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Report

Resistance to Microtubule-Stabilizing Drugs Involves Two Events

β-Tubulin Mutation in One Allele Followed by Loss of the Second Allele

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KEY WORDS

microtubules, β-tubulin, LOH, drug resistance, 6p25, epothilones, taxanes, FISH, SNP

ABBREVIATIONS

BAC	bacteria artifical chromosome
Epo	epothilone
FACS	fluoresecent activated cell sorter
FISH	fluoresecent in situ hybridization
IC50	inhibitory concentration 50%
LOH	loss of heterozygosity
MDR	multiple drug resistance
nM	nanomolar
PCR	polymerase chain reaction
Pgp	P-glycoprotein
SRP	sulforhodamine B
SNP	single nucleotide polymorphism
Ta	annealing temperature
wt	wild type

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ABSTRACT

Resistance to Taxol (paclitaxel) or the epothilones (Epo) occurs via the acquisition of point mutations in β-tubulin residues important for drug-tubulin binding. We have isolated four drug-resistant clones selected with Taxol or Epo A, which harbor distinct β-tubulin mutations. During the development of a stable drug-resistant phenotype, early clones expressing both wild-type (wt) and mutant β-tubulin sequences exhibited a 10-fold drug resistance, while more advanced clones expressing only the mutant β-tubulin sequence exhibited 30 to 50-fold drug resistance. The drug-sensitive parental 1A9 ovarian carcinoma cell line and the drug resistant clones (1A9-A8, 1A9-PTX10 and 1A9-PTX22) were evaluated for loss of heterozygosity (LOH) for β-tubulin (6p25) by single nucleotide polymorphism (SNP) and fluorescent in situ hybridization (FISH) analyses. Functional assays such as drug-induced tubulin polymerization, cell cycle analysis by FACS, DNA sequencing for β-tubulin and mitotic index by immunofluorescence were performed to correlate the β-tubulin LOH status with drug response in the early- and late-step drug-resistant clones. Late-step drug resistant clones revealed LOH in one allele for wt β-tubulin in addition to a β-tubulin mutation in the other allele leading to increased levels of drug resistance, while the early-step clones that contained both a wt and a mutant β-tubulin allele were considerably less drug resistant. The LOH and functional assays revealed cell response that was proportional to the tubulin gene and heterozygosity status. Acquired tubulin mutations in conjunction with LOH for the wt tubulin resulted in a highly resistant phenotype, revealing a new mechanism for taxane resistance.

INTRODUCTION

Microtubules are major dynamic structural components in cells. They are important for development and maintenance of cell shape, cell division, cell signaling, and cell movement. Microtubules are cytoskeletal polymers built by the self-association of α and β-tubulin dimers, existing in a constant dynamic equilibrium between their polymerized microtubule form and soluble α and β-tubulin dimer forms. Drugs that target tubulin or microtubules are one of the most effective classes of anticancer agents. These drugs bind to different sites on the tubulin dimer and within the microtubule, exerting varying effects on microtubule dynamics. However, they all block cells in mitosis at the metaphase/anaphase transition and induce cell death.[1] Among all the microtubule-targeting drugs, the taxanes, are arguably the most effective anticancer agents used to date in clinical oncology due to their remarkable activity in a broad range of malignancies.

Despite Taxol's clinical success, the emergence of drug-resistant tumor cells limits Taxol's ability to cure disease. Several mechanisms of resistance to Taxol have been described. With the exception of P-glycoprotein (Pgp)-mediated multi-drug resistance (MDR) [2,3], all these mechanisms involve alterations in tubulin. Such alterations include: (1) altered expression of β-tubulin isotypes in Taxol-resistant cells and Taxol-resistant ovarian tumors;[4-6] (2) increased microtubule dynamics in Taxol-resistant cancer cells,[7] and importantly, (3) the presence of β-tubulin mutations in Taxol-resistant cells.[8,9]

The epothilones are novel microtubule-stabilizing natural products of soil bacteria origin, that compete with Taxol for the same binding site on β-tubulin but maintain activity against Pgp-expressing MDR cells.[10,11] In an effort to better understand how the epothilones interact with microtubules and in order to ascertain the mechanism of resistance cancer cells may develop towards this new class of agents, we have isolated two epothilone-resistant human ovarian cancer cells, namely the 1A9-A8 and 1A9-B10 cells, that were selected with epothilone A and B respectively.[12] These epothilone-resistant sublines exhibit impaired epothilone-and Taxol-driven tubulin polymerization, caused by the following

acquired β-tubulin mutations in each clone: β274 (Thr → Ile) in 1A9-A8 cells and β282 (Arg → Gln) in 1A9-B10.[12] Interestingly, these mutations are located at the Taxol-binding site in the atomic model of αβ-tubulin, thus providing a clear explanation of the drug resistance mechanism.[13]

It has now become evident that acquired tubulin mutations represent the main mechanism by which cancer cells become resistant to drugs that target microtubules.[6,8,9,12,14-16] However, the temporal sequence of the molecular events that occur during the development of drug resistance to microtubule-targeting drugs is not known.

In this paper we show that during the development of drug resistance to both taxanes and epothilones, the first genetic event is acquisition of a β-tubulin point mutation at the drug's binding site in one of the two β-tubulin alleles, followed by a second genetic event involving the loss of the other (wild-type) β-tubulin allele, which occurs only after prolonged exposure to the selecting agent. This loss of heterozygosity for β-tubulin gene M40 in the presence of the protective mutant β-tubulin allele then confers still higher levels of both epothilone and Taxol resistance to these human ovarian cancer cells. Thus, loss of heterozygosity in the β-tubulin gene appears to be integral to the development of the highest level of resistance by cancer cells to these anticancer drugs.

Table 1 **Primers used for PCR amplification and sequencing of M40 β-tubulin**

Position	Sequence	Orientation	Use
M40-250	CTCCGCAAGTTGGCAGTCAAC	Forward	PCR-T_a 58°C
M40-340	GGGGATCCATTCCACAAAGTA	Reverse	
M40-253	TTGGCAGTCAACATGGTCC	Forward	Sequencing
M40-324	CGTTAAGCATCTGCTCATCGACCTCC	Reverse	

List of primers used to amplify by PCR and sequence the area around amino acid 274 of the β-tubulin M40 gene. T_a: annealing temperature.

MATERIALS AND METHODS

Cell lines, antibodies and drugs. The epothilone A resistant cell line, 1A9-A8, was selected from the human ovarian carcinoma 1A9 cells as previously described.[12] The 1A9-A8^E clone (expressing both WT and mutant alleles) was an intermediate isolate in the selection process of 1A9-A8 (mutant allele only) cells. These cells were cultured in RPMI 1640 medium (Cellgro) supplemented with 10% fetal bovine serum (Gibco BRL) and 1% Penicillin-Streptomycin (Cellgro), and grown as monolayers at 37°C in a 5% CO_2 tissue culture incubator. The mouse monoclonal anti-α-tubulin (DM1α) antibody used is from Sigma. Both epothilones A and B were a generous gift from the laboratory of K.C. Nicolaou (The Scripps Research Institute, La Jolla, CA). Paclitaxel was purchased from Sigma and vincristine from Eli-Lilly.

Drug sensitivity assay. Cytotoxicity assays using the protein-staining sulforhodamine B (SRB) method were performed in 96-well plates, as described previously.[8]

Tubulin polymerization assay. Quantitation of the degree of in vivo tubulin polymerization in response to Epothilone A was performed as previously described.[8] Briefly, cells were plated in 24-well plates. The following day, they were exposed to increasing concentrations of Epothilone A for a period of 6 h. Cells were then lysed in a hypotonic buffer [1 mM $MgCl_2$, 2 mM EGTA, 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 6.8 containing protease inhibitors (Boehringer-Mahneim)]. The lysed cells were incubated for 5 min at 37°C, and cytoskeletal and cytosolic fractions (containing polymerized (p) and soluble (s) tubulin, respectively) were separated by centrifugation. Equal loading of the fractions was resolved by electrophoresis through 10% SDS polyacrylamide gels, and immunoblotted with an antibody against α-tubulin.

β-tubulin sequencing. Total cellular RNA was isolated with the RNeasy Mini Kit (Qiagen) and the M40 β-tubulin isotype was amplified by RT-PCR using One-Step RT-PCR (Qiagen). Genomic DNA was isolated using the QIAamp DNA Mini Kit (Qiagen). For PCR amplification and sequencing of the M40 β-tubulin isotype, four overlapping sets of primers were used, as summarized in Table 1. The primers were designed to be specific for M40, using GenBank™ accession numbers AP000512 for genomic DNA and AF070600 for cDNA. PCR products were purified using the PCR Purification Kit (Qiagen) and then sent to the sequence core lab of the University of Michigan for DNA sequence analysis.

Cell cycle analysis. Cells were plated in 6-well plates. The following day, they were treated with various concentrations of epothilone A and vincristine for 18 hours. Following treatment, both adherent and floating cells were harvested and pelleted by centrifugation. Cell pellets were suspended in 1 ml of 0.1 mg/ml propidium iodide containing 0.6% NP40 (ICN Pharmaceuticals, Costa Mesa, CA) with 1 mg/ml RNase A (Sigma Chemical Co), then incubated in the dark at room temperature for 30 min. Data acquisition and analysis were performed on a FACScan instrument equipped with CellQuest software (Becton Dickinson Immunocytometry Systems). Cell cycle analysis was performed with Flowjo (Treestar). All cell cycle experiments were performed at least three times.

Mitotic index analysis. Cells were plated on glass coverslips and treated with drugs for 24 hrs. Cells were fixed with ice-cold methanol and DNA was stained with Sytox Green (Molecular Probes, Oregon). Epifluorescence microscopy was used to count a minimum of 500 cells per drug treatment and mitotic figures were scored.

Loss of heterozygosity analysis. Loss of heterozygosity for M40 β-tubulin was examined using PCR primers that amplify single nucleotide polymorphism markers (selected from the human SNP database) around the β-tubulin M40 gene (TUBB) location. A total of 45 SNPs were tested. The PCR products were purified using the PCR Purification Kit (Qiagen) and then sequenced (Sequencing Core, University of Michigan, Ann Arbor, MI) to determine if heterozygosity was present. Each PCR reaction was performed at least twice.

Fluorescence in situ hybridization (FISH) Analysis. The three cell lines (1A9, 1A9-A8 and 1A9-A8^E) were induced to be in metaphase by treatment with 0.1 μg/ml colcemid (KaryoMax, Life Technologies) for four hours at 37°C. These metaphase cell preparations were harvested and fixed in a 3:1 solution of methanol/acetic acid. One or 2 drops of this cell suspension were added onto each slide and allowed to air-dry. The BAC clone RP11-506k6 (β-tubulin, 6p25: from the RCPI-11 Human BAC Library of the Children's Hospital Oakland Reach Institute BACPAC resources) was labeled by nick translation with digoxigenin-12-dUTP (spectrum-orange, Vysis, Downers Grove, IL). Hybridization and immunodetection were performed following the manufacturer's recommendation. For the detection of chromosome 6 we used a green chromosome 6 centromeric probe (Vysis, Downers Grove, IL) Chromosomes were counterstained with Sytox Blue (Molecular Probes) and analyzed by laser scanning confocal microscopy (Zeiss LSM510 axioplasm laser scanning Confocal microscope) using a Zeiss X100 1.3 oil-immersion objective. More than 20 metaphases from each cell line were analyzed.

RESULTS

Description of drug resistance model. In order to understand the molecular events that occur during the development of drug resistance, as well as the adaptive temporal stages in the development of a stable resistance phenotype, we used a model of epothilone resistance that was previously created in our laboratory.[12] This model consists of a pair of cell lines: the parental, drug-sensitive human ovarian carcinoma cell line, 1A9, and the epothilone A-resistant clone, namely 1A9-A8. Previous characterization of 1A9-A8 cells revealed that the epothilone-resistant phenotype is due to an acquired β-tubulin mutation at residue β274 (Thr to Ala).[12] Mutation of this residue, located within the taxane-binding pocket on β-tubulin [13,17]

Table 2 **Cytotoxicity profile of Epothilone A resistant cells**

	1A9 IC_{50}	1A9-A8^E IC_{50}	Relative Resistance	1A9-A8 IC_{50}	Relative Resistance
Epothilone A	3.2	32	10	125	39
Epothilone B	0.9	8	9	29	32
Taxol	1.5	9	6	15	10

Cytotoxicity profile of Epothilone A resistant cells to drugs acting on microtubules. The IC_{50} values, expressed in nanometers, are obtained following 72-hour exposure to the drug. Relative Resistance is calculated as the ratio of the IC_{50} of each respective drug against the resistant clone divided by that obtained against the parental 1A9 cells.

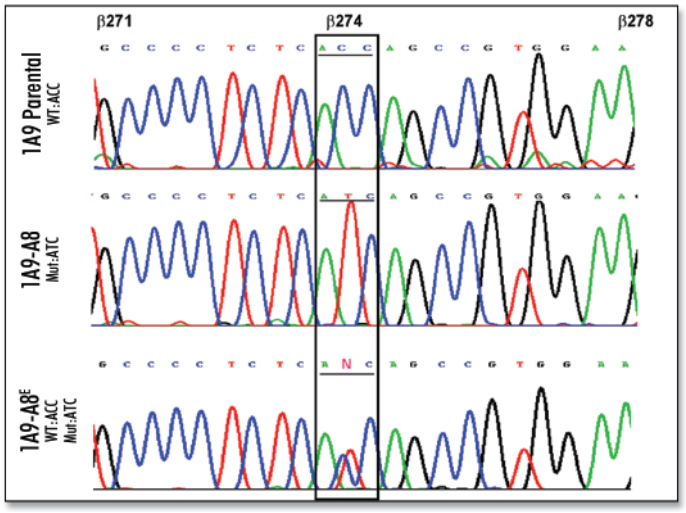

Figure 1. Sequence analysis of β-tubulin M40 cDNA from 1A9 parental and Epo A resistant cells. A portion of the sequence chromatogram of β-tubulin M40 cDNA exon 4 from 1A9 parental and both EpoR cell lines is shown. The 1A9 parental cell line displays wild type sequence for the M40 β-tubulin amino acid Thr274 (ACC) (top panel), while a homozygous point mutation at this residue β274 (ThrACC to IleATC) is seen in the late-step Epo-resistant clone 1A9-A8 (middle panel). A heterozygous point mutation for the same residue β274 (ThrACC to ThrACC/IleATC) is observed in the early-step Epo-resistant clone 1A9-A8^E (lower panel).

confers a 40-fold resistance to epothilone A (Epo A). In an effort to gain insight into the molecular evolution leading to this 40-fold drug resistance phenotype, we examined an earlier isolate of 1A9-A8 clone, which we call 1A9-A8^E. This 1A9-A8^E early-step isolate is a precursor of the 1A9-A8 late-step isolate, as it was only exposed to the selecting agent for six months, while 1A9-A8 cells endured a 15-month selection process. Growth inhibition assays revealed that this early-step isolate was only 10-fold resistant to the selecting agent, epothilone A, unlike the 40-fold resistance displayed by its later-step successor, the 1A9-A8 clone (Table 2).

The β-tubulin gene status correlates with extent of drug resistance. To examine whether alterations in the tubulin gene status could account for the differential drug sensitivity displayed by these clones, we sequenced cDNA from the predominant β-tubulin isotype (gene M40) from 1A9-A8^E cells. The results of this analysis, shown in Figure 1, clearly demonstrate that both the wild-type and mutant $^{Thr}\beta274^{Ile}$ tubulin alleles are expressed in the 1A9-A8^E cells. In contrast, the 1A9-A8 cells express only the mutant β-tubulin, consistent with our previous observations.[12] Furthermore, the heterozygous tubulin gene status appears to correlate with reduced levels of drug resistance to the microtubule-stabilizing drugs epothilone A, epothilone B, and Taxol; while significantly higher-fold resistance values are observed in the 1A9-A8 cells containing only the mutant tubulin gene (Table 2). Thus, intermediate levels of drug resistance are observed with 1A9-A8^E cells, as compared with both the 1A9 wt cells and the 1A9-A8 mutant cells.

Impaired drug-induced tubulin polymerization correlates with tubulin gene status. In order to examine whether the tubulin gene status correlates with the ability of epothilone to induce tubulin polymerization in the three related cell lines (1A9, 1A9-A8, and 1A9-A8^E) we performed cell-based tubulin polymerization assays, shown in Figure 2. After treating the cells with escalating doses of Epo A, the cells were harvested in a low salt buffer and then centrifuged to separate the pellet fraction containing the polymerized form of tubulin, from the supernatant that contains the soluble form of tubulin. Under our experimental conditions, the untreated controls from all three cell lines contained most of the cellular tubulin in the supernatant fraction, thus in the soluble or unpolymerized form. In the parental cell line (1A9), treatment with Epo A led to a dose-dependent increase in tubulin polymerization, as indicated by the shift of total tubulin from the supernatant to the pellet fractions. In sharp contrast, Epo A had almost no effect on tubulin polymerization in the late-step 1A9-A8 cells, with the majority of the tubulin remaining in the soluble form even at the highest drug concentration (1500 nM), as expected due to the mutant-only tubulin gene status. Interestingly, the intermediate selection step, represented by the 1A9-A8^E cells, showed an intermediate degree of tubulin polymerization following drug treatment, consistent with both the wild type and the mutant allele being expressed. Treatment with 150 nM of Epo A resulted in 90% of polymerized parental cell tubulin (Fig. 2A), 70% of polymerized tubulin from the early-step 1A9-A8^E cells and only 3% of polymerized tubulin from the late-step 1A9-A8 cells (Fig. 2B). Thus, the effects of Epo A on tubulin polymerization from these three cell lines correlate well with their respective tubulin gene status.

Impaired drug-induced G_2/M arrest correlates with tubulin gene status. Microtubule-targeting drugs are known to induce G_2/M arrest as a result of their binding to tubulin or microtubules, blocking cell division at mitosis. Thus, we wanted to determine epothilone's ability to induce mitotic arrest in our cell model consisting of isogenic human ovarian cancer cell lines harboring wt, wt/mut or mut only β-tubulin genes status. As shown in Figure 3, Epo A treatment resulted in a complete G_2/M arrest in the parental 1A9 cells. As expected, no change was observed in the cell cycle profile of the 1A9-A8 cells upon treatment with Epo A, while a modest G_2/M arrest was achieved in the 1A9-A8^E clone. Drug treatment with 10 nM of the microtubule-destabilizing drug vincristine, resulted in G_2/M arrest in all three cell lines, consistent with the different binding site of this drug on tubulin. Since FACS analysis cannot discriminate between G_2 arrest and mitotic arrest, we also tested the ability of epothilone ability to induce mitotic arrest in these cells lines (Table 3). The results of the mitotic index analysis fully corroborate the cell cycle analysis data as they show minimal mitotic arrest in the 1A9-A8 clone even at the highest epothilone concentration (100 nM). Collectively, these data reflect the tubulin gene status and the ability of the drug to affect tubulin polymerization (Fig. 2).

Genomic DNA sequencing indicates that wt β-tubulin gene was lost in 1A9-A8. Our data presented in Figure 1, clearly show that a tubulin mutation in one of the two alleles is acquired early on during drug selection, while following continuous selection pressure, only the mutant tubulin is expressed. Furthermore, the presence of only mutant tubulin appears to confer higher levels of drug resistance. To examine whether methylation of wt β-tubulin was responsible for the lack of wt β-tubulin expression in 1A9-A8 cells, we treated the 1A9-A8 cells with the DNA demethylating agent 5'-azacytidine and did not detect reexpression of the wild-type β-tubulin sequence (data not shown). We next examined the promoter methylation status of β-tubulin by methylation-specific PCR [18] and found it to be unmethylated (data not shown). To examine whether the gene encoding wt β-tubulin gene was present in 1A9-A8 cells, we sequenced β-tubulin M40 genomic DNA from the three cell lines. The 1A9 cell line displayed a wild-type β-tubulin sequence, as expected. The 1A9-A8 cells displayed only the mutant $^{Thr}\beta274^{Ile}$ sequence, while the intermediate clone 1A9-A8^E had both the wild type and mutant sequences (data not shown). These results

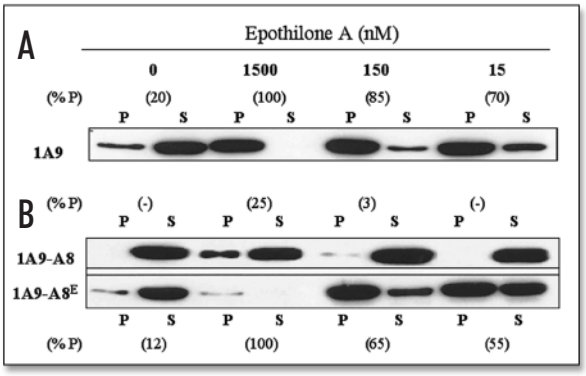

Figure 2. 1A9-A8 and 1A9-A8^E cells exhibit impaired in vivo drug-induced tubulin polymerization compared with their parental 1A9 cells. Drug-sensitive parental 1A9 (A) and the Epo A^R clones, 1A9-A8 and 1A9-A8^E (B), were treated for 5 h with or without (0) various concentrations of Epo A as indicated. After cell lysis, the polymerized (P) and the soluble (S) protein fractions were separated by centrifugation, resolved by SDS/PAGE, and immunoblotted with an antibody against alpha-tubulin. The percent of polymerized tubulin (%P) was determined by dividing the densitometric value of polymerized tubulin by the total tubulin content (the sum of P plus S). The results shown are from a representative experiment of four independent observations.

Table 3 **Mitotic index of epothilone A resistant cells**

	Mitotic Index (%)		
Epothilone A (nM)	**0**	**10**	**100**
1A9	6.3	82	96
1A9- A8^E	5.2	39	47
1A9-A8	3.2	2.7	6.1

Mitotic Index of cells treated with the indicated drug concentrations for 24 hr. Approximately 500 cells are scored per drug treatment.

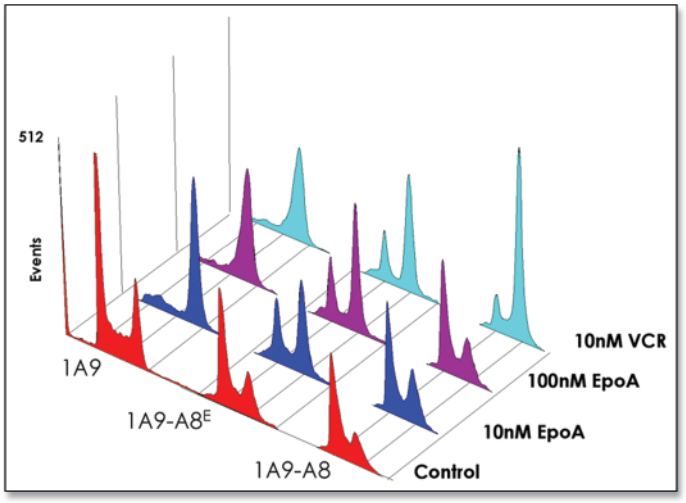

Figure 3. Impaired Epothilone-induced G_2/M arrest in the 1A9-Epo^R cells. Cell cycle analysis by flow cytometry was performed in the parental 1A9 and the 1A9-Epo^R clones, following overnight treatment with Epo A or Vincristine as indicated. The parental 1A9 cells readily arrested in G_2/M after treatment with either microtubule-stabilizing or destabilizing agents. The early step isolate 1A9-A8^E was partially arrested in G_2/M following Epo A treatment; while the late-step isolate 1A9-A8 failed to arrest in mitosis after treatment with Epo A even at the highest concentration. Both Epo^R clones were arrested in G_2/M after treatment with 10 nM Vincristine. 10000 events were recorded for each condition, the histogram is representative of three independent experiments.

suggest that the loss of wt β-tubulin in 1A9-A8 cells is a genetic event.

M40, the major isotype of β-tubulin in human cell, is located on 6p25, not on 6p21.3. Currently there are seven known isoforms of β-tubulin in the human genome. They share over a 90% nucleotide sequence similarity, with the highest degree of variation being at the C-terminus. β-tubulin M40 (also known as class I) is the most predominant of these seven isoforms, accounting for 84.7–98.7% of all expressed β-tubulin in human cancer cells, according to gene expression analysis.[19] Traditional cytogenetic mapping located M40 (gene symbol TUBB) at chromosome 6p21-6pter,[20] and M40 (GeneID 203068) was also placed at 6p21.33 in the latest release of human genome sequence (NCBI, Build 35 version 1, June 4, 2004). However, a close examination of the NCBI sequence indicated that it corresponds to the β9 tubulin isotype (GeneID 7280), not M40. On the other hand, the cDNA sequence of M40 (AF070561) mapped to 6p25 in an earlier version of the human genome sequence (NCBI, Build 30, June 2002). To resolve this conundrum we obtained BAC clones for both the 6p21.3 locus (RP11-527J5) and the 6p25 locus (RP11-506K6), and designed genomic PCR primers specific for the M40 and β-9 genes tubulin genes respectively, as seen in Figure 4A. The location of each BAC clone was verified by FISH analysis and we showed that they mapped to their respective loci (Fig. 4B). Genomic PCR analysis indicated the M40-specific PCR products only amplified from genomic DNA isolated from the RP11-506K6 BAC (located at 6p25), while β-9–specific PCR products only amplified from RP11-527J5 BAC (located at 6p21.3). To further confirm the PCR results we sequenced these PCR products and validated their identity as expected (data not shown). Hence, we concluded that M40 is located at 6p25, not 6p21.33.

Loss of heterozygosity for TUBB at 6p25 results in increased taxol and epothilone resistance. To further examine the molecular mechanism leading to loss of wt β-tubulin gene in the late-step 1A9-A8 cells, we performed loss of heterozygosity analysis (LOH). using single nucleotide polymorphic (SNP) markers. We selected forty-five SNP markers (see Table S1) from spanning 41.5 mega base pairs along 6p25, to assess the biallelic M40 status of 1A9 parental cells. The heterozygosity status of the 45 selected SNP markers was examined in 1A9 parental cells by PCR amplification of genomic DNA and sequencing. As shown in Table S1 only four from the 45 tested SNP markers were heterozygous in 1A9 cells. The remaining 41 markers that were tested in 1A9 cells and deemed uninformative by being homozygous are listed in Table S1 in the supplement along with their location on contig NT_003488. Two of these SNP markers from within the TUBB gene were not heterozygous in 1A9 cells so they could not be informative in our analysis. These four informative SNP markers were then tested in the early-step 1A9-A8^E and late-step 1A9-A8 epothilone-resistant cell lines, as well as in the late-step Taxol-resistant cells 1A9-PTX10 and 1A9-PTX22, harboring only mutant β-tubulin alleles at residues β270 and β364, respectively.[8] These results are summarized in Figure 5. The parental 1A9 and the early-step isolate 1A9-A8^E cells contain both alleles, while the late-step isolate clones 1A9-A8, 1A9-PTX10 and 1A9-PTX22 contain only one allele for all 4 SNP markers. All SNPs were located within contig NT_003488, and the deletion encompasses all of the SNP markers in this region. These results indicate that one of the wild type TUBB allele is lost in 1A9-A8, 1A9-PTX10 and 1A9-PTX22 by chromosome loss, which are consistent with our DNA sequencing analysis.

Fluorescence in situ hybridization at region 6p25. To corroborate our LOH results and to determine whether this LOH event involves the entire chromosome, we performed fluorescence in situ hybridization (FISH) using the BAC clone RP11-506K6 containing M40 at 6p25 (see scheme in Fig. 5). Before hybridization, sequence analysis confirmed the presence of M40 in this BAC clone (data not shown). As observed in Fig 6, the metaphases

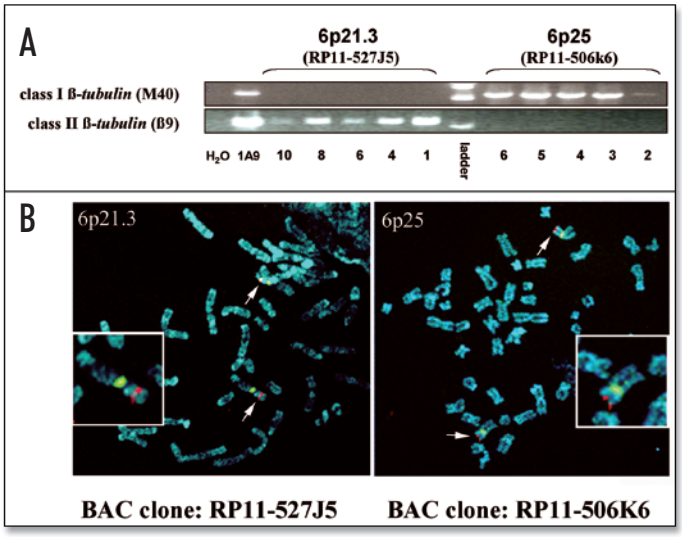

Figure 4. M40, the major isotype of β-tubulin in human cell, is located on 6p25. (A) PCR amplification from genomic DNA isolated from the BAC clones RP11-527J5 BAC (located at 6p21.3) and RP11-506K6 BAC (located at 6p25), using primers specific for M40 and β9 tubulin isotypes. The primers were designed from intron 3 to the 3′UTR region of each gene, thereby amplifying the entire exon 4. Upon bacterial expansion of the two BACs, five different clones were picked to ensure that the BACs we had received were uniform and homogeneous. As a positive control we used genomic DNA from 1A9 cells. (B) Metaphase spreads from 1A9 cells were hybridized with BAC clones RP11-527J5 (6p21.3) and RP11-506k6 (6p25) shown in orange, and a centromeric probe for chromosome 6 (green), followed by counterstaining with nucleic acid stain Sytox Blue (blue).

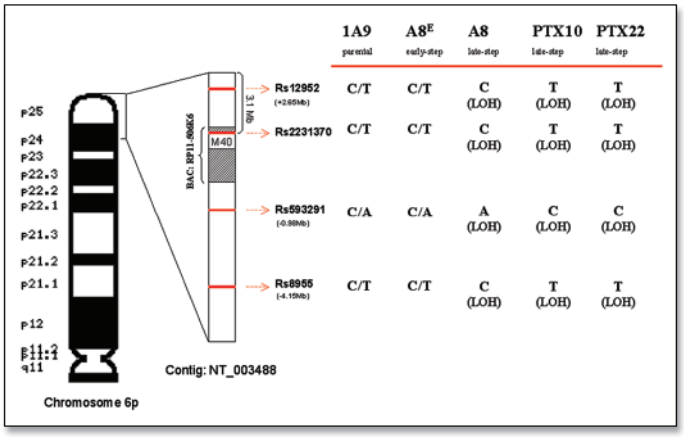

Figure 5. SNP marker analysis of 1A9 and 1A9-Resistant Cells. Left Panel: Diagram of chromosome 6p displaying the location of the SNP markers within the 9.5 Mb contig NT_003488. The β-tubulin gene M40 is highlighted within the BAC clone RP11-506k6 located within this contig at 6p25. The location of the four informative SNP markers is displayed. Right Panel: table showing the corresponding SNP nucleotides by DNA sequencing analysis in the parental 1A9 cells, and the four drug-resistant clones, as indicated.

of the parental 1A9 and the early-step 1A9-A8^{E} cells show the presence of two copies of chromosome 6 (green centromeric chromosome 6 probe), each displaying BAC hybridization (orange staining).

In contrast, FISH results from the late-step 1A9-A8 cells, depict the presence of a mixed population. Approximately 25% of 1A9-A8 cells display a pattern where the BAC probe hybridized to only one copy of chromosome 6, while the second copy of chromosome 6 is devoid of BAC hybridization (Fig. 6, left panel). In these cells, the LOH event probably involves partial chromosome loss. This result is consistent with the loss of heterozygosity for 6p25 as we previously observed. The remaining 75% of 1A9-A8 cells exhibited BAC hybridization to both copies of chromosome 6 (Fig. 6, right panel). Based on the LOH analysis showing loss of heterozygosity for 6p25, this result indicates that during drug selection the 1A9-A8 cells led to the loss of the entire chromosome containing the wt β-tubulin allele followed by duplication of the chromosome containing the mutant β-tubulin allele, as detailed in Figure 7.

DISCUSSION

Anticancer drugs select for drug resistance by killing drug-sensitive cells. Taxanes are very effective in the treatment of a wide variety of solid tumors; however, acquired resistance to taxanes limits their clinical efficacy. With continued exposure to the therapeutic drug, a cell develops a mechanism to further increase its chances of survival and expansion. We have presented the temporal mechanism by which the 1A9 ovarian carcinoma cells, upon exposure to Epo A, develop moderate drug resistance due to a mutation in one allele in the drug binding pocket domain of the target gene: β-tubulin; and subsequently lose the chromosomal area around 6p25 creating a cell type that is now highly resistant to the selecting agent albeit containing a similar, if not identical cellular background. The cells with an intermediate level of resistance are now called 1A9-A8^{E} and have approximately a ten-fold degree of resistance to Epo A, the selecting agent. The above mentioned mutation is located residue β274 (Thr → Ile). This mutation changes the binding pocket and does not allow the drug to bind as efficiently.[12] Nevertheless, the cell is still producing the wild type allele gene and protein therefore the drug can bind there and exert its effect. At some point after the acquisition of the β-tubulin mutation, 1A9-A8^{E} cells lose the chromosomal area encompassing 6p25 resulting in the loss of the wild type allele and consequently a higher degree of resistance to Epo A (40 fold). The phenomenon of acquiring a point mutation in one allele and LOH in the other allele is frequently seen in various tumor suppressor genes.

The best known example is LOH of p53. In cancer cells that lost p53 function, one p53 allele is usually mutated and the other allele is lost due to chromosomal deletion.[21] Most strikingly, a similar event: mutation of one β-tubulin allele and then loss of the other allele has been observed during evolution of resistance to Taxol. Thus, the taxane-driven selection for mutant tubulin mirrors the process of inactivation of tumor suppressors. This is consistent with the idea the genetic instability in human cancers is responsible not only for tumorigenesis but also for the development of drug-resistant clones. Our model for the development of epothilone resistance in 1A9 cancer cells foresees the acquisition of a β-tubulin mutation in one allele. As the mutation is located within the taxol-binding site, epothilone is now unable to bind to some of the M40 β-tubulins. Therefore, the cells are conferred with a moderate degree of resistance to the selecting agent, as long as the other wild-type allele is still expressed. Upon continued selection with Epo A, the expression of the wild type allele disappears, due to loss of the wild-type β-tubulin allele. Thus, Epo A is unable to effectively bind to any of the M40 β-tubulins, thus providing the cancer cells with significant growth advantage in the presence of the drug.

Several other groups have reported acquired β-tubulin mutations in response to drug selection with taxanes, epothilones and other microtubule-targeting drugs.[6,14-16,22-26] In a number of these reports, only the mutant β-tubulin gene appears to be expressed.[14-16,22,26] It

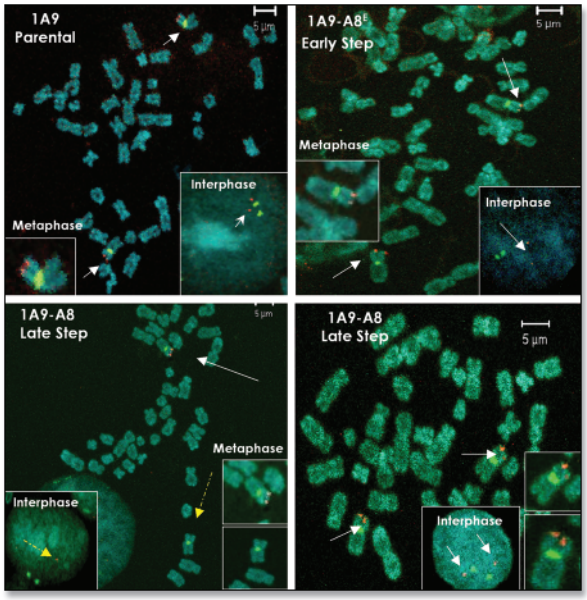

Figure 6. FISH analysis of 1A9, 1A9-A8^{E} and 1A9-A8 cells. Metaphase spreads from all three cell lines were hybridized with BAC clone RP11-506k6 (orange) containing the β-tubulin gene M40, and a centromeric probe for chromosome 6 (green), followed by counterstaining with nucleic acid stain Sytox Blue (blue). The parental 1A9 cells display two copies of chromosome 6 (white arrows) as evidenced by the chromosome 6 centromeric probe staining. Both 6 chromosomes displayed staining for the BAC clone indicating two copies of the β-tubulin gene M40. The early-step clone 1A9-A8^{E} presented a similar karyotype as the parental cells, with two copies of chromosome 6 each containing the β-tubulin BAC clone. In the late-step 1A9-A8 cells however, only one chromosome 6 stained for the BAC clone, although both copies of the chromosome 6 were present (left panel). The white arrows indicate chromosomes 6, as evidenced by the green centromeric staining, and the BAC hybridization at the tips of the chromosome. In 1A9-A8 the yellow dashed arrow indicates the chromosome 6 that has lost the chromosomal region of 6p25. Insets display either a magnification of chromosome 6 in metaphase, or interphase. DNA in interphase cells and individual chromosomes in the metaphase plate are stained in blue. Scale bar indicates 5 µm.

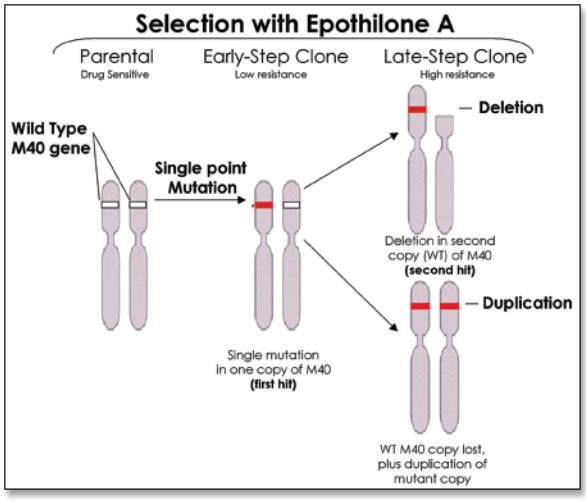

Figure 7. Temporal model for the development of high level of drug resistance to Epothilone A. Upon selection with Epothilone A a single point mutation occurs in one β-tubulin allele producing a low resistance profile in the cell. During continued selection with the drug, loss of heterozygosity of the remaining wild type allele of β-tubulin occurs along with duplication of the mutant allele generating a high resistance clone.

would be interesting to know whether the inactivation of wild-type β-tubulin allele is due to LOH or promoter methylation in these cases. In either case, tubulin mutations followed by the inactivation of the remaining wild-type tubulin allele seems to be a general mechanism of acquired resistant to microtubule targeting agents.

Most of late stage 1A9-A8 cells still have two copies of 6p25 even though our LOH analysis suggest that one of the parental allele is lost. This is probably due to the duplication of the chromosome containing the mutant β-tubulin allele after the loss of the chromosome containing the wild-type β-tubulin allele. This phenomenon has been frequently observed in association with LOH in human cancers.[27]

To date, there are seven β-tubulin isotypes described in mammalian cells.[28] Although the exact role of each of these isotypes has yet to be defined, it seems that all of them are incorporated into the microtubule polymer and contribute to the overall cellular microtubule function. Interestingly, however, all tubulin mutations identified so far occur at the major β-tubulin isotype (class βI/gene HM40),[8] the expression of which accounts for 80-95% of total tubulin mRNA in a subset of cancer cell lines from the NCI60 human cancer cell collection.[19] From a mechanistic standpoint, one wonders why the other β-tubulin isotypes in their wild-type sequence do not "take over" expression-wise, in order for the cell to escape the toxic effects of microtubule-targeting drugs that use class I β-tubulin as a target isotype. Our own experience, together with recurring data in the literature,[6,14-16,22-26] suggests that the cell's first response is to adapt to the otherwise lethal effects of the drug by acquiring tubulin mutations in sites that are important for drug-tubulin interactions. This cellular behavior of acquiring mutations versus substituting isotypes suggests that the class I β-tubulin isotype is not functionally redundant and its role in the cell simply cannot be replaced by the other isotypes. The latter constitutes a compelling hypothesis for why this is the single isotype identified in which sequence alterations occur to block the action microtubule-targeting drugs.

Losses of heterozygosity are the most common genetic alterations observed in human cancers[27] and are often associated with loss of tumor suppressor genes leading to tumorigenesis. However, no studies have correlated the occurrence of LOH with drug resistance. The results reported herein, reveal a new mechanism of taxane resistance that could be clinically important given the fact that LOH in chromosome 6p is frequently encountered in human tumors.[29-35] In addition, LOH analysis of the 6p25 region in cervical cancer has revealed two as yet unidentified tumor suppressor genes.[31] Therefore, some of these tumors may lose one copy of 6p during tumorigenesis, and they may only contain one intact copy of β-tubulin gene. Based on our model described in Figure 7, we predict that these tumors may have a high likelihood of acquiring a second β-tubulin mutation and become resistant to microtubule-polymerizing agents. This in conjunction with the unstable human cancer genome that could mutate tubulin in response to treatment with taxanes could provide a rational basis for clinical drug resistance.

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