Class III β-Tubulin Mediates Sensitivity to Chemotherapeutic Drugs in Non–Small Cell Lung Cancer

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Abstract

First line therapy for non–small cell lung carcinoma (NSCLC) commonly includes combination therapy with a tubulin-binding agent (TBA) and a DNA-damaging agent. TBAs suppress microtubule dynamics by binding to the β-tubulin subunit of α/β-tubulin, inducing mitotic arrest and apoptosis. Up-regulation of class III β-tubulin (βIII-tubulin) has been implicated in clinical resistance in NSCLC, ovarian and breast tumors treated in combination with a TBA and DNA-damaging agent. To investigate the functional significance of βIII-tubulin in resistance to both these classes of agents, small interfering RNA (siRNA) was used to silence the expression of this isotype in two NSCLC cell lines, NCI-H460 and Calu-6. Reverse transcription-PCR and immunoblotting showed that βIII-siRNA potently inhibited the expression of βIII-tubulin, without affecting the expression of other major β-tubulin isotypes. Clonogenic assays showed that βIII-siRNA cells were significantly more sensitive to TBAs, paclitaxel, vincristine, and vinorelbine, and for the first time, DNA-damaging agents, cisplatin, doxorubicin, and etoposide compared with controls. Cell cycle analysis of H460 βIII-siRNA cells showed reduced accumulation at the G_2-M boundary and an increase in the sub-G_1 population in response to TBA treatment compared with control cells. Importantly, βIII-siRNA cells displayed a significant dose-dependent increase in Annexin V staining when treated with either paclitaxel or cisplatin, compared with controls. These findings have revealed a novel role for βIII-tubulin in mediating response to both TBA and DNA-damaging agent therapy and may have important implications for improving the targeting and treatment of drug-refractory NSCLC. [Cancer Res 2007;67(19):9356–63]

Introduction

Lung cancer is the most common cancer in the world with >1 million cases diagnosed every year, and remains the leading cause of cancer death in both men and women (1). Advanced non–small cell lung carcinoma (NSCLC) accounts for >80% of lung cancer cases. More than half of these patients have developed metastasis at the time of diagnosis and chemotherapy remains the most effective treatment option (2). Tubulin-binding agents (TBA) such as paclitaxel, docetaxel, and vinorelbine are widely used in the treatment of NSCLC, either as single agents or in combination with other platinum compounds such as cisplatin and carboplatin. In fact, platinum-based doublets are considered to be the standard treatments for NSCLC (3, 4). However, the prognosis for patients remains dismal due to the emergence of drug-resistant tumors which significantly limits the clinical utility of these drugs in the treatment of lung cancer.

TBAs such as taxanes and *Vinca* alkaloids constitute the most important group of chemotherapeutic agents, with activity against a broad range of solid tumors and hematologic malignancies. These agents target the β-tubulin subunit of the α/β-tubulin heterodimers that assemble to form microtubules. Microtubules are dynamic polymers that play a key role in many fundamentally important cellular processes such as vesicular transport, maintenance of cell shape, and cell division. In mammals, there are at least six α- and seven β-tubulin isotypes, encoded by multiple genes that display differential tissue expression. Selection of cancer cells for resistance to TBAs could lead to alterations in β-tubulin isotype composition. In particular, increased expression of the neuronal specific class III β-tubulin (βIII-tubulin) has been frequently implicated in drug-resistant cancer cell lines (5, 6). Importantly, there is mounting clinical evidence that βIII-tubulin expression is involved in the resistance to taxanes and *Vinca* alkaloids in lung (7–9), breast (10, 11), and ovarian cancers (12–14). In addition, in nonneuronal cancers such as NSCLC, the expression of βIII-tubulin is associated with poorly differentiated tumor tissue and high-grade malignancy, as well as increased metastatic potential (15). Despite strong correlational evidence in preclinical and clinical studies, its role in drug response is far from clear. Stable overexpression of βIII-tubulin in human prostate cells failed to confer resistance to TBAs (16–18). In contrast, transfection of βIII-tubulin into Chinese hamster ovary (CHO) cells confers low-level resistance to paclitaxel (19). A recent study showed that CHO cells exogenously overexpressing βIII-tubulin had reduced suppression of microtubule dynamics in the presence of paclitaxel compared with control cells (20). We have previously shown that antisense oligonucleotides targeted against the βIII-tubulin isotype partially reversed paclitaxel resistance in a NSCLC cell line, A549-T12 (21). To date, the available functional data largely focuses on the role of βIII-tubulin in paclitaxel resistance, and the relevance of this isotype in resistance to other TBAs is not known. In addition, despite the common use of cisplatin and related agents in combination therapy with TBAs in NSCLC, the role of βIII-tubulin in response to these agents has not been addressed.

To identify the functional role of βIII-tubulin in response to chemotherapy in NSCLC, we employed the RNA interference technique to knock down βIII-tubulin expression in two independent lung cancer cell lines that express this isotype. Knockdown of βIII-tubulin corresponded with increased sensitivity to TBAs. Interestingly, the knockdown cells also exhibited increased sensitivity to DNA-damaging agents that are structurally and functionally unrelated to TBAs. This report shows that βIII-tubulin expression could mediate the response to microtubule-stabilizing

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and -destabilizing agents, and for the first time, DNA-damaging agents in NSCLC.

Materials and Methods

Cell culture and small interfering RNA transfection. Human NSCLC cell lines H460 and Calu-6 were maintained as monolayers in RPMI and DMEM, respectively, supplemented with 10% FCS and 2 mmol/L of L-glutamine. The cells were grown at 37°C in a humidified atmosphere with 5% CO_2.

The βIII small interfering RNA (siRNA) SMARTpool reagent was obtained from Dharmacon Research, Inc. The nonsilencing control siRNA, which has no sequence homology to any known human gene sequence, was used as a negative control in all experiments (Qiagen). Cells were transfected with βIII siRNA at a final concentration of 25 nmol/L (Calu-6) and 100 nmol/L (H460) using LipofectAMINE 2000 (Invitrogen) following the manufacturer's instructions. The optimal amount of siRNA used for transfection was determined empirically for each cell line and the lowest siRNA concentration that gave effective silencing of the targeted protein and caused minimal stress to the cells was used in all subsequent experiments. Control experiments were done in parallel by transfecting the cells with a nonsilencing control siRNA (Qiagen) at equivalent concentrations as the target siRNA. All experiments involved the use of a LipofectAMINE-only control (mock-transfected) and a nonsilencing control siRNA (negative control).

Cytotoxic drugs. Cytotoxic drugs were obtained as previously described (22). Paclitaxel (Calbiochem, Merck Biosciences) was prepared at a stock concentration of 2 mmol/L in DMSO. Vincristine (Sigma-Aldrich) was prepared at a stock concentration of 2 mmol/L in saline (0.9% wt/vol NaCl); vinorelbine (kindly provided by Dr. B. Hill, Division of Experimental Cancer Research, Pierre Fabre Research Centre, Castres, France) was solubilized in water at a stock concentration of 2 mmol/L. Doxorubucin (doxorubicin hydrochloride; Pfizer) was prepared at a stock concentration of 3.45 mmol/L in saline. Etoposide (Sigma-Aldrich) was prepared at a stock concentration of 68 mmol/L in DMSO and cisplatin (Pharmacia) was prepared at a stock concentration of 3.3 mmol/L in saline.

Reverse transcription-PCR. Total RNA was extracted from the transfected cells using the Trizol reagent (Invitrogen), according to the manufacturer's instructions. RNA samples were DNase-treated, reverse-transcribed for reverse transcription-PCR (RT-PCR) analysis using methodology and specific primers as described in detail previously (12). β2-Microglobulin was used as an internal control.

Western blot analysis. Preparation of protein lysates as well as Western blot analyses were done as described previously (23), using antibodies directed against βI-tubulin (clone SAP 4G5; Abcam, Ltd.), βII-tubulin (clone 7B9; Chemicon), βIII-tubulin (clone TUJ1, Chemicon), βIV-tubulin (clone ONS 1A6, Sigma-Aldrich), and glyceraldehyde-3-phosphate dehydrogenase (Abcam Ltd.). Enhanced chemiluminescence (GE Healthcare) was used for detection. The blots were scanned using the Typhoon scanner and quantified using ImageQuant software version 5.2 (Molecular Dynamics, Inc.).

Immunofluorescence staining. Briefly, siRNA-transfected cells were plated in glass chamber slides and allowed to reach 70% confluence. Cells were then treated with 10 nmol/L of paclitaxel and vincristine for 1 h. Drug was then removed by washing cells with PBS. Immunofluorescence staining was then done as described previously (23). For dual staining, cells were first stained with βIII-tubulin followed by Cy3 anti-mouse fluorescent-tagged antibody (GE Healthcare). This was then followed by staining with α-tubulin and Cy2 anti-mouse fluorescent-tagged antibody (GE Healthcare). Slides were mounted on a coverslip using DAPI II Counterstain (Vysis, Inc.). Immunofluorescence microscopy was done using a Zeiss Axioplan 2 Microscope (Zeiss), and images were captured using a Sensicam Charged Coupled Device camera (PCO Imaging) and the Image-Pro Plus 4.1 software (Media Cybernetics, L.P.).

Clonogenic assays. Twenty-four hours after siRNA transfection, cells were harvested and plated into six-well plates for 6 h, prior to the addition of various drugs as indicated in the figure legends. After 72 h of incubation, the drug-containing medium was removed and replaced with complete growth medium. Medium was changed every 3 days for 7 to 10 days until visible colonies formed. Colonies were simultaneously fixed and stained with 0.5% crystal violet in methanol, and manually counted. Individual stained colonies in each well were counted and the surviving fraction was calculated as follows: colony number / (number of cells seeded × plating efficiency), where plating efficiency is equivalent to the colony number divided by the number of cells seeded in the drug-free medium.

Drug accumulation assays. Cellular uptake and retention of tritiated substrate [^{3}H]paclitaxel and [^{3}H]vincristine were measured as previously described (22). Briefly, cells were transfected in 12-well plates for 48 h. Drug uptake was monitored by adding [^{3}H]paclitaxel (14.7 Ci/mmol; final concentration, 50 nmol/L; Moravek Biochemicals, Inc.) or [^{3}H]vincristine (7.1 Ci/mmol; final concentration of 12.5 nmol/L; GE Healthcare) to the transfected cells for 2 h at 37°C. Cells were washed, hydrolyzed, and counted as previously described. The amount of tritiated drug accumulated in the cells was determined for duplicate samples and expressed as picomoles of the drug per milligram of protein. At least three independent experiments were done. Relevant positive controls were included, together with vincristine-resistant neuroblastoma (BE/VCR10) and etoposide-resistant breast cancer cells (MCF7-VP16), which have previously been shown to overexpress multidrug resistance 1 (MDR1) and multidrug resistance–associated protein 1 (MRP1), respectively (23, 24).

Cell cycle analysis by flow cytometry. Cell cycle analysis was determined by transfecting H460 cells with siRNA for 72 h and harvesting (adherent and suspension) cells 24 h after drug treatment. DNA content was stained for 15 min at 37°C with a solution containing 0.4% Triton X-100 (Sigma-Aldrich), 50 μg/mL of propidium iodide (Sigma-Aldrich), and 2 μg/mL of DNase-free RNase (Roche). The cells were then analyzed for cell cycle perturbation using a FACSCalibur (Becton Dickinson). The CellQuest program was used to quantitate the distribution of cells in each cell cycle phase: sub-G_1 (apoptotic cells), G_1, S, and G_2-M.

Detection of apoptosis by Annexin V-FITC staining. Apoptosis induction was determined by transfecting H460 cells with siRNA for 72 h and harvesting (adherent and suspension) cells 48 h after drug treatment, as previously described with slight modifications (25). Briefly, 1×10^5 cells were incubated with Annexin V-FITC and propidium iodide for 15 min in the dark (Becton Dickinson), immediately followed by flow cytometry using a FACSCalibur (Becton Dickinson). Cytogram analysis was done using Cell Quest software.

Statistical analysis. A two-tailed Student's t test was used for statistical analysis of comparative data using the GraphPad Prism program. Data were expressed as means of at least three independent experiments ± SE, with $P < 0.05$ considered statistically significant.

Results

Specific silencing of βIII-tubulin in NSCLC cell lines. To examine the effects of βIII-tubulin siRNA transfection on the gene and protein expression of the isotype, RT-PCR and Western blotting were used. Treatment of H460 and Calu-6 cells with βIII-tubulin siRNA resulted in significant knockdown of βIII-tubulin mRNA levels compared with the mock- and control siRNA-transfected cells (Fig. 1*A*). This result was consistent with the decrease observed at the protein level (Fig. 1*B*). The βIII-tubulin siRNA specifically targets βIII-tubulin and has no cross-reactivity with other β-tubulin isotypes examined as shown by Western blotting (Fig. 1*C*). In addition, silencing of βIII-tubulin did not cause compensatory changes in the other isotypes examined.

Silencing of βIII-tubulin disrupts microtubules upon paclitaxel or vincristine treatment. To assess the effects of βIII down-regulation on microtubule organization, immunofluorescence staining was done on transfected Calu-6 cells. The βIII siRNA-transfected cells showed no observable changes to microtubule morphology (Supplementary Fig. S1). Consistent with

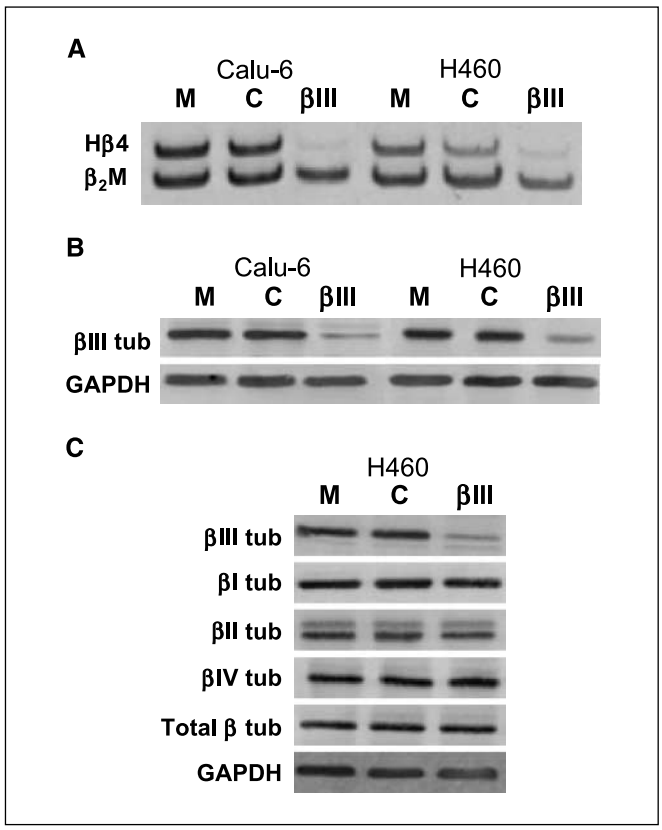

Figure 1. siRNA targeting βIII-tubulin specifically inhibits its expression. *A,* analysis of βIII-tubulin gene expression by RT-PCR following 48 h of siRNA transfection at 25 nmol/L (*Calu-6*) or 100 nmol/L (*H460*). β2-Microglobulin (β_2M) served as an internal control. *B,* protein expression of βIII-tubulin 72 h after transfection. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. *C,* specificity of βIII-tubulin siRNA. No significant changes were observed for class I, II, and IV β-tubulin isotypes and total β-tubulin at the protein level. *M,* mock; *C,* control-siRNA; *βIII,* βIII-tubulin siRNA.

the Western blotting data, a clear decrease in βIII-tubulin immunofluorescence intensity was observed in the βIII-tubulin siRNA-treated cells compared with both the control siRNA- and mock-transfected cells when imaged under identical conditions (Supplementary Fig. S1).

To examine the effect of TBAs on βIII-tubulin siRNA-treated cells, cells were exposed for 1 h to either 10 nmol/L of paclitaxel or vincristine, and cellular morphology was examined. As shown in Fig. 2, βIII siRNA-transfected Calu-6 cells showed extensive disruption of the microtubule cytoskeleton as compared with the control-treated cells. The majority of the βIII siRNA-treated cells were rounded and many of these cells displayed abnormal cellular or nuclear morphology (Fig. 2, *arrows*). The control-treated cells occasionally showed minimal bundling of microtubules normally associated with paclitaxel treatment but the frequency of rounded cells and the extent of microtubule disruption was minimal compared with βIII transfectants.

βIII-tubulin silencing increases sensitivity to TBAs. The data in the preceding section suggested that silencing βIII-tubulin expression may have increased the sensitivity of the cells to both paclitaxel and vincristine. To quantitate any change in drug sensitivity, drug-treated clonogenic assays were done. Consistent with the immunofluorescence observations, βIII-tubulin silencing resulted in a significant increase in sensitivity to paclitaxel and vincristine (Fig. 3*A* and *B*). In addition, the βIII-tubulin silenced cells exhibited enhanced sensitivity to vinorelbine compared with controls (Supplementary Fig. S2). The increased sensitivity was not due to altered accumulation of drug as there was no significant difference in the intracellular drug accumulation levels in the βIII-tubulin siRNA-treated Calu-6 or H460 NSCLC cells compared with mock and control siRNA-treated cells (Table 1).

βIII-tubulin silencing increases sensitivity to DNA-damaging agents. Previous studies modulating βIII-tubulin levels have only focused on the effects of paclitaxel sensitivity (11, 19, 21). As NSCLC is often treated with a TBA in combination with a DNA-damaging agent, such as cisplatin, we sought to determine whether the drug sensitivity effects of βIII-tubulin silencing are specific to TBAs. Therefore, drug-treated clonogenic assays were also done using DNA-damaging agents, etoposide, cisplatin, and doxorubicin. Interestingly, βIII-tubulin silencing resulted in increased sensitivity to all three DNA-damaging agents tested in H460 cells (Fig. 3*C*). Similar results were obtained with Calu-6 cells (data not shown). Because H460 cells have wild-type p53 (26) and the Calu-6 cells harbor mutated p53 (27), sensitivity to these drugs is independent of the p53 genotype.

Knockdown of βIII-tubulin abrogates paclitaxel- and vincristine-induced G_2-M arrest and induces an increase in sub-G_1 population. To determine whether βIII-tubulin silencing affects the cell cycle profiles, cell cycle analysis using flow cytometry was done. The cell cycle profiles of H460 cells were not affected by βIII-tubulin silencing (Fig. 4). To determine whether the TBAs affected the cell cycle profiles of βIII-tubulin siRNA transfectants, cells were treated with either paclitaxel or vincristine. Following 24 h of incubation with 5 nmol/L of paclitaxel, the βIII-tubulin–silenced cells had a higher sub-G_1 content (apoptotic cells) compared with the control siRNA-treated cells ($P < 0.05$; Fig. 4*A*), although both the βIII-tubulin siRNA and control siRNA-treated cells had a similar increase in G_2-M content compared with untreated samples. A major difference was observed with 40 nmol/L of paclitaxel, with the control siRNA-treated cells showing a marked G_2-M block whereas the βIII-tubulin siRNA-treated cells had a marked increase in the sub-G_1 population reflective of apoptotic cells (Fig. 4*A*). Similar results were observed when siRNA-treated cells were exposed to vincristine (Fig. 4*B*), suggesting that there is a common mechanism that enhances the effects of both the taxanes and *Vinca* alkaloids following βIII-tubulin silencing.

Knockdown of βIII-tubulin increases the sensitivity of cells to apoptosis in the presence of either paclitaxel or cisplatin. To address whether the increase in the sub-G_1 population following TBA treatment in the βIII-tubulin siRNA-treated cells was related to an increase in apoptosis induction, we did Annexin V-FITC staining followed by flow cytometry analysis. Incubation of H460 cells with paclitaxel for 48 h induced apoptosis from 1 nmol/L in βIII-tubulin siRNA-treated cells and 5 nmol/L in control siRNA-treated cells (Fig. 5*A*). Moreover, at all tested paclitaxel concentrations (1, 2, and 5 nmol/L), the percentage of apoptotic cells was significantly higher in βIII-tubulin siRNA-treated cells than in control siRNA-treated cells (Fig. 5*A*). Similarly, in βIII-tubulin siRNA-treated cells exposed to cisplatin for 48 h, there was a significant increase in the number of apoptotic cells in the 0.4 or 1 μmol/L cisplatin-treated cells compared with controls (Fig. 5*B*). Taken together, this data shows that βIII-tubulin silencing sensitized cells to apoptosis induction following TBA and DNA-damaging agent treatment in NSCLC.

Discussion

The clinical effectiveness of the chemotherapeutic agents used in the treatment of NSCLC is often negated by drug resistance. Altered expression of specific tubulin isotypes, particularly βIII-tubulin, has been strongly implicated in chemoresistance to the microtubule-stabilizing agent paclitaxel in preclinical and clinical studies. To date, many studies have been correlative, with few reports examining the role of βIII-tubulin in contributing to resistance to microtubule-destabilizing agents or indeed, resistance to other classes of agents used in the treatment of NSCLC. Herein, we show that knockdown of βIII-tubulin in two independent drug-naïve NSCLC cell lines sensitizes cells to both microtubule-stabilizing and -destabilizing TBAs. The finding that βIII-tubulin is important in the sensitivity to DNA-damaging agents is novel and shows that βIII-tubulin may be an important survival factor that mediates drug response, even to drugs that do not directly target the microtubule network.

One approach to identifying the role of specific β-tubulin isotypes in drug resistance is to overexpress these proteins in cell lines. However, altering the expression of specific tubulin isotypes is far from straightforward due to the complexity of tubulin autoregulation (28). As previously noted, overexpression of βIII-tubulin in human prostate carcinoma cells failed to confer resistance to a range of TBAs including colchicine, estramustine, paclitaxel, and vinblastine (18). Of particular note, class II and IVb tubulins were also increased collaterally in these transfected cells (18). The reasons for this nonspecific regulation were unclear, but as suggested by the investigators, might possibly be due to a compensatory mechanism that overcomes the effects of the elevated βIII levels. Expression of β-tubulin isotypes is tightly regulated by an autoregulatory mechanism through cotranslational degradation of β-tubulin mRNAs in response to an increase in the level of soluble tubulin (28, 29). Hence, it is of particular importance to analyze the total isotype profile to confirm the contribution of the target isotype in drug response studies. Importantly, the siRNA used in this study resulted in specific down-regulation of the βIII-tubulin expression with no compensatory changes observed with other β-tubulin isotypes examined, further strengthening the role of βIII-tubulin in contributing to chemosensitivity. In addition, the cell lines used in this study have not been subjected to prior drug selection, and are more likely to represent intrinsic drug resistance observed in lung carcinoma.

We have previously shown that blocking βIII-tubulin expression in drug-selected NSCLC cells by antisense oligonucleotides partially restored paclitaxel sensitivity in these paclitaxel-resistant cells (21). The results presented here using βIII-tubulin–specific siRNA supports our previous study with regard to the role of βIII-tubulin in paclitaxel response. The finding that βIII-tubulin silencing sensitizes NSCLC cells to *Vinca* alkaloids is novel. At their lowest effective concentrations, TBAs suppress microtubule dynamics in cancer cells, leading to mitotic arrest and subsequent cell death, without changing the polymer mass (30). Given the crucial role of microtubule dynamics in the pharmacologic action of these drugs, one mechanism by which βIII-tubulin has been thought to mediate resistance to TBAs is to constitutively increase microtubule dynamics (31, 32). However, a recent study showed that inducible stable overexpression of βIII-tubulin in CHO cells did not significantly alter any variables of microtubule dynamic stability, but reduced the ability of paclitaxel to suppress the microtubule dynamics in these cells (20). It remains to be determined whether down-regulation of

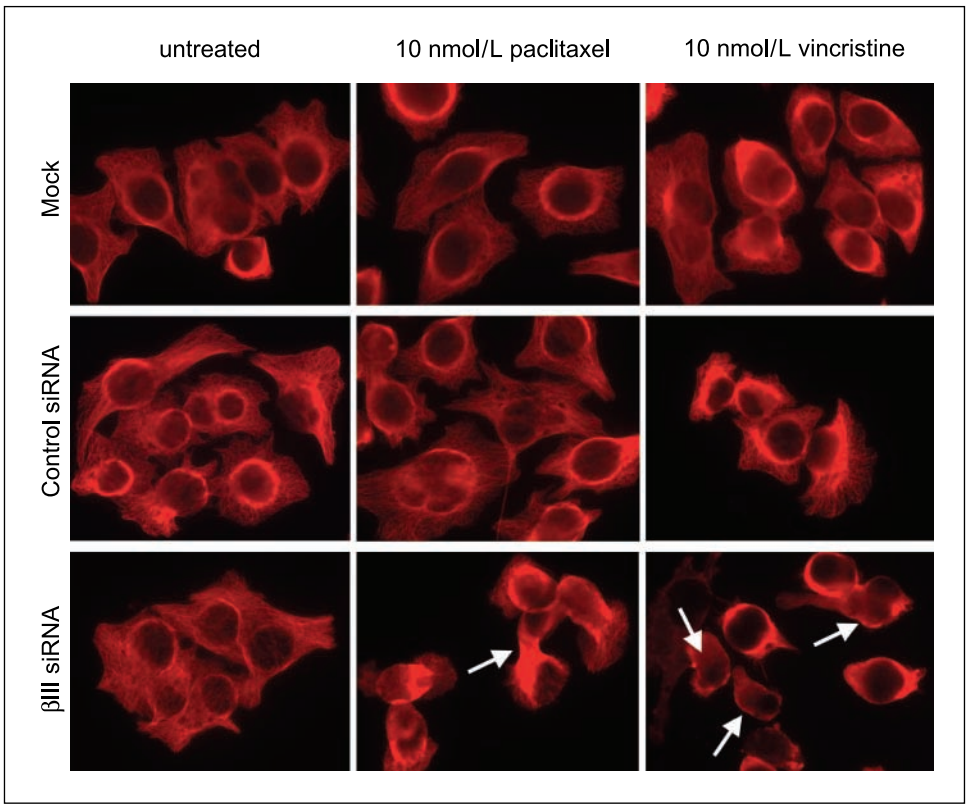

Figure 2. Microtubule morphology in siRNA-transfected Calu-6 cells incubated with 10 nmol/L of paclitaxel (*middle*) and 10 nmol/L of vincristine (*right*) for 1 h. Microtubules are shown by α-tubulin staining. Extensive microtubule disruption occurred when βIII transfectants were treated with either paclitaxel or vincristine. Cells with abnormal morphology (*arrows*). Cells were imaged on a Zeiss Axioplan 2 Immunofluorescence microscope using a 63× oil immersion objective.

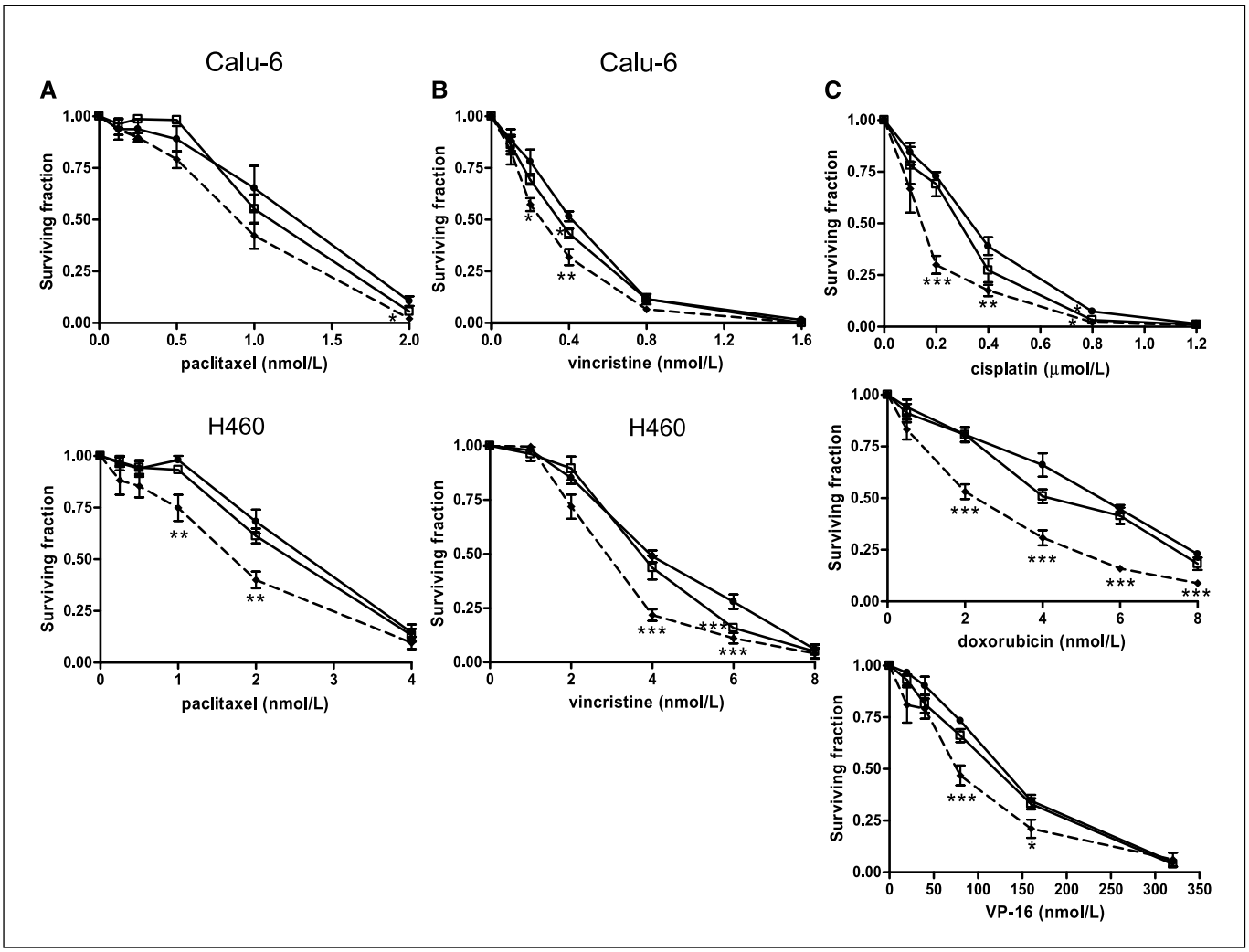

Figure 3. Clonogenic assays in the presence of TBAs or DNA-damaging agents. Clonogenic assays were done on mock- (●), control siRNA- (□), and βIII-transfected cells (- - -) as described in Materials and Methods. Clonogenic survival, expressed as surviving fractions, for treatment with TBAs, such as paclitaxel (*A*) and vincristine (*B*), and for treatment with DNA-damaging agents (*C*), such as cisplatin (*top*), doxorubicin (*middle*), and etoposide (VP-16; *bottom*). *Points,* means of at least four individual assays; *bars,* SE. Statistics were calculated by comparing the surviving fraction of the siRNA-treated cells with the mock-transfected cells at each drug concentration. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.005$.

βIII-tubulin in NSCLC cells plays a dominant role in modulating microtubule dynamics in a way that could enhance or reduce the effectiveness of TBAs.

Of particular interest is the novel finding that βIII-tubulin silencing significantly increases sensitivity to DNA-damaging agents. Although these agents are not known to directly target the tubulin/microtubule system, the results are perhaps not surprising due to frequent overexpression of βIII-tubulin in drug-refractory tumors which are commonly treated with a TBA in combination with a DNA-damaging agent (8, 13). Ferrandina et al. found that βIII-tubulin serves as a potent prognostic marker for patients with ovarian cancer, regardless of whether the chemotherapeutic regimens include a taxane (14). The βIII-tubulin–silenced NSCLC cells examined in the current study exhibited a significant increase in apoptosis induction when exposed to paclitaxel or cisplatin as revealed by Annexin-V staining. The mechanistic basis for the increased sensitivity is not clear, however, one possibility is that βIII-tubulin may serve as a survival factor to rescue tumor cells from death signals triggered by chemotherapeutic agents. In support of this hypothesis, a pool of tubulin has been shown to localize in the mitochondria membranes where it is associated with voltage-dependent anion channels (33). Interestingly, this pool of mitochondrial tubulin is enriched with βIII-tubulin, suggesting that this tubulin isotype may be involved in mitochondrial-mediated cell death. Therefore, we propose that altered expression of βIII-tubulin isotypes may provide a cellular defense not only against TBAs but also against diverse classes of DNA-targeting drugs.

TBAs are known to promote cell death by inducing a potent mitotic block causing cells to accumulate at the G_2-M phase of the cell cycle (34). In the present study, the extent of the mitotic block induced by incubation with either paclitaxel or vincristine seemed to be markedly reduced in βIII-tubulin siRNA-treated cells as compared with control siRNA-transfected cells. In contrast, the level of apoptosis induction was significantly higher in βIII-tubulin–depleted cells. These results suggest that suppression of βIII-tubulin may enhance TBA-induced apoptotic cell death via a separate pathway that is independent of classical mitotic arrest.

Table 1. Drug accumulation levels in the βIII-tubulin siRNA-treated Calu-6 and H460 NSCLC cells

Transfection	Mean [^{3}H]paclitaxel uptake (pmol/mg of protein)				Mean [^{3}H]vincristine uptake (pmol/mg of protein)			
	0 h	*P**	2 h	*P**	0 h	*P**	2 h	*P**
Drug uptake by siRNA-transfected NSCLC Calu-6 cells								
Mock	1.84 ± 0.25	(Referent)	39.3 ± 2.43	(Referent)	0.56 ± 0.11	(Referent)	16.1 ± 1.21	(Referent)
Control siRNA	1.87 ± 0.03	0.91	44.9 ± 2.04	0.15	0.39 ± 0.08	0.25	15.4 ± 0.98	0.66
βIII-Tubulin siRNA	2.05 ± 0.28	0.59	39.6 ± 2.8	0.94	0.31 ± 0.07	0.10	14.8 ± 0.88	0.43
Drug uptake by siRNA-transfected NSCLC H460 cells								
Mock	2.609 ± 0.299	(Referent)	52.898 ± 1.722	(Referent)	0.267 ± 0.075	(Referent)	8.502 ± 0.336	(Referent)
Control siRNA	3.576 ± 0.776	0.31	53.868 ± 2.841	0.78	0.312 ± 0.066	0.67	8.631 ± 0.397	0.82
βIII-Tubulin siRNA	2.781 ± 0.826	0.85	53.898 ± 1.579	0.69	0.310 ± 0.011	0.59	8.683 ± 0.859	0.85

NOTE: Data are from at least three independent experiments.
*Student's *t* test (two-sided).

Although mitotic arrest is a hallmark cellular response to TBAs, Milross et al. showed that the antitumor efficacy of paclitaxel is dependent on its ability to induce apoptosis, but not mitotic arrest (35). Previous studies with NSCLC A549 cells also found that low concentrations of paclitaxel are sufficient to induce cell death without an apparent G_2-M block (36, 37). The exact mechanism is not fully understood, although aberrant mitosis and p53 induction have been suggested as possible mechanisms (36, 38).

Thus, the sensitization effects of βIII down-regulation on apoptosis induced by TBAs may have broad significance and may also explain, in part, its hypersensitivity to DNA-damaging agents. In fact, similar results were obtained when treating the cells with

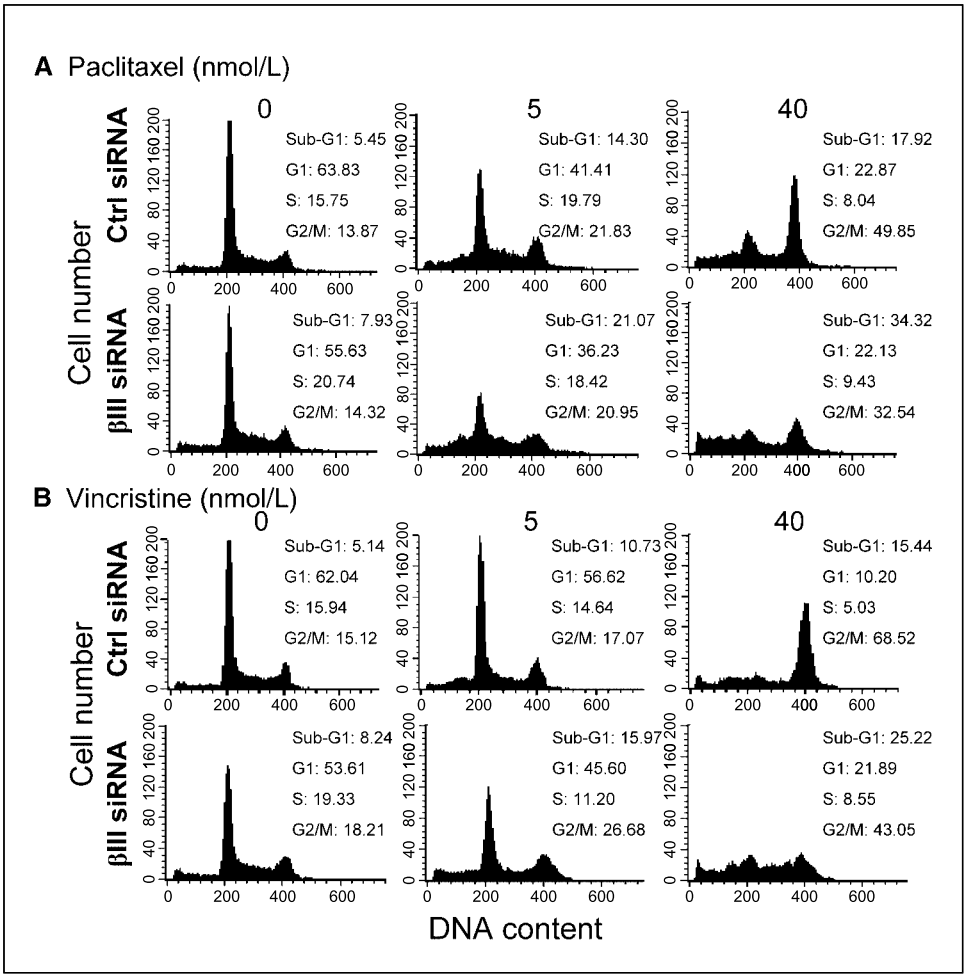

Figure 4. Cell cycle analysis of βIII-tubulin–depleted H460 cells treated with paclitaxel (*A*) or vincristine (*B*). Cells were harvested after 24 h of drug treatment and subsequently assayed for their DNA content by flow cytometry. Representative figures of multiple experiments are shown.

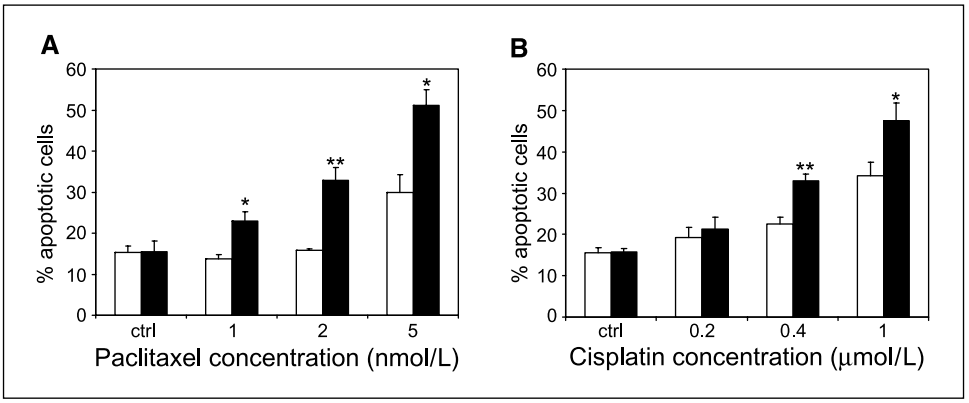

Figure 5. Apoptosis induction in control siRNA-transfected (*white columns*) and βIII-tubulin siRNA-transfected (*black columns*) H460 cells. Cells were harvested after 48 h of incubation with drug and subsequently assayed for apoptosis induction by flow cytometry using Annexin V-FITC staining. *A,* siRNA-transfected cells treated with paclitaxel. *B,* siRNA-transfected cells treated with cisplatin. *Columns,* means of at least three independent experiments; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$.

cisplatin in which βIII knockdown abrogated cisplatin-induced G_2-M arrest but showed an increase in the sub-G_1 populations (data not shown). So why does repression of mitotic block result in apoptotic cell death in βIII-tubulin–depleted cells following drug treatment? One possibility is that decreasing βIII-tubulin levels may change the expression of microtubule-associated or interacting proteins. We addressed this possibility by examining for altered expression of two important regulators of microtubule function after βIII-tubulin knockdown, microtubule-associated protein-4 and stathmin, and found no significant change (data not shown). We cannot exclude the possibility that changes in other components of the cytoskeleton are affected. Potentially, βIII-tubulin may serve as a survival factor to rescue tumor cells from death signals triggered by chemotherapeutic agents. Therefore, by knocking down this survival factor, cells are hypersensitive to drug-induced cell death. The mechanisms that enhance drug-induced cell death after suppressing βIII-tubulin expression are currently under active investigation.

Because TBAs and DNA-damaging agents have distinct cellular targets, it is also possible that βIII-tubulin may enhance the drug action of these agents via activation of signaling pathways shared by both drugs or those specific to each drug. One potential explanation for the novel enhancement of drug sensitivity by down-regulation of βIII-tubulin in NSCLC cells is through regulation of gene expression, e.g., for genes that are involved in apoptosis, DNA repair pathway, or mitosis.

The finding that βIII-tubulin is involved in the sensitivity of both taxanes and *Vinca* alkaloids highlights a common effect of this isotype on microtubules and the action of TBAs. Importantly, βIII-tubulin seems to play a broader role in chemosensitivity as shown by its effects on DNA-damaging agent response. These results have mapped out new challenges and opportunities for circumventing drug resistance to improve the efficacy of current chemotherapeutic agents in the treatment of cancer.

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References

1. Parkin, DM. Global cancer statistics in the year 2000. Lancet Oncol 2001;2:533–43.
2. Breathnach OS, Freidlin B, Conley B, et al. Twenty-two years of phase III trials for patients with advanced non-small-cell lung cancer: sobering results. J Clin Oncol 2001;19:1734–42.
3. Manegold C. Chemotherapy for advanced non-small-cell lung cancer: standards. Lung Cancer 2001;34 Suppl 2:S165–70.
4. Schiller JH, Harrington D, Belani CP, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med 2002;346:92–8.
5. Orr GA, Verdier-Pinard P, McDaid H, Horwitz SB. Mechanisms of Taxol resistance related to microtubules. Oncogene 2003;22:7280–95.
6. Verrills NM, Kavallaris M. Improving the targeting of tubulin-binding agents: lessons from drug resistance studies. Curr Pharm Des 2005;11:1719–33.
7. Rosell R, Scagliotti G, Danenberg KD, et al. Transcripts in pretreatment biopsies from a three-arm randomized trial in metastatic non-small-cell lung cancer. Oncogene 2003;22:3548–53.
8. Dumontet C, Isaac S, Souquet PJ, et al. Expression of class III β tubulin in non-small cell lung cancer is correlated with resistance to taxane chemotherapy. Bull Cancer 2005;92:Ed25–30.
9. Seve P, Isaac S, Tredan O, et al. Expression of class III {beta}-tubulin is predictive of patient outcome in patients with non-small cell lung cancer receiving vinorelbine-based chemotherapy. Clin Cancer Res 2005;11:5481–6.
10. Hasegawa S, Miyoshi Y, Egawa C, et al. Prediction of response to docetaxel by quantitative analysis of class I and III β-tubulin isotype mRNA expression in human breast cancers. Clin Cancer Res 2003;9:2992–7.
11. Tommasi S, Mangia A, Lacalamita R, et al. Cytoskeleton and paclitaxel sensitivity in breast cancer: the role of β-tubulins. Int J Cancer 2007;120:2078–85.
12. Kavallaris M, Kuo DY, Burkhart CA, et al. Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific β-tubulin isotypes. J Clin Invest 1997;100:1282–93.
13. Mozzetti S, Ferlini C, Concolino P, et al. Class III β-tubulin overexpression is a prominent mechanism of paclitaxel resistance in ovarian cancer patients. Clin Cancer Res 2005;11:298–305.
14. Ferrandina G, Zannoni GF, Martinelli E, et al. Class III β-tubulin overexpression is a marker of poor clinical outcome in advanced ovarian cancer patients. Clin Cancer Res 2006;12:2774–9.
15. Katsetos CD, Herman MM, Mork SJ. Class III β-tubulin in human development and cancer. Cell Motil Cytoskeleton 2003;55:77–96.
16. Ranganathan S, Dexter DW, Benetatos CA, Chapman AE, Tew KD, Hudes GR. Increase of β(III)- and β(IVa)-tubulin isotopes in human prostate carcinoma cells as a result of estramustine resistance. Cancer Res 1996;56:2584–9.
17. Ranganathan S, Dexter DW, Benetatos CA, Hudes GR. Cloning and sequencing of human βIII-tubulin cDNA: induction of βIII isotype in human prostate carcinoma cells by acute exposure to antimicrotubule agents. Biochim Biophys Acta 1998;1395:237–45.
18. Ranganathan S, McCauley RA, Dexter DW, Hudes GR. Modulation of endogenous β-tubulin isotype expression as a result of human β(III)cDNA transfection into prostate carcinoma cells. Br J Cancer 2001;85:735–40.
19. Hari M, Yang H, Zeng C, Canizales M, Cabral F. Expression of class III β-tubulin reduces microtubule

assembly and confers resistance to paclitaxel. Cell Motil Cytoskeleton 2003;56:45–56.
20. Kamath K, Wilson L, Cabral F, Jordan MA. βIII-Tubulin induces paclitaxel resistance in association with reduced effects on microtubule dynamic instability. J Biol Chem 2005;280:12902–7. Epub 2005 Feb 6.
21. Kavallaris M, Burkhart CA, Horwitz SB. Antisense oligonucleotides to class III β-tubulin sensitize drug-resistant cells to Taxol. Br J Cancer 1999;80:1020–5.
22. Verrills NM, Po'uha ST, Liu ML, et al. Alterations in γ-actin and tubulin-targeted drug resistance in childhood leukemia. J Natl Cancer Inst 2006;98:1363–74.
23. Don S, Verrills NM, Liaw TY, et al. Neuronal-associated microtubule proteins class III β-tubulin and MAP2c in neuroblastoma: role in resistance to microtubule-targeted drugs. Mol Cancer Ther 2004;3:1137–46.
24. Schneider E, Horton JK, Yang CH, Nakagawa M, Cowan KH. Multidrug resistance-associated protein gene overexpression and reduced drug sensitivity of topoisomerase II in a human breast carcinoma MCF7 cell line selected for etoposide resistance. Cancer Res 1994;54:152–8.
25. Pasquier E, Carre M, Pourroy B, et al. Antiangiogenic activity of paclitaxel is associated with its cytostatic effect, mediated by the initiation but not completion of a mitochondrial apoptotic signaling pathway. Mol Cancer Ther 2004;3:1301–10.
26. Mitsudomi T, Steinberg SM, Nau MM, et al. p53 gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of ras mutations and clinical features. Oncogene 1992;7:171–80.
27. Lehman TA, Bennett WP, Metcalf RA, et al. p53 mutations, ras mutations, and p53-heat shock 70 protein complexes in human lung carcinoma cell lines. Cancer Res 1991;51:4090–6.
28. Yen TJ, Gay DA, Pachter JS, Cleveland DW. Autoregulated changes in stability of polyribosome-bound β-tubulin mRNAs are specified by the first 13 translated nucleotides. Mol Cell Biol 1988;8:1224–35.
29. Theodorakis, NG Cleveland, DW. Physical evidence for cotranslational regulation of β-tubulin mRNA degradation. Mol Cell Biol 1992;12:791–9.
30. Wilson L, Jordan MA. Microtubule dynamics: taking aim at a moving target. Chem Biol 1995;2:569–73.
31. Derry WB, Wilson L, Khan IA, Luduena RF, Jordan MA. Taxol differentially modulates the dynamics of microtubules assembled from unfractionated and purified β-tubulin isotypes. Biochemistry 1997;36:3554–62.
32. Goncalves A, Braguer D, Kamath K, et al. Resistance to Taxol in lung cancer cells associated with increased microtubule dynamics. Proc Natl Acad Sci U S A 2001;98:11737–42. Epub 2001 Sep 18.
33. Carre M, Carles G, Andre N, et al. Involvement of microtubules and mitochondria in the antagonism of arsenic trioxide on paclitaxel-induced apoptosis. Biochem Pharmacol 2002;63:1831–42.
34. Jordan MA, Wilson L. Microtubules and actin filaments: dynamic targets for cancer chemotherapy. Curr Opin Cell Biol 1998;10:123–30.
35. Milross CG, Mason KA, Hunter NR, Chung WK, Peters LJ, Milas L. Relationship of mitotic arrest and apoptosis to antitumor effect of paclitaxel. J Natl Cancer Inst 1996;88:1308–14.
36. Torres K, Horwitz SB. Mechanisms of Taxol-induced cell death are concentration dependent. Cancer Res 1998;58:3620–6.
37. Das GC, Holiday D, Gallardo R, Haas C. Taxol-induced cell cycle arrest and apoptosis: dose-response relationship in lung cancer cells of different wild-type p53 status and under isogenic condition. Cancer Lett 2001;165:147–53.
38. Chen JG, Horwitz SB. Differential mitotic responses to microtubule-stabilizing and -destabilizing drugs. Cancer Res 2002;62:1935–8.

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