxel Resistant Cell Lines

A pool of cDNAs containing random mutations in β1-tubulin was prepared from pTOPHAβ1-tubulin using error-prone Taq polymerase (Invitrogen) and primers pTOPF2: 5′-GAT CCC CCA TTA TTG AAG CA-3′ and pTOPR2: 5′-CAG AAG CCA TAG AGC CCA CC-3′. The primers amplify a region of the plasmid that contains tetracycline regulated promoter elements, the full β1-tubulin coding sequence, and the 3′ untranslated region. DNA from 30, 35, and 40 PCR cycles were mixed to get a broad range of mutant products. PCR amplified DNA was purified using a GenElute PCR clean-up kit (Sigma) and then transfected into 50% confluent CHOtTA cells using Lipofectamine (Invitrogen, Carlsbad, CA). The following day, the cells were trypsinized and reseeded into 100 mm dishes containing medium with no tetracycline and with 250 nM paclitaxel. After 7–10 days, visible colonies were isolated and grown in 24-well dishes in medium containing 250 nM paclitaxel.

Immunofluorescence

Cells grown overnight (16 h) on sterile glass coverslips were rinsed briefly in PBS, pre-extracted 2–3 min on ice with microtubule stabilizing buffer (20 mM Tris-HCl, pH6.8, 1 mM $MgCl_2$, 2 mM EGTA, 0.5% NP-40 and 4 μg/ml paclitaxel), and fixed in methanol at -20°C for at least 20 min. The fixed cells were rehydrated in PBS and then

stained with mouse α-tubulin antibody DM1α (1:100) and/or rabbit HA antibody (1:50, Bethyl Laboratories, Montgomery, TX) for 30–60 min at 37°C in a humid chamber. This was followed by a 1:50 dilution of Alexa 594-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) and/or a 1:50 dilution of Alexa 488-conjugated goat antirabbit IgG containing 1 μg/ml DAPI. The microtubules were observed with an Optiphot microscope and an X60 or X40 objective (Nikon Inc., Melville, NY). Images were captured with a Magnafire digital camera (Optronics, Goleta, CA).

Sequencing Mutant HAβ1-tubulin

Genomic DNA from mutant cell lines was prepared from 80 ~90% confluent cells in 24-well dishes by lysing them in a buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, and 0.5% Tween 20. Proteins were removed by adding 0.1 mg/ml proteinase K at 55°C for 45 min followed by inactivation of the protease at 100°C for 10 min and storage at −20°C overnight. PCR amplification of the integrated HAβ1-tubulin cDNA was carried out using a 5′-UTR forward primer and an HA-tag reverse primer. The PCR-amplified products were sequenced by the Sequencing Core Facility of Baylor College of Medicine using the same primers as well as a third primer complimentary to the sequence preceding the 190th amino acid codon of β1-tubulin.

Site-directed Mutagenesis and Confirmation of Paclitaxel Resistance

Site-directed mutations were introduced into HAβ1-tubulin cDNA cloned in pTOPneo using the QuikChange kit (Agilent Technologies, Santa Clara, CA). Plasmid DNA was isolated from transformed bacteria and transfected into wild-type CHO tTA 6.6a cells as described above. To enrich for transfected cells, 2 mg/ml G418 plus 1 μg/ml tetracycline was added for 8–10 d. The surviving cells were trypsinized and reseeded into duplicate wells of a 6-well dish containing 250 nM paclitaxel with (well 1) and without (well 2) tetracycline. A third well was seeded with 1% of the previous number and grown in media with tetracycline but without G418 to measure the number of viable cells that were plated. After 7–10 days surviving colonies were stained with 0.2% methylene blue as previously described (22) and photographs were taken with a digital camera (Nikon).

Antibodies and Western Blots

HAβ1-tubulin transfected CHOtTA cells were grown in 24 well dishes to 80–100% confluence and lysed in 1% SDS. Proteins were precipitated with 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 7.5% polyacrylamide SDS minigels, and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). The membranes were blocked with 3% dry milk in PBST (PBS containing 0.05% Tween 20) and then incubated for 45–60 min in a 1:2,000 dilution of mouse monoclonal Tub 2.1 (Sigma-Aldrich) against β-tubulin or a 1:2,000 dilution of monoclonal antibody DM1A (Sigma-Aldrich) specific for α-tubulin. Actin antibody C4 (Chemicon International Inc., Temecula, CA) at a 1:2,000 dilution was also added as a loading control. The membranes were washed 3 times in PBST for 10 min and then incubated for 30–45 min in a 1:2,000 dilution of peroxidase conjugated goat antimouse IgG (Invitrogen). After 3 more washes in PBS, SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) was added to detect immunoreactive bands.

Fractionation of Microtubules

Cells were grown overnight to 70–80% confluence in triplicate wells of 24-well dishes containing αMEM but no tetracycline to allow the expression of mutant HAβ1-tubulin. The cells were lysed by scraping them in 100 μl of microtubule stabilizing buffer designed to keep the polymerized microtubules intact (23) and transferring the lysate into 1.5 ml microcentrifuge tubes. The wells were rinsed with another 100 μl microtubule buffer and combined with the lysate. To solubilize any remaining residue, 100 μl of 1% SDS was added to the wells and combined with the pellet fraction from subsequent steps. The lysates were briefly vortexed and centrifuged at 12,000 X g for 15 min at 4^0 C. Supernatants carrying the unpolymerized tubulin were transferred to fresh tubes. The pellets containing the polymerized tubulin were resuspended in 50 μl water for 20–30 min and combined with the residues solubilized in SDS from the corresponding wells. To each sample (supernatant and pellet) 10 μl of a bacterial lysate containing GST-tagged α-tubulin was also added as a quantitative control. Proteins were precipitated using 5 volumes of acetone and resuspended in 100 μl SDS sample buffer. Equal volumes of samples were resolved on 7.5% polyacrylamide SDS minigels and transferred to nitrocellulose membranes. Blots were incubated with a 1:2,000 dilution of antibody DM1A followed by a 1:2,000 dilution of Alexa 647-conjugated goat antimouse IgG (Invitrogen). Immunoreactive bands were visualized and quantified by capturing fluorescence emission with a Storm 850 scanner (Molecular Dynamics). Tubulin in supernatant and pellet fractions was calculated by first obtaining an α-tubulin to GST-α-tubulin ratio and then dividing the ratio from the pellet by the sum

of ratios from the pellet and supernatant to get a percent of tubulin in the microtubule fraction (17).

Measurement of Drug Resistance

Approximately 100–200 cells were seeded into replicate wells of 24 well dishes containing varying concentrations of paclitaxel or colcemid but no tetracycline and the dishes were incubated at 37°C until visible colonies formed (7–10 days). The cells were then stained with 0.05% methylene blue and quantification of cell growth was carried out by solubilizing the stained cells in 1% SDS and measuring the optical density at 630 nm (24).

RESULTS

Identification of New Genomic Mutations in β1-Tubulin

We previously reported the isolation of a large number of CHO cell lines selected for resistance to paclitaxel, many of which had alterations in β-tubulin as determined by two-dimensional gel electrophoresis (25). Initial sequencing of 9 of these mutants identified amino acid substitutions affecting 3 leucine residues (L215, L217, and L228) in the H6/H7 loop region of the protein (21), and a subsequent study identified 2 further mutations affecting L215 (26). This cluster of mutations was unexpected and differed from mutations reported in other laboratories (11,14,27–34). Some of these differences could have resulted from the methods used to isolate mutant cell lines. For example, we use a single step selection protocol whereas most other laboratories use multistep selections involving the exposure of cells to gradually increasing drug concentrations. The advantages and limitations of each methodology have been previously discussed (19). Alternatively, the differences could have resulted from the cell lines that were used or from other unknown factors. To further explore these issues, we sequenced additional CHO mutants to determine whether mutations outside of the H6/H7 region were present at a lower frequency. We also isolated paclitaxel resistant mutants in KB3-1 cells using the same single-step selection procedure to determine if the cell line is a major factor determining which mutations are isolated.

The results are summarized in Table I. Sequencing of additional previously isolated CHO cell lines identified 2 mutations again affecting L215 and L217 in the H6/H7 region but also 2 F270C substitutions in the M-loop of β-tubulin. Similar analysis of newly isolated paclitaxel resistant KB3-1 cells identified yet another mutation affecting L225 in the H6/H7 region as well as more dispersed mutations affecting residues L273 in the M-loop and V365 at the end of the S9-S10 loop near the paclitaxel binding site. The results confirmed that the H6/H7 region is indeed a hot spot for mutations in CHO cells and that mutations in this region can also be found in a different cell line. However, the lower frequency of such mutations in the KB3-1 cells suggested that cellular factors such as the inherent tubulin composition could be affecting the types of mutations that are isolated in single-step selections.

Table I Summary of Amino Acid Substitutions in β1-tubulin[a]

Genomic mutations		Random Mutagenesis	
CHO Cell Line	Mutation	CHO Cell Line	Mutation
Tax 4-19	L215H	Ptx 2-30-5	D26E
Tax 8-1	L217N	Ptx 2-30-6	D39G, F85L
Tax 3-13	F270C	Ptx 2-30-3	V60A
Tax 4-1	F270C	Ptx 2-30-8	V60A, E376G
		Ptx 3-35-4	D177G
		Ptx 1-40-6	D177N
		Ptx 2-35-3	T178A, N416D
		Ptx 5.4	I210T
		Ptx 3-35-7	L217P
		Ptx 1-40-3	S234G
KB3 Cell Line	Mutation	Ptx 1-40-4	V255L
KB 1	L225M	Ptx 3.1	F270C
Ptx 4-2	L273V	Ptx 2-C-2	F270V
Ptx 1-1	V365D	Ptx 1-35-1	F270V
		Ptx 2-35-5	S275C, L395D
		Ptx 1-35-6	N298S
		Ptx 1-30-2	R320C
		Ptx 3-35-14	N348S
		Ptx 2-35-6	T351A
		Ptx 1.1	A364T
		Ptx 2-40-4	F367L
		Ptx 1-35-5	T372A, Y281H
		Ptx 1-30-1	M415V

[a] Paclitaxel resistant mutants were isolated by classical genetics (genomic mutations) or by tranfection of randomly mutagenized cDNA

Random Mutagenesis Reveals a Dispersed Set of Mutations That Confer Paclitaxel Resistance

In an effort to override some of the cellular constraints that might limit the isolation of paclitaxel resistance mutations, we initiated an approach involving random mutagenesis of β1-tubulin cDNA with error-prone Taq polymerase followed by transfection into wild-type CHO cells and a single-step selection for paclitaxel resistance. The

transfected β1-tubulin cDNA encoded a C-terminal HA-tag to distinguish the protein from endogenous tubulin, and its transcription was regulated by tetracycline. Using this approach, we isolated 23 cell lines all of which produced HAβ1-tubulin as determined by immunofluorescence microscopy. HAβ1-tubulin selectively amplified from each of the resistant cell lines using primers to the 5′ UTR and HA-tag was then sequenced to reveal amino acid substitutions that were distributed throughout β1-tubulin (Table I). Most of the cell lines had single mutations in β1-tubulin, but 5 of them had 2 mutations. One cell line (not shown) had 4 mutations and was not further analyzed. The wide distribution of mutations confirmed that the random mutagenesis approach was successful in removing many of the constraints that normally limit the spectrum of genomic mutations able to cause drug resistance and provided a rapid and direct approach for mutant isolation.

Paclitaxel Resistance is Tetracycline Regulated

The isolation of resistant cell lines that express HAβ1-tubulin implies that the mutations identified in the cDNA are responsible for resistance, but it is also possible to argue that these random mutations are irrelevant and that mutations in genomic tubulin genes or other changes are actually the cause of drug resistance. As an initial step to rule out this possibility, the cell lines created by random mutagenesis were tested for paclitaxel resistance in the presence or absence of tetracycline to repress or allow the expression of mutant HAβ1-tubulin. We found that some cell lines were clearly resistant to the drug only in the absence of tetracycline, but others were resistant regardless of whether the antibiotic was present or not (Fig. 1). This was not surprising because our past experience has shown that expression of cDNAs among randomly isolated clones is not always well regulated by tetracycline. The inability of tetracycline to suppress expression in some clones could be due to mutations in the regulatory sequences, disruption of those sequences during integration into the genome, or the integration of the cDNA into regions of the genome with high endogenous promoter activity. Despite these ambiguities, the results clearly showed that the transfected HAβ1-tubulin cDNA was responsible for drug resistance in at least some of the mutant cell lines.

Mutant Confirmation by Site-directed Mutagenesis

The presence of two mutated HAβ1-tubulins in some of the cell lines left open the question of whether either or both of the mutations were needed to confer drug resistance. To address this issue, each of the mutations was recreated in HAβ1-tubulin cDNA by site-directed mutagenesis and retransfected into wild-type CHO cells. This approach was also used on some of the single mutations chosen at random to ensure that they were indeed capable of conferring drug resistance and to create stable well-regulated cell lines for further studies.

Paclitaxel resistance in these cell lines was again tested in the presence and absence of tetracycline (Fig. 2). In all clones chosen for further study, the mutation created by site-directed mutagenesis was able to confer resistance to paclitaxel and its ability to do so was tetracycline regulated.

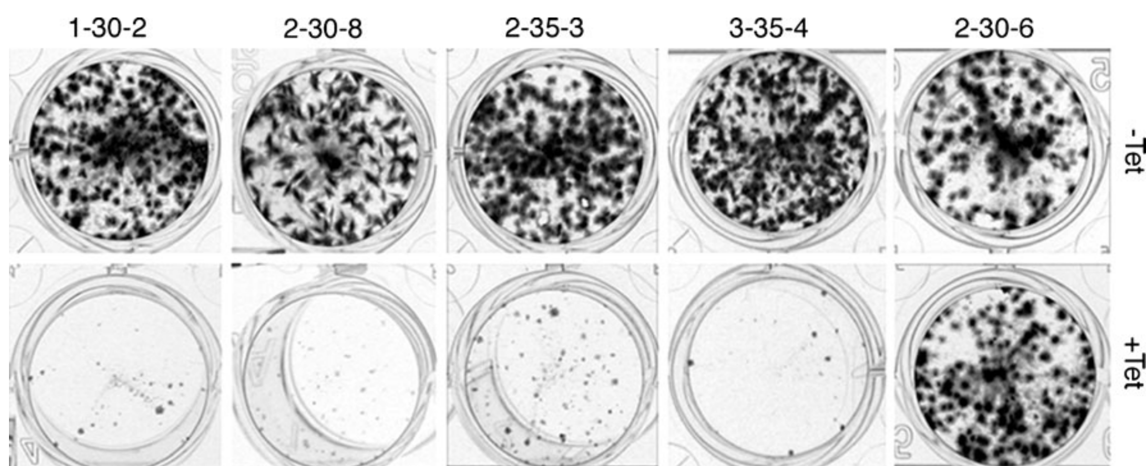

Fig. 1 The effect of ectopic tubulin expression on paclitaxel resistance. Cell lines selected for paclitaxel resistance in the absence of tetracycline (tet) were retested by plating 100–200 cells into 24-well dishes containing a toxic concentration (250 nM) of paclitaxel in the presence (inhibitory for expression) or absence (permissive for mutant tubulin expression) of tetracycline. The cells were incubated for 7–10 days and surviving colonies were stained with methylene blue. Note that many cell lines exhibited resistance to paclitaxel in the absence, but not the presence of tet. Some cell lines such as 2-30-6, however, were resistant regardless of whether tet was present or absent.

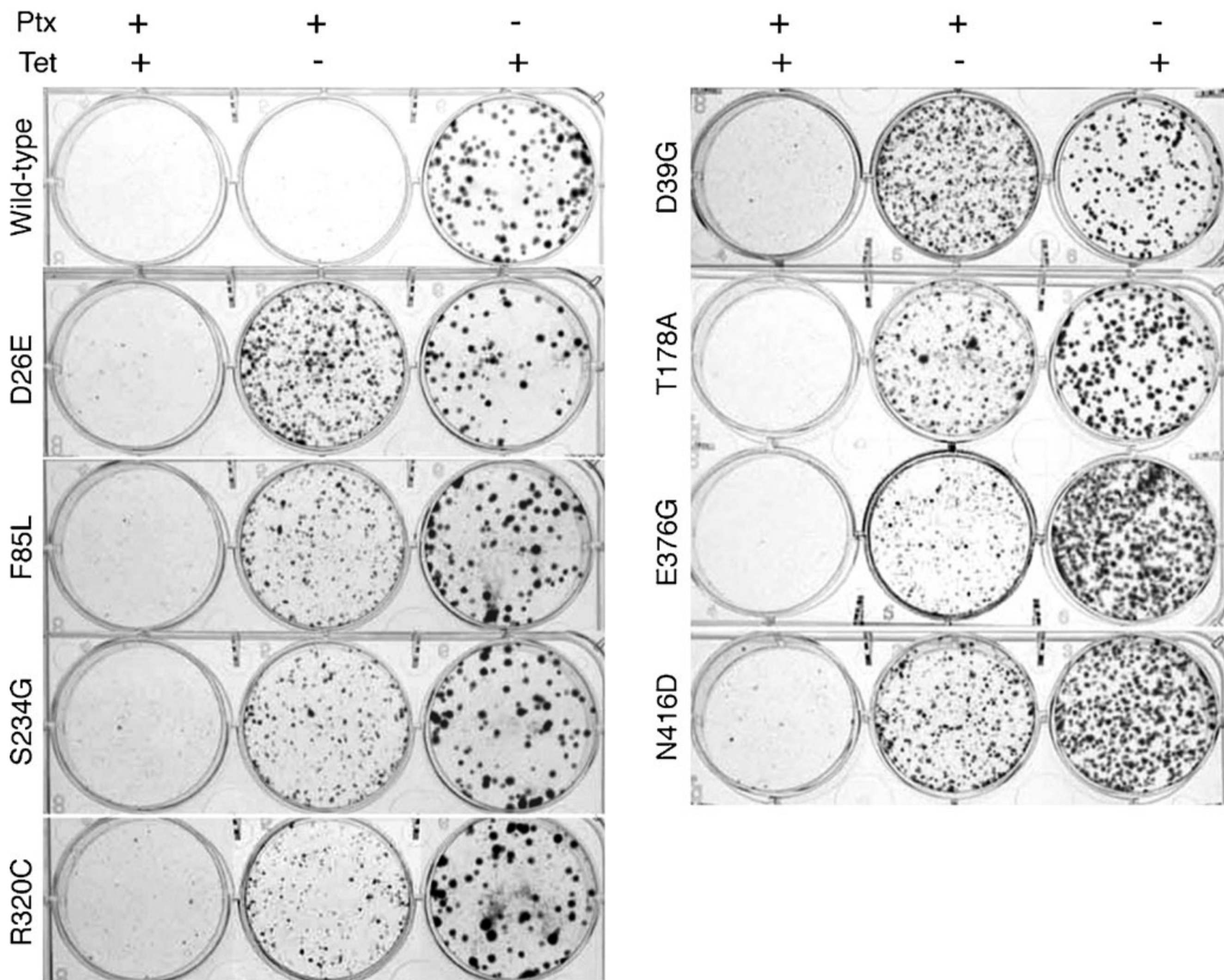

Fig. 2 Confirmation of drug resistance by site-directed mutagenesis. Mutations identified in paclitaxel resistant cell lines were recreated in HAβ1-tubulin cDNA by site-directed mutagenesis and stably transfected G418 resistant cells were seeded into replicate wells of 6-well dishes containing 250 nM paclitaxel and/or 1 μg/ml tetracycline. The plates were incubated for 7–10 days and the surviving colonies were stained with methylene blue. Note that 100-fold more cells were added to the wells that contained paclitaxel to ensure that enough transfected cells with the appropriate level of mutant tubulin expression would be present to survive the selection. The mutations tested are indicated next to the corresponding wells.

There was some variability in the growth and number of colonies in paclitaxel likely due to differences in the potency of the mutation and its level of expression in the various cell lines but the results nonetheless showed that all the tested mutations could confer drug resistance in a tetracycline regulated manner.

As a further test for the ability of the mutations to confer drug resistance we used a previously described strategy (35). We transfected small amounts of mutant HAβ1-tubulin cDNA so that most of the G418 resistant cells would have low to undetectable levels of mutant tubulin, and we then exposed the population to paclitaxel selection to ask whether cells with higher expression of mutant HAβ1-tubulin would have a survival advantage. The results (Fig. 3) showed that paclitaxel only allowed the survival of cells from the G418 resistant population that had highly elevated levels of HAβ1-tubulin. The ability of paclitaxel to kill most of the cells but not those that had elevated amounts of mutant HAβ1-tubulin strongly argues that the mutant cDNA was responsible for paclitaxel resistance. Thus, for the original cell lines that had two mutations in HAβ1-tubulin, we conclude that each of the mutations was individually capable of conferring paclitaxel resistance and that the single mutations in other cell lines were responsible for drug resistance as well.

Mutations That Confer Paclitaxel Resistance Disrupt Microtubule Assembly

Our previously isolated paclitaxel resistant cell lines with mutations in the H6/H7 region were shown to have reduced

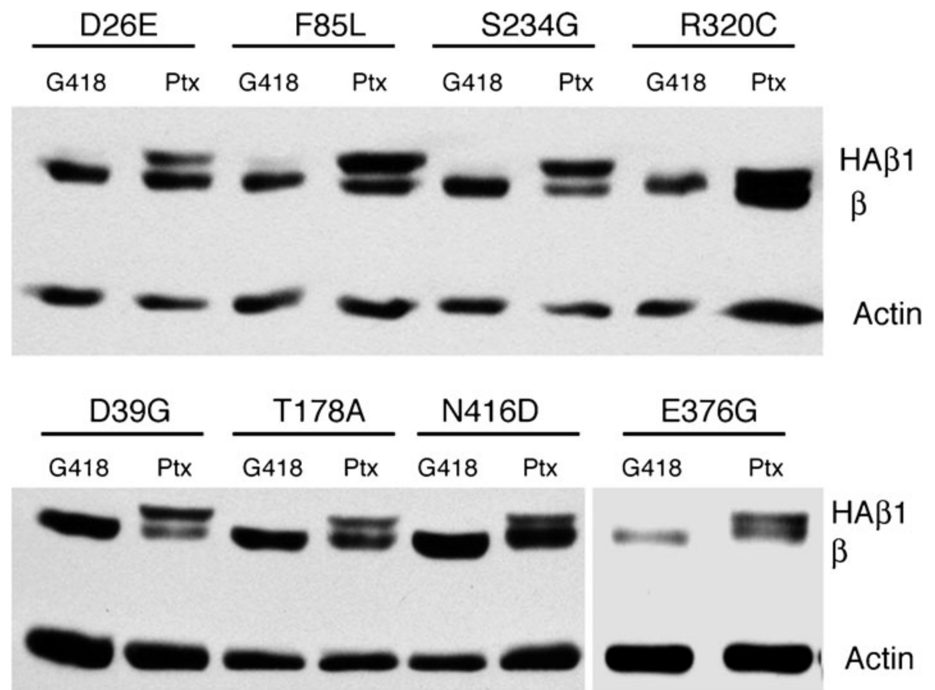

Fig. 3 Enrichment of mutant tubulin in paclitaxel selected cells. G418 resistant cells transfected with the indicated mutant HAβ1-tubulin cDNAs were reselected in 250 nM paclitaxel. Extracts of cells grown 24 h in the absence of tetracycline before and after selection in paclitaxel were compared by western blot analysis using an antibody to β-tubulin. Note the enrichment of the slower migrating mutant HAβ1-tubulin in the paclitaxel resistant cells. The faster migrating band (β) represents the endogenous isotypes of β-tubulin. An actin antibody was used as a gel loading control.

microtubule polymer consistent with a mechanism in which mutant tubulin incorporation destabilized the microtubule network (21). Subsequent mutant isolation in other laboratories using other cell lines and procedures confirmed this as common mechanism of drug resistance (18,36). To determine whether the mutations identified by our random mutagenesis approach were causing resistance by a similar mechanism, we lysed stably transfected paclitaxel resistant cell lines under conditions that preserved the intact microtubules and measured the percentage of total tubulin in the microtubule fraction as previously described (23,37). The results showed that wild-type CHO cells have approximately 40% of their tubulin assembled into microtubule polymer but that each of the paclitaxel resistant cell lines have significantly less microtubule polymer (Fig. 4). The findings are consistent with our previous studies showing equal partitioning of both wild-type and mutant subunits into the microtubule polymer, near normal tubulin levels in transfected cells, but less total microtubule assembly in cells selected for resistance to paclitaxel (38–42). Thus, mutations scattered throughout the HAβ1-tubulin sequence appear to confer paclitaxel resistance through a common mechanism that involves destabilization of the microtubule cytoskeleton.

Microtubule Organization and Cell Division are Disrupted

To determine whether the mutations that diminished the amount of polymer also produced visible changes in microtubule organization, the transfected cells were viewed by immunofluorescence after an overnight incubation without tetracycline to allow mutant HAβ1 expression (Fig. 5). Cells transfected with wild-type HAβ1-tubulin were mostly uniform in size with abundant microtubules and a single symmetrical nucleus. In contrast, many of the cells transfected with mutant HAβ1 were much more heterogeneous in size (e.g. F85L) and frequently displayed a reduced number of microtubules (e.g. D26E, D39G, and T178A) as well as bi- or multi-nucleated clusters of DNA (e.g. D26E, D39G, F85L, T178A, and E376G). There was variability in the severity of the phenotype from mutant to mutant that was likely caused by differences in the potency of the mutation and the level of the mutant tubulin accumulated during the short 16 h period. Although Fig. 5 was obtained using antibodies to the HA-tag, it should be noted that indistinguishable images were obtained using antibodies that recognize all the cellular tubulin as we previously reported for other transfected cells (41,43).

Similar changes in microtubule organization and cellular morphology are commonly seen in CHO and other cell lines that are treated with agents that interfere with spindle assembly and therefore fail to properly segregate their chromosomes (44–46). The cells are nonetheless able to exit mitosis without dividing to become large multinucleated cells. To quantify these effects, transfected cells were incubated for 3 days with and without tetracycline and the percentage of HAβ1 expressing cells with nuclear defects was determined (Fig. 6). The extent of multinucleation was low for cells transfected with wild-type HAβ1, but all of the mutant HAβ1 transfected cell lines had an elevated percentage of multinucleated cells demonstrating that all of the mutations interfered with cell division to at least some degree.

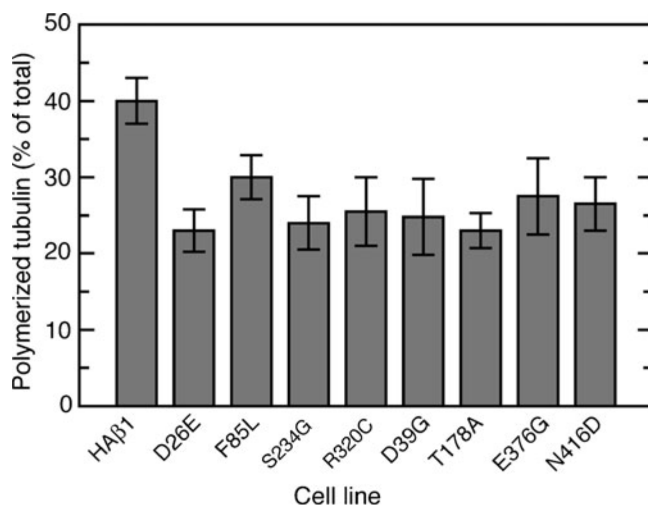

Fig. 4 Polymerized tubulin in mutant cell lines. Stably transfected cell lines expressing wild-type HAβ1-tubulin or each of the indicated mutant tubulins were grown 24 h without tetracycline and lysed in a microtubule stabilizing buffer. Polymerized microtubules were separated from free tubulin subunits by centrifugation and each fraction was analyzed by western blots using an antibody to α-tubulin. The percentage of total cellular tubulin recovered in the microtubule pellet was then calculated. An average of 3 independent experiments run in triplicate is shown for each cell line. Standard deviations are also plotted. $p < 0.05$ by the student t-test.

The changes in microtubule and nuclear morphology caused by expression of mutant tubulin were prevented by maintaining the cells in paclitaxel. As shown in supplementary Fig. S1, paclitaxel caused bundling of microtubules in wild-type CHO cells and prevented normal chromosome segregation and cell division as indicated by the presence of large and fragmented nuclei (Fig. S1B). In contrast, the disrupted morphology of cells transfected with HAβ1 mutant F85L (Fig. S1C) was prevented when the cells were maintained in paclitaxel (Fig. S1D). For a less severe mutation such as R320C, the morphological change due to mutant tubulin production was more subtle (Fig. S1E), and the addition of paclitaxel caused a slight but detectable bundling of the microtubules (Fig. S1F). The results indicate that paclitaxel is able to counteract the effects of the tubulin mutations on microtubule assembly.

Paclitaxel Resistant Mutants Have Increased Sensitivity to Colcemid

Paclitaxel and colcemid have opposing effects on microtubule assembly. Because the tubulin mutations found in the paclitaxel resistant cell lines described here were shown to have a destabilizing effect on the microtubules, it was reasonable to predict that the cells should be more sensitive to an agent such as colcemid that is known to inhibit microtubule assembly. To test this prediction, we carried out a long term growth assay in varying concentrations of drug and the results from four randomly chosen mutant cell lines are shown in Fig. 7. All four mutant cell lines were able to survive at 3–6 fold higher concentrations of paclitaxel compared to the wild-type cells (Fig. 7a). In addition, cell lines D26E and S234G grew better in the presence than in the absence of paclitaxel, demonstrating that these cells are partially drug dependent, a phenotype that has previously been described (25,47,48). By contrast, all four mutants were more sensitive to colcemid (Fig. 7b) and generally fit a pattern in which the cells that were most resistant to paclitaxel were also most sensitive to colcemid. These results are consistent with the biochemical and morphological changes seen in the mutant cell lines and further support a model in which tubulin mutations act by affecting microtubule assembly in a direction that opposes the action of the selecting drug (19,49).

DISCUSSION

Transfection of randomly mutagenized β-tubulin cDNA followed by selection for drug resistance and selective amplification of the transfected cDNA was shown to provide a rapid and efficient approach for identifying mutations that alter drug sensitivity. Here we reported the identification of 28 β1-tubulin mutations in paclitaxel selected cell lines and demonstrated that the mutations were indeed necessary for resistance using multiple independent approaches. The mutations were shown to cause microtubule disruption leading to lower polymer levels and increased sensitivity to colcemid, a drug that is known to also disrupt microtubule assembly. The mutations thus fit a pattern shared by previously isolated mutant cell lines and further support a resistance mechanism based on microtubule assembly and stability (19,49). According to this mechanism, mutations that destabilize microtubules cause resistance to agents that stabilize microtubules and increase the sensitivity to agents that destabilize the polymer. Conversely, mutations that stabilize microtubules confer resistance to agents that destabilize microtubules but increase sensitivity to drugs that stabilize them. Recent studies have indicated that the underlying molecular basis for this pattern involves changes in the stability of microtubule attachment to centrosomes. Paclitaxel and similar drugs stabilize those attachments whereas colcemid, vinblastine, and other such drugs destabilize the attachments (24,50,51).

In addition to being quick and efficient, the random mutagenesis approach also has the advantage of bypassing many of the constraints that limit which mutations may be recovered in a particular cell line. For example, mutations causing drug resistance may be influenced by the tubulin composition of the cell. If a mutation occurs in a low abundance isotype, the mutation will need to be highly disruptive in order to produce an effect on the microtubules.

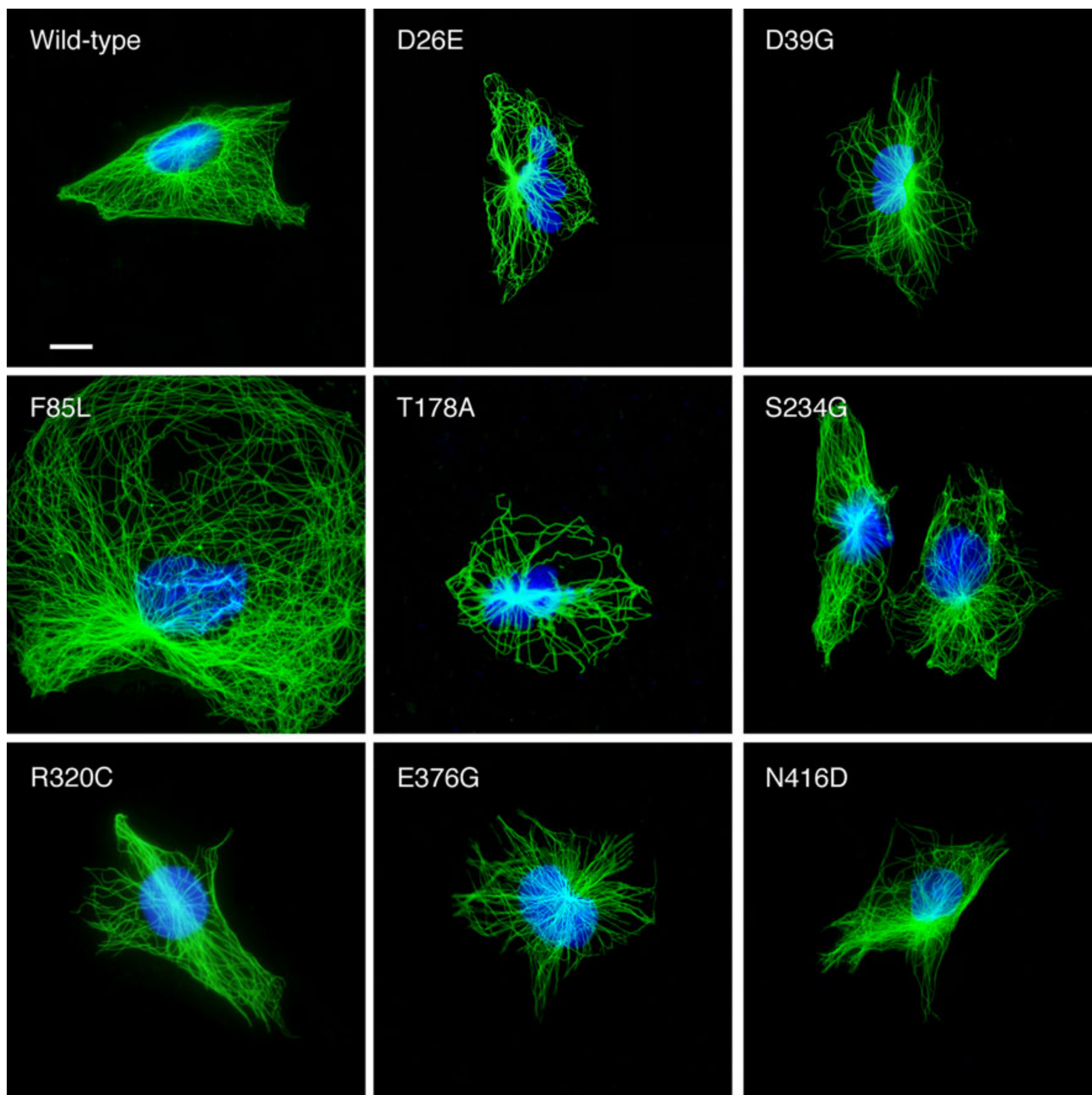

Fig. 5 Microtubule organization in mutant cells. CHO cells transfected with wild-type or the indicated mutant HAβ1-tubulin cDNAs were grown 16 h without tetracycline, fixed in methanol, and stained with an antibody specific for the HA tag as well as with DAPI to label the DNA. Bar=15 μm.

If, on the other hand, the mutation occurs in a high abundance isotype, only much weaker mutations will survive the selection because the more disruptive mutations will cause lethality. The inherent variability of ectopic gene expression in transfected cells works to the advantage of the random mutagenesis procedure by providing a heterogeneous mixture of cells with different levels of mutant protein, and at least some of these cells will have appropriate mutant tubulin levels to survive drug selection. A further advantage of this procedure is that it circumvents interference from genomic mutations in MDR1, α-tubulin, other isoforms of β-tubulin, or microtubule interacting proteins that could also cause drug resistance. Finally, the mutant DNA can be rapidly amplified by PCR and sequenced, and it is possible to rapidly determine whether the transfected cDNA is responsible for drug resistance by testing for growth in the presence or absence of tetracycline.

Unlike previously reported CHO genomic mutations for paclitaxel resistance that were clustered around the paclitaxel binding pocket (21), the newly identified mutations were scattered throughout the β-tubulin sequence (Fig. 8). Nonetheless, these new mutations caused phenotypes similar to the previous genomic mutations and appeared to cause resistance by a similar mechanism of weakening the stability of the microtubule lattice. Consistent with this view, the mutations were largely confined to regions that may be involved in subunit-subunit interactions (10). The reasons for the clustering of the genomic mutations around the paclitaxel binding site are uncertain but appear to be unrelated to drug binding (see (18,19) for discussion). More

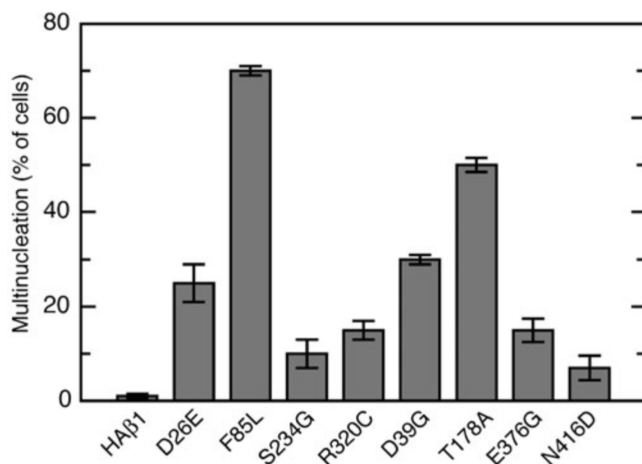

Fig. 6 Mutant tubulin causes defects in cell division. The nuclei of cells stained as described in the legend to Fig. 5 were scored as multinucleated if they were > 2 times the normal size, multilobed, or fragmented. The percentage of such cells among all the cells that express the transfected HAβ1-tubulin are plotted. Averages and standard deviations from 3 experiments are shown.

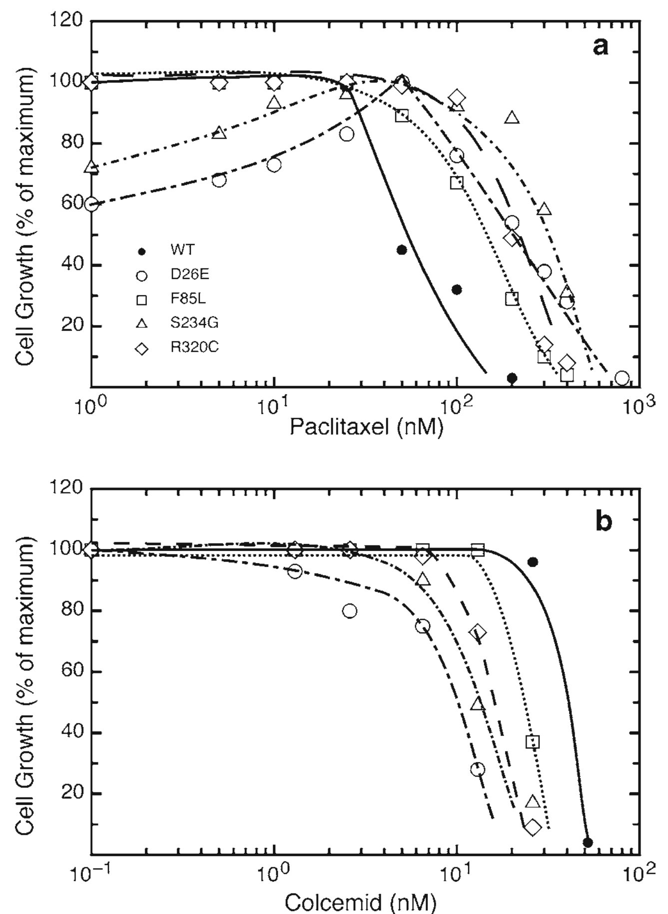

Fig. 7 Drug resistance of mutant cell lines. Cloned cell lines expressing wild-type (WT) or the indicated mutant HAβ1-tubulin cDNAs were incubated in varying concentrations of paclitaxel (**a**) or colcemid (**b**) until visible colonies formed (7–10 days). Growth was determined by staining the cells with methylene blue and quantifying the bound dye by spectroscopic analysis. Cell growth was then normalized to the maximum growth seen at any drug concentration (usually the zero drug concentration) set at 100%. Note that for two of the mutants (D26E and S234G) the maximum growth occurred in the presence of drug (panel a) indicating that these cells are partially paclitaxel dependent for growth.

likely, it may indicate that this region is especially important for controlling the assembly of microtubules because of direct participation in critical subunit interactions, or because it is central to conformational changes associated with assembly. In support of the latter explanation, the H6/H7 loop and the H7 helix in which the genomic mutations were clustered, undergo significant changes when tubulin switches from a curved (assembly unfavorable) to a straight (assembly favorable) conformation (7).

The results of this study provided some unexpected new insights into mutations that confer resistance to microtubule targeted drugs. A review of the literature revealed that roughly half of the mutations identified in the current study were previously identified in our own and in other laboratories, thus giving physiological relevance to the mutations identified by the random mutagenesis procedure (Table II). Given the relatively small number of mutations that have been reported to date, this overlap was surprising and suggests that the spectrum of mutations able to confer resistance is more limited than one might predict based on the idea that any mutation that disrupts microtubules can cause paclitaxel resistance. Alternatively, it could indicate that many mutations can cause resistance, but a smaller subset occur more frequently because of their ability to disrupt assembly sufficiently to cause resistance yet not compromise the functionality of the microtubules. This latter alternative is consistent with recent studies indicating that specific changes in the stability of microtubule attachment to centrosomes rather than more generalized changes in assembly are associated with drug action and resistance (24,50). Also of interest is the observation that 3 loci (S234, A248, and T372) can be mutated to cause resistance to paclitaxel or to colcemid. This finding reinforces a previous saturation mutagenesis study showing that P220L and P220V mutations of β1-tubulin could confer paclitaxel resistance whereas P220C, P220S, and P220T mutations conferred colcemid resistance (52). Thus, the nature of the amino acid substitution at a given locus can either stabilize or destabilize microtubules and thereby confer resistance to destabilizing or stabilizing drugs respectively.

Most unexpected was the finding that several of the mutations found to confer drug resistance have also been reported in patients with a variety of neuronal disorders (53). The mutations in these patients occurred in the β2 and β3 isotypes rather than in the β1 isotype that is most commonly mutated in drug resistance studies. This difference can probably be attributed to the fact that β1 is the most abundant isotype in most cell lines, but it is absent or

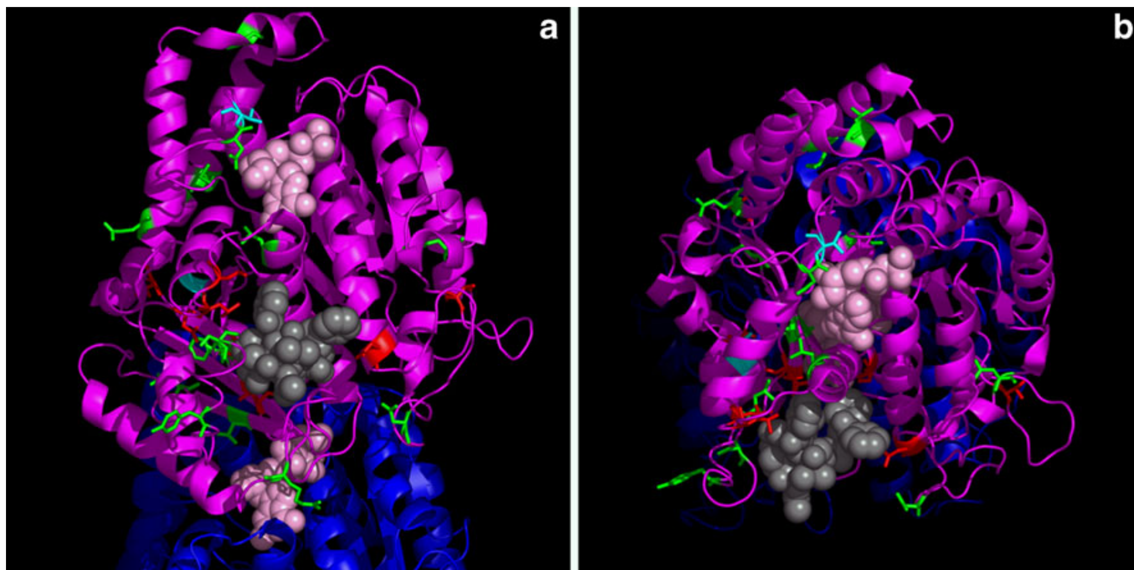

Fig. 8 Location of paclitaxel resistance mutations. The new unique mutations identified in this study are shown as green stick figures in the αβ tubulin heterodimer; mutations confirming previously reported mutations are shown in red; mutations common with those found in patients with neuronal defects are shown in light blue. The α-subunit is shown in dark blue; the β-subunit in magenta. Pink spheres, guanine nucleotides; grey spheres, paclitaxel. (**a**) Lateral view from inside the microtubule lumen. (**b**) An axial view. The figure was drawn using MacPymol (55) and atomic coordinates 1JFF (56).

present in very low abundance in neurons. Instead, neuronal cells express high levels of the β2 and β3 isotypes (1,54). Although it has yet to be tested experimentally, it is likely that the mutations in these other isotypes produce similar effects on microtubule assembly as they do when present in the β1 isotype. The observation that mutations able to stabilize (e.g. colcemid resistance mutations) or destabilize (e.g., paclitaxel resistance mutations) microtubules can be involved in neuronal defects suggests that balanced microtubule assembly is equally critical to neuronal cell

Table II Mutations Found in Multiple Studies

This study[a]	Other PtxR[b]	CmdR[c]	Neuronal[d]	References
D26E	D26E			(30)
V60A	V60A,F			(34,57)
		S172A	β2 S172P	(58,59)
T178A			β3 T178M	(60)
		D197N		(61,62)
I210T			β2 I210T	(58)
L215H	L215A,E,F,H,M,P,R			(21,26)
L217N,P	L217R			(21)
	L228F,H		β2 L228P	(21,58)
S234G		S234N		(63)
	A248V	A248T		(17,62)
F270C,V	F270V,I			(28,29)
	R282Q			(27,32)
	Q292Q,H			(31,57)
	A296T,S			(11,14)
N298S	N298S			(32)
	R306C,H			(11,14,17)
		K350M,E,N		(61–65)
A364T	A364T			(28)
V365D	V365S			(66)
T372A		T372S		(67)

[a]β-Tubulin paclitaxel resistance mutations from the present study that were previously reported elsewhere

[b]Includes resistance to other microtubule stabilizing drugs such as docetaxel, epothilone B, patupilone, and peluroside

[c]Includes resistance to other microtubule destabilizing drugs such as colchicine, vinblastine, hemiasterlin, 2-methoxyestradiol, indanocine, and BPR0L075

[d]Mutations in brain-specific β-tubulin isotypes from patients with neurological disorders

differentiation and function as it is to cell division in non-neuronal cells.

CONCLUSION

A random mutagenesis approach provides a rapid and convenient method for generating drug resistance mutations. The results using this approach have significantly expanded the number of known mutations in β-tubulin that confer paclitaxel resistance and have revealed considerable overlap with previously isolated mutations leading to the possibility that a relatively limited set of mutations may be involved in causing resistance. The observation that some of the drug resistance mutations have also been observed in patients with neuronal abnormalities indicates that similar changes in microtubule assembly underlie both drug resistance and defects in brain development.

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