

# Regulation of Class II $\beta$ -Tubulin Expression by Tumor Suppressor p53 Protein in Mouse Melanoma Cells in Response to *Vinca* Alkaloid

Katsuhiko Arai,<sup>1</sup> Yoshifumi Matsumoto,<sup>1</sup> Yuko Nagashima,<sup>1</sup> and Kazumi Yagasaki<sup>2</sup>

Departments of <sup>1</sup>Tissue Physiology and <sup>2</sup>Applied Biological Science, Tokyo University of Agriculture and Technology, Tokyo, Japan

## Abstract

**The continuous exposure of antimicrotubule drugs to tumors often results in the emergence of drug-resistant tumor cells with altered expression of several  $\beta$ -tubulin isotypes. We found that *Vinca* alkaloid enhanced expression of class II  $\beta$ -tubulin isotype (mTUBB2) in mouse B16F10 melanoma cells via alteration of the tumor suppressor p53 protein. Vincristine treatment stimulated an increase in mTUBB2 mRNA expression and promoted accumulation of this isotype around the nuclei. Transient transfection assays employing a reporter construct, together with site-directed mutagenesis studies, suggested that the p53-binding site found in the first intron was a critical region for mTUBB2 expression. Electrophoretic mobility shift assay and associated antibody supershift experiments showed that vincristine promoted release of p53 protein from the binding site. In addition, exogenous induction of TAp63 $\gamma$  (p51A), a homologue of p53, canceled the effect of vincristine on mTUBB2 expression. These results suggest that p53 protein may function as a suppressor of mTUBB2 expression and vincristine-mediated inhibition of p53 binding results in enhanced mTUBB2 expression. This phenomenon could be related with the emergence of drug-resistant tumor cells induced by *Vinca* alkaloid and may participate in determining the fate of these cells. (Mol Cancer Res 2006;4(4):247–55)**

## Introduction

Differential expression of  $\beta$ -tubulin isotypes has been observed during cellular differentiation in relation to organogenesis (1-6) and with the emergence of sublines that are resistant to antimicrotubule agents (7-10). Microtubules are composed of a heterodimer of  $\alpha$  and  $\beta$  subunits and are

dynamic structures that are constantly growing and shortening. Isoforms of  $\beta$ -tubulin manifest a characteristic cell type distribution. Among these, class II  $\beta$ -tubulin is well known as a major component of the nervous system (1). However, this isotype is also expressed in mesenchymal cells, including smooth muscle cells, during late-stage organogenesis and the perinatal period within the rat (4). Moreover, proliferating myoepithelial cells in canine mammary mixed tumors began to express this isotype during tumorigenesis (11). Microtubule dynamics and stability play important roles in many cellular events, particularly in relation to cell division (12). This has resulted in the polymerization/depolymerization system of tubulin becoming an important target for cancer chemotherapy. The effects of various antineoplastic drugs on the isotype composition and dynamics of tubulin have been reported (7), with these agents being designated as antimicrotubule drugs. Among these drugs, *Vinca* alkaloid binds the subunits of tubulin and inhibits their polymerization into microtubules (8, 13-15), whereas Taxol stabilize microtubules against depolymerization (16-18).

One major clinical problem with cancer chemotherapy is the emergence of drug-resistant tumor cells. The development of broad-spectrum drug resistance is often characterized by overexpression of the transmembrane efflux pump, P-glycoprotein, which is encoded by the *multidrug resistance-1* (*MDR1*) gene, or of the MDR-associated proteins (19-21). Additionally, alterations of microtubule proteins are thought to be associated with resistance to antimicrotubule drugs and altered expression of several  $\beta$ -tubulin isotypes in drug-resistant cells has been reported in many cases (8, 18, 22-24). Although these molecular mechanisms are not fully understood, *in vitro* studies have indicated that the isotypic composition of  $\beta$ -tubulin affects sensitivity to antimicrotubule drugs (7, 25, 26). On the other hand, the effect of tumor suppressor p53 protein on sensitivity to microtubule-targeted drugs has been investigated, with mutations in the *p53* gene being shown to confer resistance to microtubule-targeted drugs (27-29). Patients with tumors having p53 mutation often have worse prognosis than those with wild-type p53 (29-32). Recently, two genes coding for proteins homologous to p53 (i.e., *p73* and *p63/p51*) have been identified (33). Both *p63/p51* and *p73* genes contain two transcriptional start sites that are used to generate transcripts that encode proteins either with (TA form) or without ( $\Delta$ N form) a NH<sub>2</sub>-terminal transactivation domain, respectively. These transcripts can also be alternatively spliced to generate proteins with three different COOH termini, termed  $\alpha$ ,  $\beta$ , and  $\gamma$ . Whereas the TA forms have p53-like activities and

Received 9/26/05; revised 1/30/06; accepted 2/10/06.

**Grant support:** Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan grant 14560247.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Katsuhiko Arai, Department of Tissue Physiology, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Saiwai-cho 3-5-8, Fuchu, Tokyo 183-8509, Japan. Phone: 81-42-367-5789; Fax: 81-42-367-5789. E-mail: karai@cc.tuat.ac.jp

Copyright © 2006 American Association for Cancer Research.  
doi:10.1158/1541-7786.MCR-05-0183

can transactivate p53 target genes, the  $\Delta N$  splice variants function as potent inhibitors of p53 and can decrease the promoter activity of p53 target genes (34, 35). Of particular relevance to this study, these splice variants have been shown to be altered in cell lines *in vitro* following vincristine treatment (36). These results suggested that *Vinca* alkaloid could affect the function of p53 by alteration of p63 and p73 levels.

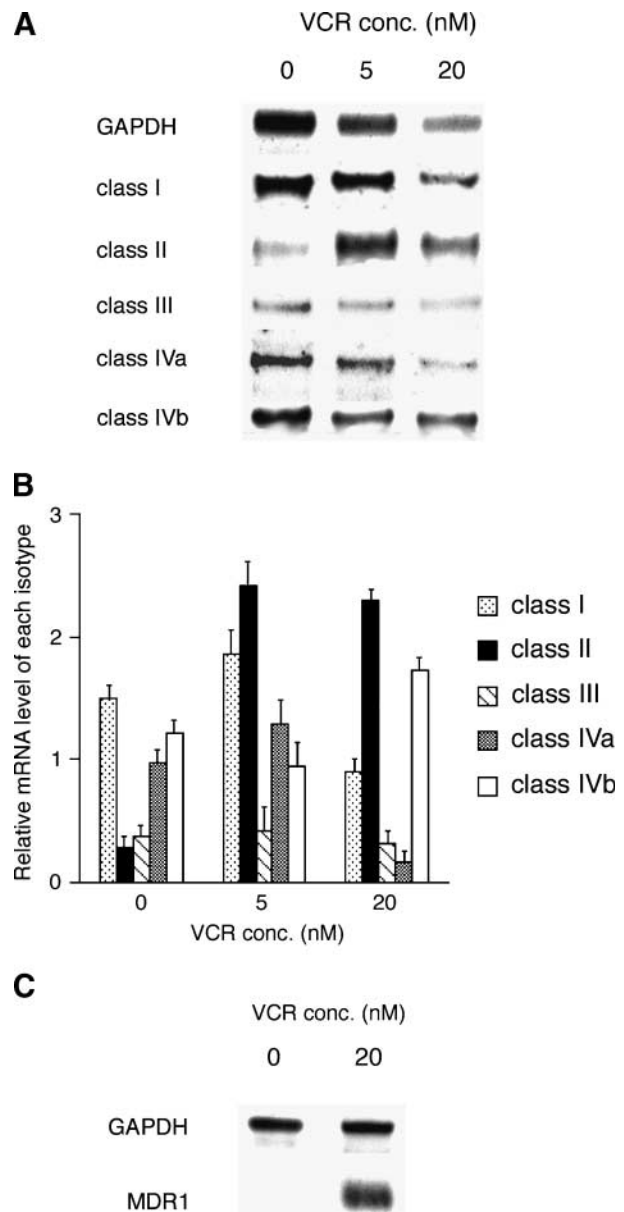
During examination of  $\beta$ -tubulin isotype expression in vincristine-resistant B16F10 cells, we found that vincristine specifically enhanced expression of the class II isotype. In the present study, we aimed to clarify the molecular mechanism of vincristine-mediated up-regulation of this isotype via promoter analysis of the class II  $\beta$ -tubulin isotype (*mTUBB2*) gene and electrophoretic mobility shift assay (EMSA). Here, we found evidence to suggest that vincristine-mediated enhancement of *mTUBB2* promoter activity could be related to inhibition of p53 binding to the response element.

## Results

### Altered Expression of $\beta$ -Tubulin Isoforms in Mouse B16F10 Melanoma Cells by Vincristine Treatment

Vincristine-resistant B16F10 melanoma cells showing active growth in the presence of 5, 10, and 20 nmol/L vincristine, respectively, were established. Drug sensitivity of vincristine-resistant subline to range of the agent was tested. The most resistant subline actively proliferated in the presence of 100 nmol/L vincristine, whereas cell division of the parental (vincristine-sensitive) cells was apparently inhibited by the treatment of 5 nmol/L vincristine (data not shown). These results indicated that 20 nmol/L vincristine-selected cells were at least 20-fold resistant to vincristine compared with the parental cells. The mRNA levels of five  $\beta$ -tubulin isotypes in the parental and vincristine-resistant sublines were examined by RNase protection assay (RPA). The expression pattern of the five  $\beta$ -tubulin isotypes in these cells is shown in Fig. 1A. Expression of the class III isotype was very low in both parental and resistant cell lines and differences in the mRNA level of class III, but not class II, between two lines was not seen. On the other hand, vincristine-resistant cell lines expressed  $\sim 10$ -fold greater amount of class II isotype compared with the parental cells, whereas class IVa was suppressed at higher doses (Fig. 1B). To examine whether vincristine-selected B16F10 overexpress P-glycoprotein, mRNA level corresponding to MDR1 between the parental and 20 nmol/L vincristine-resistant cells was compared. The resistant subline expressed a significant level of MDR1 mRNA, whereas detectable amount of the mRNA was not observed in the parental cells (Fig. 1C). These results indicated that resistance to vincristine was associated with overexpression of P-glycoprotein. Additionally, to examine whether vincristine directly stimulated mRNA expression of class II isotype, temporary exposure of vincristine to the parental B16F10 cells was done (Fig. 2A). Exposure of these cells to 5 or 20 nmol/L vincristine for 16 hours increased the mRNA level of class II isotype by 10- to 15-fold compared with the control experiments (Fig. 2B). These results indicated that vincristine directly enhanced expression of class II isotype.

To examine the effect of vincristine on cellular localization of class II isotype, immunocytochemical analysis using the class



**FIGURE 1.** Altered expression of  $\beta$ -tubulin isotypes in vincristine-resistant B16F10 sublines. **A.** Total RNA (5  $\mu$ g for GAPDH, 10  $\mu$ g for class I, II and IVb, or 50  $\mu$ g for classes III and IVa) was used for RPA. Parental B16F10 cells (0 nmol/L), 5 nmol/L vincristine-selected B16F10 cells (5 nmol/L), and 20 nmol/L vincristine-selected B16F10 cells (20 nmol/L). **B.** Densitometric analysis showing relative expression rates of five  $\beta$ -tubulin isotypes in parental B16F10 cells (0 nmol/L) and vincristine-resistant B16F10 cells (5 or 20 nmol/L). For statistical analysis, the expression level of each isotype of  $\beta$ -tubulin mRNA was normalized to GAPDH mRNA level. Representative of three independent experiments. Columns, mean; bars, SD. **C.** Total RNA (5  $\mu$ g for GAPDH) or (50  $\mu$ g for MDR1) was used for RPA. Parental B16F10 cells (0 nmol/L) and 20 nmol/L vincristine-selected B16F10 cells (20 nmol/L). VCR, vincristine.

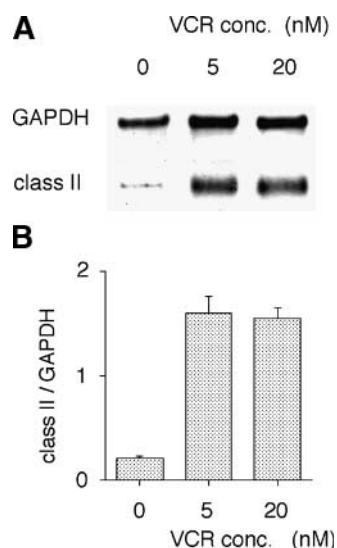
II-specific monoclonal antibody was done. Class II isotype-containing fine microtubules were weakly immunostained and ubiquitously distributed in the cytosol in untreated parental B16F10 cells (Fig. 3A). On the other hand, class II isotype was accumulated around nuclei, with dense immunostaining, in the

20 nmol/L vincristine-resistant subline (Fig. 3B). Also in the parental cells exposed to 20 nmol/L vincristine for 16 hours, class II-containing microtubules accumulated around the nuclei (Fig. 3C).

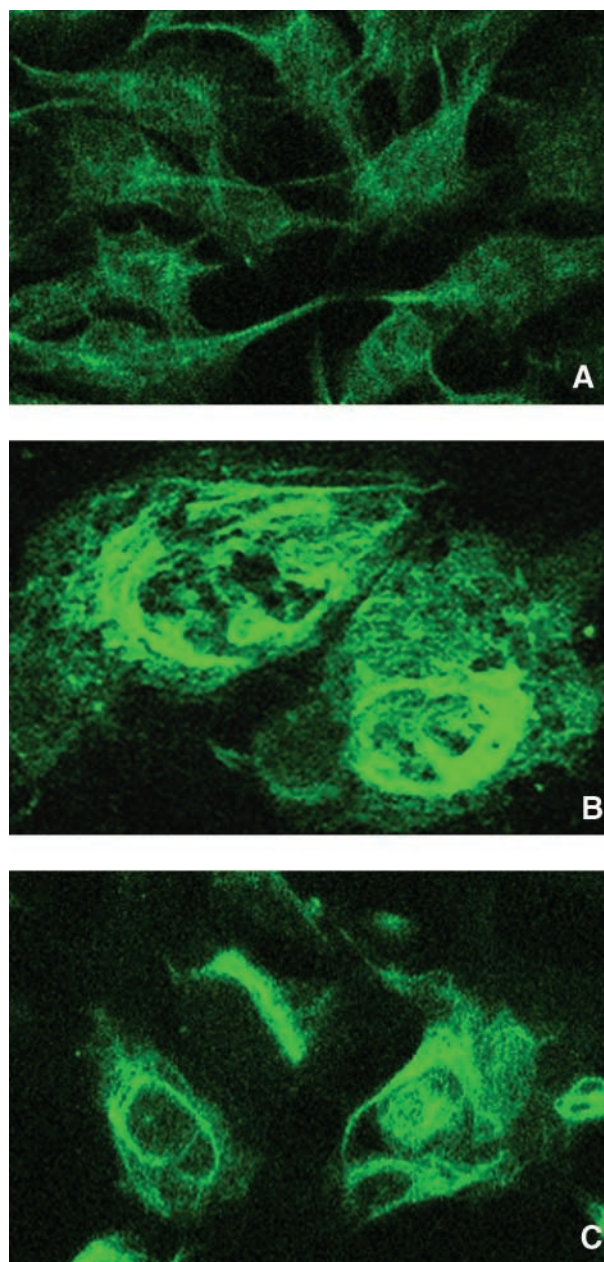
#### Characterization of Vincristine-Responsive Region in *mTUBB2*

To understand regulation of mouse class II isotype (*mTUBB2*) gene transcription in *Vinca* alkaloid-stimulated B16F10 cells, we PCR amplified and cloned 472 bp of the regulatory region of the *TUBB2* gene corresponding to -358 to +139 nucleotides (nt) relative to the +1 transcription start site (Fig. 4). To identify putative transcription factor binding sites, we employed a Web-based search engine, GenomeNet (<http://motif.genome.jp/>; Kyoto University, Kyoto, Japan), to examine this sequence (Fig. 4A). A TATA box was identified at position -108 relative to the transcription start site as well as identification of putative *cis*-regulatory elements, including one cyclic AMP response element-binding protein (-138/-130 nt) and eight Sp1 sites (-255/-246, -238/-229, -166/-153, -147/-138, -122/-114, -105/-93, -24/-15, and +62/+72 nt), in the 5'-flanking region. In addition, two copies of p53-binding sites (+60/+69 and +77/+86 nt), each having two mismatches from the p53 consensus sequence, were separated by 7 nt in first intron (Fig. 4B).

B16F10 cells transfected with pmTUBB2-luc were exposed to various concentrations of vincristine, vinblastine, and Taxol. Corrected with cytomegalovirus (CMV)-*Renilla* luciferase activity, firefly luciferase activities are shown in Fig. 5. Treatment with the *Vinca* alkaloids, vincristine and vinblastine, stimulated the promoter activity of *mTUBB2* by 2.5- to 3-fold and these activities increased in a dose-dependent manner, whereas Taxol displayed no effect on promoter activity.

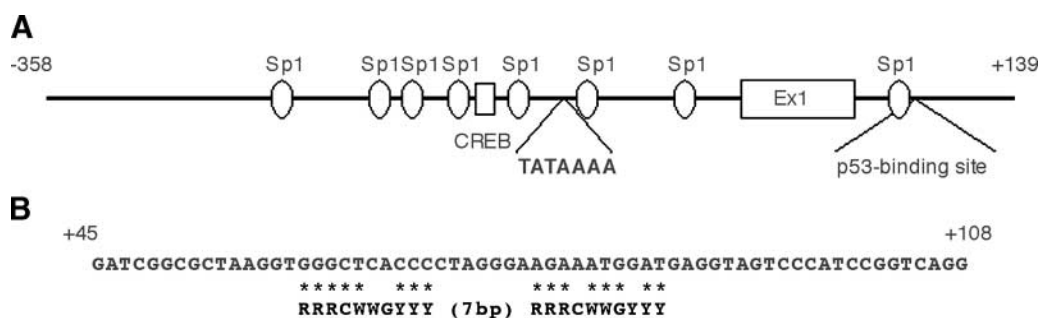


**FIGURE 2.** Dose-dependent up-regulation of class II isotype mRNA level by vincristine. Vincristine-sensitive parental B16F10 cells were treated with either 5 or 20 nmol/L vincristine for 18 hours. **A.** Cells were harvested and assayed for class II isotype mRNA using RPA. **B.** Class II isotype of  $\beta$ -tubulin mRNA was normalized to GAPDH mRNA level. Representative of three independent experiments. Columns, mean; bars, SD.  $P < 0.01$ .



**FIGURE 3.** Changes of immunocytochemical distribution of class II  $\beta$ -tubulin isotype following exposure to vincristine. **A.** Class II  $\beta$ -tubulin isotype was diffusely distributed in the cytoplasm of untreated B16F10 cells. **B.** Within the 20 nmol/L vincristine-resistant subline, this  $\beta$ -tubulin isotype was accumulated around the nuclei of largely swelled cytoplasm. **C.** Overnight exposure of vincristine to the parental cells also weakly affected microtubule structure. Fibrous class II  $\beta$ -tubulin isotype aggregation was observed surrounding the nuclei. Original magnification,  $\times 40$ .

An EMSA and a supershift assay with digoxigenin-labeled synthetic oligonucleotides corresponding to the putative p53-binding site were done using nuclear protein. Exposure to vincristine decreased the binding of nuclear protein to the p53-binding site even at the lowest concentration (Fig. 6A). To characterize further the nuclear protein involved, a competition assay using an excess amount of unlabeled p53 consensus

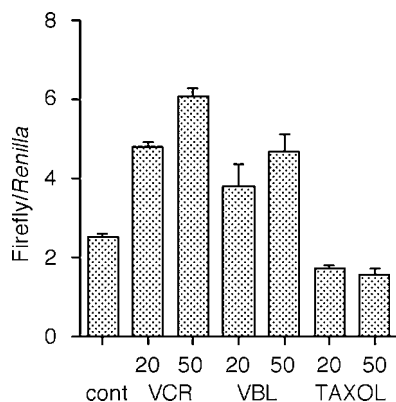


**FIGURE 4.** Putative transcription factor binding site of promoter region and first intron of the *mTUBB2* gene (Genbank accession no. NT 039579). **A.** The sequence contained within  $-359$  and  $+139$  nt of the *mTUBB2* gene was analyzed using a Web-based search engine. Putative Sp1, cyclic AMP response element-binding protein, and p53 sites are indicated and the first exon is boxed. **B.** p53-binding site region within the first intron of *mTUBB2*. Two p53-binding sites are shown separated by 7 nt. The sequence used for EMSA probe is italicized.

probe and a supershift assay using an anti-p53 polyclonal antibody were done (Fig. 6B). Binding of the nuclear extract was inhibited by an excess amount of unlabeled p53 consensus probe in a dose-dependent manner and this band was supershifted by incubation with the specific antibody to p53 (Fig. 6B, arrow). These results indicated that vincristine-mediated release of the p53 protein from the p53-binding site of the *mTUBB2* gene. Next, four mutations were made within the p53-binding site of the pmTUBB2-luc vector, with the mutated sites being confirmed by DNA sequencing. Among these mutants, whereas mt1 and mt2 had no effect on response to vincristine, mt3 showed decreased luciferase activity (Fig. 7). These results indicated that the ATG sequence in the p53-binding site (AGAAATGGAT, the mutation introduced in mt3 was italicized) was important for *mTUBB2* expression. However, weak response to vincristine still remained in mt3. Furthermore, mt4, which covered the region that was mutated

in both mt1 and mt3, showed loss of luciferase activity. It indicated that the GAAATG sequence in the p53-binding site (AGAAATGGAT, the mutation introduced in mt4 was italicized) was a critical region for *mTUBB2* expression.

As the EMSA result suggested that vincristine inhibited the function of p53, we determined whether exogenous induction of the p53 homologue, TAp63 $\gamma$ , could function as an inhibitor of vincristine-mediated up-regulation of *mTUBB2*. Figure 8 showed that cotransfection of an expression vector for TAp63 $\gamma$  abrogated the effect of vincristine on transcriptional activity of *TUBB2* in a dose-dependent manner. In relation to cotransfection with the empty vector, vincristine stimulated pmTUBB2-luc activity by 2-fold compared with untreated control. Whereas, in the presence of a low amount (100 ng) of TAp63 $\gamma$  expression vector, vincristine weakly elevated the luciferase activity by 1.2-fold compared with control, a higher amount (250 ng) of TAp63 $\gamma$  expression vector entirely suppressed the effect of vincristine. Taken together, these results indicate that overexpression of TAp63 $\gamma$  could supplement for vincristine-inactivated p53.



**FIGURE 5.** Dose-dependent stimulation of transcriptional activities of *mTUBB2* promoter by antimicrotubule drugs in B16F10 cells. B16F10 cells ( $5 \times 10^4$ ) were cotransfected with  $1 \mu\text{g}$  pmTUBB2-luc and  $2.5 \text{ ng}$  pRL-CMV for 5 hours. Medium was replaced and incubated overnight. Cells were serum starved by preincubation with medium containing 0.1% FBS for 8 hours followed by addition of various concentrations of vincristine, vinblastine, and Taxol. After 16 hours, cell layers were harvested and the luciferase activity was measured and normalized to *Renilla* luciferase activity as described in Materials and Methods. Representative of three independent experiments. Columns, mean; bars, SD.  $P < 0.01$ . VBL, vinblastine.

## Discussion

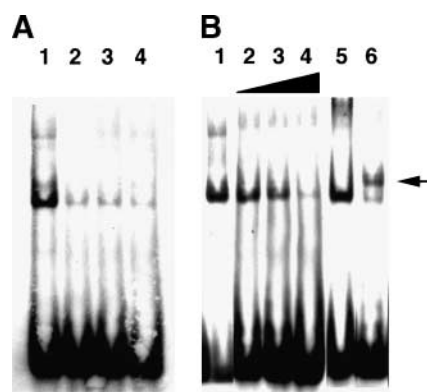
It is well known that altered  $\beta$ -tubulin expression is associated with resistance to antimicrotubule drugs (7, 8, 18, 29, 37). *Vinca* alkaloid-resistant leukemia cells have been shown to display decreased class III  $\beta$ -tubulin isotype expression (8), whereas increased mRNA expression of class I, III, and IVa  $\beta$ -tubulin isotypes in Taxol-resistant sublines of epithelial ovarian tumors (22) and significant increases in class IVa  $\beta$ -tubulin isotype expression in Taxol-resistant canine osteosarcoma (37) have been reported. These observations suggest that  $\beta$ -tubulin isotypes differ among various cell types and in response to various drug types. Although the mechanism of altered  $\beta$ -tubulin expression is not fully understood, it has been suggested to be a cellular defense against the action of microtubule drugs.

In the present study, we established vincristine-resistant B16F10 melanoma cells and showed that increased mRNA level of class II isotype of  $\beta$ -tubulin was directly induced by vincristine. In vincristine-resistant B16F10 sublines, class II  $\beta$ -tubulin isotype largely increased and class IVa isotype was decreased, whereas the relative amount of other isotypes hardly

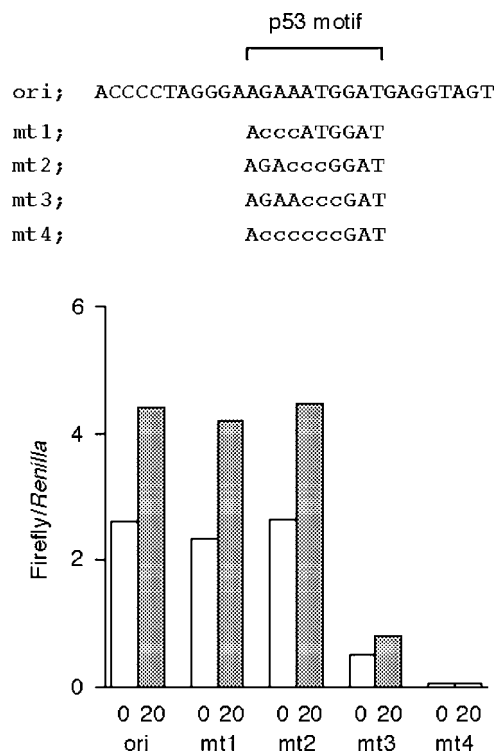
changed. Additionally, vincristine-resistant cells overexpressed *MDR1* gene, suggesting that P-glycoprotein contributed vincristine resistance of B16F10. To determine whether increased level of class II isotype was acquired phenotype in the resistant sublines or the response to the drug, change of mRNA level of this isotype in drug-sensitive parental B16F10 with a transient exposure of vincristine was examined. Overnight exposure of vincristine also increased the mRNA level of class II  $\beta$ -tubulin isotype, suggesting the response of these tumor cells to vincristine and not due to the drug resistance phenomena brought about by continuous exposure of the drug. Proteomic analysis of *Vinca* alkaloid-mediated responses in acute lymphoblastic leukemia also revealed that altered  $\beta$ -tubulin isotype expression was induced by 24-hour exposure to these agents (38). These results suggest direct effect of *Vinca* alkaloid on several gene expressions.

Next, cellular distribution of class II isotype in B16F10 cells was observed using the specific antibody. Immunofluorescence microscopy revealed that the dense accumulation of class II  $\beta$ -tubulin isotype surrounding the nuclei in vincristine-resistant sublines. In addition, aggregation of class II  $\beta$ -tubulin isotype appeared with overnight exposure of the parental cells to vincristine, with these structures appearing to accumulate around the nuclei. As it is well known that vincristine is a depolymerized agent, antibody-positive class II isotype could not be polymerized and these observations are thought to be the result of increased mRNA level. Morphologic changes in cells treated with vincristine have been reported from another group (39). They also reported cellular accumulation of class III isotype in vincristine-resistant neuroblastoma cell lines. Until now, it is unclear whether increased isotype was incorporated into the microtubule system; these results suggested that vincristine could modify the structure of the microtubule network.

We further showed that *Vinca* alkaloids stimulated gene expression of mTUBB2. By the reporter gene analysis, the



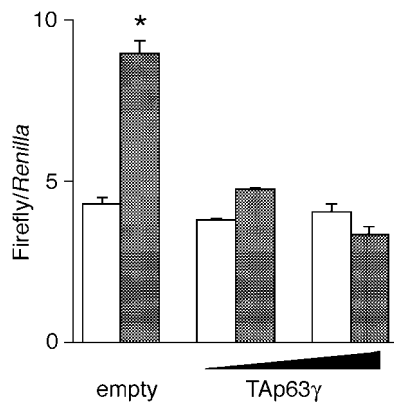
**FIGURE 6.** EMSA was done using a digoxigenin end-labeled oligonucleotide containing the putative p53-binding site found within the first intron of the *mTUBB2* gene. **A.** Nuclear protein was extracted from B16F10 cells exposed to various concentrations of vincristine and used for EMSA. Lane 1, untreated; lane 2, 5 nmol/L; lane 3, 20 nmol/L; lane 4, 50 nmol/L vincristine. **B.** Nuclear protein extracted from untreated B16F10 cells was used for EMSA and supershift assay. Competition experiments were done with 2.5-fold (lane 2), 25-fold (lane 3), and 250-fold (lane 4) excess of unlabeled p53 consensus sequence (lane 1; no addition). DNA-protein complex was further incubated with nonimmune serum (lane 5) or anti-p53 polyclonal antibody (lane 6). Arrow, supershifted band.



**FIGURE 7.** Mutation of putative p53-binding site abrogates transcriptional response by vincristine. B16F10 cells were transfected with 1  $\mu$ g pmTUBB2-luc or its mutants and 2.5 ng pRL-CMV for 5 hours. Medium was replaced and incubated overnight. Cells were serum starved by preincubation with medium containing 0.1% FBS for 8 hours followed by addition of 20 nmol/L vincristine. After 16 hours, cell layers were harvested and the luciferase activity was measured and normalized to *Renilla* luciferase activity as described in Materials and Methods. The sequence of wild-type p53 site is AGAAATGGAT, whereas those of the mutated p53 sites are AcccATGGAT (*mt1*), AGAcccGGAT (*mt2*), AGAAccGAT (*mt3*), and AccccccGAT (*mt4*). Lowercase indicates mutation. Representative of three independent experiments. Columns, mean; bars, SD.

promoter activity of mTUBB2 was stimulated by vincristine and vinblastine but not Taxol (Fig. 5), indicating that this effect is perhaps specific for *Vinca* alkaloids. Next, we found that the binding of a nuclear protein to the p53-binding site within the first intron of TUBB2 was decreased at even low concentrations (5 nmol/L) of vincristine in the parental B16F10 cells (Fig. 6A). This nuclear factor was identified as the tumor suppressor p53 protein by both competition and supershift assays (Fig. 6B). Previous reports have indicated that vinblastine induced up-regulation of c-Myc through acceleration of nuclear factor- $\kappa$ B nuclear translocation, indicating that *Vinca* alkaloid could play as a transcriptional modulator in addition to a microtubule-depolymerizing agent (40). Here, we also showed that vincristine could transcriptionally regulate the *mTUBB2* gene. Furthermore, although genomic alteration of the *p53* gene in B16F10 cells has been reported, several studies have indicated that the p53 in these cells seems to be functional (41, 42). Our results also showed that p53 protein in B16F10 cells at least retained its binding activity to the response element.

Four mutants of pmTUBB2-luc targeted to the second half site of the p53-binding motif were prepared and applied to the reporter gene assay (Fig. 7). Among these, two mutants



**FIGURE 8.** Effect of Tap63 $\gamma$  on vincristine-mediated stimulation of mTUBB2 promoter activity. B16F10 cells ( $5 \times 10^4$ ) were transfected with 1  $\mu$ g pmTUBB2-luc and 2.5 ng pRL-CMV vectors along with a pcDNA3.1 empty vector (100 ng) or an expression vector for Tap63 $\gamma$  (100 or 250 ng) for 5 hours. Medium was replaced and incubated overnight. Cells were serum starved by preincubation with medium containing 0.1% FBS for 8 hours followed by addition of 20 nmol/L vincristine. After 16 hours, cell layers were harvested and the luciferase activity was measured and normalized to *Renilla* luciferase activity as described in Materials and Methods. Representative of three independent experiments. Columns, mean; bars, SD. \*,  $P < 0.01$ .

designated as mt1 and mt2 showed no change compared with original pmTUBB2-luc, suggesting that either mt1 or mt2 was insufficient for inactivation of the element. However, the third mutant, mt3, showed decreased luciferase activity; further, mt4 lost even basal transcription activity. As the mt4 covered the region that was mutated in both mt1 and mt3, these results indicate that the GAAATG sequence in the p53-binding site (AGAAATGGAT) was one of the essential elements for basal expression. Taken together with EMSA results, p53 protein is thought to usually bind to this region to suppress mTUBB2 expression and that vincristine inhibits the binding of p53 to the response element, allowing for increased expression of class II  $\beta$ -tubulin. Other studies found that p53 transcriptionally down-regulated microtubule-associated protein 4 (43) and that human breast adenocarcinoma cells harboring a mutated *p53* gene displayed an increased level of class IV  $\beta$ -tubulin isotype and reduced levels of class I and II isotypes, suggesting that the *p53* gene may differentially regulate expression of  $\beta$ -tubulin isotypes (28, 29, 32). These findings suggest that p53 could transcriptionally regulate components of the microtubule system.

Furthermore, vincristine-mediated stimulation of mTUBB2 expression was abrogated by exogenous induction of Tap63 $\gamma$ , a homologue of p53, in a dose-dependent manner (Fig. 8). This suggests that overexpression of Tap63 $\gamma$  allows for immediate binding of the p53-binding site in the case where binding of p53 is inhibited by vincristine. Until now, although we have no data concerning the mechanism how vincristine suppressed the binding activity of p53, other investigators have indicated that chemotherapeutic drugs induced MDM2 (44), an accelerator of p53 ubiquitination, which may contribute to drug resistance by increasing p53 degradation (45). Additionally, some members of p53 family was known to be transcriptionally regulated by several factors, including chemotherapeutic drugs and MDM2

(46-48). In addition, in the context of vincristine-mediated stimulation of mTUBB2 expression, increased MDM2 level or other regulators for p53 function could be involved in an inhibitory mechanism of p53 binding.

In summary, we have shown in mouse melanoma cells a regulatory mechanism for increased class II  $\beta$ -tubulin isotype expression by vincristine. Vincristine-mediated modulation of p53 function could participate in several *Vinca* alkaloid-induced cellular events, including antineoplastic effects and the emergence of drug-resistant tumors.

## Materials and Methods

### Cell Culture

The mouse B16F10 melanoma cell line was purchased from the American Type Culture Collection (Manassas, VA). Cells were propagated in DMEM (Invitrogen Corp., Carlsbad, CA) containing 10% fetal bovine serum (FBS; Invitrogen) supplemented with 50 units/mL penicillin (Sigma Chemical Co., St. Louis, MO), 50  $\mu$ g/mL streptomycin (Sigma), and 100  $\mu$ g/mL neomycin (Sigma) in an atmosphere of humidified air and 5% CO<sub>2</sub> at 37°C. Vincristine-resistant sublines of B16F10 cells were selected in a stepwise manner according to previous reports (16, 18, 22). For this, parental B16F10 cells were first exposed to 1.5 nmol/L vincristine (Sigma) in medium, with treated cells being allowed to recover. Then, the concentration of vincristine was successfully increased to 5, 10, and 20 nmol/L. Resistant sublines were derived and maintained at respective concentrations of vincristine. Temporary exposure of antimicrotubule drugs was done as follows. Subconfluent B16F10 cells were incubated with DMEM containing 0.1% FBS for 8 hours followed by the addition of vincristine, vinblastine (Sigma), and Taxol (paclitaxel; Sigma) to final concentrations of 5, 20, or 50 nmol/L and further incubation for 18 hours. Cellular total RNA was extracted with TRIzol (Invitrogen) and nuclear extracts were prepared with a Cellytic nuclear extraction kit (Sigma) according to the manufacturer's instructions.

### RNase Protection Assay

Isotype-specific regions of five classes of mouse  $\beta$ -tubulin, MDR1, and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control were amplified using the specific primer sets shown in Table 1 and subcloned into pGEM-T easy vector (Promega, Madison, WI). DNA sequences were confirmed by a DSQ-2000L autosequencer (Shimadzu, Kyoto, Japan) using a Thermo Sequenase Primer Cycle Sequencing kit and 7-deazadGTP (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

A nonradioactive RPA using digoxigenin labeling procedure was employed followed by chemiluminescence detection. After linearization of plasmids with an appropriate restriction enzyme, antisense ribonucleotide probes corresponding to mouse GAPDH and five  $\beta$ -tubulin isotypes were synthesized with Sp6 or T7 RNA polymerase (Roche Diagnostics) and digoxigenin-UTP (Roche Diagnostics). An excess amount (~500 pg) of RNA probe was hybridized with total cellular RNA (5  $\mu$ g for GAPDH; 10  $\mu$ g for class I, II, and IVb; 50  $\mu$ g for class III and IVa and MDR1) in the presence of 80%

formamide using a RPAII kit (Ambion, Inc., Austin, TX) overnight at 42°C. After digestion of unprotected regions with the RNase A/T1 mixture, the protected fragments were separated on a 5% polyacrylamide gel containing 8 mol/L urea and electrophoretically transferred onto a positively charged nylon membrane. After the separated fragments were fixed by UV cross-linking, the positive signal was visualized using a chemiluminescence method. Briefly, the membrane was incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics) at a dilution of 1:20,000 overnight at 4°C; washed thrice in 0.15 mol/L NaCl, 0.1 mol/L maleic acid (pH 8.0); immersed in 0.2% CSPD (Roche Diagnostics) diluted with 5 mmol/L MgCl<sub>2</sub>, 100 mmol/L NaCl, 100 mmol/L Tris-HCl (pH 9.5) for 10 minutes; and exposed to X-ray film (Hyperfilm enhanced chemiluminescence, Amersham Pharmacia Biotech).

Quantification of the bands on the autoradiograms was done using densitometric analysis, with the mRNA level of each isotype being corrected by GAPDH mRNA level. For this, three independent experiments from each cell culture sample were done.

#### Construction of Luciferase Plasmid and Site-Directed Mutagenesis

Cloning of the promoter region of *mTUBB2* gene was done by PCR amplification using ExTaq DNA polymerase (Takara, Shiga, Japan). Mouse genomic DNA was prepared from C57BL/6 mouse liver and used as a template for PCR amplification. A forward primer (5'-GGCCTCGAGAATGAG-GCAGTGTGGGAGCAGAACC-3'; -358/-333) within the 5'-flanking region of the *mTUBB2* gene and a reverse primer (5'-GGCAAGCTTCCCCTACCCCTCTCCCCACGCAACC-3'; +115/+139) in the first intron were designed based on sequence derived from Genbank accession no. NT 039579. The PCR-amplified product (-358/+139; 506-bp fragment) was digested with *Xho*I and *Hind*III (*Xho*I recognition site in the forward primer and *Hind*III site in the reverse primer are italicized) and subcloned into pGL3 basic vector (Promega), with this modified vector designated as pmTUBB2-luc. Several point mutations were introduced into the potential p53-binding site (at +67/+76 nt) of pmTUBB2-luc, as shown in Fig. 7, with the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Mutation constructs were verified by DNA sequencing. These plasmids were purified by the Endofree Plasmid Maxi kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions and used for the transfection experiments described below.

#### Cell Transfection and Luciferase Assay

Transient transfection of B16F10 cells and their sublines were done using FuGENE 6 transfection reagent (Roche Diagnostics). Cells ( $5 \times 10^4$ ) were seeded in 12-well culture plates (3.8 cm<sup>2</sup>/well). Following overnight culture, cells in each well were transfected with a mixture containing 3  $\mu$ L FuGENE 6 and 1  $\mu$ g of the indicated constructs in 100  $\mu$ L Opti-MEM I (Invitrogen). To assess transfection efficiency, 2.5 ng pRL-CMV vector (Promega), which encodes a *Renilla* luciferase gene downstream from a CMV enhancer and immediate-early promoter, was systemically added to the transfection mixture. A plasmid expressing the p53 homologue, TAp63 $\gamma$  (p51A) [refs. 49, 50; generously gifted by Drs. M. Senoo (Department of Cell Biology, Harvard Medical School, Boston, MA) and S. Habu (Department of Immunology, Tokai University School of Medicine, Isehara, Japan)], was added at 100 or 250 ng to the transfection mixture. The total amount of DNA for each transfection was kept constant using the empty vector pcDNA3.1 (Invitrogen). Five hours after transfection, the culture medium was replaced with DMEM-10% FBS and cultured for an additional 15 hours. The medium was changed to DMEM containing 0.1% FBS for 8 hours followed by exposure to several concentrations of antimicrotubule drugs for 18 hours. Cell layers were harvested with 100  $\mu$ L Passive Lysis Buffer (Promega), with the cell membranes being disrupted by two freeze-thaw cycles and pelleted. The luciferase activity of cell lysates were assayed with a dual-luciferase reporter assay system (Promega) using a microtiter plate luminometer (Luminescencer JNR, ATTO, Tokyo, Japan). Activities of firefly luciferase were normalized to *Renilla* luciferase activity.

#### Electrophoretic Mobility Shift Assay

A complementary single-strand oligonucleotide containing the putative p53 site (italicized) in the first intron of *mTUBB2* (5'-CCTAGGGAGAAATGGATGAGGTAGTCC-CATCCGGT-3'; +69/+91), were annealed and 3' end-labeled with digoxigenin-11-ddUTP and terminal transferase (Roche Diagnostics). Nuclear proteins were mixed with 5 $\times$  gel shift binding buffer (Promega) and digoxigenin end-labeled duplex oligonucleotide, with the reaction subsequently incubated for 15 minutes at room temperature. To facilitate a competition assay, excess amount of unlabeled probe corresponding to the p53 consensus sequence (5'-TACAGAACATGTCTAAG-CATGCTGGGG-3', Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was incubated with nuclear extracts before being mixed with the labeled probe. For supershift experiments, the reaction mixture was further incubated with 2  $\mu$ g rabbit

**Table 1. Sequences of PCR Primer Sets Used for the Preparation of RPA Probes**

	Product size (bp)	Upper primer (5'-3')	Lower primer (5'-3')	Annealing $T_m$ (°C)
GAPDH	296	AGCCCATCACCATCTTCCAG	ATGAGCCCTTCCACAATGCC	57.4
Class I	312	TTCATTGGAAACAGCACAGC	GAAACTGGAGGACAAAGGGC	56.6
Class II	177	GTGACATCCCTCCTCGTG	CTTCCCTAACCTGTCTTG	55.5
Class III	244	CAACAGCACGGCCATCCAGG	CTTGGGGCCCTGGCCCTCCGA	56.6
Class IVa	271	GGACGAGATGGAGTTTACGC	AGCAAATAGAAGCAACCTGG	55.8
Class IVb	344	GTCCAAAACAAGAACAGCAG	CTCTAAGCCACCTCCTCTCT	55.2
MDR1	258	TCGCAAAAAGCATCTGTGG	AAGTTGCTGTCTGCCGC	53.2

polyclonal antibody to p53 (Active Motif, Carlsbad, CA) at 4°C for 1 hour followed by electrophoresis. DNA-protein complexes were separated on 5% nondenaturing polyacrylamide gels supplemented with 3.33% Rhinohide Polyacrylamide Gel Strengthener (Molecular Probes, Inc., Eugene, OR) in 0.25× Tris borate/EDTA at 4°C and 50 V. Gels were transferred onto a positively charged nylon membrane and the complexes were visualized in the same way as for RPA.

#### Immunocytochemistry

Immunofluorescence microscopy was done to analyze the cellular distribution of class II  $\beta$ -tubulin protein. The parental B16F10 cells and 20 nmol/L vincristine-resistant subline were seeded onto chamber slides (Nunc, Inc., Naperville, IL). The parental cells were also transiently exposed to 20 nmol/L vincristine. After fixation with absolute ethanol at -20°C overnight, slide-grown cells were incubated with an anti-class II  $\beta$ -tubulin monoclonal antibody (4), in which the epitope was identified as EEEEGED (corresponding to the COOH-terminal sequence of class II isotype), at 4°C overnight followed by FITC-conjugated secondary antibody (Chemicon International, Inc., Temecula, CA) for 1 hour at room temperature. Resulting slides were mounted with aqueous mounting medium (Mount-Quick Aqueous, Daido Sangyo Co. Ltd., Tokyo, Japan). Images were acquired with a TCS NT confocal laser scanning microscope (Leica, Wetzlar, Germany).

#### Statistical Analysis

Statistical differences between the values of the respective experimental groups and controls were determined by Mann-Whitney's *U* test and *P* < 0.01 was considered significant.

#### Acknowledgments

We thank Drs. M. Senoo and S. Habu for providing the TAp63 $\gamma$  (p51A) expression plasmid.

#### References

- Burgoyne RD, Cambay-Deakin MA, Lewis SA, Sarkar S, Cowan NJ. Differential distribution of  $\beta$ -tubulin isotypes in cerebellum. *EMBO J* 1988;7:2311–9.
- Jensen-Smith HC, Luduena RF, Hallworth R. Requirement for the  $\beta$ I and  $\beta$ IV tubulin isotypes in mammalian cilia. *Cell Motil Cytoskeleton* 2003;55:213–20.
- Jensen-Smith HC, Eley J, Steyger PS, Luduena RF, Hallworth R. Cell type-specific reduction of  $\beta$  tubulin isotypes synthesized in the developing gerbil organ of Corti. *J Neurocytol* 2003;32:185–97.
- Arai K, Shibutani M, Matsuda H. Distribution of the class II  $\beta$ -tubulin in developmental and adult rat tissues. *Cell Motil Cytoskeleton* 2002;52:174–82.
- Hallworth R, Luduena RF. Differential expression of  $\beta$  tubulin isotypes in the adult gerbil cochlea. *Hear Res* 2000;148:161–72.
- Katsetos CD, Karkavelas G, Herman MM, et al. Class III  $\beta$ -tubulin isotype ( $\beta$ III) in the adrenal medulla. I. Localization in the developing human adrenal medulla. *Anat Rec* 1998;250:335–43.
- Kamath K, Wilson L, Cabral F, Jordan MA.  $\beta$ III-tubulin induces paclitaxel resistance in association with reduced effects on microtubule dynamic instability. *J Biol Chem* 2005;280:12902–7.
- Kavallaris M, Tait AS, Walsh BJ, et al. Multiple microtubule alterations are associated with *Vinca* alkaloid resistance in human leukemia cells. *Cancer Res* 2001;61:5803–9.
- Burkhardt CA, Kavallaris M, Band Horwitz S. The role of  $\beta$ -tubulin isotypes in resistance to antimetabolic drugs. *Biochim Biophys Acta* 2001;1471:O1–9.
- Carles G, Braguer D, Dumontet C, et al. Differentiation of human colon cancer cells changes the expression of  $\beta$ -tubulin isotypes and MAPs. *Br J Cancer* 1999;80:1162–8.

- Arai K, Nakano H, Shibutani M, Naoi M, Matsuda H. Expression of class II  $\beta$ -tubulin by proliferative myoepithelial cells in canine mammary mixed tumors. *Vet Pathol* 2003;40:670–6.
- Giannakakou P, Nakano M, Nicolaou KC, et al. Enhanced microtubule-dependent trafficking and p53 nuclear accumulation by suppression of microtubule dynamics. *Proc Natl Acad Sci U S A* 2002;99:10855–60.
- Leonetti C, Scarsella M, Semple SC, et al. *In vivo* administration of liposomal vincristine sensitizes drug-resistant human solid tumors. *Int J Cancer* 2004;110:767–74.
- Kivela T, Suci S, Hansson J, et al. Bleomycin, vincristine, lomustine and dacarbazine (BOLD) in combination with recombinant interferon  $\alpha$ -2b for metastatic uveal melanoma. *Eur J Cancer* 2003;39:1115–20.
- Pyrhonen S, Hahka-Kemppinen M, Muhonen T, et al. Chemoimmunotherapy with bleomycin, vincristine, lomustine, dacarbazine (BOLD), and human leukocyte interferon for metastatic uveal melanoma. *Cancer* 2002;95:2366–72.
- Balachandran R, Welsh MJ, Day BW. Altered levels and regulation of stathmin in paclitaxel-resistant ovarian cancer cells. *Oncogene* 2003;22:8924–30.
- Martello LA, McDaid HM, Regl DL, et al. Taxol and discodermolide represent a synergistic drug combination in human carcinoma cell lines. *Clin Cancer Res* 2000;6:1978–87.
- Ranganathan S, Benetatos CA, Colarusso PJ, Dexter DW, Hudes GR. Altered  $\beta$ -tubulin isotype expression in paclitaxel-resistant human prostate carcinoma cells. *Br J Cancer* 1998;77:562–6.
- Zhan M, Yu D, Liu J, et al. Transcriptional repression of protein kinase C $\alpha$  via Sp1 by wild type p53 is involved in inhibition of multidrug resistance 1 P-glycoprotein phosphorylation. *J Biol Chem* 2005;280:4825–33.
- Kang CD, Ahn BK, Jeong CS, et al. Downregulation of JNK/SAPK activity is associated with the cross-resistance to P-glycoprotein-unrelated drugs in multidrug-resistant FM3A/M cells overexpressing P-glycoprotein. *Exp Cell Res* 2000;256:300–7.
- Shabbits JA, Mayer LD. P-glycoprotein modulates ceramide-mediated sensitivity of human breast cancer cells to tubulin-binding anticancer drugs. *Mol Cancer Ther* 2002;1:205–13.
- Kavallaris M, Kuo DYS, Burkhardt CA, et al. Taxol-resistant epithelial ovarian tumors are associated with altered expression of specific  $\beta$ -tubulin isotypes. *J Clin Invest* 1997;100:1282–93.
- Montgomery RB, Guzman J, O'Rourke DM, Stahl WL. Expression of oncogenic epidermal growth factor receptor family kinases induces paclitaxel resistance and alters  $\beta$ -tubulin isotype expression. *J Biol Chem* 2000;275:17358–63.
- Blade K, Menick DR, Cabral F. Overexpression of class I, II or IV  $\beta$ -tubulin isotypes in CHO cells is insufficient to confer resistance to paclitaxel. *J Cell Sci* 1999;112:2213–21.
- Geyp M, Ireland CM, Pittman SM. Increased tubulin acetylation accompanies reversion to stable ploidy in vincristine-resistant CCRF-CEM cells. *Cancer Genet Cytogenet* 1996;87:116–22.
- Derry WB, Wilson L, Khan IA, Luduena RF, Jordan MA. Taxol differentially modulates the dynamics of microtubules assembled from unfractionated and purified  $\beta$ -tubulin isotypes. *Biochemistry* 1997;36:3554–62.
- Schmidt M, Bachhuber A, Victor A, et al. p53 expression and resistance against paclitaxel in patients with metastatic breast cancer. *J Cancer Res Clin Oncol* 2003;129:295–302.
- Chang JT, Chang GC, Ko JL, et al. Induction of tubulin by docetaxel is associated with p53 status in human non small cell lung cancer cell lines. *Int J Cancer* 2006;118:317–25.
- Galmari CM, Kamath K, Vanier-Viomery A, et al. Drug resistance associated with loss of p53 involves extensive alterations in microtubule composition and dynamics. *Br J Cancer* 2003;88:1793–9.
- Giannakakou P, Poy G, Zhan Z, et al. Paclitaxel selects for mutant or pseudo-null p53 in drug resistance associated with tubulin mutations in human cancer. *Oncogene* 2000;19:3078–85.
- Cassinelli G, Supino R, Perego P, et al. A role for loss of p53 function in sensitivity of ovarian carcinoma cells to taxanes. *Int J Cancer* 2001;92:738–47.
- Galmari CM, Falette N, Tabone E, et al. Inactivation of wild-type p53 by a dominant negative mutant renders MCF-7 cells resistant to tubulin-binding agent cytotoxicity. *Br J Cancer* 2001;85:902–8.
- Yang A, Kaghad M, Wang Y, et al. p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 1998;2:305–16.
- Melino G, Lu X, Gasco M, Crook T, Knight RA. Functional regulation of p73 and p63: development and cancer. *Trends Biochem Sci* 2003;28:663–70.



35. Levrero M, De Laurenzi V, Costanzo A, et al. Structure, function and regulation of p63 and p73. *Cell Death Differ* 1999;6:1146–53.
36. Vayssade M, Faridoni-Laurens L, Benard J, Ahomadegbe JC. Expression of p53-family members and associated target molecules in breast cancer cell lines in response to vincristine treatment. *Biochem Pharmacol* 2002;63:1609–17.
37. Arai K, Maruo K, Ara KY, Uehara K, Matsuda H. Characterization of isotype-specific regions of five classes of canine  $\beta$ -tubulin and their expression in several tissues and cell culture. *J Vet Med Sci* 2001;63:1297–302.
38. Verrills NM, Walsh BJ, Cobon GS, Hains PG, Kavallaris M. Proteome analysis of *Vinca* alkaloid response and resistance in acute lymphoblastic leukemia reveals novel cytoskeletal alterations. *J Biol Chem* 2003;278:45082–93.
39. Don S, Verrills NM, Liaw TY, et al. Neuronal-associated microtubule proteins class III  $\beta$ -tubulin and MAP2c in neuroblastoma: role in resistance to microtubule-targeted drugs. *Mol Cancer Ther* 2004;3:1137–46.
40. Bourgarel-Rey V, Vallee S, Rimet O, et al. Involvement of nuclear factor  $\kappa$ B in c-Myc induction by tubulin polymerization inhibitors. *Mol Pharmacol* 2001;59:1165–70.
41. Koma YI, Ito A, Watabe K, Kimura SH, Kitamura Y. A truncated isoform of the PP2A B56 $\gamma$  regulatory subunit reduces irradiation-induced Mdm2 phosphorylation and could contribute to metastatic melanoma cell radioresistance. *Histol Histopathol* 2004;19:391–400.
42. Melnikova VO, Bolshakov SV, Walker C, Ananthaswamy HN. Genomic alterations in spontaneous and carcinogen-induced murine melanoma cell lines. *Oncogene* 2004;23:2347–56.
43. Zhang CC, Yang JM, Bash-Babula J, et al. DNA damage increases sensitivity to *Vinca* alkaloids and decreases sensitivity to taxanes through p53-dependent repression of microtubule-associated protein 4. *Cancer Res* 1999;59:3663–70.
44. Rokhlin OW, Glover RA, Taghiyev AF, et al. Bisindolylmaleimide IX facilitates tumor necrosis factor receptor family-mediated cell death and acts as an inhibitor of transcription. *J Biol Chem* 2002;277:33213–9.
45. Tang HJ, Qian D, Sondak VK, Stachura S, Lin J. A modified p53 enhances apoptosis in sarcoma cell lines mediated by doxorubicin. *Br J Cancer* 2004;90:1285–92.
46. Zheng L, Ren JQ, Li H, et al. Downregulation of wild-type p53 protein by HER-2/*neu* mediated PI3K pathway activation in human breast cancer cells: its effect on cell proliferation and implication for therapy. *Cell Res* 2004;14:497–506.
47. Calabro V, Mansueto G, Parisi T, et al. The human MDM2 oncoprotein increases the transcriptional activity and the protein level of the p53 homolog p63. *J Biol Chem* 2002;277:2674–81.
48. Toh WH, Siddique MM, Boominathan L, Lin KW, Sabapathy K. c-Jun regulates the stability and activity of the p53 homologue, p73. *J Biol Chem* 2004;279:44713–22.
49. Senoo M, Seki N, Ohira M, et al. A second p53-related protein, p73L, with high homology to p73. *Biochem Biophys Res Commun* 1998;248:603–7.
50. Senoo M, Matsumura Y, Habu S. TAp63 $\gamma$  (p51A) and dNp63 $\alpha$  (p73L), two major isoforms of the p63 gene, exert opposite effects on the vascular endothelial growth factor (VEGF) gene expression. *Oncogene* 2002;21:2455–65.

# Molecular Cancer Research

## Regulation of Class II $\beta$ -Tubulin Expression by Tumor Suppressor p53 Protein in Mouse Melanoma Cells in Response to *Vinca* Alkaloid

Katsuhiko Arai, Yoshifumi Matsumoto, Yuko Nagashima, et al.

*Mol Cancer Res* 2006;4:247-255. Published OnlineFirst March 23, 2006.

**Updated version** Access the most recent version of this article at:  
doi:[10.1158/1541-7786.MCR-05-0183](https://doi.org/10.1158/1541-7786.MCR-05-0183)

**Cited articles** This article cites 49 articles, 15 of which you can access for free at:  
<http://mcr.aacrjournals.org/content/4/4/247.full#ref-list-1>

**E-mail alerts** [Sign up to receive free email-alerts](#) related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at [pubs@aacr.org](mailto:pubs@aacr.org).

**Permissions** To request permission to re-use all or part of this article, use this link  
<http://mcr.aacrjournals.org/content/4/4/247>.  
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.